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Glutamate receptor-mediated regulation of c-fos expression in cultured microglia

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Abstract

It has been recently shown that the expression of various types of neurotransmitter receptors is not restricted to neurons but also observed in a majority of glial cells. However, their function in glial cells is not known well in both physiological and pathological conditions. Here, we investigated the role of glutamate receptor on c-fos gene expression in primary cultured and BV-2 microglia. Our results demonstrated that both c-fos mRNA and protein were dramatically induced following treatment with various glutamate receptor agonists (500 μM); *N*-methyl-D-aspartic acid, kainic acid, (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and (*RS*)-3,5-dihydroxyphenylglycine. The responses were significantly suppressed by specific antagonists and also by calcium chelating agents EGTA and BAPTA-AM. Our results suggest that glutamate receptor activation regulates c-fos gene expression by modifying intracellular calcium levels in microglia. These findings might provide an insight in to understanding the function of microglial glutamate receptors in neuron-to-glial interaction under the excitotoxic conditions.

Keywords: Glutamate receptor; Microglia; c-fos; c-jun; Immediate-early gene; Calcium; Gene expression

Glutamate receptor family includes ligand-gated ionotropic receptors/channels and G-protein-coupled metabotropic receptors linked to phospholipase C (PLC) (group I) or adenylyl cyclase (groups II and III). The ionotropic glutamate receptors are further classified into three groups according to their interactions with non-endogenous analogues, *N*-methyl-D-aspartic acid (NMDA), (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid (KA). Metabotropic glutamate receptors (mGluRs) are also classified into three different groups depending on the compositions of subunits; mGluR1 and 5 for group I, mGluR2 and 3 for group II, and mGluR4 and 6–8 for group III. These glutamate receptors have been implicated in the synaptic transmission and plasticity, and in the refinement of neuronal connections during neural development [1–4]. On the other hand, excess stimulations of glutamate receptors could cause neuroinflammation and neurodegeneration, which have been implicated in major areas of brain pathology such as ischemia, epilepsy, and Alzheimer's disease [5–8].

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The understandings of glutamate receptor function in neurons have been gradually extended to glial cells in the central nervous system (CNS).

Recent methodological advances in molecular biology and electrophysiology have brought new insights into glial cell physiology [9,10]. In fact, it has been recently shown that expression of either ionotropic or metabotropic glutamate receptors is found in neurons and also in majority of glial cells [10–13]. However, their precise functions and underlying action mechanisms have not been clearly understood yet. Nevertheless, the recent data on glial glutamate receptors raise a possibility that glutamate, the dominant excitatory neurotransmitter, might play a role as an important mediator in "neuron-to-glia interaction," as well as in "neuron-to-neuron interaction" in both physiological and pathological conditions [9,14–17].

Glutamate is severely accumulated in the excitotoxic focus in the pathological conditions such as epilepsy and ischemia, which could result in activation of glial glutamate receptors on the adjacent glial cells [5,14]. Regarding the glial cell responses to glutamate, it has been shown previously that glutamate treatment induced the expression of immediate-early genes (IEGs) including c-fos, c-jun, and zif/268 in oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells [18] and furthermore modulated the proliferation and differentiation of O-2A cells [9,19]. These findings suggest that the excitatory neurotransmitter released from neurons might play a role to be one of the environmental signals regulating glial cell development.

Microglial cells are a subtype of glia and have been implicated in neuroinflammation and neurodegeneration. Regarding the role of glutamate in activating microglial cells, only a few studies have been reported addressing glutamate-induced proliferation and release of NO, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) [5,10].

Here, we present the evidences that the induction of c-fos and c-jun, the genes required for activator protein 1 (AP1: c-Fos/c-Jun), was observed in microglia following the glutamate treatment. Generally, IEGs are activated rapidly and transiently in response to environmental insults and regulate expression of the later transcribed subset of genes named delayed-response genes or target genes [20]. Therefore, the glutamate-stimulated induction of IEGs expression shown in this study might be closely related with the expression of genes responsible for microglial activation processes in the pathological conditions.

Because our experimental approach using rather high concentration of glutamate, which is lethal to most of neurons, might mimic the excitotoxic conditions [29,30], our study could provide an insight to understand the pathophysiology of brain diseases related with excitotoxic lesions.

