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Abbreviations used: AP1, activator protein-1; APICA, (RS)-1-amino-5-phosphonpindan-1-carboxylic acid; BAPTA-AM; 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester; DCG-IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; DHPG, 3,5-dihydroxyphenylglycine; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle Medium; DTT, dithiothreitol; FCS, fetal calf serum; Glu, glutamate; HKR, HEPES-Krebs Ringer; IBMX, 3-isobutyl-1-methylxanthine; iGluR, ionotropic glutamate receptor; IP_3, inositol (1,4,5)-trisphosphate; L-AP4, L-2-amino-4-phosphonobutyrate; mGluR, metabotropic glutamate receptor; NaGP, sodium β-glycerophosphate; NMDA, N-methyl-D-aspartate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecylsulfate.

Abstract

Activation of ionotropic glutamate (Glu) receptors, such as N-methyl-D-aspartate receptors, is shown to modulate the gene transcription mediated by the transcription factor activator protein-1 (AP1) composed of Fos and Jun family proteins in the brain, while little attention has been paid to the modulation of AP1 expression by metabotropic Glu receptors (mGluRs). In cultured rat cortical neurons, where constitutive expression was seen with all group I, II and III mGluR subtypes, a significant and selective increase was seen in the DNA binding activity of AP1 120 min after the brief exposure to the group II mGluR agonist (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) for 5 min. In cultured rat cortical astrocytes, by contrast, a significant increase was induced by a group I mGluR agonist, but not by either a group II or III mGluR agonist. The increase by DCG-IV was significantly prevented by a group II mGluR antagonist as well as by either an intracellular Ca^{2+} chelator or a voltage-sensitive Ca^{2+} channel blocker, but not by an intracellular Ca^{2+} store inhibitor. Moreover, DCG-IV significantly prevented the increase of cAMP formation by forskolin in cultured neurons. Western blot analysis revealed differential expression profiles of Fos family members in neurons briefly exposed to DCG-IV and NMDA. Prior or simultaneous exposure to DCG-IV led to significant protection against neuronal cell death by NMDA. These results suggest that activation of the group II mGluR subtype would modulate the gene expression mediated by AP1 through increased intracellular Ca^{2+} levels in cultured rat cortical neurons.

1. Introduction

Glutamate (Glu) receptors are categorized into the two major subclasses, ionotropic (iGluR) and metabotropic (mGluR) receptors, on the basis of intracellular signal transduction systems as well as nucleotide sequence homology. The mGluRs are a family of type III G-protein-coupled receptors activated by the excitatory amino acid neurotransmitter Glu, and classified into three major subtypes (group I, mGluR1 and 5 isoforms; group II, mGluR2 and 3 isoforms; group III, mGluR4, 6, 7 and 8 isoforms) based on sequence homology, signal transduction pathway and pharmacology (Conn and Pin, 1997; Schoepp et al., 1999; Zhai et al., 2003). The group I mGluR subtype is coupled to stimulatory G_q proteins to activate phospholipase C, which catalyzes the production of diacylglycerol and inositol (1,4,5)-trisphosphate (IP_3) for subsequent activation of protein kinase C and release of Ca^{2+} from intracellular stores, respectively. Both group II and III mGluR subtypes are coupled to the inhibitory $G_{i/o}$ protein to negatively regulate the activity of adenylyl cyclase, which decreases intracellular concentrations of cAMP (Cartmell and Schoepp, 2000; Schoepp, 2001; Moldrich et al., 2003; Kenny and Markou, 2004).

These mGluR subtypes show an enormous diversity of effects on neurons and glia (Winder and Conn, 1996; Conn and Pin, 1997; Anwyl, 1999; Cartmell and Schoepp, 2000; Schoepp, 2001). Immunocytochemical studies reveal that the neuronal location varies in relation to the density of synaptic clefts with mGluR subtypes in the brain (Shigemoto and Mizuno, 2000; Moldrich et al., 2003). Group I mGluR isoforms are predominantly found at the postsynaptic location adjacent to the margins in the synaptic cleft, for example, while mGluR5, but not mGluR1, isoform is found in glia. The mGluR1α isoform is particularly localized to GABAergic interneurons in hippocampus and cerebellum (Shigemoto et al., 1997), whereas mGluR5 isoform is highly expressed in the limbic cortex and basal ganglia.

The group II mGluR subtype predominates in presynaptic locations, with strong evidence for the location of mGluR2 isoform on presynaptic axons in cerebellum, neocortex and thalamus. While mGluR3 isoform shares a similar distribution pattern with mGluR2 isoform throughout the brain, this mGluR3 isoform is also expressed in glia (Tamaru et al., 2001). Although the group III mGluR subtype is basically presynaptic with the localization of mGluR4, mGluR7 and mGluR8 isoform mRNA to the basal ganglia motor loop (Messenger et al., 2002), however, mGluR6 isoform expression appears to be largely restricted to retinal neurons (Moldrich et al., 2003).

The transcription factor activator protein-1 (AP1) is a homo- or hetero-dimer between Jun and Fos family members with affinity for the core nucleotide sequence TGACTCA at the upstream or downstream of inducible target genes (Curran and Franza, 1988). Increased AP1 DNA binding would lead to long-lasting and sometimes permanent alterations of a variety of neuronal activities, such as neuronal plasticity and delayed neuronal death, following transient activation of iGluRs located at membranes in the brain. In fact, several independent lines of evidence indicate that activation of N-methyl-D-aspartate (NMDA) and kainate subtypes of iGluRs leads to a marked increase in the DNA binding activity of AP1 through up-regulation of particular Jun and Fos family member proteins in the brain *in vivo* and *in vitro*. However, relatively little attention has been paid to the modulation of expression of AP1 complex in response to activation of mGluR subtypes in the brain to date.

In this article, therefore, we have evaluated AP1 DNA binding following the activation of different mGluR subtypes to investigate the possible participation of gene transcription by the AP1 complex in mechanisms underlying the signal input mediated by mGluR subtypes across plasma membranes in primary cultured rat cortical neurons.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies against c-Fos, FosB, Fra-2, c-Jun, JunB and JunD proteins were all provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). An anti-rabbit IgG antibody conjugated with peroxidase, ECL^{TM} detection reagents, a QuickPrep Micro mRNA Purification Kit and a cAMP enzyme immunoassay system were supplied by Amersham Biosciences (Piscataway, NJ, USA). Versene, Dulbecco's Modified Eagle Medium (DMEM) and DMEM: Nutrient Mixture F-12 (DMEM/F-12) 1:1 Mixture were purchased from GIBCO BRL (Gaithersburg, MD, USA). Fetal calf serum (FCS) was obtained from JRH Biosciences, Inc. (Lenexa, KS, USA). [α-^{32}P]deoxy-ATP (111 MBq/mmol) was provided by PerkinElmer Life Sciences (Waltham, MA, USA). rTaq DNA Polymelase was supplied by TAKARA BIO, Inc. (Otsu, Japan). (RS)-1-Amino-5-phosphonpindan-1-carboxylic acid (APICA), (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), 3,5-dihydroxyphenylglycine (DHPG), L-2-amino-4-phosphonobutyrate (L-AP4) and NMDA were purchased from Tocris Cookson (Bristol, UK). Other chemicals used were all of the highest purity commercially available.

2.2. Preparation of neuronal cultures

This study was carried out in compliance with the Guideline for Animal Experimentation at Kanazawa University with an effort to minimize the number of animals used and their suffering. Primary neuronal cultures were prepared from cerebral neocortex of

18-day-old embryonic rats as originally described by di Porzio et al. (1980) with several modifications (Nakamichi et al., 2002a, 2002b). In brief, cerebral neocortex was dissected from embryonic Wistar rats and incubated with Versene at room temperature for 12 min. Cells were then mechanically dissociated with a fire-narrowed Pasteur pipette in the culture medium, and plated at a density of 2.5×10^5 cells/cm^2 on a 6-well or 24-well dish (NUNC, Roskilde, Denmark) after counting cell numbers with a Trypan Blue exclusion test. Prior to use, dishes were sequentially coated with 75 μg/ml poly-L-lysine. The culture medium contained basal DMEM/F-12 with supplementation by 10% FCS, 33 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES and 0.11% sodium bicarbonate. Cells were treated with 10 μM cytosine arabinoside for 24 h during 2 to 3 days *in vitro* (DIV). Cultures were kept in serum free medium, basal DMEM with supplementation by 25.5 mM glucose, 0.5 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.11% sodium bicarbonate, 50 μg/ml transferrin, 500 ng/ml insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium seleniate and 100 μM putrescine after 3 DIV. The culture medium was replaced with freshly prepared culture medium of the same composition every 3 days. Cultures were always maintained at 37°C in a 5% CO_2/95% air-humidified incubator. Cortical neurons cultured for 14 DIV were usually used in this study unless otherwise indicated.

