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Original Article

Group 1 metabotropic glutamate receptor 5 is involved in synaptically-induced Ca^{2+}-spikes and cell death in cultured rat hippocampal neurons

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ABSTRACT Group 1 metabotropic glutamate receptors (mGluRs) can positively affect postsynaptic neuronal excitability and epileptogenesis. The objective of the present study was to determine whether group 1 mGluRs might be involved in synaptically-induced intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) spikes and neuronal cell death induced by 0.1 mM Mg^{2+} and 10 μM glycine in cultured rat hippocampal neurons from embryonic day 17 fetal Sprague–Dawley rats using imaging methods for Ca^{2+} and *3-(4,5*-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays for cell survival. Reduction of extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) to 0.1 mM induced repetitive $[Ca^{2+}]_i$ spikes within 30 sec at day 11.5. The mGluR5 antagonist 6-Methyl-2-(phenylethynyl) pyridine (MPEP) almost completely inhibited the $[Ca^{2+}]_i$ spikes, but the mGluR1 antagonist LY367385 did not. The group 1 mGluRs agonist, 3,5-dihydroxyphenylglycine (DHPG), significantly increased the $[Ca^{2+}]_i$ spikes. The phospholipase C inhibitor U73122 significantly inhibited the $[Ca^{2+}]_i$ spikes in the absence or presence of DHPG. The IP_3 receptor antagonist 2-aminoethoxydiphenyl borate or the ryanodine receptor antagonist 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate also significantly inhibited the $[Ca^{2+}]_i$ spikes in the absence or presence of DHPG. The TRPC channel inhibitors SKF96365 and flufenamic acid significantly inhibited the $[Ca^{2+}]_i$ spikes in the absence or presence of DHPG. The mGluR5 antagonist MPEP significantly increased the neuronal cell survival, but mGluR1 antagonist LY367385 did not. These results suggest a possibility that mGluR5 is involved in synaptically-induced $[Ca^{2+}]_i$ spikes and neuronal cell death in cultured rat hippocampal neurons by releasing Ca^{2+} from IP_3 and ryanodine-sensitive intracellular stores and activating TRPC channels.

INTRODUCTION

Glutamate can activate non-N-methyl-D-aspartate (non-NMDA) receptors, leading to depolarize membranes in neurons. This depolarization can induce Ca^{2+} influx from the extracellular space by decreasing Mg^{2+} block of N-methyl-D-aspartate (NMDA) receptors and activating voltage-gated Ca^{2+} channels. Glutamate can also induce the release of Ca^{2+} from intracellular stores through metabotropic glutamate receptor (mGluR)-induced activation of phospholipase C. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) can be further increased by Ca^{2+}-induced Ca^{2+} release through ryanodine receptors [1].

Reduction of extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) elicits excitatory patterns of electrical activity [2] and repetitive $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons [3,4]. These low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes have been reported to depend on glutama-

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tergic synaptic transmission [3]. The Ca^{2+} spikes are synchronized in an active network and induce neuronal cell death [5,6]. In addition, reduction of $[Mg^{2+}]_o$ has been characterized as an *in vitro* model for mechanistic investigation of seizures [7-9].

All eight subtypes of mGluR which are classified into 3 groups, are expressed in the hippocampus [10]. The group II mGluRs (mGluR2, mGluR3) and group III mGluRs (mGluR4, mGluR6, mGluR7, mGluR8) are negatively linked to adenylnyl cyclase and suppress excitatory synaptic transmission at presynaptic sites [11]. The group 1 mGluRs (mGluR1, mGluR5) are coupled to inositol phospholipid hydrolysis and induce subsequent $[Ca^{2+}]_i$ increases by releasing Ca^{2+} from IP_3-sensitive intracellular stores [12,13], which can induce sustained depolarization through activation of TRPC channels [14]. mGluR1 and mGluR5 have been reported to be concentrated at postsynaptic and perisynaptic sites, suggesting that they are involved in the modulation of synaptic transmission [15,16]. However, little is known about whether group 1 mGluRs are involved in synaptically-induced $[Ca^{2+}]_i$ spikes and neuronal cell death by 0.1 mM $[Mg^{2+}]_o$ in cultured rat hippocampal neuronal cells.

Reduction of $[Mg^{2+}]_o$ to 0.1 mM rather than omitting it has been reported to induce a stable pattern of repetitive $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons [3]. Therefore, in the present study we determined how group 1 mGluRs might be involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes and neuronal cell death in cultured rat hippocampal neurons. We found a possibility that mGluR5 could be involved in synaptically-induced $[Ca^{2+}]_i$ spikes and neuronal cell death in cultured rat hippocampal neurons by releasing Ca^{2+} from IP_3 and ryanodine-sensitive intracellular stores and activating TRPC channels.

METHODS

Materials

Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS), and horse serum (HS) were obtained from Gibco-BRL (Grand island, NY, USA). Fura-2 acetoxymethyl ester (AM) was purchased from Molecular Probes (Eugene, OR, USA). 6-Methyl-2-(phenylethynyl) pyridine hydrochloride, 3,5-dihydroxyphenylglycine (DHPG), and other chemicals were purchased from Sigma (St. Louis, MO, USA).

Culture of primary rat hippocampal cells

Rat hippocampal cells were cultured as previously described [6] with minor modifications. Briefly, primary cells were obtained from the hippocampus of embryonic day 17 adult maternal Sprague-Dawley rats (250–300 g). Fetuses were removed on embryonic day 17 from rats that had been anesthetized with urethane (1.3 g/kg body weight, i.p.). All procedures of animal research were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experiment provided by the Institutional Animal Care and Use Committee in the College of Medicine, The Catholic University of Korea (approval number: 2015-0171-03). Hippocampi were dissected and placed in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) adjusted to pH 7.4 with NaOH. Cells were dissociated by trituration through a 5-ml pipette and a flame-narrowed Pasteur pipette. The cell suspension was centrifuged at 201 g for 3 min. Cells were then resuspended in DMEM without glutamine supplemented with 10% FBS and penicillin/streptomycin (100 U/ml and 100 μg/ml). Dissociated cells were plated into six-well culture plates at a density of 50,000 cells/well onto 25-mm-round cover glasses (Fisher Scientific, Pittsburgh, PA, USA) that were previously coated with Matrigel (0.2 mg/ml) (BD Bioscience, San Jose, CA, USA). Neurons and glial cells were grown in a humidified atmosphere of 10% CO_2/90% air (pH 7.4) at 37°C. The medium was replaced with DMEM supplemented with 10% HS and penicillin/streptomycin at 72 to 90 h after plating and fed by an exchange of 25% of the medium after 7 days. These cells were cultured without mitotic inhibitors for a minimum of 12 days and used between 10 and 12 days of culture for fluorescent dye-based digital imaging.