Materials and methods

Microglial cultures. Primary microglial cells were prepared from cerebral cortices of 1-day-old Sprague–Dawley rats as described previously [21,22]. Briefly, the cortices were triturated into single cells with fire-polished Pasteur pipettes. The cells were plated into 75 cm^2-T-flasks (Corning) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Life Technologies) containing 10% fetal bovine serum (FBS, Life Technologies) for 2 weeks at 37 °C in a humidified atmosphere of 5% CO_2. Microglial cells were then separated from the underlying astrocytic monolayer by mild shaking of the flasks, filtered through a nylon mesh to remove astrocytes, and seeded in 6-well plates (0.3×10^6 cells/well, Corning) or 60 mm culture dishes (0.8×10^6 cells/well, Corning). One hour after cell-seeding, unattached cells and loosely attached cells were removed to obtain pure microglial cells. BV-2 cells [40], immortalized murine microglia, were obtained from Dr. E. Joe (Ajou University, Korea). BV-2 microglia were cultured in DMEM containing 10% FBS and kept at 37 °C in a humidified atmosphere of 5% CO_2. The cells were seeded in 6-well plates (1.2×10^6 cells/well, Corning) or 60 mm culture dishes (3×10^6 cells/well, Corning).

Reagents. L-Glutamic acid, ionomycin, and BAPTA-AM (1,2-bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester) were purchased from Sigma. The following reagents were purchased from Tocris Cookson; (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid (KA), *N*-methyl-D-aspartic acid (NMDA), (*RS*)-3,5-dihydroxyphenylglycine (DHPG), γ-aminobutyric acid (GABA), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ((+)-MK-801 maleate), (*RS*) 1-aminoindan-1,5-dicarboxylic acid (AIDA), and 6-chloro-3,4-dihydro-3-(2-norbornen-5-yl)-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide (cyclothiazide). Glutamate, AIDA, BAPTA-AM, and ionomycin were dissolved with HCl (1 N), NaOH (1 N), dimethyl sulfoxide (DMSO), and ethanol (EtOH), respectively, while other chemicals were dissolved with sterile water.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from microglial cells was prepared using TRIZOL (Molecular Research Center). Briefly, 0.5 μg of total RNA was incubated in a tube (20 μl) containing 100 U of SuperScript II Reverse Transcriptase (Invitrogen), 100 U RNaseOUT (Invitrogen), 250 μM dNTPs, and 0.5 μM oligo$(dT)_{12-18}$ primer for 1 h at 42 °C. The reaction was terminated by heating the mixture at 70 °C for 5 min followed by cooling on ice. The RNA strands in the RNA–DNA hybrid were removed by adding 10 U RNaseH (Invitrogen) and incubating for 1 h at 37 °C.

Then, polymerase chain reaction (PCR) was performed in a total volume of 50 μl containing 3 μl cDNA, 200 μM dNTPs, 20 pmol of each primer pair, and 3 U *Taq* DNA polymerase (Bioneer, Korea) in *Taq* DNA polymerase reaction buffer as provided by the supplier. For the PCR, a total of 30 cycles were run under the following schemes; a denaturing at 94 °C for 40 s, a primer annealing at 50 °C (TNF-α), 53 °C (c-fos and c-jun), 55 °C (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin), and 58 °C (iNOS) for 40 s, and an extension step at 72 °C for 40 s in the GeneAmp PCR Thermocycler System 9700 (PE Biosystems). The following sets of primers were used in the PCR amplifications: c-fos (forward 5′-AGACAGATCAACTTGAAGA-3′, reverse 5′-AAACAAGAAGTCATCAAA-3′); c-jun (forward 5′-CTACAGTAACCCTAAGAT-3′, reverse 5′-TTGAAGTTGCTGAGGTT-3′); TNF-α (forward 5′-ATGAGCACAGAAAGCATGAT-3′, reverse 5′-TGACTTTCTCCTGGTATGA-3′); iNOS (forward 5′-ACCAAACTGTGTGCCTGGAGGTT-3′, reverse 5′-AGTAGCCTGTGTGCACCTGGAA-3′); rat GAPDH (forward 5′-GTCCACATTGTTGCCATCAACGAC-3′, reverse 5′-TTTCTCGTGGTTCACACCCATCAC-3′); and mouse β-actin (forward 5′-ATCCGTAAAGACCTCTA-3′, reverse 5′-AACGCAGCTCAGTAACAGTC-3′). The expected PCR products were 320 bp for c-fos, 451 bp for