2.3. Preparation of astrocyte cultures

Astrocytes were prepared as described previously (Murakami et al., 2003). In brief, brain cortices from 20-day-old embryos of Wistar rats were cleared of meninges, cut into about 1-mm^3 blocks, and treated with 0.25% trypsin in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) containing 5.5 mM glucose for 20 min at 37°C with gentle shaking. An equal

volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate trypsin. Then, tissues were centrifuged at 1,500×*g* for 5 min. The tissue sediments were triturated through a Pasteur pipette with DMEM containing 10% FCS, 100 mg/l streptomycin, and 5×10^4 unit/l penicillin. After filtration of cell suspensions through a lens-cleaning paper (Fuji Photo, Tokyo, Japan), cells were plated on polyethyleneimine-coated 100 mm-diameter plastic dishes (NUNC, Roskilde, Denmark) at a density of $0.8\text{-}1.3\times10^5$ cells/cm^2. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C with changing medium every 3 days. After one week, astrocytes were replated to remove neurons. On days 12 to 14, they were again replated onto a 6-well dish using an ordinary trypsin-treatment technique at a density of $0.8\text{-}1.3\times10^5$ cells/cm^2 and cultured for additional one week.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

We used a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences; Piscataway, NJ, USA) to extract mRNA from rat whole brain, cultured cortical neurons and astrocytes, and mRNA was then subjected to the synthesis of cDNA. The individual cDNA species were amplified in a reaction mixture containing a cDNA aliquot, PCR buffer, dNTPs, the relevant sense and antisense primers (mGluR1 subunit: 5'-CCAGTGATGTTCTCCATACC-3' and 5'-CACTCTGGGTAGACTTGAGTG-3'; mGluR2 subunit: 5'-TTTAGGTCAGAAGCCAGAGT-3' and 5'-CAGTAACCATCCTCTCTATCC-3'; mGluR3 subunit: 5'-TATTCTCAGTCCTCTGCAAG-3' and 5'-TTGTAGCACATCACTACATACC-3'; mGluR4 subunit: 5'-TCATTTTCTCTTCTGTTCCC-3' and 5'-GACATGCTACACATCAGAGAC-3'; mGluR5 subunit:

5’-CCCCAAACTCTCCAGTCT-3’ and 5’-ATTTTTCACCTCGGGTTC-3’; mGluR6 subunit: 5’-CAAGTAGCAAGGTTGAGTGT-3’ and 5’-GGTTGTAGTGTTGGATCAAG-3’; mGluR7 subunit: 5’-GAACTCTGTGAAAATGTAGACC-3’ and 5’-TTAGGGAGTCCAGAATTACAG-3’; mGluR8 subunit: 5’-CGAGGGTTATAACTACCAGGT-3’ and 5’-TAGGTGCTGTGACAGATTTCT-3’) and rTaq DNA Polymerase. Reactions were initiated by incubating at 95°C for 5 min and PCR (denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 75°C for 2 min, respectively) was performed for a suitable number of cycles with a final extension at 75°C for 10 min. The PCR products were determined on 2% agarose gel electrophoresis and visualized by ethidium bromide.

2.5. Preparation of nuclear extracts

Nuclear fractions were prepared according to the method of Schreiber et al. (1989), with minor modifications (Yoneda and Ogita, 1994; Hirai et al., 2002). Cortical neurons were exposed to one of mGluR agonists for 5 min, followed by washing and subsequent further incubation for 120 min. Cells were harvested with a rubber policeman and homogenized in 500 μl of 10 mM HEPES-NaOH buffer (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT), 10 mM NaF, 10 mM sodium β-glycerophosphate (NaGP) and 1 μg/ml of following protease inhibitors; (*p*-amidinophenyl)methanesulfonyl fluoride, benzamidine, leupeptin and antipain. Following the addition of 10% Nonidet P-40 to make a final concentration of 0.6%, homogenates were centrifuged at 20,000×*g* for 5 min. Pellets were suspended in 20 μl of 50 mM Tris-HCl buffer (pH 7.5) containing 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10% (v/v) glycerol, 10 mM NaF, 10 mM NaGP and the aforementioned protease inhibitors, followed by the centrifugation at 20,000×*g* for 5 min.

Supernatants thus obtained were stored at -80°C as nuclear extracts until use. Protein concentration was determined by Bio-Rad Protein assay kit (Bio-Rad; Hercules, CA, USA) and adjusted at 3 μg in 8 μl of incubation buffer.

2.6. Preparation of probe

The probe for determination of AP1 binding was double stranded oligonucleotides with 22 mer (5'-CTAGTGATGAGTCAGCCGGATC-3'/ 3'-GATCACTACTCAGTCGGCCTAG-5') that was radiolabeled with [α-^{32}P]deoxy-ATP using Klenow fragment of DNA polymerase I in 10 mM Tris–HCl buffer (pH 7.4) containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 50 μM deoxy-GTP, deoxy-CTP and deoxy-TTP at 25°C for 30 min, followed by the purification with gel filtration chromatography on a Nick column (Ogita and Yoneda, 1994). The term "AP1 DNA binding" was used to refer to binding of a radiolabeled probe for AP1 complex throughout the paper.

2.7. Gel retardation electrophoresis

An aliquot of nuclear extracts (3 μg protein in 8 μl) was mixed with 10 μl of 0.1 mg/ml poly(dI-dC), 50 mM Tris–HCl buffer (pH 7.5), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10% glycerol and 1 μg/ml protease inhibitors, followed by the incubation with 2 μl of 20 fmol of radiolabeled probe (0.5-5.0 $\times$ 10^6 cpm/pmol) at 25°C for 30 min (Ogita and Yoneda, 1994). Bound and free probes were separated by electrophoresis on a 6% polyacrylamide gel in 50 mM Tris-HCl (pH 8.5), 0.38 M glycine and 2 mM EDTA at a constant voltage of 11 V/cm for 1.5 h in an ice bath. Gels were fixed, dried and exposed to an X-ray film for autoradiography. Gels were always exposed to X-ray films for different periods

to obtain autoradiograms adequate for quantitative densitometric analysis.

2.8. Quantification of cAMP

Cortical neurons cultured for 14 DIV were washed twice with PBS and incubated in PBS containing 1 mM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase, for 20 min at 37°C under 5% CO_2, followed by the addition of 10 μM forskolin in either the presence or absence of DCG-IV at 100 μM in PBS containing 1 mM IBMX for 5 min at 37°C under 5% CO_2, as described previously (Fujimori et al., 2002). After the stimulation, cells were sonicated in lysis buffer [0.25% solution of dodecyltrimethylammonium bromide in 0.05 M acetate buffer; 0.02% (w/v) bovine serum albumin; 0.01% (w/v) preservative]. Measurement of cAMP was conducted by enzyme immunoassay according to the manufacturer's instructions.

2.9. Western blotting

Cortical neurons were exposed to NMDA at 100 μM in the absence of Mg^{2+} ions or DCG-IV at 100 μM in the presence of Mg^{2+} ions for 5 min, followed by washing and subsequent further incubation for 120 min. Cells harvested with a rubber policeman were homogenized in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM NaGP, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 1 μg/ml of various protease inhibitors [(*p*-amidinophenyl)methanesulfonyl fluoride, leupeptin, antipain and benzamidine], followed by the addition of 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecylsulfate (SDS), 0.01% bromophenol blue and 5% 2-mercaptoethanol (SDS sample buffer) at a volume ratio of 4:1 and subsequent boiling at

100°C for 10 min (Manabe et al., 2001). Each aliquot of 20 μg proteins was loaded on a 7.5% polyacrylamide gel for the electrophoresis at a constant current of 15 mA/plate for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was reacted with an antibody against c-Fos, FosB, Fra-2, c-Jun, JunB or JunD, adequately diluted with the buffer containing 1% skim milk, followed by the reaction with an anti-rabbit IgG antibody conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of ECL^{TM} detection reagents through exposure to X-ray films.

2.10. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Cellular viability was assessed by the MTT reduction assay for the mitochondrial activity as described previously (Nakamichi et al., 2005). Cultured cells were washed once with PBS and incubated with 0.5 mg/ml MTT in PBS for 1 h at 37°C, followed by the addition of 0.04 M HCl in isopropyl alcohol to the well and subsequent shaking of the mixture for 10 min to dissolve the formazan. The dissolved suspension was subjected to an enzyme-linked immunosorbent assay reader and the absorbance at a wavelength of 550 nm was measured.

