

Ionic and signaling mechanisms involved in the excitation of entorhinal neurons by group I mGluRs



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ARTICLE INFO

Handling Editor: Dr T. Di Paolo

Keywords:
Excitability
Action potential
 K^+ channels
G protein
Synapse
Receptor

ABSTRACT

Activation of metabotropic glutamate receptors (mGluRs) modulates neuronal excitability, synaptic transmission and plasticity in the brain. While group I mGluRs in layer III pyramidal neurons in the entorhinal cortex (EC) are implicated in persistent firing which is considered as a cellular mechanism for working memory, the underlying ionic and signaling mechanisms have not been determined. Here, we showed that application of (S)-3,5-dihydroxyphenylglycine (DHPG), the selective agonist for group I mGluRs, excited layer III pyramidal neurons of the EC via activation of both mGluR1 and mGluR5. DHPG excited layer III pyramidal neurons by activating TRPC5 channels and depressing GIRK type of inwardly rectifying K^+ (Kir) channels. The functions of G proteins, phospholipase C β (PLC β) and PLC β -mediated depletion of PIP₂ were required for group I mGluR-mediated activation of TRPC5 and depression of GIRK channels, whereas intracellular Ca^{2+} release and PKC were not involved in DHPG-elicited excitation of layer III pyramidal neurons. We also found that diacylglycerol was involved in DHPG-elicited activation of TRPC5 channels. Our results may serve as a signaling and ionic mechanism to explain the physiological functions of group I mGluRs *in vivo*.

1. Introduction

The entorhinal cortex (EC) serves as an interface between the hippocampus and other cortical areas (Witter et al., 1989, 2000a, 2000b). Sensory inputs converge onto the superficial layers (layers II–III) of the EC (Burwell, 2000) which give rise to dense projections to the hippocampus; the axons of the stellate neurons in layer II of the EC form the perforant path that innervates the dentate gyrus and CA3 (Steward and Scoville, 1976), whereas those of the pyramidal neurons in layer III form the temporoammonic pathway synapsing onto the distal dendrites of pyramidal neurons in CA1 and the subiculum (Steward and Scoville, 1976; Witter et al., 2000a, 2000b). Moreover, neurons in the deep layers of the EC (layers V–VI) relay a large portion of hippocampal output projections back to the superficial layers of the EC (Dolorfo and Amaral, 1998a, 1998b; Kohler, 1986; van Haeften et al., 2003) and to other cortical areas (Witter et al., 1989). The EC is part of a network that is closely related to the consolidation and recall of memories (Haist et al., 2001; Squire et al., 2004), Alzheimer's disease (Hyman et al., 1984; Kotzbauer et al., 2001), schizophrenia (Arnold et al., 1991; Falkai et al., 1988; Joyal et al., 2002; Prasad et al., 2004) and temporal lobe epilepsy (Avoli et al., 2002; Spencer and Spencer, 1994).

Glutamate is the major excitatory neurotransmitter in the brain where it interacts with ionotropic (NMDA, AMPA, and kainate) and metabotropic glutamate receptors (mGluRs). While the ionotropic glutamate receptors mediate the fast excitatory synaptic transmission, mGluRs normally play a modulatory role in the brain. mGluRs are G protein-coupled receptors that are linked to various intracellular second messenger cascades. Group I mGluRs comprise mGluR1 and mGluR5 that are coupled to $G\alpha_{q/11}$, resulting in activation of phospholipase C β (PLC β) to increase intracellular Ca^{2+} release and the activity of protein kinase C (PKC) (Ritzen et al., 2005). Group II (mGluR2, mGluR3) and Group III (mGluR4, mGluR6–8) mGluRs are coupled to $G\alpha_{i/o}$ proteins. These two groups of receptors are negatively linked to adenylate cyclase leading to a reduction in the intracellular level of cyclic AMP and an inhibition of protein kinase A (PKA) (Ritzen et al., 2005).

The EC expresses a variety of subtypes of mGluRs (Fotuhi et al., 1994; Lujan et al., 1996; Shigemoto et al., 1997). Activation of group I mGluRs promotes persistent firing of layer III pyramidal neurons in a manner like carbachol-induced persistent firing (Yoshida et al., 2008). Furthermore, the functions of mGluRs also modulate synaptic transmission at many synapses in the EC. Group I mGluRs augment GABAergic transmission onto superficial layer II/III principal neurons

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by depolarizing local interneurons in an mGluR5-dependent manner but agonists for group II/III mGluRs do not alter GABAergic transmission (Deng et al., 2010). Whereas activation of group I/II mGluRs reduces the amplitude of evoked excitatory postsynaptic currents in layer III (Iserhot et al., 2004), selective activation of group II mGluRs depresses glutamatergic transmission by reducing the glutamate release probability via inhibition of P/Q voltage-gated Ca^{2+} channels (Wang et al., 2012). Group II mGluR activation in layer III neurons directly reduces their intrinsic excitability by increasing a K^+ conductance and inhibiting a Na^+ -permeable cation channel (Zhang et al., 2015). Group III mGluRs suppress, in the superficial layers, but facilitate, in the deep layers, glutamatergic transmission (Evans et al., 2000). Activation of group III mGluRs in layer V depresses the amplitude of stimulus-evoked excitatory postsynaptic currents, but concurrently enhanced the frequency of spontaneous excitatory currents (Woodhall et al., 2007). For GABAergic transmission, activation of group III mGluRs does not alter spontaneous inhibition in the superficial layers (Deng et al., 2010), but reduces spontaneous inhibition onto layer V neurons (Woodhall et al., 2001). However, the cellular and molecular mechanisms underlying mGluR-mediated neural modulation in the EC have not been fully determined. In the present study, we probed the effects of group I mGluRs on the excitability of layer III pyramidal neurons by using their selective agonist, (S)-3,5-dihydroxyphenylglycine (DHPG). Our results demonstrate that activation of group I mGluRs excites layer III pyramidal neurons by activation of TRPC5 channels and depressing the GIRK type of the inwardly rectifying K^+ (Kir) channels via PLC β -mediated depletion of PIP₂. We also found that diacylglycerol (DAG) participated in DHPG-induced activation of TRPC5 channels as application of the selective DAG lipase inhibitor, RHC 80267, to decrease the degradation of DAG significantly enhanced DHPG-elicited inward holding currents. Our results may provide a cellular and molecular mechanism to explain the physiological roles of group I mGluRs *in vivo* such as persistent firing and memory.

2. Materials and methods

2.1. Slice preparation

Horizontal brain slices (350 μm) were prepared from male and female Sprague-Dawley rats (21–35 days old) purchased from Envigo RMS, INC. (Indianapolis, IN), knockout (KO) and wild-type (WT) mice purchased from The Jackson Laboratory. The following 3 strains of KO mice (1–2 months) and their corresponding age-matched WT mice were used: TRPV1 KO mice (B6.129X1-*Trpv1*^{tm1Jul}/J, strain 003770) vs. WT mice (C57BL/6J, strain 000664); TRPC4 KO mice (129S1/SvlmJ-*Trpc4*^{tm1.1clph}/J, strain 030802) vs. WT mice (129S1/SvlmJ, strain 002448); TRPC5 KO mice (129S1/SvlmJ-*Trpc5*^{tm1.1clph}/J, strain 030804) vs. WT mice (129S1/SvlmJ, strain 002448). The animals were housed in the Center for Biomedical Research in the University of North Dakota with food and water available *ad libitum*. The animal rooms were maintained on a 14/10 h light-dark cycle (lights on at 7:00 a.m.), with a room temperature of 22 °C. All procedures and experiments presented in this study were approved by the Institutional Animal Use and Care Committee of the University of North Dakota and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The numbers of males and females for each experiment were kept as equal as possible. After being deeply anesthetized with isoflurane, animals were decapitated and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1.5 MgCl₂, and 10 glucose, saturated with 95 % O₂ and 5 % CO₂, pH 7.3. Slices were then incubated in the above solution at 35 °C until use.

2.2. Recordings of action potentials, resting membrane potentials and holding currents from layer III pyramidal neurons of the medial EC

Whole-cell recordings using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in current- or voltage-clamp mode were made from layer III pyramidal neurons in the medial EC visually identified with infrared video microscopy (Olympus BX51WI) and differential interference contrast optics. During recordings, the bath temperature was maintained at 33–34 °C by an in-line heater and an automatic temperature controller (TC-324C, Warner Instruments). The recording electrodes were filled with (in mM) 120 K⁺-gluconate, 10 KCl, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 2 ATPNa₂, 0.4 GTPNa, and 5 phosphocreatine (osmolarity: 290–300 mOsm/kg, pH 7.3). For the Cs⁺-containing intracellular solution, K⁺-gluconate and KCl in the abovementioned solution were replaced with the same concentration of Cs⁺-gluconate and CsCl, respectively. The recording extracellular solution was the abovementioned cutting solution. Action potentials (APs) were recorded in the extracellular solution supplemented with kynurenic acid (1 mM) to block ionotropic glutamatergic receptors and picrotoxin (100 μM) to block GABA_A receptors. Resting membrane potentials (RMPs) and holding currents (HCs) at -60 mV were recorded in the extracellular solution containing tetrodotoxin (TTX, 0.5 μM), kynurenic acid (1 mM) and picrotoxin (100 μM) to block AP firing, glutamatergic and GABAergic transmission, respectively. For the experiments of using potassium-containing intracellular solution, data were collected only from those cells showing initial RMPs more negative than -57 mV, whereas for the experiments with cesium-containing intracellular solution, only the cells showing initial holding currents of less than -150 pA at -60 mV after the formation of whole-cell configuration were used. Pharmacological inhibitors were applied to the cells either extracellularly or intracellularly via the recording pipettes. For extracellular application, slices were pretreated in the extracellular solution supplemented with the inhibitors and bubbled with 95 % O₂ and 5 % CO₂ in a beaker for at least 2 h to ensure permeation of reagents into the cells and the bath solution continuously contained the same concentrations of the reagents. Control slices were treated in the same fashion with the vehicle (0.1 % DMSO). For intracellular application, we waited for >15 min after the formation of whole-cell configuration to ensure the diffusion of the inhibitors into the cells. We selected the concentrations of pharmacological reagents by referring to their IC₅₀ and published effective concentrations. The group I mGluR agonist, (S)-3,5-dihydroxyphenylglycine (DHPG), was dissolved in the extracellular solution and bath-applied to the slices. To avoid potential desensitization induced by repeated applications of the agonist, one slice was limited to only one application of DHPG. Data were filtered at 2 kHz, digitized at 10 kHz, acquired and analyzed subsequently using pCLAMP 10.7 software (Molecular Devices, Sunnyvale, CA). For the experiments involving voltage clamp, series resistance was compensated by ~80 % and monitored by applying a -5 mV voltage step to measure the current transient prior to and at the end of experiments.

2.3. Data analysis

Data are presented as the means \pm S.E.M. N numbers in the text were the numbers of cells used for each experiment. Because we recorded data from only one cell in each slice, the cell numbers could also be regarded as the slice numbers. To minimize potential influences of variations from individual animals, each experiment was performed in slices attained from at least 4 animals and One-way ANOVA was performed to ensure that there was no significant difference for the data obtained from individual animals under the same treatment. We measured the peak response of DHPG for statistical analysis. For normally distributed data, dual comparisons were made by student's t-test. Differences between multiple means were assessed by one-way or two-way ANOVA followed by appropriate post-hoc tests. For data that were not normally distributed, non-parametric tests, including Wilcoxon matched-pairs signed

rank test (abbreviated as Wilcoxon test in the text) and Mann-Whitney test were used. P values were reported throughout the text and significance was set as $P < 0.05$. Frequency of APs was measured by Clampfit 10.7 with “Event Detection” and “Threshold Search”. The data were then output to Excel and binned per minute with a custom formula in Excel. The concentration-response curve of DHPG was fit by Hill equation: $I = I_{max} \times \{1/[1 + (EC_{50}/[ligand])^n]\}$, where I_{max} is the maximum response, EC_{50} is the concentration of ligand producing a half-maximal response, and n is the Hill coefficient.