c-jun, 507 bp for TNF-α, 744 bp for iNOS, 200 bp for β-actin, and 336 bp for GAPDH, respectively. Optical densities of the PCR bands for c-fos, c-jun, TNF-α, and iNOS were quantified with Image J analyzer (http://rsb.info.nih.gov/ij/) and normalized with those of GAPDH or β-actin.

Double-labeling immunocytochemistry. Primary cultured microglial cells plated on the 12-mm diameter cover glasses (0.3×10^5 cells/glass) were incubated with glutamate (500 μM) for 30 min, followed by 5 h incubation without glutamate. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 15 min and permeabilized in 0.2% Triton X-100 in PBS for 15 min. The nonspecific bindings were blocked with 5% normal goat serum in PBS for 30 min. c-Fos protein expression was examined using rabbit polyclonal antibody against c-Fos (1:100, Calbiochem) and Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:400, Molecular Probes). Alexa Fluor 594-conjugated isolectin IB_4 (1:500, Molecular Probes) was used as a microglial cell marker. Nuclear counterstaining was performed with Hoechst 33342 (1:2000, Molecular Probes). Prolong kit (Molecular Probes) was used to reduce fading. Image acquisition was performed using Axiovision software (Carl Zeiss) and a charge-coupled device camera (AxioCam-HRm, Carl Zeiss) attached to a microscope (Axioskop, Carl Zeiss).

Western blotting analysis. Cells were washed once with PBS and lysed with modified RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The lysate was centrifuged at 12,000*g* for 15 min at 4 °C and the supernatant was used for the experiment. An aliquot (60 μg) of protein was separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto 0.2 μm nitrocellulose membrane using Towbin transfer buffer (192 mM glycine, 25 mM Tris, and 20% methanol; pH 8.3). The membrane was preincubated with PBS containing 5% skim milk and probed with rabbit anti-c-Fos antibody (1:500, Calbiochem) or goat anti-actin antibody (1:500, Santa Cruz Biotechnology) in PBS containing 5% skim milk for 1 h at room temperature. The membrane was then washed with PBS containing 0.03% Tween 20 and incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. After several washes, the blot was developed using an enhanced chemiluminescence reagent (ECL, Amersham Biosciences) according to the manufacturer's instructions. The protein concentration was determined by the BCA method (Sigma). Optical densities of the band were quantified with Image J analyzer and normalized with those of actin.

Ca^{2+} imaging. Calcium indicator fluo-4 exhibits large increase of fluorescence intensity upon Ca^{2+} binding. Unlike the ultraviolet light-excitable indicators fura-2 and indo-1, there is no accompanying spectral shift. The fluorescence intensity is known to be increased (>100-fold) upon Ca^{2+} binding. Another advantage of fluo-4 is an excitation spectrum compatible with 488 nm argon-ion laser source. Cells were loaded with a membrane-permeable form of fluorescent Ca^{2+} indicator fluo-4 AM (10 μM, Molecular Probes) in normal Tyrode's solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.35) for 60 min. Then, cells were washed and placed in the dye-free normal Tyrode's solution for at least 30 min for de-esterification of the indicator. Following the treatment of several glutamate receptor agonists, fluo-4 was excited by an argon laser at 488 nm and the fluorescent images were acquired at 15 s intervals on a confocal laser scanning microscope (μRadiance 2000, Bio-Rad). This system was operated using a personal computer running Lasersharp 2000 software (Bio-Rad). Phase contrast images were visualized using laser differential interference optics. The Ca^{2+} images are pseudocolored according to fluorescence intensity; with red representing high Ca^{2+} concentrations and blue representing low Ca^{2+} concentrations.

Statistics. Data are expressed as mean values ± standard error of mean (SEM). Statistical analyses were performed using Student's *t* test. The differences between groups were considered to be significant when $p < 0.05$.