2.11. Drug treatments

Cultured cells were washed twice with HEPES-Krebs Ringer buffer (HKR; 130 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 5.6 mM glucose, 50 mM HEPES), followed by the prior incubation at 37°C for 15 min in HKR under the

humidified atmosphere of 5% CO_2. For NMDA treatment, $MgSO_4$ was omitted from HKR during the incubation. A test drug was then added into the incubation medium for the brief exposure for 5 min, followed by the rapid washing of cells with ice-cold Ca^{2+}, Mg^{2+}-free PBS and subsequent placement of culture plates on ice. The cell monolayer was rapidly scraped in ice-cold extract or lysis buffer. Drugs were freshly made on the day of the experiment. All mGluR agonists were used at 100 μM unless otherwise indicated, for DCG-IV was most efficient at 100 μM in increasing AP1 DNA binding as shown below.

2.12. Data analysis

Densitometric data were subjected to calculation of the area under the curve using Scion Image β 4.02 software (Scion Co., Frederick, MD, USA). For samples on the same gel, the area was directly used as a densitometric unit for quantitative analysis. Results are all expressed as the mean ± S.E. and the statistical significance was determined by the one-way analysis of variance (ANOVA) with Bonferroni/Dunnett post hoc test.

3. Results

3.1. Changes in AP1 DNA binding by mGluR agonists

In order to examine the expression of mRNA for mGluR isoforms during *in vitro* maturation, RT-PCR analysis was done using primers specific for each mGluR isoform in rat cortical neurons cultured for 3 to 14 DIV. As shown in Fig. 1A, constitutive mRNA expression was seen for all mGluR isoforms in neurons cultured for 3 to 14 DIV. Cortical neurons cultured for 14 DIV were then exposed to one of the mGluR agonists at 100 μM for 5

min, followed by the further incubation for an additional 120 min and subsequent cell harvest for the preparation of nuclear extracts to measure AP1 DNA binding on gel retardation electrophoresis. Of agonists tested, the group II mGluR agonist DCG-IV, but neither the group I mGluR agonist DHPG nor the group III mGluR agonist L-AP4, significantly increased AP1 DNA binding in cultured neurons (Fig. 1B). In cultured rat cortical astrocytes, by contrast, mRNA expression was only seen with mGluR5, mGluR3 and mGluR6 isoforms, but not with other mGluR isoforms (Fig. 2A). Of mGluR agonists tested, only DHPG was efficient in significantly increasing AP1 DNA binding with other mGluR agonists being ineffective in astrocytes (Fig. 2B).

3.2. Activation of group II mGluR subtype in neurons

The brief exposure to DCG-IV significantly increased AP1 DNA binding in a concentration-dependent manner at a concentration range of 1 to 100 μM (Fig. 3A). This increase by DCG-IV was prevented to the control level by the prior addition of the group II antagonist APICA, while APICA alone did not markedly affect AP1 DNA binding (Fig. 3B). Quantitative analysis clearly revealed that DCG-IV significantly increased AP1 DNA binding in an APICA-sensitive manner (Fig. 3C). In cortical neurons cultured for 14 DIV, the cAMP level was 13.7±2.6 fmol/well when determined in the presence of the phosphodiesterase inhibitor IBMX alone. The addition of 10 μM forskolin drastically increased the endogenous level of cAMP to 1139.9±295.9 fmol/well, while the further addition of DCG-IV significantly inhibited the increase by forskolin at 100 μM (424.9±74.4 fmol/well) (Fig. 3D).

3.3. AP1 complex

In order to compare the constituent member proteins of the inducible AP1 complex, cortical neurons were exposed to 100 μM NMDA in the absence of Mg^{2+} ions or 100 μM DCG-IV in the presence of Mg^{2+} ions for 5 min, followed by the further incubation for an additional 120 min and subsequent cell harvest for the determination of AP1 component proteins on Western blot analysis. Both NMDA and DCG-IV drastically increased immunoreactivities to c-Fos protein, while expression of immunoreactive Fra-2 protein was significantly increased by NMDA but not by DCG-IV (Fig. 4A). Neither NMDA nor DCG-IV significantly affected expression of immunoreactive FosB protein. Both NMDA and DCG-IV significantly increased immunoreactivities to JunB protein, while neither DCG-IV nor NMDA significantly affected the expression of immunoreactive c-Jun and JunD (Fig. 4B).

3.4. Possible involvement of intracellular calcium

Cortical neurons were exposed to different calcium blockers 10 min before the exposure to 100 μM DCG-IV for 5 min, followed by the further incubation for an additional 120 min and subsequent cell harvest for the determination of AP1 DNA binding on gel retardation electrophoresis. Prior addition of either the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM) (Fig. 5A) or the L-type voltage-sensitive Ca^{2+} channel blocker nifedipine (Fig. 5C) significantly prevented the increase in AP1 DNA binding by DCG-IV at concentrations above 50 μM. However, the inhibitor of Ca^{2+} release across ryanodine-sensitive Ca^{2+} channel from intracellular stores dantrolene was ineffective in significantly inhibiting the increase by DCG-IV even at the highest concentration used (Fig. 5B). Either BAPTA-AM or nifedipine at 100 μM was also effective in significantly decreasing AP1 DNA binding alone, with dantrolene alone being ineffective.

3.5. Protection against cell death by NMDA

In order to confirm the neuroprotective property of the group II mGluR subtype, cellular viability was determined in neurons exposed to 100 μM NMDA for 30 min in either the presence or absence of 100 μM DCG-IV, followed by the further incubation for an additional 24 h and subsequent determination of MTT reduction. In neurons exposed to NMDA alone in the presence of Mg^{2+}, a slight and statistically insignificant decrease was seen in the MTT reduction activity (Fig. 6A). In the presence of Mg^{2+}, however, a significant increase was induced in the MTT reduction activity by both prior and simultaneous exposures to DCG-IV, but not by the later exposure, compared to the activity in neurons exposed to NMDA alone. In cells exposed to NMDA alone in the absence of Mg^{2+}, by contrast, a significant decrease was seen in the MTT reducing activity, which was significantly prevented by prior and simultaneous, but not later, exposure to DCG-IV (Fig. 6B). Exposure to DCG-IV alone did not significantly affect the activity irrespective of the presence of Mg^{2+}.

4. Discussion

The essential importance of the present findings is that activation of the group II mGluR subtype led to a significant increase in AP1 DNA binding through expression of particular Fos and Jun family member proteins in association with increased intracellular free Ca^{2+} levels in cultured rat neocortical neurons. The data from pharmacological evaluation give support for the possible involvement of voltage-sensitive Ca^{2+} channels, rather than intracellular Ca^{2+} stores, in mechanisms underlying the increase of AP1 DNA binding after activation of the group II mGluR subtype. However, Western blot analysis suggests that the

group II mGluR subtype would increase AP1 DNA binding through a mechanism different from that by NMDA receptor channels highly permeable to this divalent cation. There is accumulating evidence for the correlation between activation of the group I mGluR subtype and expression of the AP1 complex. Activation of the group I mGluR subtype could affect Ca^{2+} homeostasis at multiple levels with expression of both *c-fos* mRNA and c-Fos protein in striatal neurons (Mao and Wang, 2003), for example, while pretreatment with a group I mGluR subtype antagonist completely blocks morphine-induced c-Fos protein expression in the caudate-putamen (Garcia et al., 2003). Selective activation of the mGluR5 isoform induces a rapid and transient phosphorylation of c-Jun N-terminal kinase, followed by the induction of c-Jun protein and subsequent increase in AP1 expression in cultured neurons (Yang et al., 2006). 1S,3R-1-Amino-cyclopentane-1,3-dicarboxylate, a non-selective agonist for mGluRs, induces a rapid and transient expression of c-*fos*, *fosB* and *junB* mRNA, with increasing AP1 DNA binding in primary astroglial cultures (Condorelli et al., 1993). Therefore, it is highly conceivable that activation of the group I mGluR subtype would lead to upregulation of the AP1 complex composed of both Fos and Jun family member proteins through a mechanism related to an increased free Ca^{2+} level mediated by the promoted release from intracellular stores after the activation of this subtype. Since supershift analysis is often insufficient for the clear demonstration of the differential induction profiles, Western blot analysis was employed for the direct demonstration of differential expression profiles of Fos and Jun family member proteins in response to the activation of group II mGluR and NMDA receptor subtypes in the present study.