2.4. Chemicals

The following chemicals were products of R&D Systems: DHPG, TTX, kynurenic acid, picrotoxin, LY456236, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), ML 133, tertiapin-Q, ML297, U73122, heparin, thapsigargin, chelerythrine, bisindolylmaleimide II (Bis II), RHC 80267. GDP- β -S and dioctanoyl phosphatidylinositol 4,5-bisphosphate (dic8-PIP₂) were purchased from BIOLOGICAL Life Science

Institute and Echelon Biosciences, respectively. Drugs were initially prepared in stock solution, aliquoted and stored at -20°C . For those chemicals requiring dimethyl sulfoxide (DMSO) as a solvent, the concentration of DMSO was less than 0.1 %.

3. Results

3.1. Activation of group I mGluRs excites layer III pyramidal neurons in the EC

We examined the effects of group I mGluRs on the excitability of layer III pyramidal neurons because group I mGluRs in entorhinal layer III neurons can drive persistent firing (Yoshida et al., 2008), a cellular model for working memory (Constantinidis et al., 2018; Curtis and Sprague, 2021; Hasselmo and Eichenbaum, 2005; Hasselmo and Stern, 2006; Lin et al., 2020). The extracellular solution was supplemented with kynurenic acid (1 mM) to block glutamatergic transmission and picrotoxin (100 μM) to block GABAergic transmission. We recorded APs

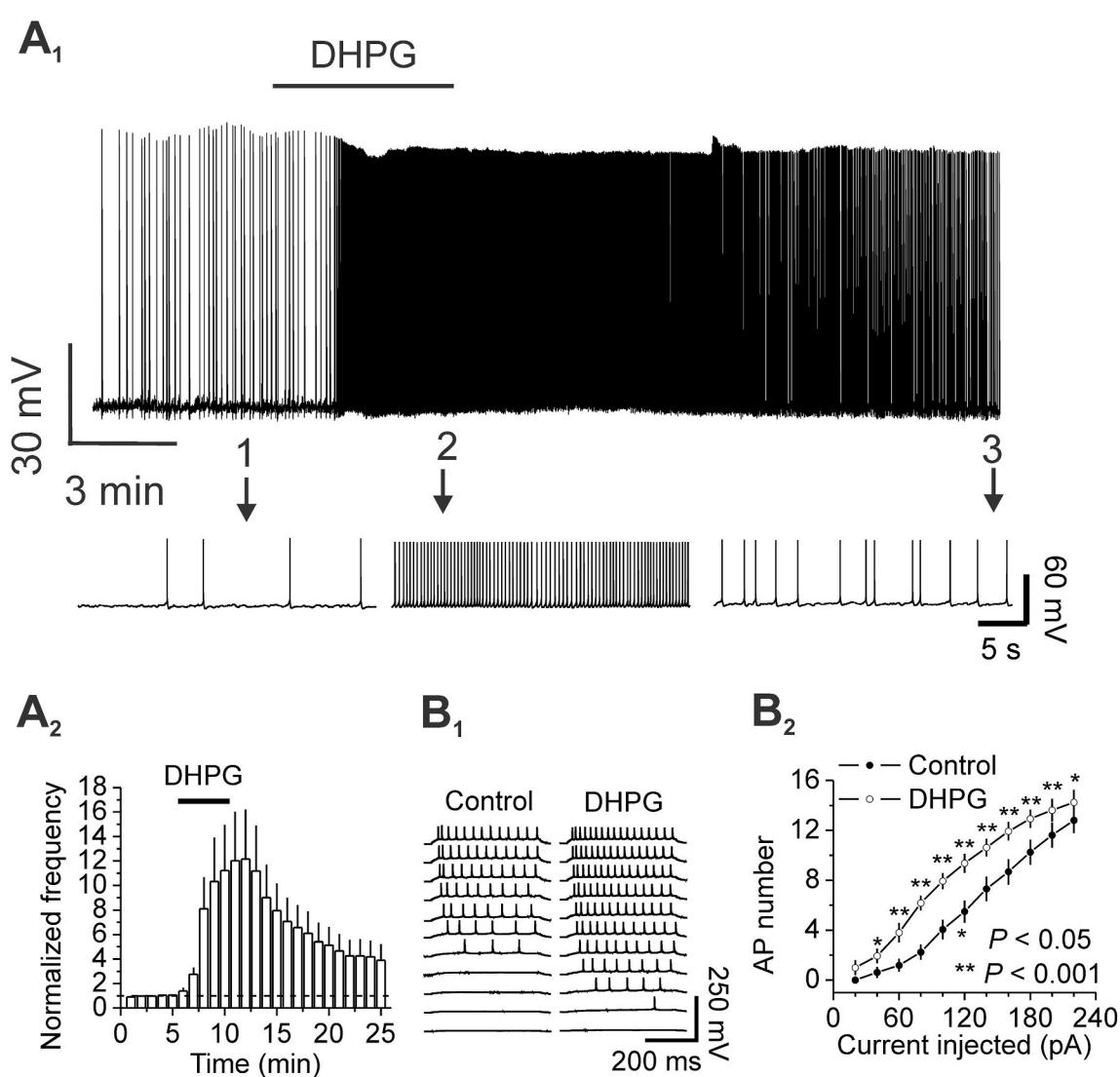


Fig. 1. DHPG excites entorhinal layer III pyramidal neurons. **A₁-A₂**, DHPG augmented AP firing frequency in layer III pyramidal neurons evoked by persistent positive current injection. **A₁**, AP trace recorded from a layer III pyramidal neuron prior to, during and after the application of DHPG (10 μM). Low panel showed the APs recorded at the times indicated in the figure. **A₂**, Summarized time course of APs in response to bath application of DHPG ($n = 18$). **B₁-B₂**, DHPG enhanced AP firing frequency in layer III pyramidal neurons elicited by injecting a series of positive currents. **B₁**, AP traces recorded from a layer III pyramidal neuron prior to (left) and during (right) the application of DHPG (10 μM) evoked by injecting positive currents from 20 pA (bottom) to 220 pA (top) at an increment of 20 pA and a duration of 600 ms. **B₂**, Summarized current-AP number relationship in response to DHPG ($n = 16$, two-way repeated-measures ANOVA followed by Sidak multiple comparison tests, Drug: $F_{(1,15)} = 51.61$, $P < 0.0001$; Current: $F_{(10,150)} = 94.21$, $P < 0.0001$; Drug x Current: $F_{(10,150)} = 5.784$, $P < 0.0001$; * $P < 0.05$, ** $P < 0.001$).

from layer III pyramidal neurons in the EC by injecting a persistent positive current (43.2 ± 3.0 pA, $n = 18$) to elevate the resting membrane potentials to just above the threshold to induce sparse AP firing. Bath application of the selective group I mGluR agonist DHPG (10 μ M) significantly increased the AP firing frequency to 1215 ± 405 % of control (Control: 0.628 ± 0.129 Hz, DHPG: 2.932 ± 0.309 Hz, $n = 18$, $P < 0.0001$, Wilcoxon test, Fig. 1A₁-A₂). We further measured the effect of DHPG on AP firing evoked by injecting a series of positive currents from 20 to 220 pA at an interval of 20 pA with a duration of 600 ms. Bath application of DHPG (10 μ M) significantly enhanced the number of AP firing ($n = 16$, $F_{(1,15)} = 51.61$, $P < 0.0001$, two-way repeated-measures ANOVA followed by Sidak multiple comparison tests, Fig. 1B₁-B₂). These results together demonstrate that activation of group I mGluRs excites layer III pyramidal neurons in the medial EC.

We included TTX (0.5 μ M) in the extracellular solution to block AP firing and examined the effect of DHPG on the RMPs. Bath application of DHPG (10 μ M) induced significant depolarization of layer III pyramidal neurons (Control: -67.4 ± 1.5 mV, DHPG: -61.0 ± 2.2 mV, net depolarization: 6.3 ± 1.1 mV, $t(14) = 6.002$, $P < 0.0001$, Fig. 2A₁-A₂). In voltage clamp, application of DHPG (10 μ M) induced an inward holding current at -60 mV (baseline: 19.3 ± 1.8 pA, DHPG: -6.7 ± 2.6 pA, net current: -26.0 ± 1.7 pA, $n = 30$, $P < 0.0001$ vs. baseline, Wilcoxon test, Fig. 2B₁, 2B₂), suggesting that DHPG depolarizes layer III pyramidal neurons. As the ages of the rats used in this study were 21–35 days, we tested whether the effects of DHPG were age-dependent by correlating the inward holding currents versus the corresponding ages of the rats. There was no significant correlation between the ages of the rats with the DHPG-elicited net currents ($r = 0.064$, $P = 0.738$, data not shown), excluding the possibility that DHPG-elicited excitation of layer III pyramidal neurons was age-dependent. The EC₅₀ of DHPG was measured to be 1.9 μ M (Fig. 2B₂). We used DHPG at 10 μ M for the remaining experiments.

3.2. DHPG enhances both the input resistance and membrane time constants of layer III pyramidal neurons

We examined the effects of DHPG on the input resistance (R_{in}) and membrane time constants of layer III pyramidal neurons. We injected negative current steps from 0 to -60 pA with 20 pA steps for a duration of 600 ms before and after the application of DHPG when the maximal effect of DHPG was achieved. We fit the I-V relationship with a linear function for each cell to obtain R_{in}, which equals the slope of the linear fitting (Fig. 2C₁-C₃). Application of DHPG significantly enhanced R_{in} (Control: 167 ± 18 M Ω , DHPG: 209 ± 21 M Ω , $t(10) = 4.283$, $P = 0.002$, Fig. 2C₃). DHPG also increased the membrane time constants attained by fitting a single exponential function to the voltage transient elicited by -60 pA current step (Control: 25.3 ± 3.8 ms, DHPG: 35.1 ± 4.4 ms, $t(10) = 6.439$, $P < 0.0001$, Fig. 2D₁-D₃).

3.3. Both mGluR1 and mGluR5 are involved in DHPG-mediated excitation of layer III pyramidal neurons

The group I mGluRs include mGluR1 and mGluR5 subtypes. We next identified the roles of mGluR1 and mGluR5 in DHPG-induced increases in inward currents. Pretreatment of slices with and continuous bath application of the selective mGluR1 antagonist LY456236 (5 μ M) significantly reduced but did not block DHPG-induced inward currents (-13.1 ± 1.9 pA, $t(11) = 6.993$, $P < 0.0001$ vs. baseline; $F_{(3,68)} = 31.89$, $P < 0.0001$ vs. DHPG alone, Fig. 3A, B, 3E), suggesting that mGluR1 subtype is partially accountable for DHPG-mediated excitation of layer III pyramidal neurons. In a similar fashion, DHPG induced a significantly smaller inward current in slices treated with the selective mGluR5 antagonist, MPEP (5 μ M) (-16.0 ± 2.1 pA, $t(15) = 7.572$, $P < 0.0001$ vs. baseline; $F_{(3,68)} = 31.89$, $P < 0.001$ vs. DHPG alone, Fig. 3C and E), suggesting that mGluR5 subtype is partially responsible for DHPG-elicited excitation of layer III pyramidal neurons. However, when

slices were treated with both LY456236 and MPEP, DHPG failed to evoke inward currents significantly (-1.1 ± 1.6 pA, $t(13) = 0.691$, $P = 0.502$ vs. baseline; $F_{(3,68)} = 31.89$, $P < 0.0001$ vs. DHPG alone, Fig. 3D and E). These results together suggest that both mGluR1 and mGluR5 are required for DHPG-elicited excitation of layer III neurons in the EC.

