Cellular/Molecular

The Scaffold Protein Homer1b/c Links Metabotropic Glutamate Receptor 5 to Extracellular Signal-Regulated Protein Kinase Cascades in Neurons

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Group I metabotropic glutamate receptors (mGluRs) increase cellular levels of inositol-1,4,5-triphosphate (IP_3) and thereby trigger intracellular Ca^{2+} release. Also, group I mGluRs are organized with members of Homer scaffold proteins into multiprotein complexes involved in postreceptor signaling. In this study, we investigated the relative importance of the IP_3/Ca^{2+} signaling and novel Homer proteins in group I mGluR-mediated activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in cultured rat striatal neurons. We found that selective activation of mGluR5, but not mGluR1, increased ERK1/2 phosphorylation. Whereas the IP_3/Ca^{2+} cascade transmits a small portion of signals from mGluR5 to ERK1/2, the member of Homer family Homer1b/c forms a central signaling pathway linking mGluR5 to ERK1/2 in a Ca^{2+}-independent manner. This was demonstrated by the findings that the mGluR5-mediated ERK1/2 phosphorylation was mostly reduced by a cell-permeable Tat-fusion peptide that selectively disrupted the interaction of mGluR5 with the Homer1b/c and by small interfering RNAs that selectively knocked down cellular levels of Homer1b/c proteins. Furthermore, ERK1/2, when only coactivated by both IP_3/Ca^{2+}- and Homer1b/c-dependent pathways, showed the ability to phosphorylate two transcription factors, Elk-1 and cAMP response element-binding protein, and thereby facilitated c-Fos expression. Together, we have identified two coordinated signaling pathways (a conventional IP_3/Ca^{2+} vs a novel Homer pathway) that differentially mediate the mGluR5-ERK coupling in neurons. Both the Ca^{2+}-dependent and -independent pathways are corequired to activate ERK1/2 to a level sufficient to achieve the mGluR5-dependent synapse-to-nucleus communication imperative for the transcriptional regulation.

Key words: **CREB; Elk-1; Fos; calcium; PSD-95; striatum; nucleus accumbens**

Introduction

L-Glutamate (glutamate) is a major excitatory neurotransmitter in brain. Through interacting with ionotropic or G-protein-coupled receptors, glutamate vigorously modulates cell survival, synaptic plasticity, gene expression, and many other neuronal activities (Ozawa et al., 1998; Dingledine et al., 1999). Among three groups of metabotropic glutamate receptors (mGluRs), group I mGluRs (mGluR1/5) have drawn the most attention as a result of their wide distribution in brain and active roles in regulating multiple signaling systems. Agonist binding of these receptors increases hydrolysis of membrane phosphoinositide (PI) via activating phospholipase Cβ1 (PLCβ1). This yields diacylglycerol (DCG), which activates protein kinase C (PKC), and inositol-1,4,5-triphosphate (IP_3), which releases intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Nakanishi, 1994; Conn and Pin, 1997; Mao and Wang, 2003a). Altered PKC and Ca^{2+} signals could then engage in the modulation of various metabotropic activities.

In addition to the conventional PI-dependent signaling, group I mGluRs are organized with other scaffolding/signaling proteins into multiprotein complexes in the postsynaptic density (PSD) (Xiao et al., 2000; Sheng and Kim, 2002; Thomas, 2002). A prominent organizing protein in complexes is Homer (Brakeman et al., 1997). Through long C-terminal intracellular tails, group I mGluRs bind the N-terminal EVH1 (Enabled/vasodilator-stimulated phosphoprotein homology 1) domain of all members of three Homer families, including an inducible immediate early gene, *Homer1a*, and the constitutively expressed longer form of Homer proteins, Homer1b/c, Homer2a/b, and Homer3 (Brakeman et al., 1997; Xiao et al., 1998, 2000). Because the longer form of Homer proteins contains a C-terminal coiled-coil structure and leucine zipper motifs rendering a capability for self-assembly (Xiao et al., 1998), these proteins are thought to link group I mGluRs to specific cellular regions for the regulation of a specific signaling activity. Indeed, emerging evidence indicates that the coordinated interaction of group I mGluRs with adaptor Homer proteins plays essential roles in the membrane trafficking of mGluR1α/5 (Ciruela et al., 1999, 2000; Roche et al., 1999; Tadokoro et al., 1999; Ango et al., 2000, 2002), the coupling of mGluR1/5 with IP_3 receptors (Tu et al., 1998), the signaling from mGluRs to the N-type Ca^{2+} channel and M-type K^+ channel (Kammermeier et al., 2000), and the development of spines,