Results

Glutamate-stimulated IEGs induction in microglia

Microglial cells were treated with glutamate (500 μM) for the indicated time (15, 30, and 120 min) and immediately prepared for RT-PCR analyses. We examined here gene expression of c-fos and c-jun, inducible nitric oxide synthase (iNOS), and TNF-α. Their basal mRNA expressions were barely detectable. However, following glutamate treatment, their expression levels were dramatically increased, peaked at 30 min-time point (for c-fos, 23.1 ± 5.56-fold, $p < 0.05$ shown in Fig. 1B), and decreased to basal level at 2 h-time point in case of immediate-early gene c-fos and c-jun. On the other hand, TNF-α and iNOS expression levels were peaked at 2 h-time point (Fig. 1A). As a control, effects of vehicle treatment (1 mM HCl) were negligible.

For immunocytochemical detection of c-Fos protein, primary cultured microglial cells were incubated with glutamate for 30 min in culture medium followed by 2-h incubation without agonist. Cells were then fixed in 4% paraformaldehyde for 15 min. Our results show that c-Fos expression was dramatically upregulated dominantly in nuclei on isolectin IB_4-positive microglia. In addition, cells were morphologically transformed to hypertropic reactive forms (Fig. 1C).

Involvement of various subtypes of glutamate receptors in c-fos induction

All subtypes of ionotropic glutamate receptor agonists (500 μM AMPA, 500 μM KA, and 500 μM NMDA) and group I mGluR agonist (500 μM DHPG) were treated in primary cultured microglia to examine which subtypes of glutamate receptor agonists are responsible for the induction of c-fos gene. Out of various mGluR agonists, we tested DHPG, a group I mGluR agonist, in this study because mGluR5a subunit was previously shown to be predominantly expressed in microglial cells whereas other mGluR subunits were barely detectable [12,13].

Expression of c-fos mRNA was markedly induced following all the excitatory agonist treatments (Fig. 2A). In contrast, inhibitory neurotransmitter GABA (500 μM) did not show any significant effect on the expression of c-fos mRNA. Fig. 2B shows dose–response relations for the c-fos mRNA expression. Dose–response patterns were similar to each other among glutamate analogues. The responses were peaked at 500 μM and almost decreased to basal level at 1 mM except KA. Fig. 2C shows that the AMPA-induced c-fos gene expression was significantly potentiated ($p < 0.01$) in the presence of 100 μM cyclothiazide (CTZ), which is known to enhance AMPA/KA-mediated inward currents by inhibiting the unique desensitization behavior