Culture of primary rat pure hippocampal neurons

Isolation of hippocampal neurons was performed using the same procedures mentioned above. Dissociated cells were then plated at a density of 40,000 cells per well onto 96-well plates previously coated with Matrigel (0.2 mg/ml). Cells were grown in neurobasal medium (Gibco/Life Technologies, St. Petersburg, FL, USA) supplemented with 2% B27, 1% penicillin/streptomycin, 0.5 mM glutamine, and 25 μM glutamate at 37°C in 10% CO_2. One-half of the culture medium was changed every 3–4 days without glutamate [17]. For cytotoxicity assays, cells were used between 11 and 12 days of culture.

Calcium imaging

Calcium imaging was performed as described by Kim *et al.* [6]. Briefly, hippocampal cells were loaded with 12 μM fura-2 AM in HEPES-buffered HBSS containing 0.5% BSA for 45 min. The HEPES-buffered HBSS contained the following: 20 mM HEPES, 137 mM NaCl, 1.26 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 5 mM KCl, 0.4 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 3 mM $NaHCO_3$, and 5.6 mM glucose. Fura-2-loaded cells were alternately excited at 340 and 380 nm. Digital fluorescence images (510 nm) were captured with a computer-controlled, cooled, charge-coupled device camera (1,280 × 1,035 binned to 256 × 207 pixels, Quantix; Photometrics, Tucson, AZ, USA). Ratios of 340/380 nm were obtained from background-subtracted digital images. Background

images were collected at the beginning of each experiment after removing cells from another area to the coverslip. $[Ca^{2+}]_i$ spikes were defined as rapid elevations of $[Ca^{2+}]_i$ equal to or > 20% of basal levels [18]. The frequency and the area under the curve of $[Ca^{2+}]_i$ spikes were calculated from data collected during a 10 min window before application of drug or vehicle (1st), a 10 min window at 5 min after application of drug or vehicle (2nd), and a 10 min window at 5 min after wash (3rd). Inhibition of $[Ca^{2+}]_i$ spikes was calculated with the following formula: 2nd / 1st. After identifying $[Ca^{2+}]_i$ spikes, the area under the curve (AUC) was measured between signals and basal line (Y = 0) for individual cells [19].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

Neuronal cell viability was quantified by measuring the reduction of MTT known to produce a purple formazan product by mitochondrial dehydrogenase in viable cells [17]. In brief, neurons were treated with glutamate and/or drugs at 37°C for 24 h in HEPES-buffered HBSS. After treatment, fresh HEPES-buffered HBSS containing MTT at a final concentration of 0.5 mg/ml was added to each well and incubated at 37°C for 3 h. After removing the medium, dimethyl sulfoxide was used to dissolve the purple formazan product. Absorbance was measured at a wavelength of 570 nm using a microplate multi-label counter system (Perkin Elmer, Boston, MA, USA). The absorbance of formazan that had formed in non-treated cells grown in culture medium (control) represented 100% viability.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Significance was determined using either a non-paired Student's t-test or a one-way analysis of variance (ANOVA) followed by a Bonferroni test.

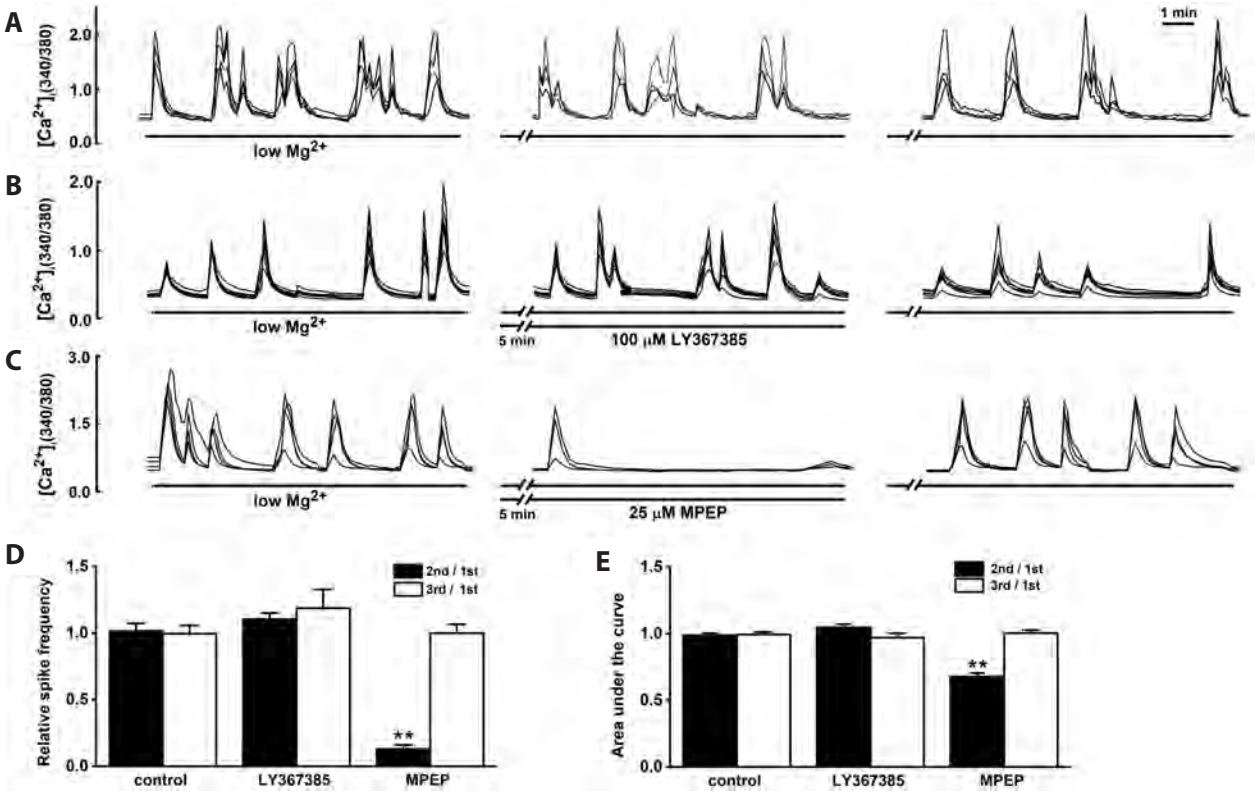

Fig. 1. The mGluR5 antagonist MPEP, but not the mGluR1 antagonist LY367385, inhibits 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (A) 0.1 mM $[Mg^{2+}]_o$ induced synchronized and repetitive $[Ca^{2+}]_i$ spikes within 30 sec at day 11.5. (B) LY367385 (100 μM) did not inhibit 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ responses. (C) MPEP (25 μM) inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (D, E) Graph summarizing the frequency (D) and the area under the curve (E) of 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in non-treated (control, n = 28), MPEP-treated (n = 21), and LY367385-treated (n = 27) cells. Relative spike frequencies or area under curves (2nd /1st: drug or non-treatment; 3rd / 1st: 0.1 mM $[Mg^{2+}]_o$ wash) were shown as a ratio of an initial $[Ca^{2+}]_i$ spike frequency and area under curves for 1st 0.1 mM $[Mg^{2+}]_o$ treatment. The frequency and the area under the curve of $[Ca^{2+}]_i$ spikes were calculated from data collected during a 10 min window before application of drug or vehicle (1st), a 10 min window at 5 min after application of drug or vehicle (2nd), and a 10 min window at 5 min after wash (3rd). Data are expressed as means ± SEM. mGluR, metabotropic glutamate receptor; MPEP, 6-Methyl-2-(phenylethynyl) pyridine. **p < 0.01 relative to respective control and LY367385 (ANOVA with Bonferroni test).