Received Oct. 20, 2004; revised Jan. 13, 2005; accepted Jan. 31, 2005.

This work was supported by National Institutes of Health Grants R01DA010355 (J.Q.W.) and R01MH061469 (J.Q.W.).

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DOI:10.1523/JNEUROSCI.4360-04.2005

 0270-6474/05/252741-12$15.00/0

axons, and synapses (Shiraishi et al., 1999, 2003; Foa et al., 2001; Sala et al., 2001).

Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are expressed in postmitotic neurons and are activated through phosphorylation on their Thr^{202} and Tyr^{204} sites in response to various extracellular stimuli (Nozaki et al., 2001; Peyssonnaux and Eychene, 2001; Volmat and Pouyssegur, 2001). Glutamate is among effective extracellular signals that readily activate ERK1/2 (Wang et al., 2004). Agonist ligands for ionotropic NMDA or AMPA receptors activate ERK1/2 (Sgambato et al., 1998; Mao et al., 2004), probably via a signaling mechanism involving Ca^{2+} and Ca^{2+}-sensitive kinases (Perkinton et al., 1999, 2002). Similarly, the group I mGluR selective agonists activate ERK1/2 in neurons and cell lines (Vanhoutte et al., 1999; Thandi et al., 2002). However, the postreceptor signaling cascades transmitting group I mGluR signals to ERK1/2 are poorly understood at present.

In this study, we examined the relative importance of group I mGluR-linked PI hydrolysis/Ca^{2+} release and adaptor Homer proteins in mediating the group I mGluR signals to ERK1/2 in cultured striatal neurons. We found that Homer1b/c proteins are significantly involved in linking mGluR5 to ERK1/2. Furthermore, ERK1/2 activated simultaneously by both PI/Ca^{2+} and Homer pathways participate in an efficient cascade for a synapse-to-nucleus communication implicated in the facilitatory regulation of gene expression.

Materials and Methods

Primary striatal neuronal cultures. Standardized procedures in this laboratory were used to prepare primary striatal neuronal cultures from 18 d rat embryos or neonatal 1-d-old rat pups (Charles River Laboratories, New York, NY) (Mao and Wang, 2002; Yang et al., 2004; Mao et al., 2005). Predominant GABAergic neurons were obtained using the procedures as evidenced by the fact that > 90% of total cells were immunoreactive to glutamic acid decarboxylase-65/67, GABA, and the specific marker for neurons [microtubule-associated protein-2a + 2b (MAP2)] but not for glia (glial fibrillary acidic protein). Cells were cultured for 15–18 d before use.