In addition to the aforementioned group I mGluR subtype linked to the formation of IP_3 responsible for the release of Ca^{2+} from intracellular stores, other mGluR subtypes are also shown to be involved in the expression of AP1 complex in the brain. Administration of a potent and selective agonist for the group II mGluR subtype decreases stress-induced

increases in c-Fos protein expression in the CA3 subfield of the hippocampus, but significantly increases c-Fos protein expression in several other stress-sensitive brain regions (Linden et al., 2004). Systemic administration of an mGluR8 isoform agonist significantly increases c-Fos protein in stress-related brain regions in wild-type mice, but not in mGluR8-null mice (Linden et al., 2003). To our knowledge, this is the first direct demonstration of upregulation of the AP1 complex consisting of c-Fos and JunB proteins through a mechanism relevant to increased intracellular free Ca^{2+} levels after activation of the group II mGluR subtype negatively coupled to the formation of cAMP in cultured rat cortical neurons. From the data cited in the present study, the involvement of voltage-sensitive Ca^{2+} channels in the upregulation is highly conceivable. The exact mechanism as well as the functional significance of the transformation of cAMP signals mediated by the group II mGluR subtype into intracellular Ca^{2+} signals, however, remains to be elucidated in future studies. The presence of two different bands of mGluR3 mRNA in cultured astrocytes could be account for by taking into consideration the possible differential expression between neurons and astrocytes of alternative splicing variants of mGluR3 isoform (Sartorius et al., 2006).

Several independent lines of evidence indicate the facilitation of Ca^{2+} influx across voltage-sensitive Ca^{2+} channels through the phosphorylation mediated by cAMP-dependent protein kinase (PKA). For instance, phosphorylation of the pore-forming subunit (=α1 subunit) (Hell et al., 1993) is shown to induce enhancement of whole-cell Ca^{2+} currents carried by the L-type Ca^{2+} channels in different tissues (Gross et al., 1990; Trautwein and Hescheler, 1990; Hartzell et al., 1991). The present idea that activation of the group II mGluR subtype leads to the promotion of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels toward subsequent induction of the AP1 complex in cultured neurons, by contrast, is not prevailing. The group II mGluR subtype has been indeed believed to decrease the phosphorylation

mediated by PKA through a decreased cAMP level after the negative regulation of adenylyl cyclase in an inhibitory $G_{i/o}$ protein-dependent manner (Cartmell and Schoepp, 2000; Schoepp, 2001; Moldrich et al., 2003; Kenny and Markou, 2004). The differential profile of AP1 complex induction by group II and III mGluR subtypes in cultured neurons and astrocytes, therefore, argues in favor of an idea that the mechanism underlying the transformation of cAMP signals into Ca^{2+} signals in the cytoplasm could be different from cell to cell depending on prevailing conditions.

The reason why the decreased intracellular cAMP level is associated with an increased intracellular free Ca^{2+} level is not clarified so far. Although an unidentified protein could mediate the signal flow from cAMP to voltage-sensitive Ca^{2+} channels in cortical neurons, but not in astrocytes, in addition to PKA, the possibility that the decreased cAMP level is not relevant to the increased intracellular free Ca^{2+} level after activation of the group II mGluR subtype is not ruled out. However, it is unlikely that βγ subunits of the inhibitory $G_{i/o}$ protein would be responsible for the increased intracellular free Ca^{2+} level after activation of the group II mGluR subtype in neurons. In case of $GABA_B$ receptors similarly linked to the inhibitory $G_{i/o}$ protein, for example, an agonist could lead to the inhibition of voltage-sensitive Ca^{2+} channels and the activation of voltage-sensitive K^+ channels through the βγ subunits, in addition to the inhibition of adenylyl cyclase by the α subunit, toward subsequent hyperpolarization in neurons (Bowery et al., 2002). Taken together, cAMP formation should be under the tonic stimulation by particular intracellular signals for an agonist of the group II mGluR subtype negatively linked to adenylyl cyclase to elicit pharmacological actions in any types of cells. An alternative possibility that the group II mGluR subtype might be linked to the inhibitory $G_{i/o}$ protein toward subsequent activation of voltage-sensitive Ca^{2+} channels, nevertheless, is inconceivable.

It thus appears that activation of the group II mGluR subtype would induce the AP1

complex through a mechanism related to an increased intracellular free Ca^{2+} level after the promoted influx across the nifedipine-sensitive channels in cultured neurons. As neuronal cell death would undoubtedly involve mechanisms relevant to overshooting of intracellular free Ca^{2+} levels, elucidation of the protection mechanism could give us a new point of view toward the therapy and treatment of a variety of neurodegenerative disorders in human beings.

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Figure legends

Fig. 1. Effects of mGluR agonists on AP1 DNA binding in cultured neurons. (A) Cortical neurons were cultured for 3 to 14 DIV, followed by extraction of mRNA for RT-PCR using primers specific for each mGluR isoform. PCR products were determined on 2% agarose gel electrophoresis and visualized by ethidium bromide. A representative observation is shown with similar results in 3 independent experiments. (B) Cortical neurons were cultured for 14 DIV and exposed to the mGluR agonists DHPG (group I), DCG-IV (group II) and L-AP4 (group III) at 100 μM for 5 min. After washing, neurons were further incubated for an additional 120 min and harvested for preparation of nuclear extracts to determine AP1 DNA binding on gel retardation electrophoresis. $F_{(3,12)} = 7.756$, $P<0.01$. Values are the mean ± S.E. of percentages over the control value obtained in cells not exposed to any mGluR agonists in 4 independent determinations. **$P<0.01$, significantly different from the control group.

Fig. 2. Effects of mGluR agonists on AP1 DNA binding in cultured astrocytes. (A) Cortical astrocytes were subjected to extraction of mRNA for RT-PCR using primers specific for each mGluR isoform. PCR products were determined on 2% agarose gel electrophoresis and visualized by ethidium bromide. A representative observation is shown with similar results in 3 independent experiments. (B) Cortical astrocytes were exposed to the mGluR agonists DHPG (group I), DCG-IV (group II) and L-AP4 (group III) at 100 μM for 5 min. After washing, cells were further incubated for 120 min and harvested for preparation of nuclear extracts to determine AP1 DNA binding on gel retardation electrophoresis. $F_{(3,12)} = 11.657$, $P<0.01$. Values are the mean ± S.E. of percentages over the control value obtained in astrocytes not exposed to any mGluR agonists in 4 independent determinations. *$P<0.05$, significantly different from the control group.

Fig. 3. Activation of group II mGluR in cultured neurons. Cortical neurons were cultured for 14 DIV, followed by exposure to DCG-IV at a concentration range of 1 to 200 μM for 5 min (A) or DCG-IV at 100 μM for 5 min in either the presence or absence of APICA at 100 μM (B, C). After washing, neurons were further incubated for an additional 120 min and harvested for preparation of nuclear extracts to determine AP1 DNA binding activity on gel retardation electrophoresis. Typical autoradiogram is shown in the panel (B), while quantitative dare are shown as the mean ± S.E. of percentages over the control value obtained in neurons not exposed to DCG-IV in 4 independent determinations in the panel (C). $F_{(3,12)}$ = 6.971, $P<0.01$. *$P<0.05$, significantly different from each control values. $^{\#}P<0.05$, significantly different from the value obtained in neurons exposed to DCG-IV alone. (D) Cortical neurons cultured for 14 DIV were exposed to forskolin (FK) at 10 μM in either the presence or absence of DCG-IV at 100 μM for 5 min. Cells were washed, followed by cell harvest and subsequent measurement of cAMP concentrations. $F_{(2,9)} = 21.859$, $P<0.01$. Values are the mean ± S.E. of percentages over the control value obtained in neurons not exposed to FK in 4 independent determinations. *$P<0.05$, significantly different from each control value. $^{\#}P<0.05$, significantly different from the value obtained in neurons exposed to FK alone.