RESULTS

Roles of mGluRs in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes

It has been reported that a reduction of $[Mg^{2+}]_o$ to 0.1 mM can elicit an intense pattern of $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons driven by glutamatergic synaptic transmission [3,4]. Group I mGluRs composed of mGluR1 and mGluR5 are known to be expressed in the hippocampus [15,20,21]. Thus, we investigated whether group 1 mGluR antagonists could affect 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. An exposure to 0.1 mM $[Mg^{2+}]_o$ induced synchronized and repetitive $[Ca^{2+}]_i$ spikes within 30 sec at day 11.5 (Fig. 1A). Treatment with the mGluR5 antagonist MPEP [22] (25 μM) almost completely blocked the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 1C). However, the mGluR1 antagonist LY367385 [23] (100 μM) had no effect on the $[Ca^{2+}]_i$ spikes (Fig. 1B). The frequency and the area under the curve of the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in non-treated (control), LY367385-treated and MPEP-treated cells are summarized in Fig. 1D and E. MPEP, but not LY367385, significantly inhibited the frequency (Fig. 1D) and the area under the curve (Fig. 1E) of the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. These results suggest that mGluR 5 might be involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. We indirectly confirmed the effects of group 1 mGluR antagonists by using a selective group 1 mGluR agonist DHPG [24] on the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. DHPG (1 μM) increased the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. DHPG significantly increased both the frequency (control = 0.90 ± 0.06, n = 23; DHPG = 1.74 ± 0.10, n = 29; $p < 0.01$) and the area under the curve (control = 0.96 ± 0.01, n = 23; DHPG = 1.16 ± 0.04, n = 29; $p < 0.01$) of the $[Ca^{2+}]_i$ spikes.

Roles of phospholipase C in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes

mGluR-induced activation of phospholipase C can hydrolyse phosphatidylinositol 4,5 bisphosphate into IP_3 and diacylglycerol. IP_3 induces a release of Ca^{2+} from IP_3-senstive intracellular stores. Binding of Ca^{2+} to ryanodine receptors further increases $[Ca^{2+}]_i$ by Ca^{2+}-induced Ca^{2+} release [1]. We tested whether the phospholipase C inhibitor U73122 could inhibit 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 2). Treatment with U73122 (1 μM) inhibited the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 2A, B). As shown in the summary of the frequency and the area under the curve of the $[Ca^{2+}]_i$ spikes (Fig. 2C, D), U73122 significantly inhibited both the frequency (Fig. 2C) and the area under the curve (Fig. 2D) of the $[Ca^{2+}]_i$ spikes. U73122 also significantly inhibited both the frequency (DHPG = 1.74 ± 0.10, n = 29; U73122 = 0.66 ± 0.05, n = 26; $p < 0.01$) and the area under the curve (DHPG = 1.16 ± 0.04, n = 29; U73122 = 0.90 ± 0.03, n = 26; $p < 0.01$) of the $[Ca^{2+}]_i$ spikes in the presence of DHPG (1 μM).

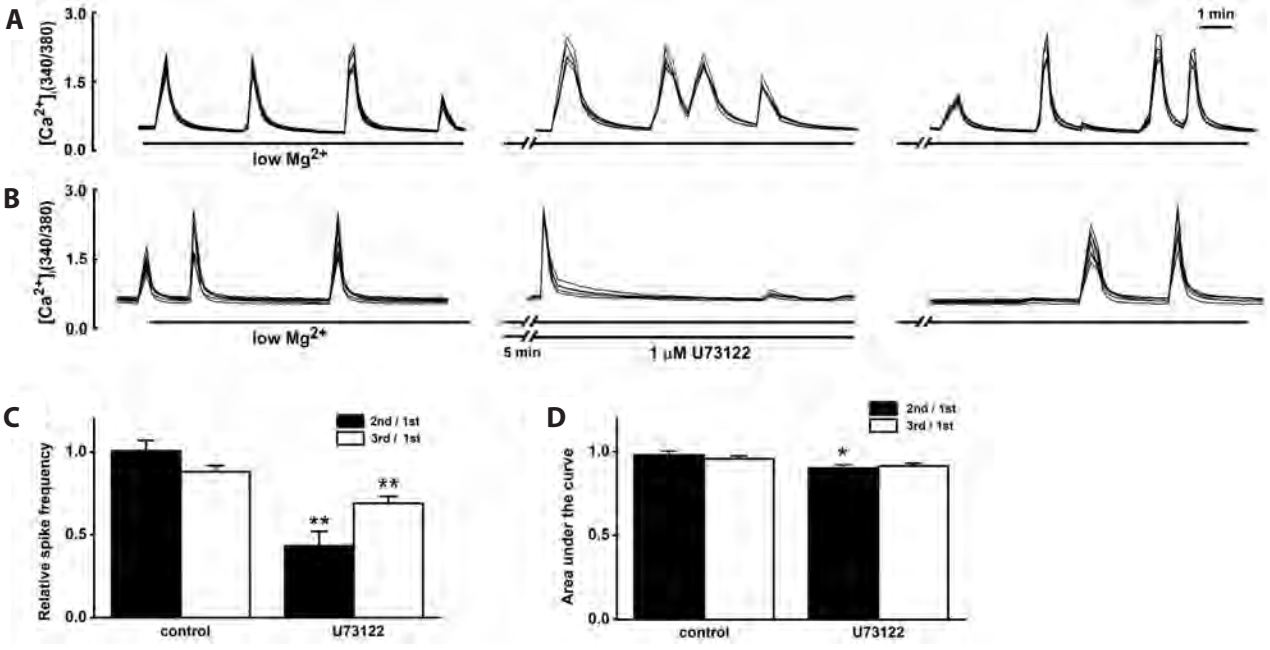

Fig. 2. The phospholipase C inhibitor U73122 (1 mM) inhibits 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (A) 0.1 mM $[Mg^{2+}]_o$ induced synchronized and repetitive $[Ca^{2+}]_i$ spikes within 30 sec at day 11.5. (B) 1 μM U73122 inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (C, D) Graph summarizing the frequency (C) and the area under the curve (D) of 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in non-treated (control, n = 25) and U73122-treated (n = 24) cells. Relative spike frequencies or area under curves (2nd / 1st: drug or non-treatment; 3rd / 1st: 0.1 mM $[Mg^{2+}]_o$ wash) were shown as a ratio of an initial $[Ca^{2+}]_i$ spike frequency and area under curves for 1st 0.1 mM $[Mg^{2+}]_o$ treatment. Data are expressed as means ± SEM. **$p < 0.01$ relative to respective control (non-paired Student's t-test), *$p < 0.05$ relative to respective control (non-paired Student's t-test).