Western blot analysis. Cell lysates from cultures were sonicated, and protein concentrations were determined. The equal amount of protein (20 μg/20 μl per lane) was separated on NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk in PBS and 0.1% Tween 20) for 1 h. The blots were incubated in primary rabbit polyclonal antibodies against phosphorylated ERK (pERK) 1/2(Thr^{202}/Tyr^{204}) (1:1000; Cell Signaling Technology, Beverly, MA), ERK1/2 (1:1000; Cell Signaling Technology), phosphorylated cAMP response element-binding protein (pCREB) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), CREB (1:500; Santa Cruz Biotechnology), pElk-1 (1:100; Santa Cruz Biotechnology), Elk-1 (1:500; Santa Cruz Biotechnology), c-Fos (1:1000; Oncogene Research Products, San Diego, CA), Homer2a/b (1:500; Santa Cruz Biotechnology), or rat antibodies against Homer1b/c (1:1000; Chemicon, Temecula, CA) overnight at 4°C. This was followed by 1 h incubation in goat anti-rabbit or anti-rat horseradish peroxidase (HRP)-linked secondary antibodies (Jackson ImmunoResearch, West Grove, PA) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ) and captured into Kodak (Eastman Kodak, Rochester, NY) Image Station 2000R. Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software, and all bands were normalized to percentages of control values.

Immunofluorescent labeling. Single or double immunofluorescent labeling on eight-chamber glass slides was performed as described previously (Mao and Wang, 2002; Yang et al., 2004). Briefly, cultures were fixed in cold 4% paraformaldehyde (10 min), followed by incubation in 4% normal donkey serum and 1% bovine serum album (20 min) to block nonspecific staining. The cells were treated with primary antibodies against mGluR5 (rabbit; 1:400; Upstate Biotechnology, Charlottesville, VA), Homer1b/c (rat; 1:400; Chemicon), or Shank 1/2/3 (C-20) (goat; 1:500; Santa Cruz Biotechnology) for 2 nights at 4°C. Sections were then incubated for 1 h with donkey anti-rabbit, anti-rat, or anti-goat secondary antibodies conjugated to FITC at 1:200. For double labeling, the cells were treated with a mixture of primary antibodies containing rabbit anti-pERK1/2 or anti-ERK1/2 antibodies (1:100; Cell Signaling Technology) and mouse anti-MAP2 antibodies (1:250; Chemicon) overnight at 4°C. Sections were then incubated for 1 h with donkey anti-rabbit secondary antibodies conjugated to FITC (Jackson ImmunoResearch) and donkey anti-mouse secondary antibodies conjugated to tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch) at 1:200. The immunofluorescent images were analyzed using confocal microscopy (Nikon C1 laser scanning confocal microscope; Nikon, Tokyo, Japan).

Coimmunoprecipitation. Rat striatal cell proteins were prepared under weakly denaturing conditions to permit the mGluR5/Homer interaction (Takagi et al., 2000). Briefly, striatal cultures were scraped into a microtube containing ice-cold sample buffer containing the following (in mM): 10 Tris-HCl, pH 7.4, 5 NaF, 1 Na_3VO_4, 1 EDTA, and 1 EGTA and were homogenized by sonication. The homogenate was centrifuged at 800 × *g* (10 min) at 4°C. The supernatant was again centrifuged at 11,000 × *g* at 4°C for 30 min to obtain the P2 pellet, a fraction enriched with synaptic structures. The P2 pellet was resuspended in sample buffer and solubilized in 1% sodium deoxycholate. After incubation at 37°C for 30 min, Triton X-100 was added to a final concentration of 0.1%. Insoluble proteins were sedimented at 100,000 × *g* at 4°C for 20 min. The supernatants were used for coimmunoprecipitation. Group I mGluR subunits were then precipitated using rabbit polyclonal antibodies against mGluR1 or mGluR5 (Upstate Biotechnology) and 50% protein A agarose/Sepharose bead slurry (Amersham Biosciences). Proteins were separated on Novex 4–12% gels and probed with rabbit antibodies against mGluR1 (Upstate Biotechnology), mGluR5 (Upstate Biotechnology), or Homer2a/b (Santa Cruz Biotechnology) or rat anti-Homer1b/c (Chemicon) antibodies. HRP-conjugated secondary antibodies and enhanced chemiluminescence were used to detect proteins. Negative controls with antigen preabsorption were performed for antibodies used in immunoprecipitation.