3.4. DHPG-elicited excitation of layer III pyramidal neurons is mediated by activation of a cationic conductance and depression of an inwardly rectifying K⁺ channel

We examined the ionic mechanisms underlying DHPG-induced excitation of layer III pyramidal neurons by constructing the current-voltage (I-V) relationship of the currents generated by DHPG. The abovementioned extracellular solution contained TTX (0.5 μ M) to block voltage-gated Na⁺ channels. Cells were held at -60 mV and stepped from -140 mV to -40 mV for 400 ms at a voltage interval of 10 mV every 5 s. Steady-state currents were measured within 5 ms prior to the end of the step voltage protocol. In 7 of the 15 cells recorded, DHPG elicited a persistent inward current at the voltage range between -140 mV and -40 mV (Fig. 4A₁-A₃), suggestive of the involvement of cation channels (Fig. 4A₃). The I-V curve of the DHPG-elicited currents in the remaining 8 cells resembled that of the inwardly rectifying K⁺ (Kir) channels with a reversal potential of -101.6 ± 3.7 mV ($n = 8$, Fig. 4B₁-B₃), close to the calculated K⁺ reversal potential (-94.2 mV). These results demonstrate that activation of group 1 mGluRs excites layer III pyramidal neurons by two ionic mechanisms, i. e., activating a cationic conductance and depressing a Kir channel.

We further extended the voltage range from -100 mV to $+50$ mV to measure the reversal potential of the cationic currents elicited by DHPG with intracellular solution containing Cs⁺-gluconate to block the Kir channels. The extracellular solution contained TTX (0.5 μ M) to block voltage-gated Na⁺ channels and CdCl₂ (200 μ M) to prevent contamination of voltage-gated Ca²⁺ channel currents. Under these circumstances, DHPG-elicited net currents showed a reversal potential of -24.6 ± 5.6 mV with outward rectification in 10 out of 10 cells examined (Fig. 4C₁-C₃), further confirming the involvement of cationic channels.

As two receptors (mGluR1 and mGluR5) and two channels (Kir and cation channels) are involved in DHPG-mediated excitation of layer III pyramidal neurons, we tested whether mGluR1 and mGluR5 receptors interact with distinct ion channels to excite layer III pyramidal neurons. If so, blockade of one receptor would annul the contribution of the corresponding channel, highlighting the role of the other mGluR subtype and ion channel. We pretreated slices with the selective mGluR1 antagonist, LY456236 (5 μ M), and the same concentration of the antagonist was bath-applied continuously. Under these circumstances, the I-V curve of DHPG-elicited net currents resembled a cation channel in 9 out of 21 cells examined (Fig. 4D₁-D₂). The remaining 12 cells displayed an I-V curve similar to Kir channels (Fig. 4 D₃-D₄), suggesting that activation of mGluR5 receptors interacts with both cation channels and Kir channels to excite layer III pyramidal neurons. We then treated the slices with the selective mGluR5 antagonist, MPEP (5 μ M), in the same fashion to test the role of mGluR1 in activation of cation channels and depression of Kir channels. The I-V curve of DHPG-elicited net currents resembled cation channels in 10 out of 20 cells examined (Fig. 4E₁-E₂) and Kir channels in the remaining 10 cells (Fig. 4E₃-E₄), suggesting that mGluR1 activation excites layer III pyramidal neurons via interacting with both cation channels and Kir channels. Together, these results suggest that there is no one-to-one relationship between the two receptor types (mGluR1 and mGluR5) and ion channels (cation and Kir).

3.5. TRPC5 channels are involved in DHPG-elicited excitation of layer III pyramidal neurons

We tried to identify the type of cation channels involved in DHPG-elicited excitation of layer III pyramidal neurons in the EC. The I-V

curve of the DHPG-sensitive currents with intracellular Cs^+ (Fig. 4C₃) resembles that of TRPV1, TRPC4 and TRPC5 channels (Wu et al., 2010). Both TRPV1 (Cavanaugh et al., 2011) and TRPC5 (Fowler et al., 2007; von et al., 2005) channels are expressed in the EC. We used TRPV1 KO mice to determine the roles of TRPV1 channels in DHPG-induced excitation of layer III pyramidal neurons. Application of DHPG elicited an inward current in slices cut from TRPV1 KO mice ($-25.6 \pm 5.4 \text{ pA}$, $n = 19$, $P < 0.0001$ vs. baseline, Wilcoxon test, Fig. 5A₁), which was not significantly different ($P = 0.172$, Mann-Whitney test, Fig. 5A₃) from that in slices cut from WT mice ($-59.5 \pm 19.7 \text{ pA}$, $n = 16$, $P < 0.0001$ vs. baseline, Wilcoxon test, Fig. 5A₂). These results together indicate that TRPV1 channels are not critical for DHPG-elicited excitation of layer III pyramidal neurons.

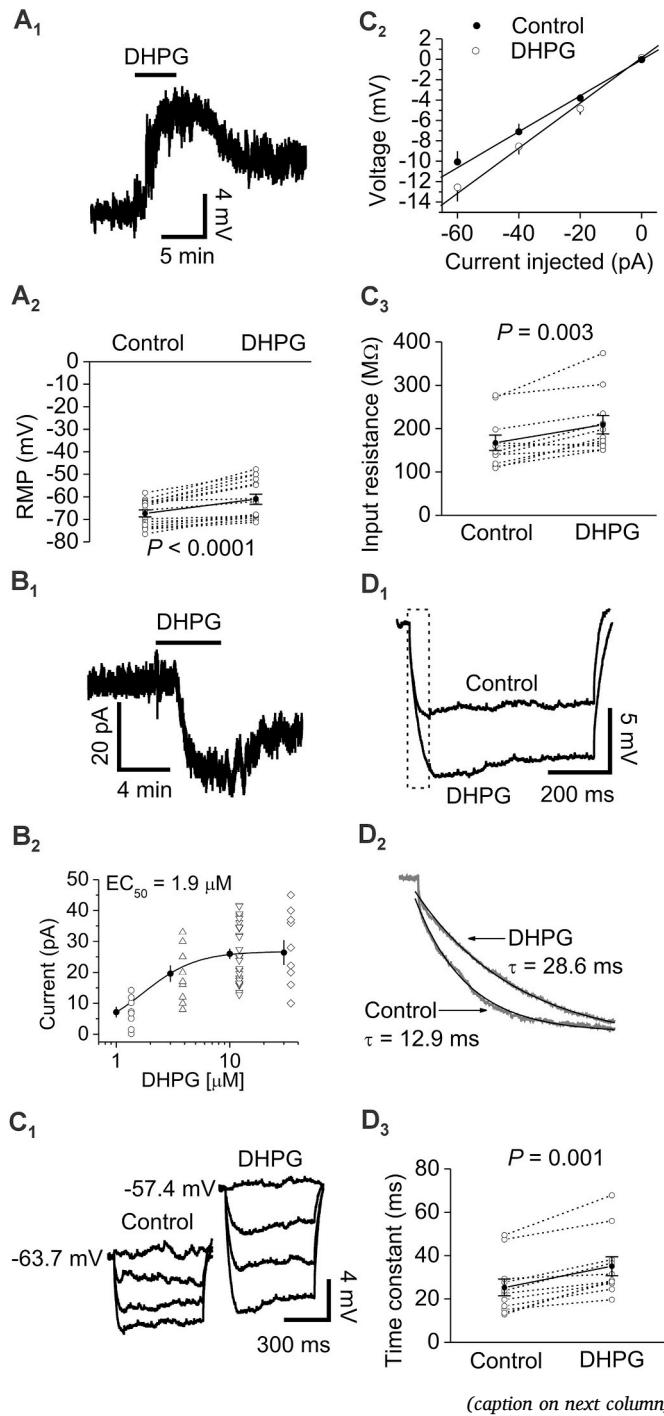


Fig. 2. DHPG depolarizes layer III principal neurons and increases both the input resistance and membrane time constants of layer III pyramidal neurons. **A₁-A₂**, DHPG elicited depolarization of layer III pyramidal neurons. **A₁**, RMP recorded from a layer III pyramidal neuron in response to bath application of DHPG. **A₂**, Summary graph showing DHPG-induced depolarization of layer III pyramidal neurons ($n = 15$). **B₁-B₂**, DHPG induced an inward holding current at -60 mV recorded from layer III pyramidal neurons. **B₁**, Current trace recorded at the holding potential of -60 mV from a layer III pyramidal neuron in response to bath application of DHPG. **B₂**, Concentration-response curve of DHPG. Empty symbols were the values from individual cells and the solid symbols were their averages. **C₁-C₃**, DHPG increased the input resistance of layer III pyramidal neurons. **C₁**, Voltage responses evoked by injection of negative currents from 0 to -60 pA at an interval of 20 pA before (left) and during (right) the application of DHPG from the same cell. **C₂**, Current-voltage relationship averaged from 11 cells. Input resistance was obtained by linear fitting of the current-voltage relationship. **C₃**, Summary graph for input resistance before and during the application of DHPG ($n = 11$). **D₁-D₃**, DHPG increased the time constants of layer III pyramidal neurons. **D₁**, Voltage response evoked by injection of -60 pA before and during the application of DHPG. **D₂**, Expansion of the scaled voltage transients shown in the box in D₁ (gray) to show DHPG-induced increase in membrane time constants attained by fitting with one-exponential function (black). **D₃**, Summary graph for membrane time constants before and during the application of DHPG.

We then tested the roles of TRPC4 and TRPC5 channels in DHPG-mediated excitation of layer III pyramidal neurons by using TRPC4 and TRPC5 KO mice. Application of DHPG evoked an inward current in slices cut from TRPC4 KO mice ($-24.9 \pm 2.7 \text{ pA}$, $t(14) = 9.223$, $P < 0.0001$ vs. baseline, Fig. 5B₁), which was comparable ($t(27) = 1.649$, $P = 0.111$, Fig. 5B₃) to that recorded from slices cut from WT mice ($-40.5 \pm 9.3 \text{ pA}$, $t(13) = 4.334$, $P = 0.0008$ vs. baseline, Fig. 5B₂), suggesting that TRPC4 channels are not essential for DHPG-mediated depolarization. However, DHPG induced a significantly smaller inward current in slices cut from TRPC5 KO mice ($-11.3 \pm 2.1 \text{ pA}$, $t(24) = 5.328$, $P < 0.0001$ vs. baseline, Fig. 5C₁), compared with WT mice ($-45.5 \pm 8.3 \text{ pA}$, $t(14) = 5.485$, $P < 0.0001$ vs. baseline; WT mice vs. TRPC5 KO mice: $t(38) = 4.932$, $P < 0.0001$, Fig. 5C₂-C₃). These results together indicate that TRPC5 channels are essential for DHPG-elicited excitation of layer III pyramidal neurons.

3.6. GIRK channels are involved in DHPG-mediated excitation of layer III pyramidal neurons

Because micromolar concentration of Ba^{2+} has been shown to block Kir channels (Hu et al., 2017; Lacey et al., 1988; Li et al., 2019b), we included $300 \mu\text{M}$ Ba^{2+} in the extracellular solution to inhibit Kir channels. Bath application of $300 \mu\text{M}$ Ba^{2+} by itself induced an inward current ($-52.2 \pm 3.3 \text{ pA}$, $t(19) = 15.62$, $P < 0.0001$ vs. baseline, Fig. 6A-E) and significantly reduced DHPG-elicited inward currents ($-16.5 \pm 1.5 \text{ pA}$, $t(19) = 11.36$, $P < 0.0001$ vs. baseline; $P < 0.001$ vs. DHPG alone, one-way ANOVA followed by Dunnett's multiple comparison test, Fig. 6A and F), further confirming the involvement of Kir channels.