Roles of IP_3 receptors and ryanodine receptors in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes

Since phospholipase C inhibitor inhibited low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in this study, we tested whether the IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB) or the ryanodine receptor antagonist 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) could affect 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 3). Treatment with 2-APB (30 μM) inhibited the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 3A, B) and significantly inhibited the frequency (Fig. 3D) and the area under the curve (Fig. 3E) of the $[Ca^{2+}]_i$ spikes. Treatment with TMB-8 (10 μM) also inhibited the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 3A, C) and significantly inhibited both the frequency (Fig. 3D) and the area under the curve (Fig. 3E) of the $[Ca^{2+}]_i$ spikes. 2-APB also significantly inhibited both the frequency (DHPG = 1.74 ± 0.10, n = 29; 2-APB = 0.52 ± 0.06, n = 19; $p < 0.01$) and the area under the curve (DHPG = 1.16 ± 0.04, n = 29; 2-APB = 0.91 ± 0.03, n = 19; $p < 0.01$) of the $[Ca^{2+}]_i$ spikes in the presence of DHPG. In addition, TMB-8 also significantly inhibited both the frequency (DHPG = 1.74 ± 0.10, n = 29; TMB-8 = 0.10 ± 0.03, n = 24; $p < 0.01$) and the area under the curve (DHPG = 1.16 ± 0.04, n = 29; TMB-8 = 0.54 ± 0.03, n = 24; $p < 0.01$) of the $[Ca^{2+}]_i$ spikes in the presence of DHPG.

Roles of TRPC channels in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes

It has been suggested that mGluR-induced activation of phospholipase C can induce the activation of TRPC channels in hippocampus [25]. TRPC channels can modulate neuronal excitability and promote excitotoxicity [26-29]. TRPC1, 3, 4, 5, and 6 are known to be expressed in rat hippocampal neurons [30-32]. Therefore, we tested whether these TRPC channels might be involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 4). The relatively specific inhibitor SKF96365 (10 μM) for TRPC 3, 6, 7 channels almost completely inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 4A, B). The relatively specific inhibitor flufenamic acid (100 μM) of TRPC 1, 4, 5 channels also inhibited 0.1 mM $[Mg^{2+}]_o$-

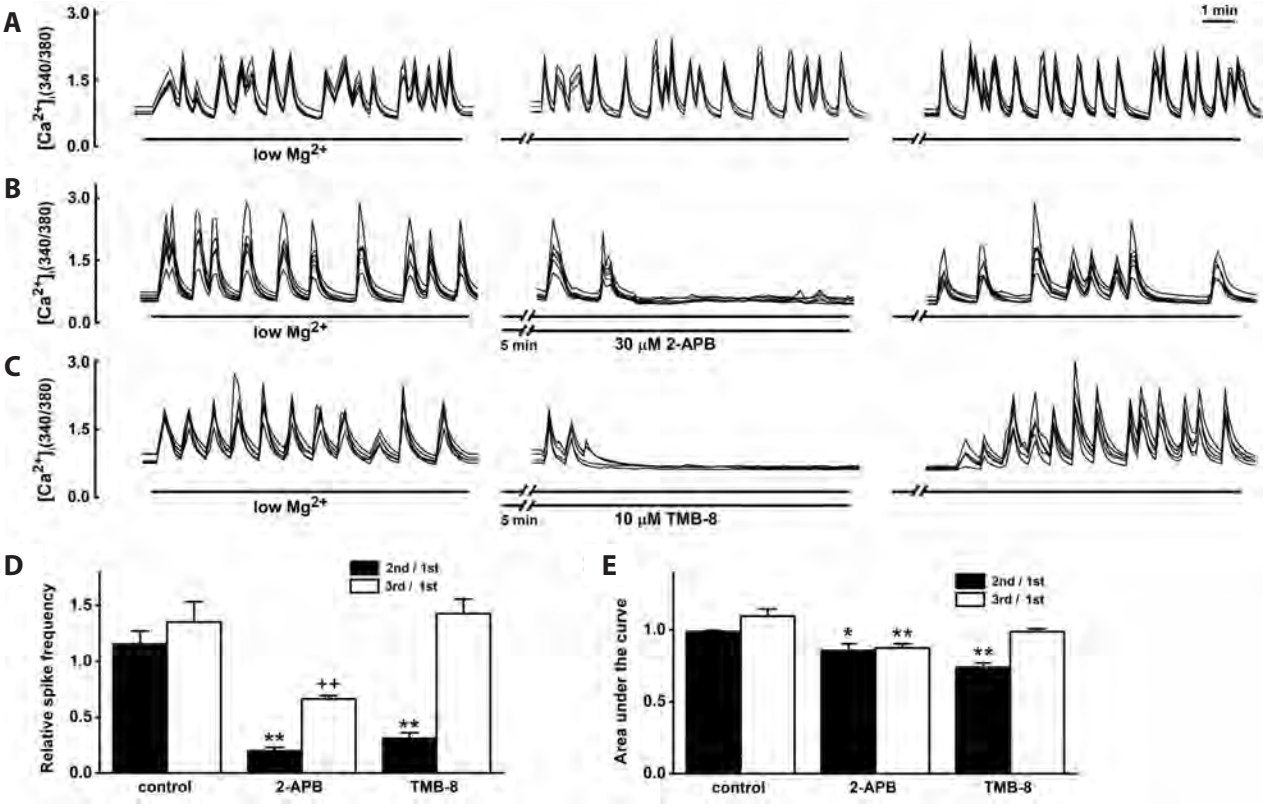

Fig. 3. The IP_3 receptor antagonist 2-APB and the ryanodine receptor antagonist TMB-8 inhibits 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (A) 0.1 mM $[Mg^{2+}]_o$-induced synchronized and repetitive $[Ca^{2+}]_i$ spikes within 30 sec at day 11.5. (B) 2-APB (30 μM) inhibits 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (C) TMB-8 (10 μM) inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (D, E) Graph summarizing the frequency (D) and the area under the curve (E) of 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in non-treated (control, n = 21), 2-APB-treated (n = 21), and TMB-8-treated (n = 21) cells. Relative spike frequencies or area under curves (2nd /1st: drug or non-treatment; 3rd / 1st: 0.1 mM $[Mg^{2+}]_o$ wash) were shown as a ratio of an initial $[Ca^{2+}]_i$ spike frequency and area under curves for 1st 0.1 mM $[Mg^{2+}]_o$ treatment. Data are expressed as means ± SEM. IP_3, inositol-1,4,5-trisphosphate; 2-APB, 2-aminoethoxydiphenyl borate; TMB-8, 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate. **$p < 0.01$ relative to respective control (ANOVA with Bonferroni test), *$p < 0.05$ relative to respective control (ANOVA with Bonferroni test), $^{++}p < 0.01$ relative to respective control and TMB-8 (ANOVA with Bonferroni test).