PI hydrolysis. PI hydrolysis was analyzed by measuring the concentration of IP_3 with the IP_3 assay supplement kit from Perkin-Elmer (Palo Alto, CA), according to manufacturer's instructions. Cells were washed with 0.5 ml of HEPES-buffered balanced salt solution (HBS) consisting of the following (in mM): 154 NaCl, 5.6 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 5.5 glucose, and 20 HEPES-KOH or HEPES-NaOH, pH 7.4, and were incubated in this solution. LiCl (10 mM) was added for 30 min before agonist addition. After drug treatment, the solutions were aspirated, and ice-cold methanol was added to terminate the reaction. Cells were scraped, and the samples were sonicated briefly before the aqueous and organic phases were separated by centrifugation at 4000 rpm for 10 min. The upper aqueous phase was analyzed for the concentration of IP_3 with an addition of glutathione *S*-transferase (GST)-tagged IP_3 binding protein followed by an addition of detection mix containing a biotinylated IP_3 analog, streptavidin-coated donor beads, and anti-GST-conjugated acceptor beads.

$[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ measurements were performed with fura-2 fluorescence according to our previous procedures (Mao and Wang, 2002; Yang et al., 2004). The $[Ca^{2+}]_i$ concentration was calculated from ratios of the intensities of emitted fluorescence at two excitation wavelengths (*F*340/*F*380) with Northern Eclipse Image software (Empix Imaging, Mississauga, Ontario, Canada). When needed, fluorescence ratios (340/380) were converted to an absolute $[Ca^{2+}]_i$ concentration using the following equation: $[Ca^{2+}]_i = K_d(F_{min}/F_{max})[(R - R_{min})/(R_{max} - R)]$.

Cell viability assay. Cell viability was measured using a double fluorescein diacetate/propidium iodide staining procedure (Jones and Senft, 1985). Fluorescein diacetate freely enters intact cells, in which it is converted to membrane-impermeable green fluorescein, exhibited only by live cells. Propidium iodide is nonpermeable to live cells but penetrates the membranes of dying/dead cells, showing red fluorescence. Cells were