Fig. 4. Effects of DCG-IV and NMDA on expression of AP1 complex proteins. Cortical neurons cultured for 14 DIV were exposed to DCG-IV or NMDA at 100 μM for 5 min, followed by washing and subsequent culture for an additional 120 min toward SDS-PAGE for Western blotting using antibodies against (A) Fos family member proteins (c-Fos, FosB and Fra2) and (B) Jun family member proteins (c-Jun, JunB and JunD). $F_{(2,9)} = 19.577$, $P<0.01$ for c-Fos; $F_{(2,9)} = 8.999$, $P<0.01$ for Fra-2; $F_{(2,9)} = 42.553$, $P<0.01$ for JunB. Values are the mean ± S.E. of percentages over the control value obtained in neurons not exposed to a GluR

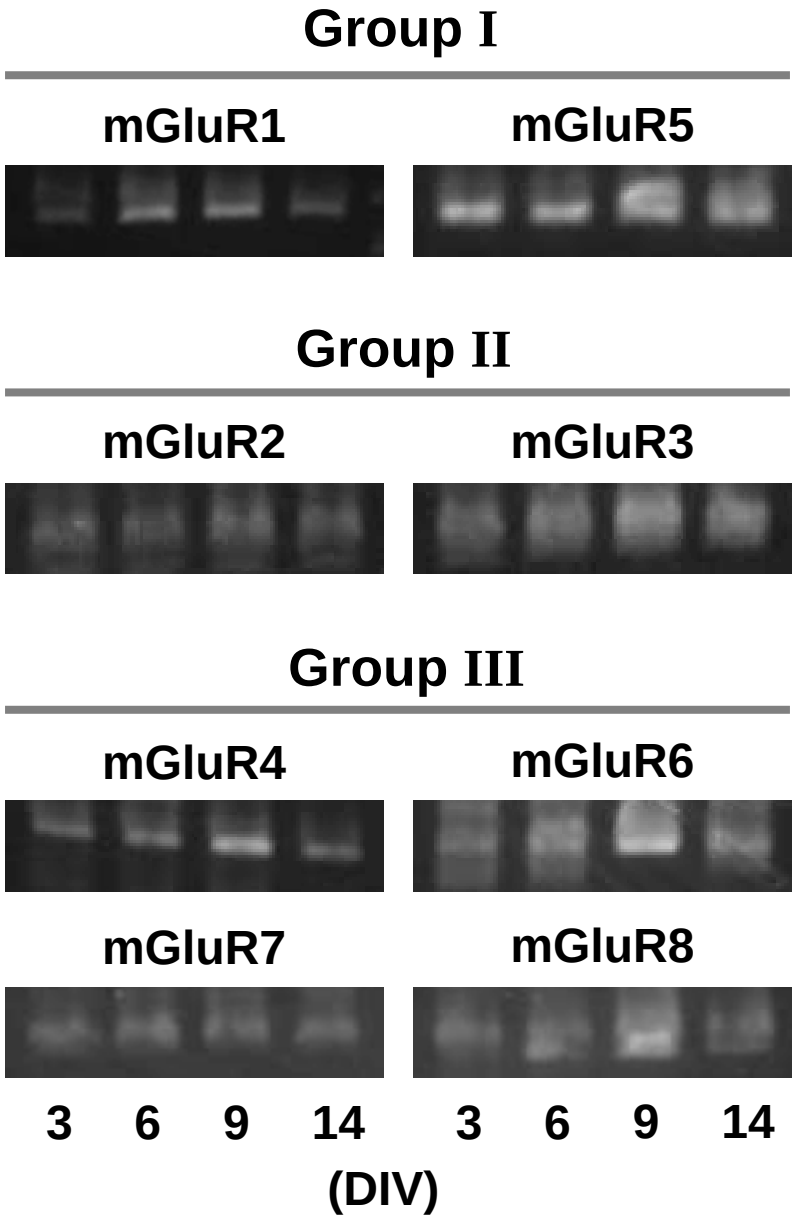
agonist in 4 independent determinations. *P<0.05, **P<0.01, significantly different from each control value.

Fig. 5. Effects of calcium blockers on DCG-IV-induced increase in AP1 DNA binding. Cortical neurons cultured for 14 DIV were exposed to DCG-IV at 100 μM for 5 min in either the presence or absence of (A) BAPTA-AM, (B) dantrolene and (C) nifedipine at a concentration range of 10 to 100 μM. After washing, neurons were further incubated for an additional 120 min and harvested for preparation of nuclear extracts to determine AP1 DNA binding on gel retardation electrophoresis. $F_{(5,18)}$ = 9.381, P<0.01 for BAPTA-AM; $F_{(5,24)}$ = 15.759, P<0.01 for dantrolene; $F_{(5,18)}$ = 3.459, P<0.05 for nifedipine. Values are the mean ± S.E. of percentages over the control value obtained in neurons not exposed to either DCG-IV or Ca^{2+} blocker in 4 or 5 independent determinations. *P<0.05, **P<0.01, significantly different from the control value. $^{\#}$P<0.05, $^{\#\#}$P<0.01, significantly different from the value obtained in neurons exposed to DCG-IV alone.

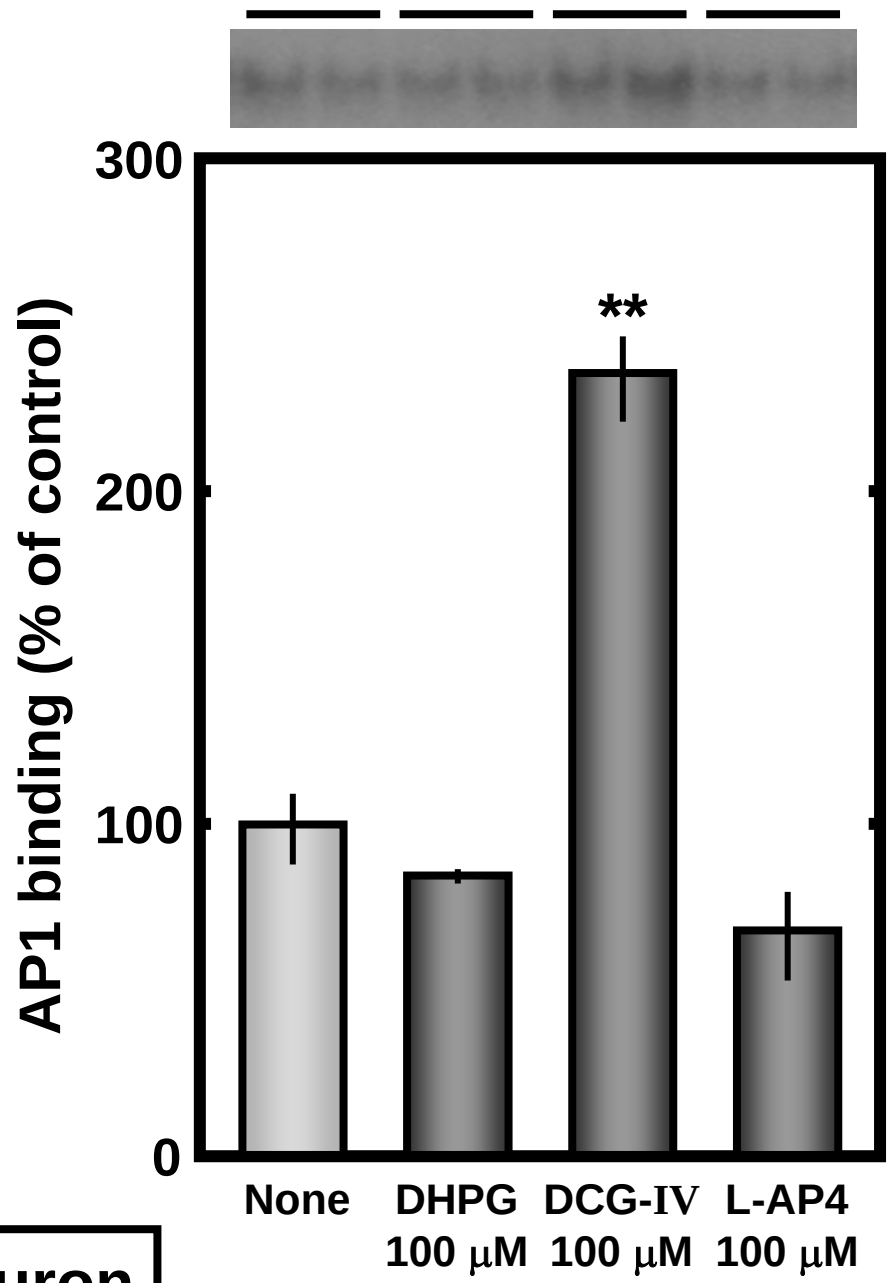
Fig. 6. Effects of DCG-IV on NMDA-induced cell death. Cortical neurons cultured for 14 DIV were exposed to 100 μM NMDA for 30 min in either the (A) presence or (B) absence of Mg^{2+}, followed by the further incubation for an additional 24 h and subsequent determination of cellular viability with MTT reduction assays. Neurons were also exposed to 100 μM DCG-IV 10 min before (pre), simultaneously (sim) or immediately (post) after the exposure to NMDA. $F_{(5,18)}$ = 4.489, P<0.01 for the presence of Mg^{2+}; $F_{(5,18)}$ = 3.562, P<0.05 for the absence of Mg^{2+}. Values are the mean ± S.E. of percentages over the control value obtained in neurons not exposed to either NMDA or DCG-IV in 4 independent determinations. **P<0.01, significantly different from each control value. $^{\#}$P<0.05, $^{\#\#}$P<0.01, significantly different from the value obtained in neurons exposed to NMDA alone.