We then identified the subtypes of the Kir channels responsible for DHPG-elicited excitation of layer III pyramidal neurons. There are seven Kir channel subfamilies that can be classified into four functional groups: (i) Kir2 subfamily including Kir2.1, Kir2.2, Kir2.3 and Kir2.4 form the classical Kir channels and are constitutively active; (ii) Kir3 subfamily comprising Kir3.1 (GIRK1), Kir3.2 (GIRK2), Kir3.3 (GIRK3) and Kir3.4 (GIRK4) encoded by the genes KCNJ3, KCNJ6, KCNJ9, and KCNJ5, respectively, constitute the GIRK channels; (iii) Kir6 subfamily encompassing Kir6.1 and Kir6.2 form the ATP-sensitive K^+ (K_{ATP}) channels; and (iv) K^+ transport channels include Kir1.1, Kir4.1, Kir4.2 and Kir7.1 (Hibino et al., 2010). ML 133, is a specific antagonist for Kir2 subfamily channels (Ford and Baccei, 2016; Huang et al., 2018; Kim et al., 2015; Sonkusare et al., 2016; Wang et al., 2011a) and the EC expresses low level of Kir2 subfamily channel mRNAs including Kir2.1, Kir2.2 and Kir2.3 (Karschin et al., 1996). Bath application of ML 133 by

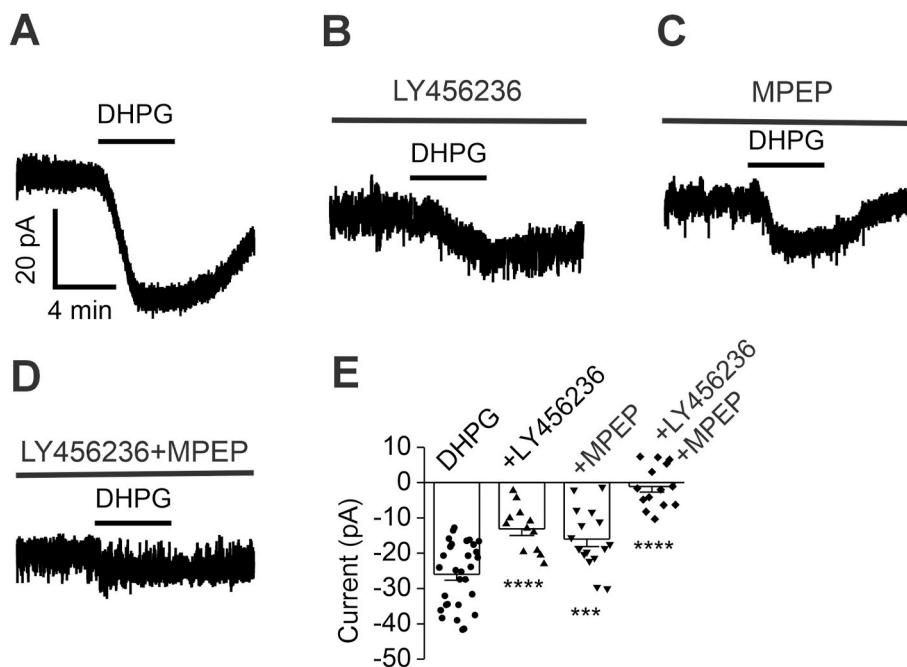


Fig. 3. DHPG-mediated excitation of layer III pyramidal neurons requires the functions of both mGluR1 and mGluR5. **A**, Current trace recorded at -60 mV from a layer III pyramidal neuron in control condition. Scale bar is the same for all figures. **B**, Current trace recorded at -60 mV from a layer III pyramidal neuron in response to bath application of DHPG in a slice pretreated with the selective mGluR1 antagonist, LY456236 ($5\text{ }\mu\text{M}$) and the extracellular solution contained the same concentration of LY456236. **C**, DHPG-elicted inward current in a slice treated with the selective mGluR5 antagonist, MPEP ($5\text{ }\mu\text{M}$). **D**, Current trace in a slice treated with both LY456236 and MPEP in response to DHPG. **E**, Summary graph to show the effects of mGluR1 and mGluR5 antagonists on DHPG-induced inward currents (one-way ANOVA followed by Dunnett's multiple comparison tests, $F_{(3,68)} = 31.89$, $P < 0.0001$; *** $P < 0.001$, **** $P < 0.0001$ vs. DHPG alone).

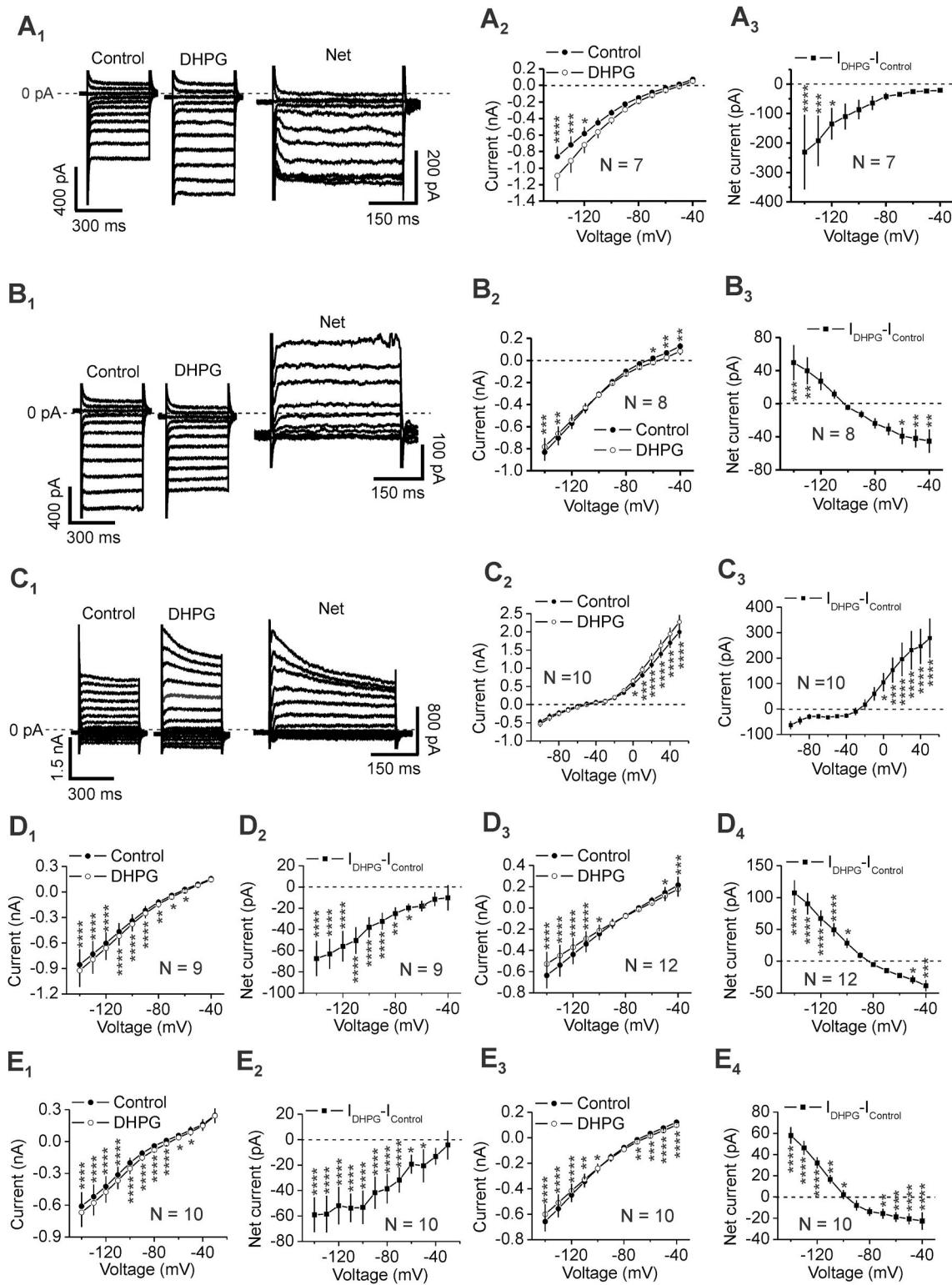
itself did not significantly alter holding currents ($-5.6 \pm 4.2\text{ pA}$, $t(11) = 1.355$, $P = 0.202$ vs. baseline, Fig. 6B-E) and failed to change DHPG-elicted inward currents ($-31.2 \pm 3.1\text{ pA}$, $t(11) = 10.01$, $P < 0.0001$ vs. baseline; $F_{(3,69)} = 11.20$, $P = 0.210$ vs. DHPG alone, one-way ANOVA followed by Dunnett's multiple comparison test, Fig. 6B and F), suggesting that the function of Kir2 subfamily channels is not required for DHPG-induced inward currents.

We further tested the roles GIRK channels by using tertiapin-Q to block GIRK channels (Jin et al., 1999; Jin and Lu, 1999). Bath application of tertiapin-Q (500 nM) by itself elicited an inward current ($-15.6 \pm 5.6\text{ pA}$, $n = 11$, $P = 0.001$ vs. baseline, Wilcoxon test, Fig. 6C and E) and significantly reduced DHPG-induced inward currents ($-15.9 \pm 2.4\text{ pA}$, $F_{(3,69)} = 11.20$, $P = 0.004$ vs. DHPG alone, one-way ANOVA followed by Dunnett's multiple comparison test, Fig. 6C and F), suggesting that GIRK channels are required for DHPG-elicted excitation of layer III pyramidal neurons. The results that bath application of Ba^{2+} or tertiapin-Q by themselves induced an inward current suggest that GIRK channels are tonically active, as demonstrated previously (Chen and Johnston, 2005; Gonzalez et al., 2018). As tonic adenosine A1 receptor-mediated activation of GIRK channels has been shown to be responsible for the more hyperpolarized resting membrane potential observed in dorsal hippocampal CA1 neurons (Kim and Johnston, 2015), we wondered whether the tonic activity of GIRK channels in the layer III neurons was due to the tonic activation of adenosine A1 receptors by ambient adenosine in the slices. Bath application of the selective adenosine A1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) at 100 nM , an effective concentration (Kim and Johnston, 2015), did not significantly alter the holding currents recorded at -60 mV ($1.3 \pm 3.2\text{ pA}$, $t(7) = 0.4$, $P = 0.698$ vs. baseline, Fig. 6E), suggesting that the tonic activity of GIRK channels in layer III pyramidal neurons of the EC is not mediated by activation of adenosine A1 receptors. Consistent with the involvement of GIRK channels, bath application of ML297 ($10\text{ }\mu\text{M}$), an activator of GIRK1-containing channels (Kaufmann et al., 2013), induced an outward current ($39.5 \pm 6.0\text{ pA}$, $t(8) = 6.602$,

$P = 0.0002$, Fig. 6D and E). In line with our electrophysiological data, GIRQ1 channels are expressed in the EC (DePaoli et al., 1994; Karschin et al., 1996; Sosulina et al., 2008).