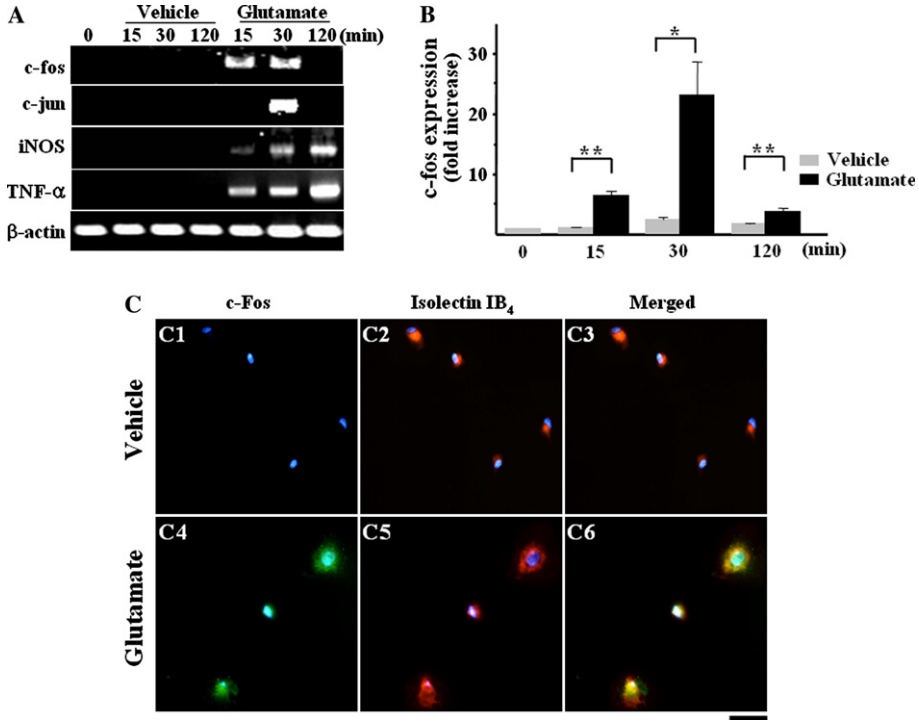

Fig. 1. Glutamate-stimulated induction of c-fos, c-jun, TNF-α, and iNOS mRNA expressions. (A) Representative data on c-fos, c-jun, TNF-α, and iNOS mRNA expressions. BV-2 microglial cells were treated with glutamate (500 μM) for the indicated time. RT-PCR data indicated that c-fos, c-jun, iNOS, and TNF-α mRNA levels were dramatically increased following glutamate treatment but with slightly different time courses. Treatment of vehicle (1 mM HCl) alone did not induce any noticeable mRNA induction. At least four experiments were independently performed. (B) Quantitative analysis on glutamate-stimulated mRNA expression levels. Values are means ± SEM of three samples in one independent experiment. $*p < 0.05$; $**p < 0.001$ as compared to vehicle treatment at each time point. (C) Double-labeling immunocytochemistry data. Primary cultured microglial cells were incubated with glutamate (500 μM) for 30 min, followed by 2-h incubation without glutamate. Cells were morphologically transformed to hypertropic reactive forms. c-Fos (green) was dramatically upregulated dominantly in nuclei on microglial cell marker isolectin IB_4 (red)-labeled microglial cells. Nuclear counterstaining signals with Hoechst 33342 (blue) were shown in C1–C6. Merged images of c-Fos and isolectin IB_4 are shown in C3 (vehicle-treated) and C6 (glutamate-treated). Scale bar, 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

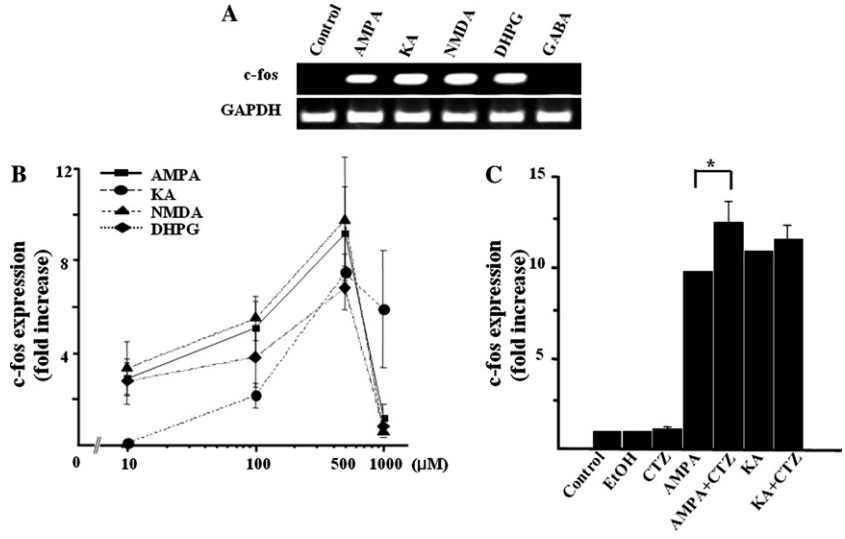

Fig. 2. Involvement of various subtypes of glutamate receptors in c-fos induction. (A) RT-PCR data indicate that c-fos mRNA expression was dramatically induced in primary cultured microglia following the treatment (500 μM, 30 min) of several subtypes of glutamate receptor agonists, whereas inhibitory neurotransmitter GABA did not induce the c-fos mRNA expression. (B) Dose–response relations of glutamate analogue-stimulated c-fos mRNA expression in BV-2 microglia. (C) Cyclothiazide (100 μM, CTZ) significantly potentiated AMPA-induced c-fos mRNA expression in BV-2 microglia. Data are representative of three independent experiments. Values are means ± SEM of three samples in one independent experiment. $*p < 0.01$ as compared to AMPA treatment alone.