Fig. 1

(A)



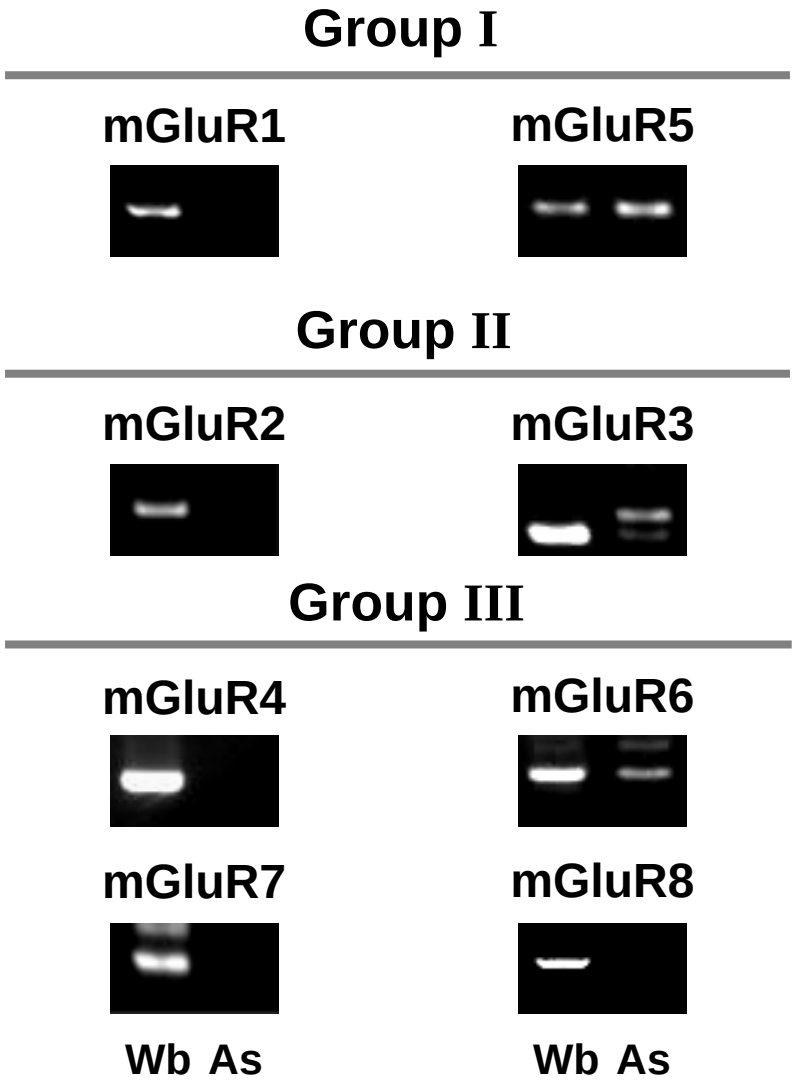
(B)



Cortical neuron

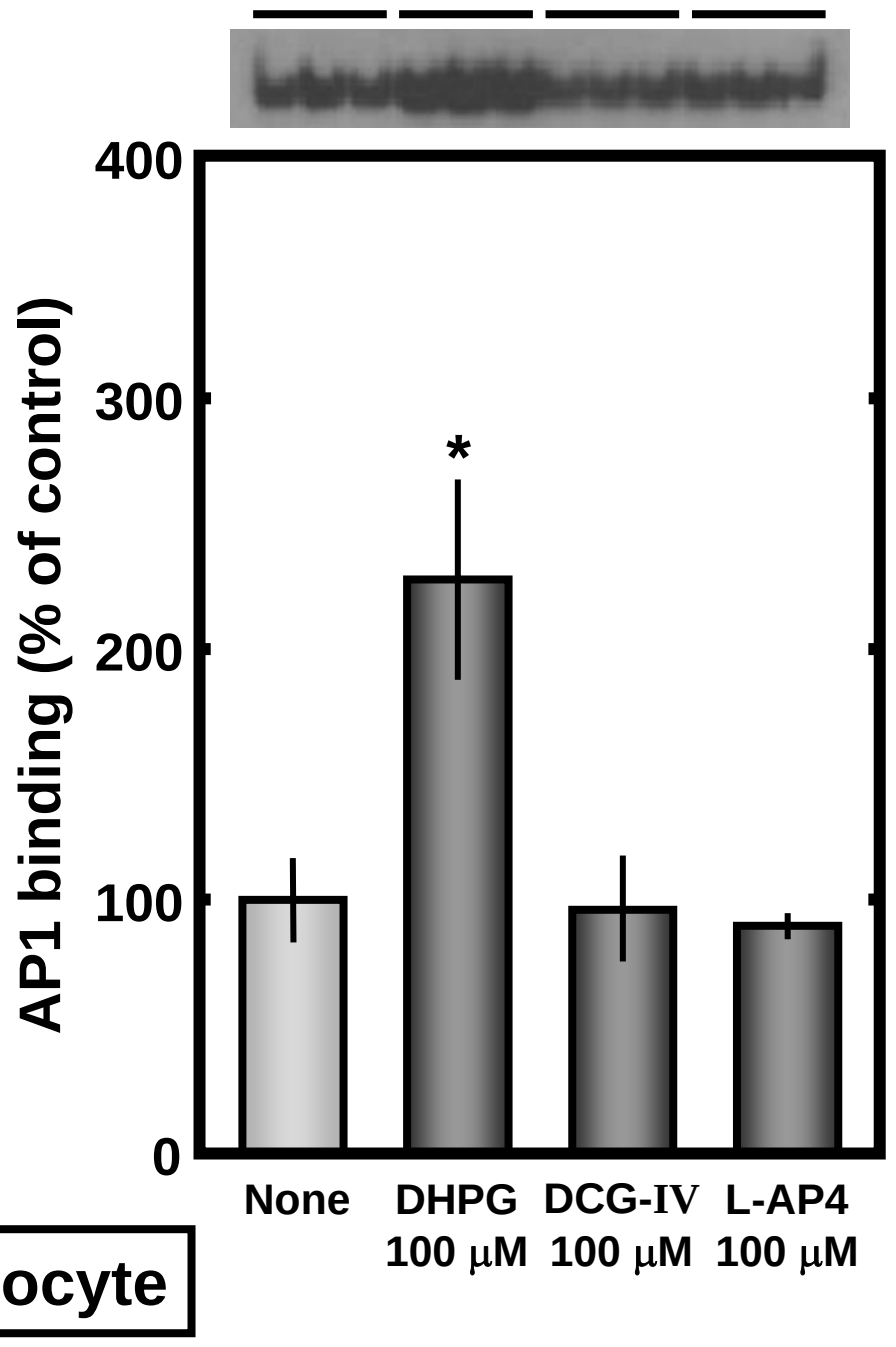
Fig. 2

(A)



Wb: Whole brain As: Astrocyte

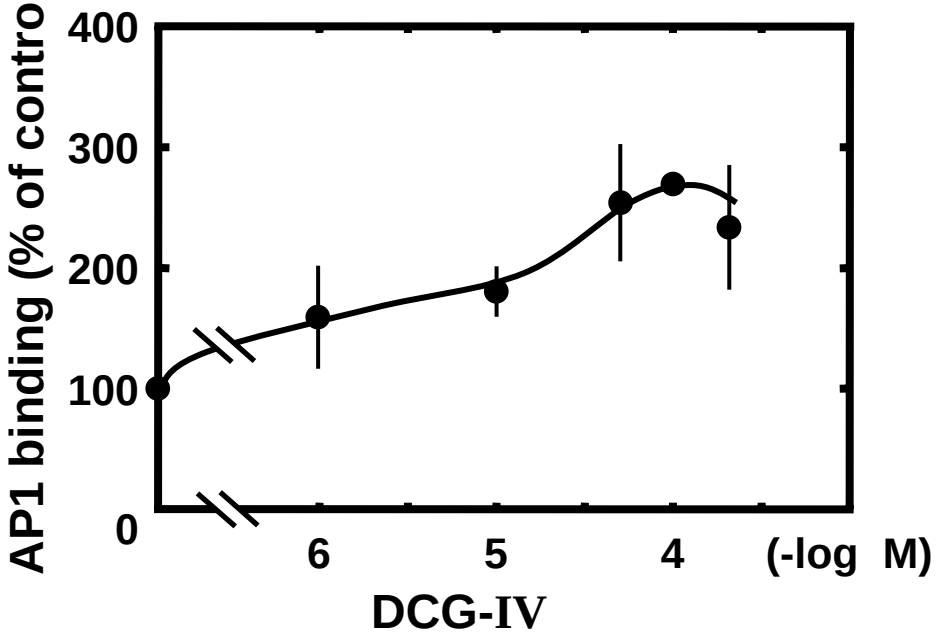
(B)