3.7. Signaling mechanisms underlying DHPG-mediated depression of Kir channels in layer III pyramidal neurons

We further identified the properties of the remaining currents elicited by DHPG in slices cut from TRPC5 KO mice. The I-V curve of the remaining currents evoked by DHPG showed inward rectification with a reversal potential at $-89.5 \pm 3.2\text{ mV}$ in 11 out of 11 cells (Fig. 7A1-A3), suggesting that the remaining currents elicited by DHPG in TRPC5 KO mice were mediated by Kir channels. We then took the advantage that the remaining currents in slices cut from the TRPC5 KO mice were mediated by Kir channels to probe the signaling mechanisms whereby DHPG depresses Kir channels in layer III pyramidal neurons by using the TRPC5 KO mice to exclude the contamination of TRPC5 channels. The intracellular solution was the K^+ -gluconate-containing solution. Under these circumstances, DHPG-mediated inward currents should be mediated by depression of Kir channels. Because both G protein-dependent (Congar et al., 1997; Gee et al., 2003; Kubota et al., 2014) and -independent (Heuss et al., 1999) mechanisms are implicated in group I mGluR signaling, we determined the roles of G proteins in DHPG-elicted depression of Kir channels. Inclusion of the G protein inactivator, GDP- β -S (0.5 mg/ml), in the recording pipettes blocked DHPG-induced currents in slices cut from TRPC5 KO mice ($-0.74 \pm 1.08\text{ pA}$, $t(13) = 0.683$, $P = 0.506$ vs. baseline; $F_{(2,51)} = 14.88$, $P < 0.0001$ vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7B, C, 7E), suggesting the involvement of G proteins in DHPG-mediated depression of GIRQ channels. Because group I mGluRs are $\text{G}\alpha_q$ -coupled, we next determined whether PLC β was involved in DHPG-elicted depression of Kir channels. Pretreatment of slices with and continuous bath application of the PLC inhibitor, U73122 ($5\text{ }\mu\text{M}$), significantly reduced DHPG-induced currents in slices cut from TRPC5 KO mice ($-2.89 \pm$



(caption on next page)

Fig. 4. DHPG-elicited excitation of layer III pyramidal neurons is mediated by opening a cation channel and inhibiting a Kir channel. A₁-A₃, DHPG opened a cation channel. A₁, Currents elicited by a voltage-step protocol before (*left*) and during (*middle*) bath application of DHPG and the net current obtained by subtraction (*right*) from a layer III pyramidal neuron. Note the differences of the scale bars. The dash line was the zero current level. A₂, I-V curve averaged from 7 cells before and during application of DHPG (Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 6) = 6.755, P = 0.041; Voltage: F_(10, 60) = 39.45, P < 0.0001; Drug x Voltage: F_(10, 60) = 2.92, P = 0.005; *P < 0.05, ***P < 0.001). A₃, I-V curve of the net current obtained by subtracting the currents in control condition from those during the application of DHPG. B₁-B₃, DHPG depressed a Kir channel. B₁, Currents elicited by the voltage-step protocol before (*left*) and during (*middle*) bath application of DHPG and the net current obtained by subtraction (*right*) from a layer III pyramidal neuron. The dash line was the zero current level. B₂, I-V curve averaged from 8 cells before and during application of DHPG (Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 7) = 6.881, P = 0.034; Voltage: F_(10, 70) = 88.12, P < 0.0001; Drug x Voltage: F_(10, 70) = 8.751, P < 0.0001; *P < 0.05, **P < 0.01, ***P < 0.001). B₃, I-V curve of the net current obtained by subtracting the currents in control condition from those during the application of DHPG. C₁-C₃, DHPG opened a double-rectified cation channel recorded in the extracellular solution containing 0.5 μM TTX and 200 μM CdCl₂ and intracellular solution contained Cs⁺-gluconate. C₁, Currents elicited by the voltage-step protocol (from -100 mV to +50 mV) before (*left*) and during (*middle*) bath application of DHPG and the net current obtained by subtraction (*right*) from a layer III pyramidal neuron. The dash line was the zero current level. C₂, I-V curve averaged from 10 cells before and during application of DHPG (Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 9) = 6.707, P = 0.029; Voltage: F_(15, 135) = 115.2, P < 0.0001; Drug x Voltage: F_(15, 135) = 12.75, P < 0.0001; *P < 0.05, ***P < 0.001, ****P < 0.0001). C₃, I-V curve of the net current obtained by subtracting the currents in control condition from those during the application of DHPG. D₁-D₄, DHPG-induced net currents in the presence of mGluR1 antagonist, LY456236 (5 μM) showed cation channel-like I-V curve in 9 out of 21 cells examined (D₁-D₂, Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 8) = 16.05, P = 0.004; Voltage: F_(10, 80) = 23.7, P < 0.0001; Drug x Voltage: F_(10, 80) = 11.48, P < 0.0001; *P < 0.05, **P < 0.01, ***P < 0.0001) and Kir channel-like I-V curve in 12 out of 21 cells examined (D₃-D₄, Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 11) = 15.96, P = 0.002; Voltage: F_(10, 110) = 17.8, P < 0.0001; Drug x Voltage: F_(10, 110) = 30.62, P < 0.0001; *P < 0.05, ***P < 0.001, ****P < 0.0001). E₁-E₄, DHPG-induced net currents in the presence of mGluR5 antagonist, MPEP (5 μM) showed cation channel-like I-V curve in 10 out of 20 cells examined (E₁-E₂, Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 9) = 13.04, P = 0.006; Voltage: F_(11, 99) = 20.31, P < 0.0001; Drug x Voltage: F_(11, 99) = 9.815, P < 0.0001; *P < 0.05, ****P < 0.0001) and Kir channel-like I-V curve in 10 out of 20 cells examined (E₃-E₄, Two-way repeated measures ANOVA followed by Sidak multiple comparison tests; Drug: F_(1, 9) = 1.654, P = 0.231; Voltage: F_(10, 90) = 66.52, P < 0.0001; Drug x Voltage: F_(10, 90) = 50.01, P < 0.0001; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

1.36 pA, t(14) = 2.126, P = 0.052 vs. baseline; F_(2, 51) = 14.88, P = 0.0004 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7D and E), suggesting the requirement of PLCβ. We tested the roles of intracellular Ca²⁺ and PKC in DHPG-elicited inhibition of Kir channels. Intracellular application of the IP₃ receptor blocker, heparin (0.5 mg/ml), an effective concentration (Saleem et al., 2014), did not significantly alter DHPG-induced inward currents in slices cut from TRPC5 KO mice (-13.1 ± 1.8 pA, t(14) = 7.143, P < 0.0001 vs. baseline; F_(5, 96) = 4.648, P = 0.996 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7F and K). Likewise, intracellular perfusion of thapsigargin (10 μM), an inhibitor of sarco/endoplasmic reticulum Ca²⁺-ATPase, did not significantly change DHPG-elicited inward currents (-15.9 ± 2.3 pA, t(14) = 6.849, P < 0.0001 vs. baseline; F_(5, 96) = 4.648, P = 0.515 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7G and K). These results together suggest that intracellular Ca²⁺ release is not necessary for DHPG-induced depression of Kir channels. Pretreatment of slices with and continuous bath application of the selective PKC inhibitor, chelerythrine (10 μM), failed to block DHPG-mediated inward currents (-12.6 ± 2.0 pA, t(14) = 6.252, P < 0.0001 vs. baseline; F_(5, 96) = 4.648, P = 0.997 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7H and K). Similarly, application of another PKC inhibitor, Bis II (2 μM), in the same fashion, had no significant effect on DHPG-mediated inward currents (-14.7 ± 2.8 pA, t(13) = 5.247, P = 0.0002 vs. baseline; F_(5, 96) = 4.648, P = 0.829 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7I and K), suggesting that PKC is not involved in DHPG-elicited depression of Kir channels in the EC. Because PIP₂ has been shown to modulate numerous ion channels (Rodríguez-Menchaca et al., 2012; Suh and Hille, 2008), we further probed the roles of PIP₂ depletion induced by activation of PLCβ in DHPG-mediated depression of Kir channels. Application of short-chain, water-soluble analog dioctanoyl (diC8)-PIP₂ (diC8-PIP₂, 20 μM) via the recording pipettes significantly reduced DHPG-induced inward current (-3.8 ± 1.2 pA, t(17) = 3.185, P = 0.005 vs. baseline; F_(5, 96) = 4.648, P = 0.007 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 7J and K), suggesting that depletion of PIP₂ is involved in DHPG-mediated depression of Kir channels in the EC.

3.8. Signaling mechanisms underlying DHPG-induced activation of TRPC5 channels in layer III pyramidal neurons

Because activation of TRPC5 channels is another ionic mechanism

whereby DHPG excites layer III pyramidal neurons, we next probed the signaling mechanisms whereby DHPG activates TRPC5 channels in layer III pyramidal neurons. We used Cs⁺-containing intracellular solution to exclude the contamination of K⁺ channels in rat slices. Under these circumstances, DHPG-mediated inward currents showed an I-V curve suggestive of TRPC5 channels (Fig. 4C₁-C₃). Intracellular application of the G protein inactivator GDP-β-S (0.5 mM) via the recording pipettes significantly reduced DHPG-induced increases in inward currents (-1.9 ± 1.4 pA, t(17) = 1.325, P = 0.203 vs. baseline; F_(5, 96) = 4.648, P < 0.0001 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8A, B, 8F), suggesting that functions of G proteins are required for DHPG-induced activation of TRPC5 channels. Furthermore, pretreatment of slices with and continuous bath application of the PLC inhibitor U73122 (5 μM), blocked DHPG-elicited inward currents (-2.5 ± 2.1 pA, t(18) = 1.201, P = 0.245 vs. baseline; F_(5, 96) = 4.648, P < 0.0001 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8C and F), suggesting that PLCβ is required for DHPG-mediated activation of TRPC5 channels. We further tested the roles of intracellular Ca²⁺ release in DHPG-elicited activation of TRPC5 channels in layer III pyramidal neurons. Dialysis of the IP₃ receptor blocker, heparin (0.5 mg/ml) via the recording pipettes, did not block DHPG-induced inward currents (-24.8 ± 4.1 pA, n = 14, P = 0.0001 vs. baseline, Wilcoxon test; F_(5, 96) = 4.648, P > 0.999 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8D and F), suggesting that intracellular Ca²⁺ release from IP₃ store is not involved in DHPG-elicited activation of TRPC5 channels in layer III pyramidal neurons. Furthermore, intracellular application of thapsigargin (10 μM) via the recording pipettes did not alter significantly DHPG-induced inward currents (-26.7 ± 4.8 pA, t(12) = 5.526, P = 0.0001 vs. baseline; F_(5, 96) = 4.648, P = 0.99 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8E and F). These results indicate that intracellular Ca²⁺ release is not required for DHPG-elicited activation of TRPC5 channels. We then tested the roles of PKC in DHPG-induced activation of TRPC5 channels. Pretreatment of slices with and continuous bath application of the selective PKC inhibitor, chelerythrine (10 μM), did not alter significantly DHPG-elicited inward current (-20.2 ± 3.3 pA, n = 17, P < 0.0001 vs. baseline, Wilcoxon test; F_(5, 96) = 4.648, P = 0.469 vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8G and K). Likewise, pretreatment of slices with and continuous bath application of another selective PKC inhibitor, Bis II (2 μM), did not alter significantly DHPG-elicited inward current (-17.0 ± 2.5 pA, t(15) = 6.904, P < 0.0001 vs. baseline; F_(5, 96) = 4.648, P = 0.121