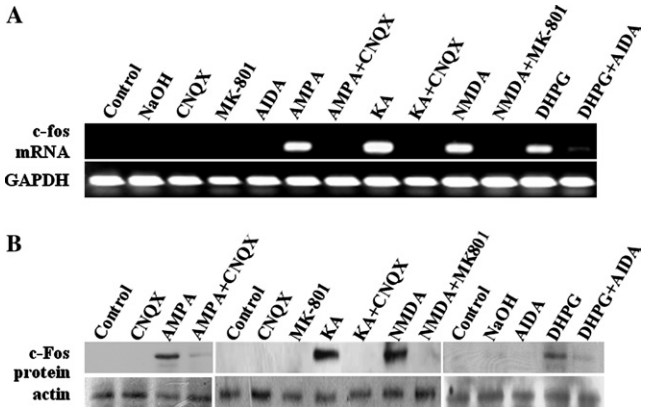

Fig. 3. Blockade of the c-fos induction by specific glutamate receptor antagonists. Both RT-PCR (A) and Western blotting data (B) indicate that various subtypes of glutamate receptor agonist-stimulated c-fos inductions were almost completely blocked by the specific antagonists CNQX (200 μM), MK 801 (10 μM), and AIDA (100 μM) in BV-2 microglia. Treatment of antagonists or vehicle (1 mM NaOH) alone did not show any c-fos induction. At least four experiments were independently performed and representative data are shown.

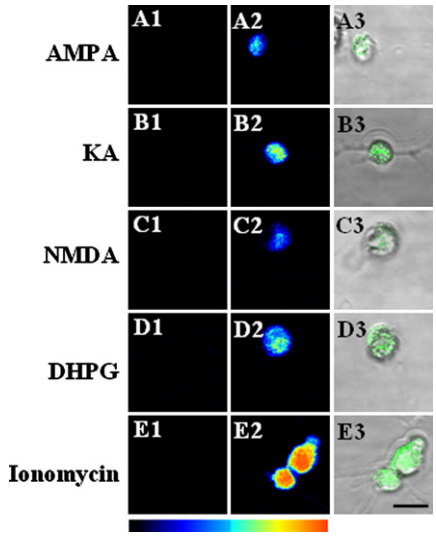

Fig. 4. Glutamate-stimulated intracellular calcium ion concentration ($[Ca^{2+}]_i$) elevation monitored with calcium imaging. Intracellular Ca^{2+}-bound fluo-4 was excited at 488 nm using an argon laser and the fluorescent signals were monitored at 15 s intervals following the treatments using a confocal laser scanning microscope. Intracellular Ca^{2+} levels were rapidly increased in primary cultured microglia within 15-s time point and decreased to basal levels within 2 min-time point by glutamate receptor agonists tested here and ionomycin as well. Images of left (A1, B1, C1, D1, and E1) and median lanes (A2, B2, C2, D2, and E2) represent before and after the treatment (15 s-time point), respectively. Phase contrast images merged with fluo-4 signals (green) are shown at the right lane (A3, B3, C3, D3, and E3). The Ca^{2+} images are pseudocolored according to fluorescence intensity; with red representing high Ca^{2+} concentrations and blue representing low Ca^{2+} concentrations. Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

[10,23] (Fig. 2C). However, KA response was not significant in our study.

We further investigated whether glutamate receptor antagonist could prevent glutamate-stimulated induction of c-fos mRNA and protein. Cells were treated with glutamate analogues (500 μM, 30 min) in the presence of the well-known specific antagonist of glutamate receptors CNQX (for AMPA/KA receptor, 200 μM), MK 801 (for NMDA receptor, 10 μM), and AIDA (for group I mGluR, 100 μM) after pretreatment of these antagonists for 30 min. Microglia were incubated for 5 h in culture medium after the glutamate treatment and then prepared for Western blot analysis, while total RNA was prepared for RT-PCR analysis immediately after the treatment. We found that expression levels of c-fos mRNA (Fig. 3A) and protein (Fig. 3B) were almost completely blocked by treatments of glutamate receptor antagonists. Treatment of antagonists alone and vehicle for DHPG (1 mM NaOH) did not show any significant effect on c-fos induction. These data indicate the direct involvement of all the subtypes of glutamate receptors tested here in c-fos induction without noticeable differences among them.