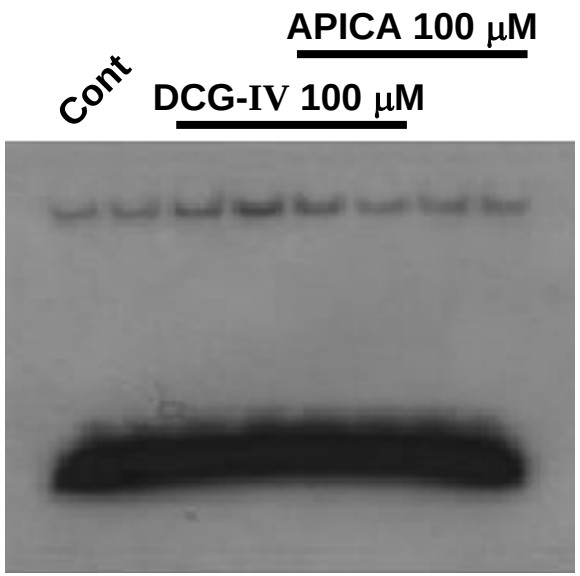
Cortical astrocyte

Fig. 3

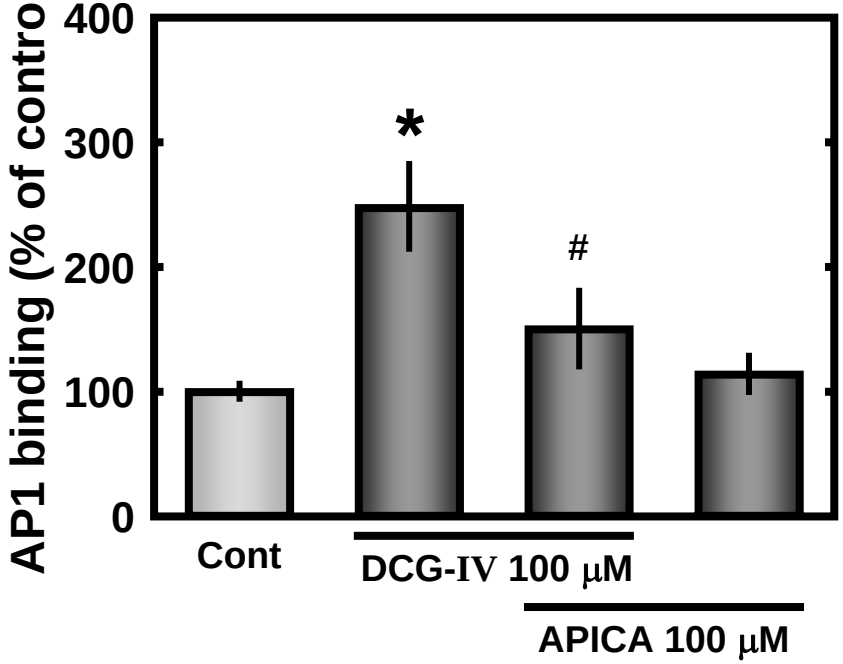
(A)


AP1 binding (% of control)
400
300
200
100
0
6
5
4
(-log M)
DCG-IV

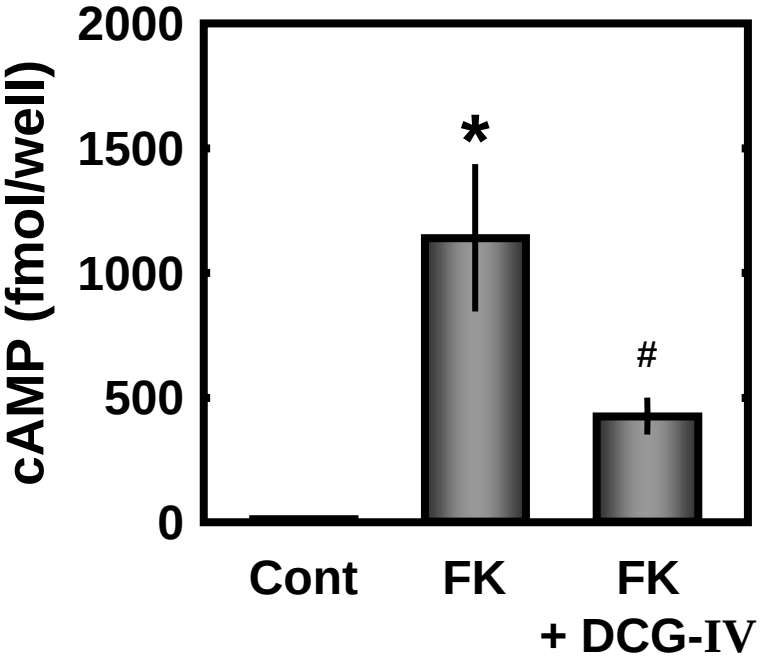
(B)


APICA 100 μM
Cont
DCG-IV 100 μM

(C)


AP1 binding (% of control)
400
300
200
100
0
*
#
Cont
DCG-IV 100 μM
APICA 100 μM

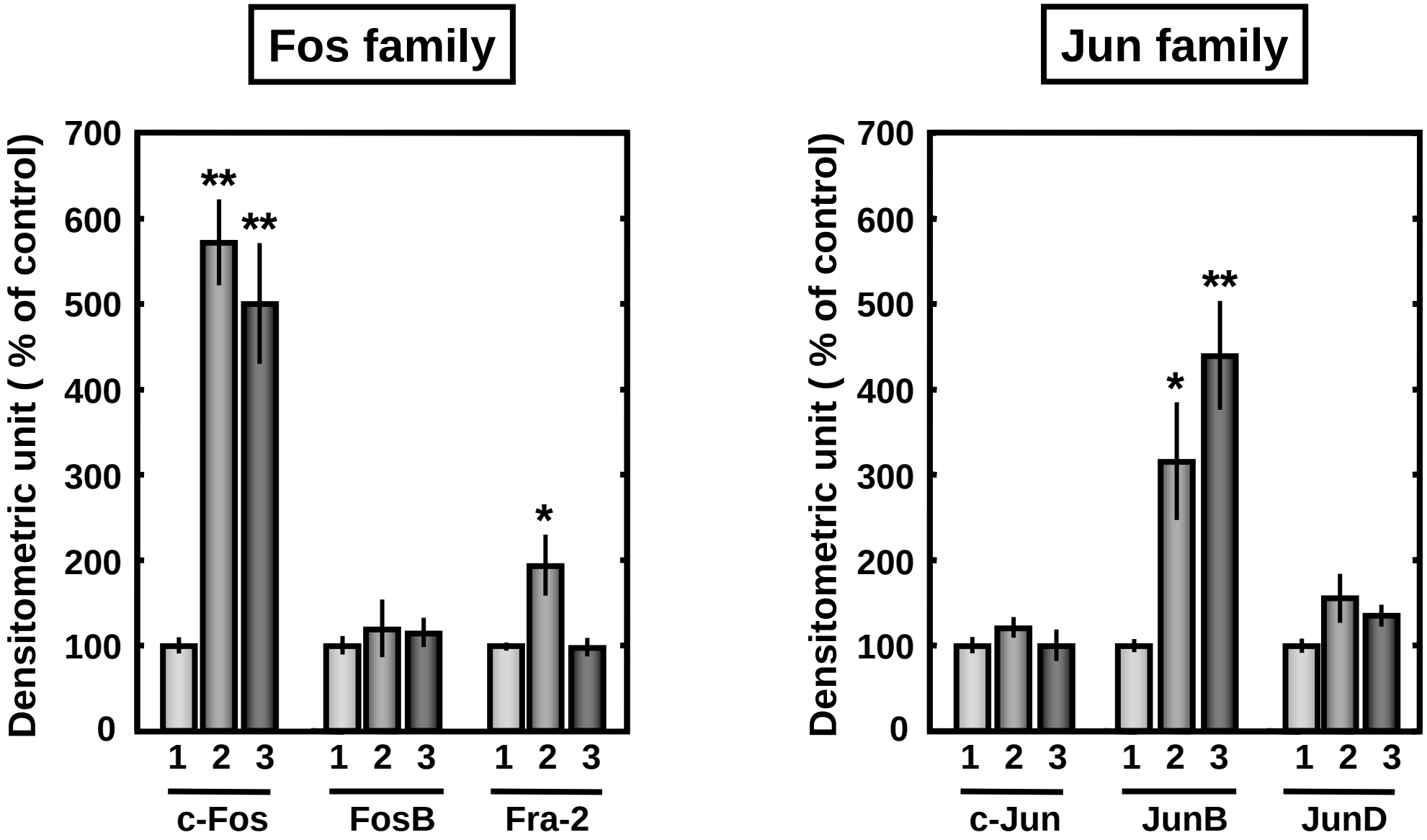
(D)


cAMP (fmol/well)
2000
1500
1000
500
0
*
#
Cont
FK
FK
+ DCG-IV

(A)