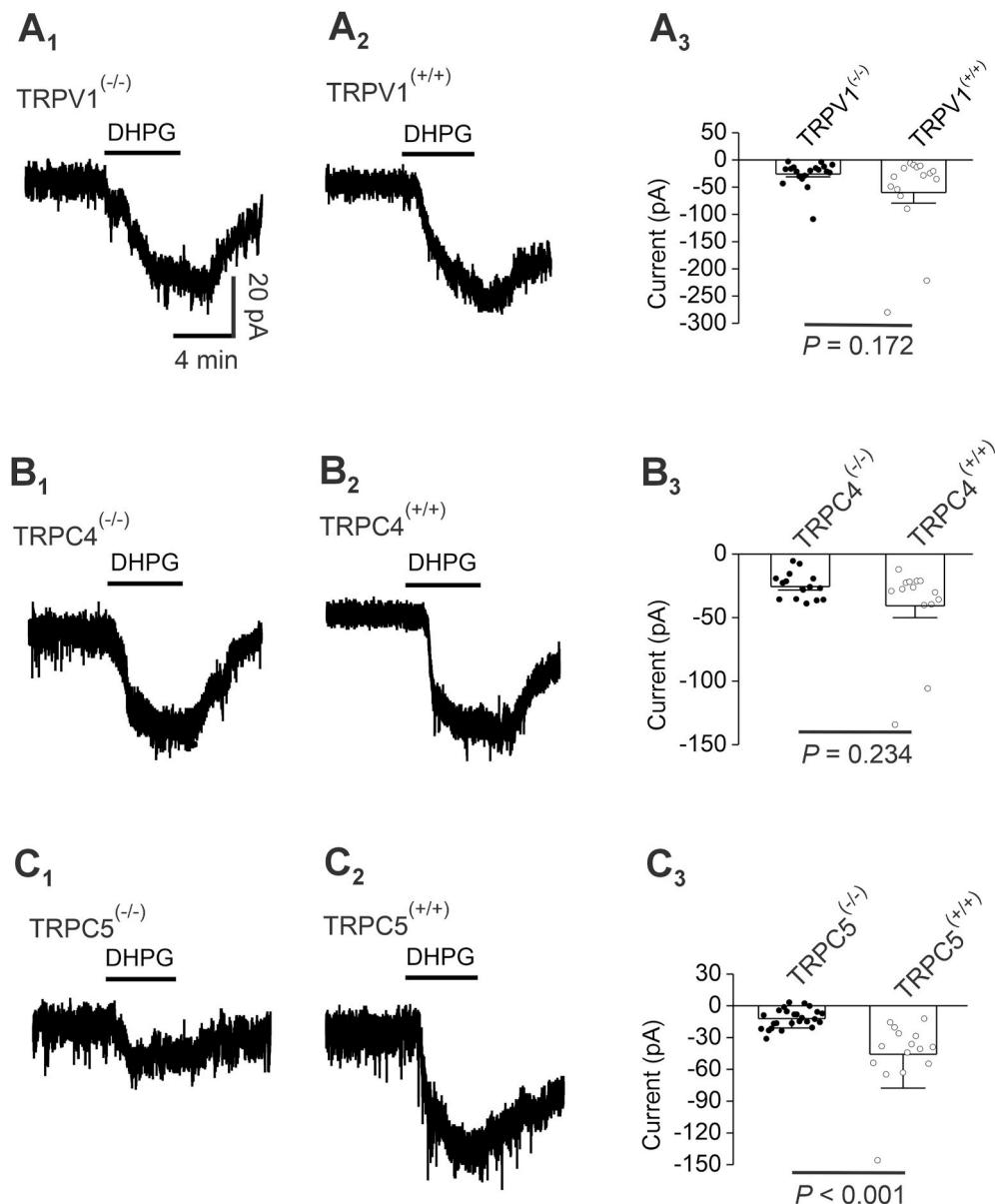


Fig. 5. TRPC5 channels are involved in DHPG-elicted excitation of layer III pyramidal neurons. A₁-A₃, Application of DHPG (10 μM) induced comparable inward currents recorded from layer III pyramidal neurons of EC in slices cut from TRPV1 KO mice and corresponding WT mice. A₁, Inward currents evoked by DHPG recorded from a layer III pyramidal neuron of EC in a slice cut from a TRPV1 KO mice. Scale bare is the same for all the current traces of the figure. A₂, Inward currents evoked by DHPG recorded from a layer III pyramidal neuron of EC in a slice cut from a WT mice. A₃, Summary results showed no significant difference for DHPG-induced inward currents between TRPV1 KO mice and WT mice. B₁-B₃, Application of DHPG (10 μM) induced comparable inward currents recorded from layer III pyramidal neurons of EC in slices cut from TRPC4 KO mice and corresponding WT mice. B₁, Inward currents induced by DHPG recorded from a layer III pyramidal neuron of EC in a slice cut from a TRPC4 KO mice. B₂, Inward currents elicited by DHPG recorded from a layer III pyramidal neuron of EC in a slice cut from a WT mice. B₃, Summary results showed no significant difference for DHPG-induced inward currents between TRPC4 KO mice and WT mice. C₁-C₃, Application of DHPG (10 μM) elicited significantly smaller inward currents recorded from layer III pyramidal neurons of EC in slices cut from TRPC5 KO mice, compared with the corresponding WT mice. C₁, Inward currents evoked by DHPG recorded from a layer III pyramidal neuron of EC in a slice cut from a TRPC5 KO mice. C₂, Inward currents evoked by DHPG recorded from a layer III pyramidal neuron of EC in a slice cut from a WT mice. C₃, Summary results showed significantly smaller DHPG-induced inward currents in TRPC5 KO mice compared with WT mice.

vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8H and K). These results together suggest that PKC is not required for DHPG-elicted activation of TRPC5 channels in layer III pyramidal neurons of the EC. We further probed the roles of PIP₂ depletion induced by activation of PLCβ in DHPG-elicted activation of TRPC5 channels. Inclusion of diC8-PIP₂ (20 μM) in the recording pipettes significantly reduced DHPG-induced inward currents (-5.5 ± 0.7 pA, t(19) = 7.716, $P < 0.0001$ vs. baseline; $F_{(5,96)} = 4.648$, $P < 0.0001$ vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8I and K), suggesting that depletion of PIP₂ is required for DHPG-elicted activation of TRPC5

channels in layer III pyramidal neurons of the EC. Consistent with our results, PLC-mediated PIP₂ depletion is involved in TRPC4 and -5 channel activation (Otsuguro et al., 2008; Trebak et al., 2009).

In addition to PIP₂ depletion, TRPC5 channels might be DAG-sensitive as well (Lee et al., 2003; Storch et al., 2017; Venkatachalam et al., 2003). A plausible action model is that during receptor activation, PIP₂ at the plasma membrane is degraded by PLC resulting in an active TRPC5 conformation characterized by C-terminal rearrangements and the ensuing dissociation of the scaffolding proteins Na⁺/H⁺ exchanger regulatory factor NHERF1 and -2, thereby conferring DAG sensitivity

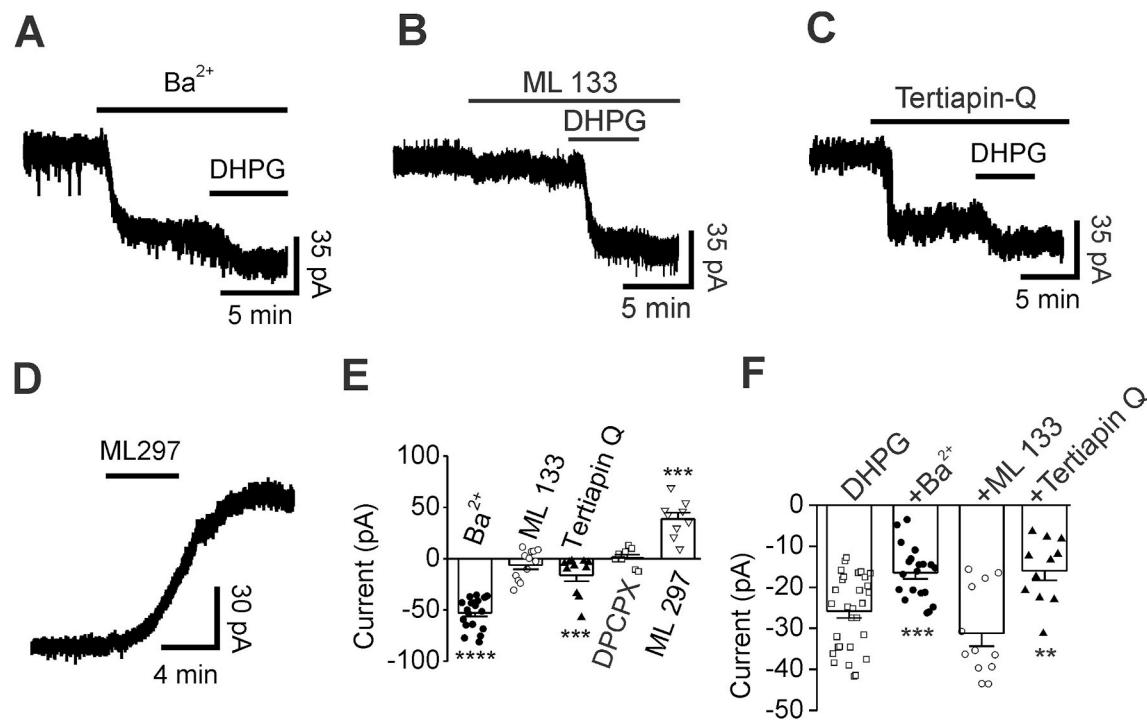


Fig. 6. Involvement of Girk type of Kir channels in DHPG-elicited excitation of layer III pyramidal neurons in the EC. A, Bath application of 300 μ M Ba²⁺ by itself induced an inward current and reduced DHPG-evoked inward currents recorded from a layer III pyramidal neuron. B, Holding currents recorded from a layer III pyramidal neuron in response to ML 133 (30 μ M) alone and together with DHPG. C, Holding currents recorded from a layer III pyramidal neuron in response to tertiapin-Q (500 nM) alone and together with DHPG. D, bath application of ML297 (10 μ M) induced an outward current recorded from a layer III pyramidal neuron. E, Summary graph showing the effects of Kir channel modulators on holding currents recorded from layer III pyramidal neurons. ***P < 0.0001, **P < 0.001 vs. baseline, Wilcoxon test. Bath application of the selective adenosine A1 antagonist, DPCPX (100 nM) did not significantly alter holding currents in layer III pyramidal neurons ($t(7) = 0.4$, $P = 0.698$ vs. baseline). F, Summary graph showing DHPG-elicited inward currents in the presence of Kir channel blockers (one-way ANOVA followed by Dunnett's test, $F_{(3,69)} = 11.20$, $P < 0.0001$; ***P < 0.001, **P < 0.01 vs. DHPG alone).

upon TRPC4 and -5 channels (Storch et al., 2017). If so, application of DAG lipase inhibitors to decrease the degradation of DAG would elevate DAG concentration, augmenting DHPG-mediated effect. Consistent with our expectation, bath application of the α and β DAG lipase inhibitor, RHC 80267 (25 μ M) by itself did not significantly alter the holding currents (0.04 ± 1.21 pA, $n = 12$, $P = 0.97$ vs. baseline, Wilcoxon test, Fig. 8J and K), but significantly enhanced DHPG-elicited holding currents (-56.0 ± 5.9 pA, $n = 12$, $P < 0.0001$ vs. DHPG alone, one-way ANOVA followed by Dunnett's test, Fig. 8J and K), suggesting that both PIP₂ and DAG are involved in DHPG-mediated excitation of layer III pyramidal neurons.

4. Discussion

Whereas group I mGluRs have been implicated in the persistent firing of layer III neurons in the EC (Yoshida et al., 2008) which could be a cellular mechanism for memory (Constantinidis et al., 2018; Curtis and Sprague, 2021; Hasselmo and Eichenbaum, 2005; Hasselmo and Stern, 2006; Lin et al., 2020), the cellular and molecular mechanisms underlying group I mGluR-mediated excitation of layer III pyramidal neurons have not been determined. In this study, we showed that application of DHPG, a group I mGluR agonist excites layer III pyramidal neurons via activation of both mGluR1 and mGluR5 in the EC. DHPG-mediated excitation of layer III pyramidal neurons was mediated by activating TRPC5 channels and depressing Kir channels. The functions of G proteins, PLC β and PLC β -mediated depletion of PIP₂ are involved in DHPG-elicited activation of TRPC5 channels and depression of Kir channels, whereas DAG is also involved in DHPG-mediated activation of TRPC5 channels. Our results may provide a cellular and molecular mechanism whereby activation of group I mGluRs excites layer III pyramidal neurons in the EC.