Increases of intracellular calcium levels through various subtypes of glutamate receptors

To confirm the increases of intracellular calcium ion concentration ($[Ca^{2+}]_i$) through these above glutamate receptors, we examined intracellular calcium levels with fluorescent calcium indicator fluo-4 using a confocal laser scanning microscope. Fluo-4 images were monitored at 15 s intervals following glutamate treatment. Calcium imaging data demonstrated that intracellular calcium levels were rapidly increased within 15 s-time point and decreased to basal level within 2 min-time point in primary cultured microglia by all the subtypes of glutamate receptor agonists tested in the present study and ionomycin as well (Fig. 4).

Blockade of c-fos induction by extra- and intracellular calcium chelation

Microglia were treated with NMDA (500 μM, 30 min) or DHPG (500 μM, 30 min) in the presence of the chelating agents EGTA (3 mM) or BAPTA-AM (50 μM) in culture medium after pretreatment of these chelating agents for 10 min [18,24]. NMDA-stimulated c-Fos protein induction was markedly suppressed by EGTA ($p < 0.001$, Fig. 5A), suggesting the involvement of calcium ion influx through NMDA receptor. In addition, DHPG-stimulated c-Fos induction was significantly attenuated by BAPTA-AM ($p < 0.01$, Fig. 5B), suggesting that group I mGluR-mediated c-Fos expression might be associated with increases of the intracellular calcium ions probably released from intracellular

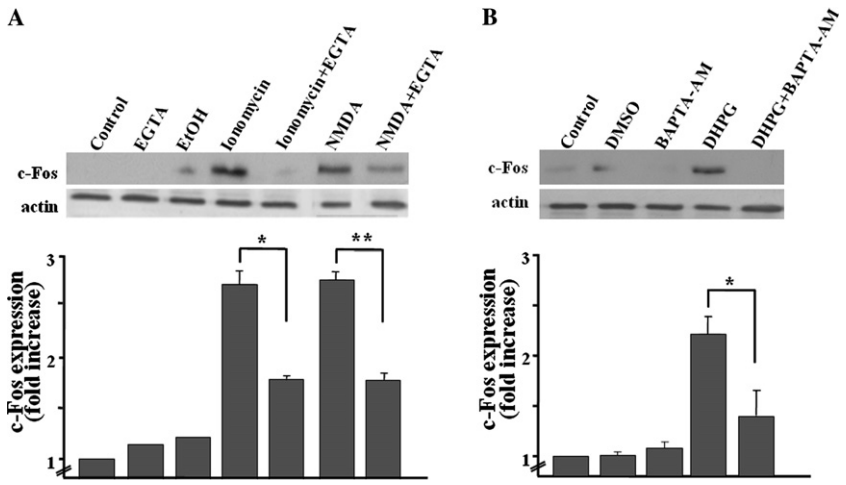

Fig. 5. Blockade of the c-Fos protein induction by extra- and intracellular calcium chelation. (A) Effects of extracellular calcium depletion by EGTA (3 mM) on NMDA (500 μM) and ionomycin (1 μM)-stimulated c-Fos induction in BV-2 microglia. (B) Effects of intracellular calcium chelation by BAPTA-AM (50 μM) on DHPG (500 μM)-stimulated c-Fos induction in BV-2 microglia. Treatment of vehicle (EtOH and DMSO) alone or calcium chelating agents alone did not induce any significant induction. Figure shows the representative Western blots of three independent experiments and their quantitative analyses. Values are means ± SEM of three samples in one independent experiment. $*p < 0.01$; $**p < 0.001$ as compared to treatments with NMDA, DHPG, and ionomycin in the absence of calcium chelating agents.

calcium stores through inositol triphosphate (IP_3) calcium release channels. Interestingly, c-Fos expression was dramatically induced even by treatment of ionomycin (1 μM), a membrane ionophore which could increase intracellular calcium ion concentration ($[Ca^{2+}]_i$) through calcium ion influx [24]. In addition, the chelation of extracellular calcium ions with EGTA treatment (3 mM) significantly reduced ionomycin-induced c-Fos induction ($p < 0.01$, Fig. 5A).