(B)

Fig. 4



1. Control 2. NMDA 100 μM 3. DCG-IV 100 μM

Fig. 5

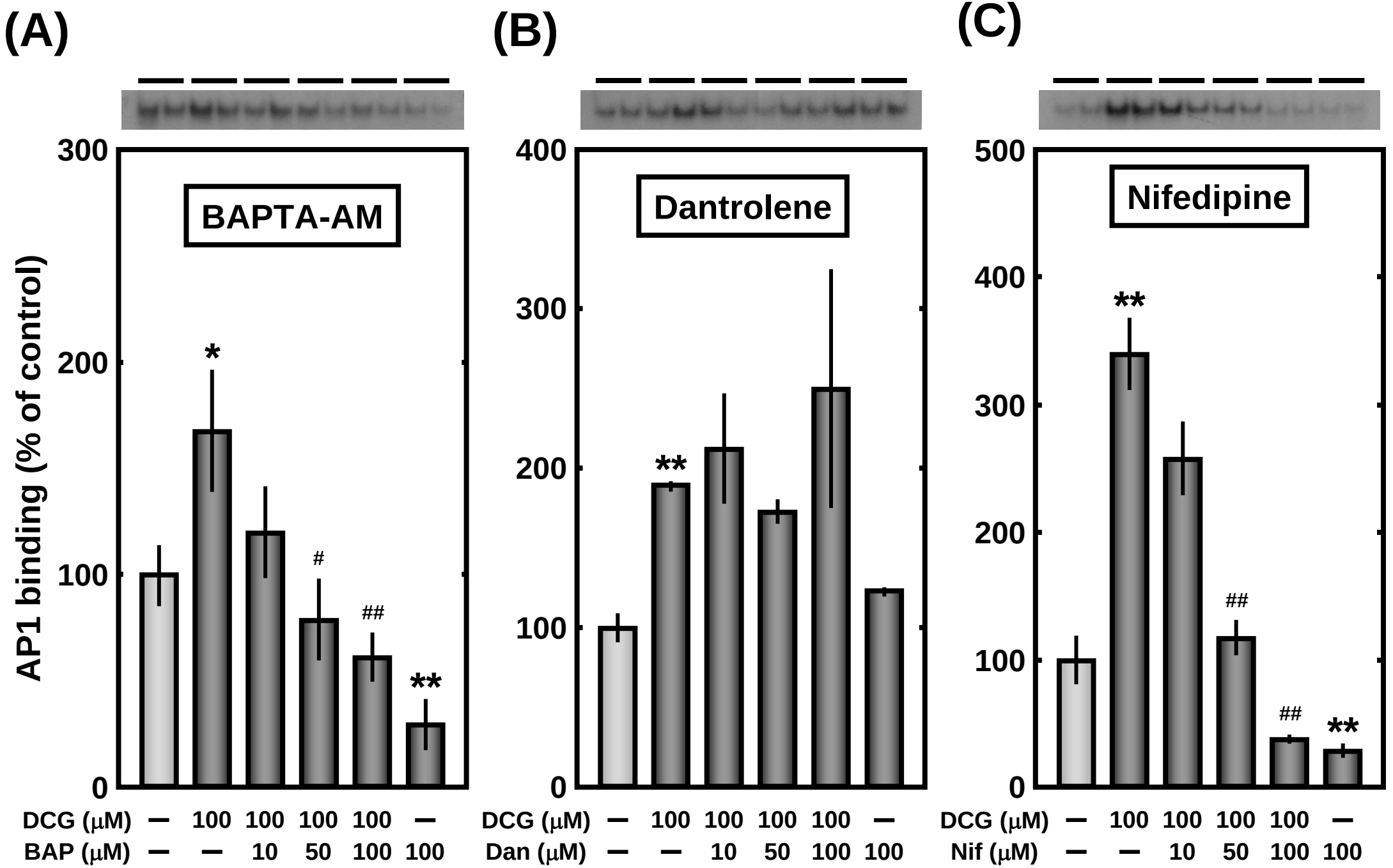
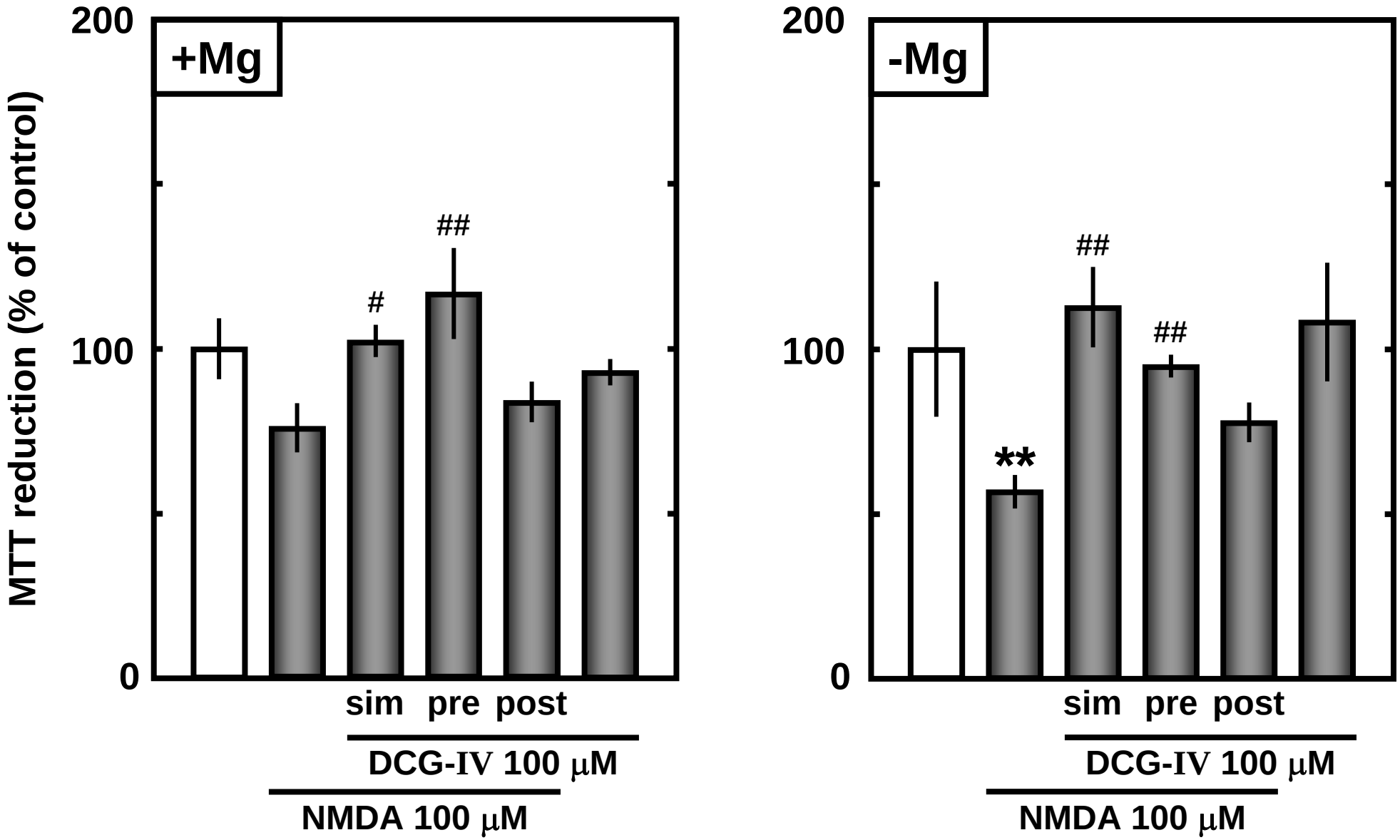

(A)
(B)
(C)
BAPTA-AM
Dantrolene
Nifedipine
AP1 binding (% of control)
300
200
100
0
400
500
*
**
#
##
DCG (μM) — 100 100 100 100 —
BAP (μM) — — 10 50 100 100
Dan (μM) — — 10 50 100 100
Nif (μM) — — 10 50 100 100

Fig. 6


(A)
+Mg
200
100
0
MTT reduction (% of control)
#
##
sim
pre
post
DCG-IV 100 μM
NMDA 100 μM
(B)
-Mg
200
100
0
**
##
##
sim
pre
post
DCG-IV 100 μM
NMDA 100 μM