Activation of group I mGluRs increases neuronal excitability via a variety of ionic mechanisms depending on neuronal types. Depressions of a Ca²⁺-dependent K⁺ channel (Charpak et al., 1990; Mannaioni et al., 2001) and a voltage-gated K⁺ channel (Charpak et al., 1990; Chuang et al., 2002) are involved in the excitation of hippocampal neurons elicited by group I mGluRs. Alternatively, group I mGluRs increase excitability by increasing cation influx via voltage-gated Ca²⁺ channels (Park et al., 2010), Ca²⁺-dependent cation channels (Congar et al., 1997; Gee et al., 2003; Kim et al., 2003), Ca²⁺-independent cation channels (Guerineau et al., 1995), persistent Na⁺ channels (D'Ascenzo et al., 2009), and/or activation of the Na⁺-Ca²⁺ exchanger (Keele et al., 1997). TRPC channels are also implicated in group I mGluR-induced increases in excitability. Indeed, carbachol-induced persistent firing in layer V neurons might involve TRPC 1/4/5 subunit-containing cation channels (Zhang et al., 2011) although slices prepared from constitutive knockout animals for all TRPC subunits failed to abolish persistent firing (Egorov et al., 2019). Added to this spectrum of ion channels, our results demonstrate that activation of group I mGluRs excites layer III pyramidal neurons by activation of TRPC5 channels and depression of Girk type of Kir channels. Because DHPG-elicited net currents in the intracellular solution containing Cs⁺ to block Girk channels showed only an I-V curve resembling cation channels in all the cells examined (Fig. 4C1-C3) and the DHPG-evoked net currents in slices cut from the TRPC5 KO mice displayed an I-V curve featuring Kir channels in all the cells probed (Fig. 7A1-A3), one explanation for the dimorphic forms of I-Vs observed in Fig. 4A1-B3 is that both TRPC5 and Girk channels are expressed in the same neurons, whereas there is a predominance of expression of these two channel types in individual neurons.

Activation of TRPC5 channels should theoretically decrease the input resistance. However, as demonstrated previously, the negative or flat slope of the currents generated by cation channels including the TRPC

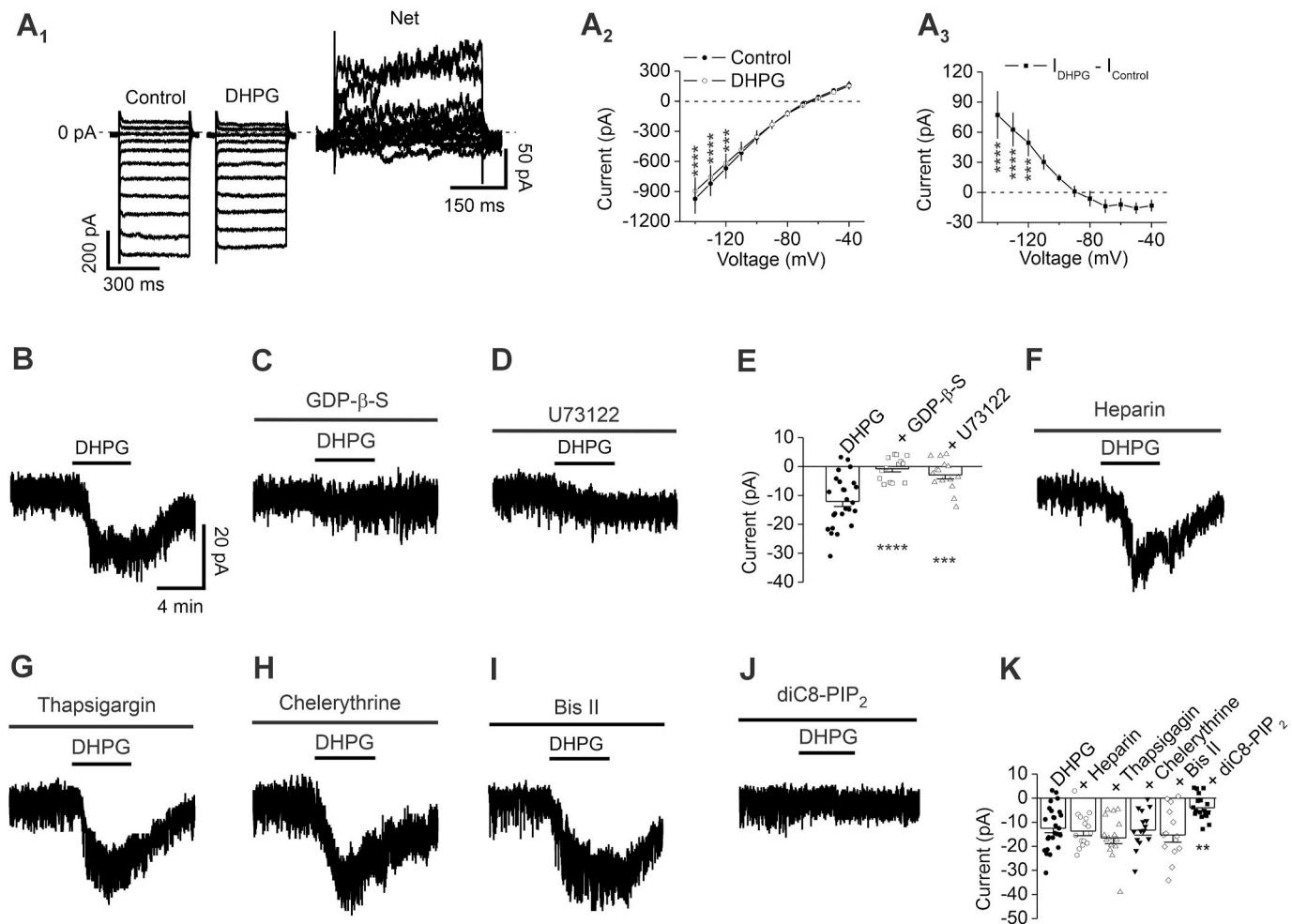


Fig. 7. G proteins, PLC β and PIP₂ depletion are required for DHPG-evoked depression of Girk channels in slices cut from TRPC5 KO mice. **A₁-A₃**, DHPG-elicited net currents in slices cut from TRPC5 KO mice were mediated by Kir channels. **A₁**, Current traces recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse prior to (left) and during (middle) the application of DHPG and the net currents obtained by subtraction (right). **A₂**, I-V curves averaged from 11 cells before and during application of DHPG (Two-way repeated measures ANOVA followed by Sidak multiple comparison test; Drug: $F_{(1, 10)} = 20.40, P = 0.001$; Voltage: $F_{(10, 100)} = 42.46, P < 0.0001$; Drug x Voltage: $F_{(10, 100)} = 9.514, P < 0.0001$; *** $P < 0.001$, **** $P < 0.0001$). **A₃**, I-V curve of the net current obtained by subtracting the currents in control condition from those during the application of DHPG. **B**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse prior to, during and after the application of DHPG. Scale bar is applicable to each current trace in figure. **C**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with GDP- β -S (0.5 mM). **D**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with U73122 (5 μ M) and the extracellular solution continuously perfused with the same concentration of U73122. **E**, Summary graph showing that the functions of G proteins and PLC β are required for DHPG-induced inward currents in slices cut from TRPC5 KO mice (one-way ANOVA followed by Dunnett's test, $F_{(2,51)} = 14.88, P < 0.0001$, **** $P < 0.0001$, *** $P < 0.001$ vs. DHPG alone). **F**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with heparin (0.5 mg/ml). **G**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with thapsigargin (10 μ M). **H**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with chelerythrine (10 μ M). **I**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with Bis II (2 μ M). **J**, Current trace recorded from a layer III pyramidal neuron in a slice cut from a TRPC5 KO mouse pretreated with diC8-PIP₂ (20 μ M). **K**, Summary graph showing the roles of intracellular Ca²⁺ release, PKC and PIP₂ depletion in DHPG-evoked inward currents in slices cut from TRPC5 KO mice (one-way ANOVA followed by Dunnett's test, $F_{(5,96)} = 4.648, P = 0.0008$; ** $P < 0.01$ vs. DHPG alone).

channels within the negative voltage ranges could result in an increase in input resistance (Haj-Dahmane and Andrade, 1996; Wang et al., 2011b). Simultaneously, DHPG-elicited depression of Kir channels should increase the input resistance of layer III pyramidal neurons. Our results that DHPG increased the input resistance and membrane time constants of layer III pyramidal neurons could be due to the combined interactions of depressed Kir channels and activated cation channels. Congruous to our electrophysiological data, the EC neurons express TRPC5 channels (Fowler et al., 2007; von et al., 2005) and Girk

channels including Girk1, Girk2, Girk3 (Karschin et al., 1996). Another prerequisite for the involvement of Kir channels is that the Kir channels must be tonically active. Consistently, Kir2 subfamily channels (Hibino et al., 2010) and Girk channels (Chen and Johnston, 2005; Gonzalez et al., 2018) are tonically active. Adenosine A1 receptor-mediated tonic activation of Girk channels by the ambient adenosine is responsible for the more hyperpolarized resting membrane potentials of dorsal hippocampal CA1 neurons (Kim and Johnston, 2015). However, this mechanism is not applicable to the layer III