Discussion

We demonstrated here that all the subtypes of glutamate receptor agonists examined (NMDA, KA, AMPA, and DHPG) dramatically increased both c-fos mRNA and protein levels in cultured microglial cells (Fig. 2). These effects were significantly suppressed by the specific glutamate receptor antagonists MK-801, CNQX, and AIDA (Fig. 3) and also by calcium chelating agents EGTA and BAPTA-AM (Fig. 5). To our knowledge, our findings are the first to demonstrate glutamate-stimulated c-fos induction and involvement of all the subtypes of ionotropic and group I metabotropic glutamate receptors in cultured microglia.

We are particularly interested in the differences in dose–response behaviors between neurons and non-neuronal microglial cells shown in the present study. The glutamate concentration used here (500 μM) is about 10- to 50-fold higher than the dose often used for physiological activation of neurons [26,27]. Microglial cells looked morphologically healthy at this high concentration, which is lethal to most of the neuronal cells [28,29]. In addition, we could not find any indication of cell death in microglia after glutamate treatment when examined by MTT viability assay (data not shown). These findings could be compatible with the fact that resting microglial cells are strongly activated in pathological conditions in vivo [30,31] whereas the cells are barely activated at the physiological glutamate concentration [26,27]. One possible reason for different dose responsiveness between neuron and microglia may be due to different expression levels of glutamate receptors. Microglial cells probably express fewer glutamate receptors than neurons. Other possible explanations may be the presence of different regulatory mechanisms and different subsets of each subtype of glutamate receptor.

Our calcium imaging data with fluo-4 demonstrated that the activations of all the subtypes of glutamate receptors tested here resulted in intracellular calcium level increases rapidly and transiently in primary cultured microglia (Fig. 4). The results suggest that even the short events of intracellular calcium increases may lead to the consequential calcium-dependent signaling cascades during the relatively long period [25]. The calcium chelating agents EGTA and BAPTA-AM could prevent increases of intracellular calcium ion concentration ($[Ca^{2+}]_i$) by either depletion of extracellular calcium ions or rapid chelation of calcium ions released from intracellular calcium stores. Both EGTA (3 mM) and BAPTA-AM (50 μM) significantly suppressed NMDA or DHPG-stimulated c-fos induction (Fig. 4). Interestingly, ionomycin which could induce calcium influx through membrane pores mimicked glutamate analogues (Fig. 4A). Taken together, these findings suggest that increases of intracellular calcium ion concentration ($[Ca^{2+}]_i$) mediated by glutamate receptor activation might regulate glutamate-stimulated c-fos induction in

microglia, regardless of calcium pathways, either calcium influx from extracellular space or calcium release from intracellular calcium stores.

The mechanism underlying the c-fos induction in microglial cells is not understood well but could be learned from neuronal cells where the regulatory mechanisms are rather clearly understood. In one pathway, Ca^{2+}/calmodulin (CaM) activates CaM kinase II, and in turn it activates extracellular stimuli-response kinase (ERK 1/2) and ribosomal S6 kinase (RSK). In the other pathway, Ca^{2+}/CaM is translocated to nucleus and activates nuclear CaM kinase IV followed by phosphorylation of CREB at Ser133 [32–36]. However, it remains to be further elucidated whether these signaling pathways are not restricted to neurons but also work in microglial cells.

Glutamate-stimulated IEGs induction may be temporally correlated with late-response genes such as TNF-α and iNOS (Fig. 1A). Glutamate treatment was recently shown to stimulate microglia [17] and induce release of NO, IL-1β, and TNF-α [5,10,17]. We also confirmed the similar effects on cytokines TNF-α and iNOS (Fig. 1A) which seem to have AP-1 binding sites in the promoter region [41,42]. In addition, we found severe cellular morphological changes which could be explained as another consequence of the microglial activation (Fig. 1C). In summary, our results suggest that glutamate-stimulated induction of transcription factors such as c-Fos and c-Jun may cause expression of several essential genes for microglial activation processes [20,36–39]. Our results provide an insight to extend the understandings of the physiological roles of microglial glutamate receptors in terms of neuron-to-glial interaction under the sustained neuronal hyperexcitation or glutamate-toxic pathological conditions.

Acknowledgments

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