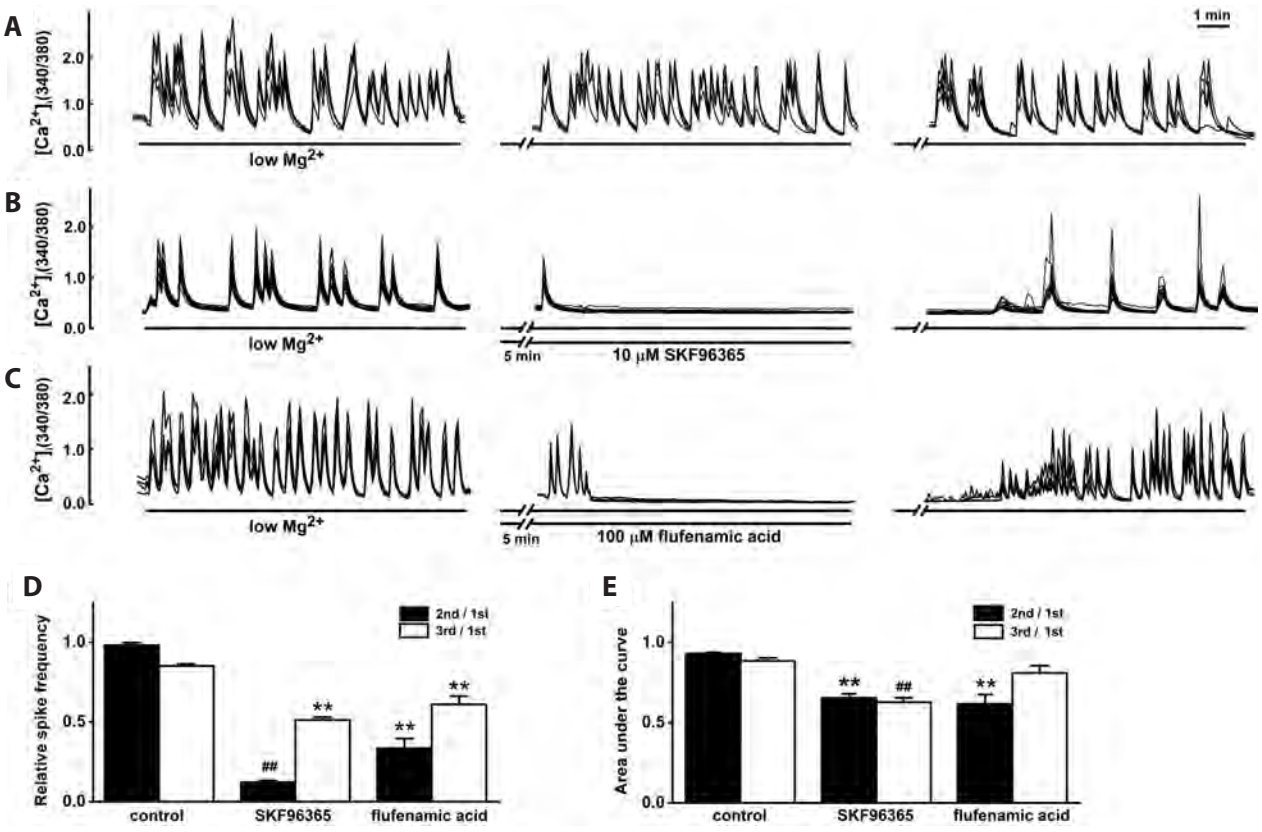

Fig. 4. Effects of TRPC channel inhibitors such as SKF96365 and flufenamic acid on 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (A) 0.1 mM $[Mg^{2+}]_o$ induced synchronized and repetitive $[Ca^{2+}]_i$ spikes within 30 sec at day 11.5. (B) SKF96365 (10 μM) almost completely inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (C) flufenamic acid (100 μM) inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. (D, E) Graph summarizing the frequency (D) and the area under the curve (E) of 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in non-treated (control, n = 19), SKF96365-treated (n = 22), and flufenamic acid-treated (n = 20) cells. Relative spike frequencies or area under curves (2nd /1st: drug or non-treatment; 3rd / 1st: 0.1 mM $[Mg^{2+}]_o$ wash) were shown as a ratio of an initial $[Ca^{2+}]_i$ spike frequency and area under curves for 1st 0.1 mM $[Mg^{2+}]_o$ treatment. Data are expressed as means ± SEM. **p < 0.01 relative to respective control (ANOVA with Bonferroni test), ##p < 0.01 relative to respective control and flufenamic acid (ANOVA with Bonferroni test).

induced $[Ca^{2+}]_i$ spikes (Fig. 4A, C). As shown in the summary of the frequency and the area under the curve of the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (Fig. 4D, E), SKF96365 and flufenamic acid significantly inhibited both the frequency (Fig. 4D) as well as the area under the curve (Fig. 4E) of the $[Ca^{2+}]_i$ spikes. SKF96365 also significantly inhibited both the frequency (DHPG = 1.74 ± 0.10, n = 29; SKF96365 = 0.11 ± 0.05, n = 21; p < 0.01) and the area under the curve (DHPG = 1.16 ± 0.04, n = 29; SKF96365 = 0.58 ± 0.02, n = 21; p < 0.01) of the $[Ca^{2+}]_i$ spikes in the presence of DHPG. In addition, flufenamic acid also significantly inhibited both the frequency (DHPG = 1.74 ± 0.10, n = 29; flufenamic acid = 0.17 ± 0.04, n = 18; p < 0.01) and the area under the curve (DHPG = 1.16 ± 0.04, n = 29; flufenamic acid = 0.89 ± 0.05, n = 18; p < 0.01) of the $[Ca^{2+}]_i$ spikes in the presence of DHPG.