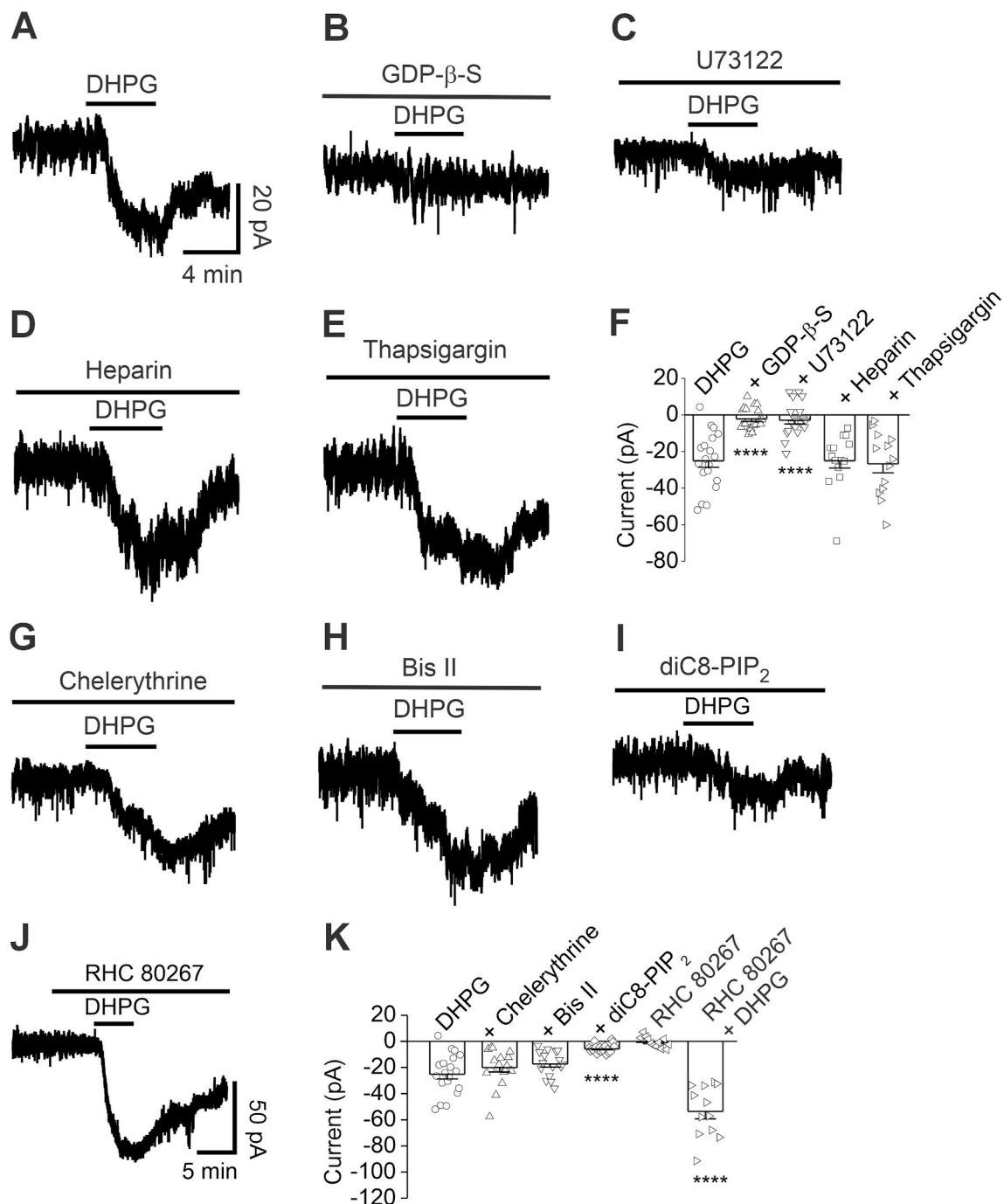


Fig. 8. Signaling mechanisms underlying DHPG-mediated activation of TRPC5 channels by using Cs⁺-containing intracellular solution in rat slices. A, Current trace recorded with Cs⁺-containing intracellular solution prior to, during and after the application of DHPG. Scale bar is applicable to each current trace in figure. B, Intracellular dialysis of GDP- β -S (0.5 mM) via the recording pipette blocked DHPG-induced inward currents. C, Pretreatment of slices with and continuous bath application of U73122 (5 μ M) reduced DHPG-evoked inward current. D, Intracellular perfusion of heparin (0.5 mg/ml) via the recording pipette did not block DHPG-induced inward current. E, Intracellular application of thapsigargin (10 μ M) via the recording pipette failed to block DHPG-elicited inward currents. F, Summary graph (one-way ANOVA followed by Dunnett's test, $F_{(5,96)} = 4.648$, $P = 0.0008$, **** $P < 0.0001$ vs. DHPG alone). G, Pretreatment of slices with and continuous bath application of chelerythrine (10 μ M) did not block DHPG-mediated inward currents. H, Pretreatment of slices with and continuous bath application of Bis II (2 μ M) did not block DHPG-mediated inward currents. I, Intracellular application of diC8-PIP₂ (20 μ M) via the recording pipette reduced DHPG-elicited inward currents. J, Bath application of the DAG lipase inhibitor, RHC 80267 (25 μ M) alone had no effect, but significantly enhanced DHPG-induced inward holding currents. K, Summary graph (one-way ANOVA followed by Dunnett's test, $F_{(4,79)} = 28.86$, $P < 0.0001$, **** $P < 0.0001$ vs. DHPG alone).

pyramidal neurons of the EC, as bath application of the selective A1 antagonist, DPCPX, failed to alter significantly the holding currents (Fig. 6E). Our results therefore support other mechanisms underlying the tonic activation of GIRK channels including basal G $\beta\gamma$ activity, continuous presence of PIP₂ in the cell membrane, modulation by

endogenous substances such as cholesterol and certain intracellular ions (Na^+ and Mg^{2+}) and/or presence of low level of endogenous neurotransmitters (Dascal and Kahanovitch, 2015; Kahanovitch et al., 2014; Luo et al., 2022; Rishal et al., 2005).

The group I mGluRs, mGluR1 and mGluR5, exhibit a high degree of

sequence homology and frequently colocalize in the same neurons throughout the CNS (Marino et al., 2002; Poisik et al., 2003; Wu et al., 2004) (for a review, see (Valenti et al., 2002)). Functionally, tremendous evidence suggests that both subtypes of receptors are involved in DHPG-mediated physiological responses. For instance, DHPG-induced suppression of evoked IPSCs in CA1 pyramidal neurons required the blockade of both mGluR1 and mGluR5 (Mannaioni et al., 2001); DHPG-induced depolarizations of striatal (Pisani et al., 2001) and septohippocampal (Wu et al., 2004) cholinergic interneurons were blocked only by coadministration of mGluR1 and mGluR5 antagonists; both mGluR1 and mGluR5 play a role in mediating DHPG-induced increase in intracellular Ca^{2+} (Marino et al., 2002). In agreement with this scenario, our results indicate that DHPG-elicited excitation of layer III pyramidal neurons involves both mGluR1 and mGluR5. Congruent with our electrophysiological results, group I mGluRs are expressed in the EC (Fotuhi et al., 1994; Lujan et al., 1996; Shigemoto et al., 1997). Whereas group II and group III mGluRs are predominantly localized to presynaptic side, group I mGluRs (mGluR1 and mGluR5) are localized to postsynaptic elements including the cell bodies and dendrites (Lujan et al., 1996; Shigemoto et al., 1997; Yeung et al., 2022). Both mGluR1 and mGluR5 are located outside the postsynaptic membrane specialization (Lujan et al., 1996), suggesting that activation of group I mGluRs predominantly modulates postsynaptic neuronal excitability. Our results that both mGluR1 and mGluR5 are required for DHPG-mediated excitation of layer III pyramidal neurons are further supported by the co-expression of mGluR1 and mGluR5 in the outer (superficial) layers (layer II and III) of the EC, although the density of mGluR5 is higher than that of mGluR1 (Fotuhi et al., 1994).

Whereas group I mGluRs are coupled to $\text{G}\alpha_q$, both G protein-dependent (Congar et al., 1997; Gee et al., 2003; Kubota et al., 2014) and -independent (Heuss et al., 1999) mechanisms are implicated in group I mGluR signaling. Our results demonstrate that G proteins are required for DHPG-mediated excitation of layer III pyramidal neurons. Activation of group I mGluRs increases the activity of PLC β resulting in hydrolysis of PIP₂ generating IP₃ to increase intracellular Ca^{2+} release and DAG to activate PKC. Our results suggest that PLC β is required for group I mGluRs-mediated activation of TRPC5 channels and depression of GIRK channels, whereas intracellular Ca^{2+} release and PKC are not involved. Consistent with this scenario, activation of $\text{G}\alpha_{q/11}$ -coupled receptors results in activation of TRPC5 channels (Plant and Schaefer, 2005; Rohacs, 2013) and depression of GIRK channels (Cho et al., 2001; Hatcher-Solis et al., 2014; Hibino et al., 2010; Karschin, 1999; Keselman et al., 2007; Lei et al., 2003; Meyer et al., 2001; Niemeyer et al., 2019). TRPC5 activation elicited by $\text{G}\alpha_{q/11}$ -coupled receptors depends upon PLC β , but does not involve IP₃ (Schaefer et al., 2000). Intracellular administration of heparin to inhibit IP₃ receptors has no effects on receptor-mediated activation of currents in cells transfected with TRPC5 channels (Plant and Schaefer, 2005). TRPC5 channels could still be activated via a muscarinic receptor in DT-40 B cells lacking all three IP₃ receptor subtypes (Venkatachalam et al., 2003). Similar to homomeric TRPC4 and TRPC5 channels, heteromers formed between TRPC1 and TRPC5 are not activated by infusion of IP₃ but still respond to subsequent muscarinic receptor activation (Strubing et al., 2001). However, the roles of PKC in the depression of GIRK channels in response to $\text{G}\alpha_{q/11}$ -coupled receptors are controversial. PKC has been shown to phosphorylate and depress GIRK channels (Adney et al., 2015; Mao et al., 2004; Niemeyer et al., 2019; Stevens et al., 1999). Other studies demonstrate that the function of PKC is not required for the depression of GIRK channels induced by $\text{G}\alpha_{q/11}$ -coupled receptors (Lei et al., 2003; Mark and Herlitze, 2000).

PIP₂ has been shown to regulate the functions of a variety ion channels including TRPC and Kir channels (Rodriguez-Menchaca et al., 2012; Suh and Hille, 2008). Intracellular application of PIP₂ strongly inhibits TRPC4 α but not TRPC4 β channels (Otsuguro et al., 2008).

Depletion of PIP₂ activates TRPC5 channels, whereas intracellular perfusion of PIP₂ via the patch pipettes inhibited TRPC5 currents

expressed in HEK293 cells (Trebak et al., 2009), consistent with our results showing that activation of group I mGluRs excites layer III pyramidal neurons by PLC β -mediated depletion of PIP₂. Because PIP₂ is also involved in DHPG-elicited inward currents in layer III pyramidal neurons in slices cut from the TRPC5 KO mice, it is reasonable to speculate that PLC β -mediated degradation of PIP₂ is responsible for DHPG-induced inhibition of GIRK channels. Congruous to this speculation, GIRK channels are activated by PIP₂ (Huang et al., 1998; Li et al., 2019a; Suh and Hille, 2008), but inhibited by PLC β -mediated degradation of PIP₂ (Cho et al., 2001, 2005; Meyer et al., 2001).

In addition to PIP₂ depletion, TRPC5 channels might be DAG-sensitive as well (Lee et al., 2003; Storch et al., 2017; Venkatachalam et al., 2003). A hypothetical action model is that during receptor activation, PIP₂ at the plasma membrane is degraded by PLC resulting in an active TRPC5 conformation characterized by C-terminal rearrangements and the ensuing dissociation of the scaffolding proteins Na^+/H^+ exchanger regulatory factor NHERF1 and -2, thereby conferring DAG sensitivity upon TRPC4 and -5 channels (Storch et al., 2017). Our results support this action model, as application of the selective DAG lipase inhibitor, RHC 80267, significantly augmented DHPG-elicited response, revealing a latent role of DAG.

Persistent firing is observed in the EC (Frank and Brown, 2003; Major and Tank, 2004). Persistent firing in the EC is believed to be an important mechanism for working memory (Fransen et al., 2002, 2006; Hasselmo and Eichenbaum, 2005; Hasselmo and Stern, 2006) and dependent on group I mGluRs in layer III pyramidal neurons (Yoshida et al., 2008). Our results that activation of group I mGluRs excites layer III pyramidal neurons by activating TRPC5 channels and depressing GIRK channels suggest an ionic mechanism to explain some of the physiological functions of group I mGluRs in the EC. As the ages of the animals used in this study were 21–35 days, the signaling and ionic mechanisms discovered could be limited to the functions of group I mGluRs in young age, although the expressions of some targeting molecules such as GIRK (Fernandez-Alacid et al., 2011) and TRPC5 (Huang et al., 2007) channels have reached maturity at this age.

5. Conclusion

Whereas group I mGluRs are implicated in persistent firing, the cellular and molecular mechanisms underlying mGluRs-mediated neural modulation have not been determined. We demonstrated that both mGluR1 and mGluR5 receptors are involved in DHPG-elicited excitation of layer III pyramidal neurons. Activation of group I mGluRs excites layer III pyramidal neurons by depressing the GIRK type of the Kir channels and activating TRPC5 channels. PLC β and PLC β -mediated depletion of PIP₂ are involved in, whereas PKC and intracellular Ca^{2+} release are not required for mGluRs-mediated excitation of layer III pyramidal neurons. DAG is also involved in group I mGluRs-mediated activation of TRPC5 channels. Our results may provide one of the cellular and molecular mechanisms to explain the physiological functions of group I mGluRs *in vivo*.

CRediT authorship contribution statement

Saobo Lei: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chidiebele S. Oraegbuna:** Investigation, Formal analysis, Data curation. **Cody A. Boyle:** Investigation, Formal analysis, Data curation. **Morgan R. Mastrud:** Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that there is no interest of conflict for this work.

Acknowledgments

This work was supported by the National Institute Of General Medical Sciences (NIGMS) and National Institute Of Mental Health (NIMH) grant R01MH118258 to S.L.

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