mGluR5 is involved in 0.1 mM $[Mg^{2+}]_o$-induced neuronal cell death

The low $[Mg^{2+}]_o$-induced Ca^{2+} spikes are synchronized in an active network and can induce neuronal cell death [5,6,33]. In the present study, mGluR 5 antagonist MPEP inhibited 0.1 mM $[Mg^{2+}]_o$-induced synchronized and repetitive $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons. We determined whether mGluR 5 and mGluR 1 might be involved in 0.1 mM $[Mg^{2+}]_o$-induced cell death in cultured pure rat hippocampal neurons (Fig. 5). Hippocampal neurons at 11 days of culture were exposed to HEPES-buffered HBSS containing 0.1 mM Mg^{2+} and 10 μM glycine for 24 h in the presence or absence of the mGluR5 antagonist MPEP or the mGluR1 antagonist LY367385. MTT reduction assay was performed to observe the neuroprotective effects of mGluR antagonists (Fig. 5A). Treatment with 0.1 mM Mg^{2+} and 10 μM glycine for 24 h significantly decreased neuronal cell survival. The mGluR5 antagonist MPEP (25 μM), but not LY367385 (100 μM), significantly increased neuronal cell survival. Treatment with MPEP or LY367385 alone for 24 h did not affect the low $[Mg^{2+}]_o$-induced cell death. Photographs shown in Fig. 5B demonstrate the effects of mGluR antagonists on 0.1 mM Mg^{2+}-induced cell death. Treatment with the 0.1 mM $[Mg^{2+}]_o$ solution for 24 h destroyed the cell bodies of hippocampal neurons as well as the processes of neurons compared with vehicle control. Co-treatment of MPEP

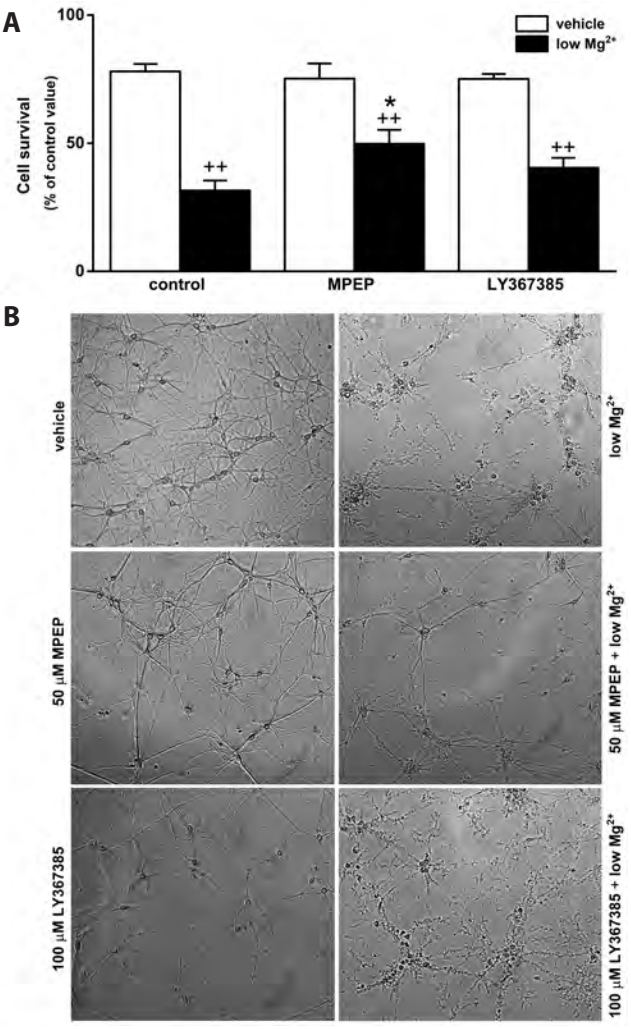

Fig. 5. Effects of the mGluR5 antagonist MPEP and the mGluR1 antagonist LY367385 on 0.1 mM $[Mg^{2+}]_o$-induced neurotoxicity in cultured pure rat hippocampal neurons. Cells were exposed to HEPES-buffered HBSS containing 0.1 mM Mg^{2+} and 10 μM glycine for 24 h in the presence or absence of MPEP (25 μM) or LY367385 (100 μM). Neuronal cell survival was measured by MTT reduction assay at 12 days after culture. Cell survival was shown as a percentage of control value. The absorbance of formazan that had formed in non-treated cells grown in culture medium (control) represented 100% viability. (A) Bar graph showing MTT reduction in HEPES-buffered HBSS (control) (vehicle, n = 6; 0.1 mM Mg^{2+}, n = 6), MPEP (vehicle, n = 6; 0.1 mM Mg^{2+}, n = 6), LY367385 (vehicle, n = 6; 0.1 mM Mg^{2+}, n = 6)-treated cells. (B) Representative phasecontrast photomicrographs showing cultured pure rat hippocampal neurons at 24 h following co-treatment of mGluR antagonists with 0.1 mM $[Mg^{2+}]_o$ solution for 11 days in culture. Data are expressed as means ± SEM. mGluR, metabotropic glutamate receptor; MPEP, 6-Methyl-2-(phenylethynyl) pyridine; HBSS, Hank's balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. $^{++}p < 0.01$ relative to respective vehicle (non-paired Student's t-test), $^{*}p < 0.05$ relative to respective control (ANOVA with Bonferroni test).

with the 0.1 mM $[Mg^{2+}]_o$ solution inhibited the destruction of the cell bodies and the processes of hippocampal neurons. However, co-treatment of LY367385 with the 0.1 mM $[Mg^{2+}]_o$ solution did not. Treatment with MPEP or LY367385 alone for 24 h did not affect the cell bodies and the processes of hippocampal neurons.

DISCUSSION

Results of the present study showed that mGluR5 of group 1 mGluRs was involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons by releasing Ca^{2+} from intracellular store *via* IP_3 receptors and ryanodine receptors and by activating PKC and TRPC channels through activation of phospholipase C in the absence or presence of DHPG. In addition, the mGluR5 antagonist MPEP significantly increased neuronal cell survival.

In the present study, group 1 mGluR agonist DHPG increased 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons. MPEP, an mGluR5 antagonist, but not mGluR1 antagonist LY367385, inhibited the $[Ca^{2+}]_i$ spikes. In our previous study, DHPG induced $[Ca^{2+}]_i$ increase in cultured rat hippocampal neurons [6]. The DHPG-induced $[Ca^{2+}]_i$ increase were almost blocked by MPEP, but not by LY36385. mGluR5 and mGluR1 have been reported to be expressed in the hippocampus. mGluR 5 is expressed in the all hippocampal principal neurons including highly expressed CA1 regions [15,34], whereas mGluR1 is highly expressed on some pyramids in the CA3 regions and weakly in granule cells but not in the CA1 regions [15,35]. mGluR5 is particularly concentrated at the postsynaptic and perisynaptic sites [15]. These data suggesting a possibility that mGluR5 is involved in the modulation of glutamate synaptic transmission. These results suggest a possibility that mGluR 5 is involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes in cultured rat hippocampal neurons. mGluR5 can be coupled to activate phospholipase C [34]. Activation of mGluRs induces mobilization of Ca^{2+} from intracellular stores *via* IP_3 receptors through activation of phospholipase C and Ca^{2+}-induced Ca^{2+} release from the same stores by IP_3 receptors and ryanodine receptors [1]. In the present study, phospholipase C inhibitor U73122 inhibited the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. The IP_3 receptor antagonist 2-APB or the ryanodine receptor antagonist TMB-8 also inhibited the $[Ca^{2+}]_i$ spikes. These data suggest a possibility that mGluR 5 is involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes by releasing Ca^{2+} from intracellular stores *via* IP_3 receptors and ryanodine receptors following activation of phospholipase C.

In this study, mGluR 5 was involved in the low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes by releasing Ca^{2+} from intracellular stores following activation of phospholipase C. Underlying each low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spike is an intense burst of action potentials [5]. mGluRs are known to regulate hippocampal CA1 pyramidal neuron excitability *via* Ca^{2+}-dependent activation of small-conductance K^+ channels and TRPC channels [14]. In the present study, the relatively specific inhibitor SKF96365 of TRPC 3, 6, 7 channels completely inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. The relatively specific inhibitor flufenamic acid of TRPC 1, 4, 5 chan-

nels also significantly inhibited 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. In rat hippocampus, TRPC1, 3, 4, 5 channels are expressed in the granule cells of the dentate gyrus and the pyramidal neurons of the CA1 and CA3 regions [30-32]. TRPC 6 channels are also found in the molecular layer of the dentate gyrus [36]. All these data suggest that multiple TRPC channels are mixed and expressed in the cultured rat hippocampal neurons in the present study. In this study, we could not tell which TRPC channels were involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. These data suggest that mGluR5 is involved in 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes by activating TRPC channels.

We measured the low $[Mg^{2+}]_o$-induced Ca^{2+} spikes in the soma of cultured hippocampal neurons. In the hippocampus, mGluR 5 is expressed in the all principal neurons including the highly expressed CA1 regions [15,34]. mGluR 5 is particularly concentrated at the postsynaptic and perisynaptic sites [15]. These data suggesting a possibility that mGluR5 is involved in the modulation of glutamate synaptic transmission. mGluR5 is coupled to the hydrolysis of PIP2 into IP3 and DAG *via* activation of phospholipase C [34]. It has been suggested that a concomitant activation of perisynaptic mGluRs and TRPC 1/4 channels further depolarizes the postsynaptic terminal and induces influx of Ca^{2+} [28,37]. However, we did not show direct evidence that mGluR5 is involved in synaptically-induced $[Ca^{2+}]_i$ spikes by releasing Ca^{2+} from IP3 and ryanodine-sensitive intracellular stores and activating TRPC channels. All these information suggests a possibility that mGluR 5 is involved in synaptically-induced Ca^{2+} spikes in cultured rat hippocampal neurons by releasing Ca^{2+} from intracellular stores *via* IP_3 receptors and ryanodine receptors and activating TRPC channels.

Blocking 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes has been reported to inhibit low $[Mg^{2+}]_o$-induced neuronal cell death [5,6,38]. These low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes depend on glutamatergic synaptic transmission [3]. A NMDA receptor antagonist can block low $[Mg^{2+}]_o$-induced neuronal cell death [5]. In the same study, a L-type Ca^{2+} channel antagonist nifedifine can inhibit neuronal cell death when it used at concentrations that can block synaptic activity. In the present study, mGluR5 antagonist MPEP inhibited low $[Mg^{2+}]_o$-induced neuronal cell death as well as $[Ca^{2+}]_i$ spikes. Besides NMDA receptor antagonist and L-type Ca^{2+} channel antagonist, mGluR5 antagonist can also block cell death by inhibiting $[Ca^{2+}]_i$ spikes as shown in the present study. These results are supported by reports that mGluR is involved in glutamate-induced cell death by large $[Ca^{2+}]_i$ increase *via* TRPC channels in hippocampal cell line HT22 [27] and mouse hippocampal slice culture [39]. It has been also suggested that TRPC 1, 4. 5 channels are involved in glutamate-induced cell death as well as mGluR-induced epileptiform firing burst [26,40]. All these data suggest that mGluR5 is involved in 0.1 mM $[Mg^{2+}]_o$-induced neuronal cell death by releasing Ca^{2+} from intracellular stores and activating TRPC channels. Thus, mGluR5 may be involved in synaptically-induced neuronal cell death following brain ischemia and epilepsy. The detailed mechanisms of mGluR5 in the $[Ca^{2+}]_i$ spikes and neuronal cell death should be further studied.

In conclusion, our results suggest that mGluR5 may be involved in synaptically-induced-induced $[Ca^{2+}]_i$ spikes and neuronal cell death in cultured rat hippocampal neurons by releasing Ca^{2+} from IP_3 and ryanodine-sensitive intracellular stores and activating TRPC channels.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Simpson PB, Challiss RA, Nahorski SR. Neuronal Ca^{2+} stores: activation and function. *Trends Neurosci.* 1995;18:299-306.
2. Abele AE, Scholz KP, Scholz WK, Miller RJ. Excitotoxicity induced by enhanced excitatory neurotransmission in cultured hippocampal pyramidal neurons. *Neuron.* 1990;4:413-419.
3. Shen M, Piser TM, Seybold VS, Thayer SA. Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J Neurosci.* 1996;16:4322-4334.
4. Kim HJ, Yang JS, Yoon SH. Brief low $[Mg^{2+}]$o-induced Ca^{2+} spikes inhibit subsequent prolonged exposure-induced excitotoxicity in cultured rat hippocampal neurons. *Korean J Physiol Pharmacol.* 2016;20:101-109.
5. McLeod JR Jr, Shen M, Kim DJ, Thayer SA. Neurotoxicity mediated by aberrant patterns of synaptic activity between rat hippocampal neurons in culture. *J Neurophysiol.* 1998;80:2688-2698.
6. Kim HJ, Kim TH, Choi SJ, Hong YJ, Yang JS, Sung KW, Rhie DJ, Hahn SJ, Yoon SH. Fluoxetine suppresses synaptically induced $[Ca^{2+}]_i$ spikes and excitotoxicity in cultured rat hippocampal neurons. *Brain Res.* 2013;1490:23-34.
7. Mody I, Lambert JD, Heinemann U. Low extracellular magnesium induces epileptiform activity and spreading depression in rat hippocampal slices. *J Neurophysiol.* 1987;57:869-888.
8. Sombati S, Delorenzo RJ. Recurrent spontaneous seizure activity in hippocampal neuronal networks in culture. *J Neurophysiol.* 1995;73:1706-1711.

9. Gibbs JW 3rd, Sombati S, DeLorenzo RJ, Coulter DA. Physiological and pharmacological alterations in postsynaptic $GABA_A$ receptor function in a hippocampal culture model of chronic spontaneous seizures. *J Neurophysiol.* 1997;77:2139-2152.
10. Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N. Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J Neurosci.* 1997;17:7503-7522.
11. Pin JP, Duvoisin R. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology.* 1995;34:1-26.
12. Nicoletti F, Meek JL, Iadarola MJ, Chuang DM, Roth BL, Costa E. Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J Neurochem.* 1986;46:40-46.
13. Sugiyama H, Ito I, Hirono C. A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature.* 1987;325:531-533.
14. El-Hassar L, Hagenston AM, D'Angelo LB, Yeckel MF. Metabotropic glutamate receptors regulate hippocampal CA1 pyramidal neuron excitability via Ca^{2+} wave-dependent activation of SK and TRPC channels. *J Physiol.* 2011;589(Pt 13):3211-3229.
15. Lujan R, Nusser Z, Roberts JD, Shigemoto R, Somogyi P. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur J Neurosci.* 1996;8:1488-1500.
16. Forsythe ID, Barnes-Davies M. Synaptic transmission: well-placed modulators. *Curr Biol.* 1997;7:R362-R365.
17. Yang JS, Perveen S, Ha TJ, Kim SY, Yoon SH. Cyanidin-3-glucoside inhibits glutamate-induced Zn^{2+} signaling and neuronal cell death in cultured rat hippocampal neurons by inhibiting Ca^{2+}-induced mitochondrial depolarization and formation of reactive oxygen species. *Brain Res.* 2015;1606:9-20.
18. Shim EY, Kim HJ, Kim MJ, Rhie DJ, Jo YH, Kim MS, Hahn JS, Lee MY, Yoon SH. Desensitization of somatostatin-induced inhibition of low extracellular magnesium concentration-induced calcium spikes in cultured rat hippocampal neurons. *Brain Res.* 2006;1111:61-71.
19. Lévêque M, Penna A, Le Trionnaire S, Belleguic C, Desrues B, Brinchault G, Jouneau S, Lagadic-Gossmann D, Martin-Chouly C. Phagocytosis depends on TRPV2-mediated calcium influx and requires TRPV2 in lipids rafts: alteration in macrophages from patients with cystic fibrosis. *Sci Rep.* 2018;8:4310.
20. Martin LJ, Blackstone CD, Huganir RL, Price DL. Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron.* 1992;9:259-270.
21. Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, Mizuno N. Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. *Neurosci Lett.* 1993;163:53-57.
22. Gasparini F, Lingenhöhl K, Stoehr N, Flor PJ, Heinrich M, Vranesic I, Biollaz M, Allgeier H, Heckendorn R, Urwyler S, Varney MA, Johnson EC, Hess SD, Rao SP, Sacaan AI, Santori EM, Veliçelebi G, Kuhn R. 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology.* 1999;38:1493-1503.
23. Bruno V, Battaglia G, Kingston A, O'Neill MJ, Catania MV, Di Grezia R, Nicoletti F. Neuroprotective activity of the potent and selective mGlu1a metabotropic glutamate receptor antagonist, (+)-2-methyl-4 carboxyphenylglycine (LY367385): comparison with LY357366, a broader spectrum antagonist with equal affinity for mGlu1a and mGlu5 receptors. *Neuropharmacology.* 1999;38:199-207.
24. Schoepp DD, Goldsworthy J, Johnson BG, Salhoff CR, Baker SR. 3,5-dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. *J Neurochem.* 1994;63:769-772.
25. Gee CE, Benquet P, Gerber U. Group I metabotropic glutamate receptors activate a calcium-sensitive transient receptor potential-like conductance in rat hippocampus. *J Physiol.* 2003;546(Pt 3):655-664.
26. Phelan KD, Shwe UT, Abramowitz J, Wu H, Rhee SW, Howell MD, Gottschall PE, Freichel M, Flockerzi V, Birnbaumer L, Zheng F. Canonical transient receptor channel 5 (TRPC5) and TRPC1/4 contribute to seizure and excitotoxicity by distinct cellular mechanisms. *Mol Pharmacol.* 2013;83:429-438.
27. Narayanan KL, Subramaniam S, Bengston CP, Irmady K, Unsicker K, von Bohlen und Halbach O. Role of transient receptor potential channel 1 (TRPC1) in glutamate-induced cell death in the hippocampal cell line HT22. *J Mol Neurosci.* 2014;52:425-433.
28. Lepannetier S, Gualdani R, Tempesta S, Schakman O, Seghers F, Kreis A, Yerna X, Slimi A, de Clippele M, Tajeddine N, Voets T, Bon RS, Beech DJ, Tissir F, Gailly P. Activation of TRPC1 channel by metabotropic glutamate receptor mGluR5 modulates synaptic plasticity and spatial working memory. *Front Cell Neurosci.* 2018;12:318.
29. Yerna X, Schakman O, Ratbi I, Kreis A, Lepannetier S, de Clippele M, Achouri Y, Tajeddine N, Tissir F, Gualdani R, Gailly P. Role of the TRPC1 channel in hippocampal long-term depression and in spatial memory extinction. *Int J Mol Sci.* 2020;21:1712.
30. Strübing C, Krapivinsky G, Krapivinsky L, Clapham DE. TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron.* 2001;29:645-655.
31. Fowler MA, Sidiropoulou K, Ozkan ED, Phillips CW, Cooper DC. Corticolimbic expression of TRPC4 and TRPC5 channels in the rodent brain. *PLoS One.* 2007;2:e573.
32. Chung YH, Ahn HS, Kim D, Shin DH, Kim SS, Kim KY, Lee WB, Cha CI. Immunohistochemical study on the distribution of TRPC channels in the rat hippocampus. *Brain Res.* 2006;1085:132-137.
33. Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S, Attkisson E, DeLorenzo RJ. Time course and mechanism of hippocampal neuronal death in an in vitro model of status epilepticus: role of NMDA receptor activation and NMDA dependent calcium entry. *Eur J Pharmacol.* 2008;583:73-83.
34. Abe T, Sugihara H, Nawa H, Shigemoto R, Mizuno N, Nakanishi S. Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca^{2+} signal transduction. *J Biol Chem.* 1992;267:13361-13368.
35. Shigemoto R, Nakanishi S, Mizuno N. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. *J Comp Neurol.* 1992;322:121-135.
36. Reboreda A, Theissen FM, Valero-Aracama MJ, Arboit A, Corbu MA, Yoshida M. Do TRPC channels support working memory? Comparing modulations of TRPC channels and working memory through G-protein coupled receptors and neuromodulators. *Behav Brain Res.* 2018;354:64-83.
37. Gualdani R, Gailly P. How TRPC channels modulate hippocampal function. *Int J Mol Sci.* 2020;21:3915.

38. Shen M, Thayer SA. Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. *Mol Pharmacol.* 1998;54:459-462.
39. Narayanan KL, Irmady K, Subramaniam S, Unsicker K, von Bohlen und Halbach O. Evidence that TRPC1 is involved in hippocampal glutamate-induced cell death. *Neurosci Lett.* 2008;446:117-122.
40. Phelan KD, Mock MM, Kretz O, Shwe UT, Kozhemyakin M, Greenfield LJ, Dietrich A, Birnbaumer L, Freichel M, Flockerzi V, Zheng F. Heteromeric canonical transient receptor potential 1 and 4 channels play a critical role in epileptiform burst firing and seizure-induced neurodegeneration. *Mol Pharmacol.* 2012;81:384-392.