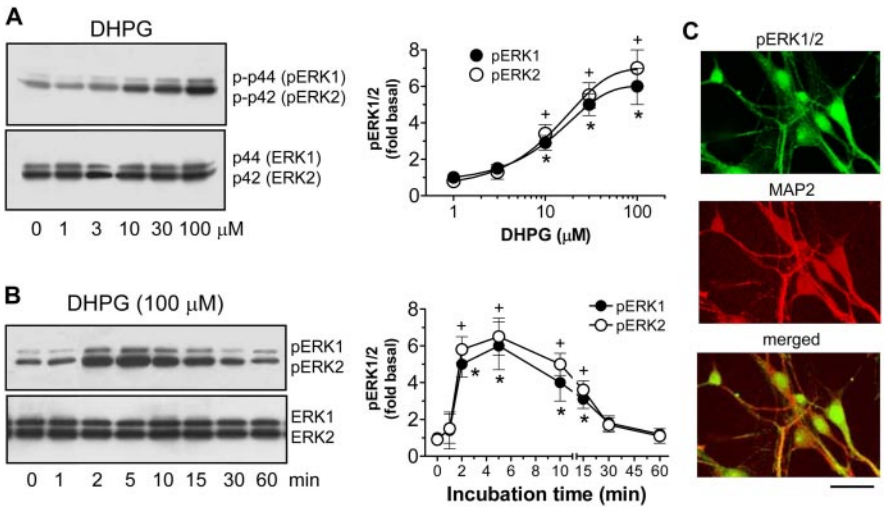

Figure 1. ERK1/2 phosphorylation by activation of group I mGluRs with DHPG in cultured rat striatal neurons. ***A***, DHPG (1–100 μM; 5 min) concentration-dependently increased pERK1/2, but not ERK1/2, levels. Representative immunoblots are shown left of the quantified data of pERK1/2 analyzed from separate experiments (mean ± SEM; $n = 6$). ***B***, DHPG caused a rapid and transient increase in ERK1/2 phosphorylation without altering ERK1/2 levels. DHPG at 100 μM was added to cultures and incubated for different durations. Representative immunoblots are shown left of the quantified data (mean ± SEM; $n = 5$). ***C***, Confocal immunofluorescent images illustrating subcellular distributions of pERK1/2 induced by DHPG (100 μM; 5 min). Strong pERK1/2 immunostaining (green) was seen in the nucleus and weak to moderate staining in the cytoplasm and neural processes of neurons were identified by the neuron-specific marker MAP2 (red). Scale bar, 30 μm. * (pERK1) and + (pERK2) represent $p < 0.05$ versus basal levels.

rinsed twice with 1× PBS and incubated at 37°C for 5 min with 1× PBS (0.5 ml per well) containing 10 μg/ml fluorescein diacetate (Sigma, St. Louis, MO) and 5 μg/ml propidium iodide (Sigma). Cultures were washed once with PBS and examined under fluorescent light microscopy. The total numbers of viable cells stained by green fluorescein and dead cells stained by red propidium iodide were determined by counting cells in five random fields. Positive control was produced by treating cultures with kainic acid (500–1000 μM; 24 h)

Transient transfections of small interfering RNAs. The small interfering RNA (siRNA) duplexes were synthesized and annealed by Qiagen (Valencia, CA) with 5′ phosphate, 3′ hydroxyl, and two base (dTdT) overhangs on 3′ of each strand. The siRNA duplex sequences target the Homer1b/c mRNA (Brakeman et al., 1997), sense 5′-UCAGUCUAGACGGCUCAAA-3′ and antisense 5′-UUUGAGCCGUCUAGACUGA-3′, and the Homer2a/b mRNA (Kato et al., 1998), sense 5′-UCGAGACGUCAAGUAAUCA-3′ and antisense 5′-UGAUUACUUGACGUCUCGA-3′. Both the antisense and sense sequences were analyzed by an advanced basic local alignment search tool search for matches with other known genes, and results show no substantial similarity to other sequences present in the National Center for Biotechnology Information database. The control siRNAs were purchased from Qiagen.

Transfections of siRNAs were made by an optimized procedure described in our previous report (Yang et al., 2004). Briefly, the transfections were made on a 24-well plate with the cationic lipid Lipofectamine 2000 (Invitrogen). Following the manufacturer's instructions, 1 μl of Lipofectamine was diluted in 50 μl of antibiotic-free and serum-reduced Opti-MEM I medium (Invitrogen) and incubated for 5 min at room temperature. A total of 1 μg of siRNAs was mixed in Opti-MEM I (50 μl) and was added to the Lipofectamine mixture, and incubation continued for 20 min at room temperature. Cells were washed quickly with Opti-MEM I three times before addition of the above mixed medium. Cells were transfected for 2–4 h at 37°C, followed by three rinses in Opti-MEM I. Cells were then incubated in growth medium until analysis. Cells were lysed for examining Homer1b/c or Homer2a/b levels or used to detect ERK1/2 phosphorylation in response to drug treatments 24 h after transfections.

Drug treatments. Cultures were washed with PBS and preincubated at 37°C in HBS for 60 min. For NMDA treatments, $MgSO_4$ was omitted from, and 10 μM glycine was added to, the HBS. For extracellular Ca^{2+} dependency studies, $CaCl_2$ was removed from the HBS with substitution of a Ca^{2+} chelator EGTA (2 mM). To have parameters comparable, none of the HBSs contained sodium bicarbonate. Cells were treated by adding freshly made drugs to the HBS. At the end of drug treatment, the cells were washed quickly with ice-cold PBS, pH 7.4 (Ca^{2+}-free), and placed immediately on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer. Drugs were dissolved in 1× PBS with or without dimethyl sulfoxide (DMSO). Whenever DMSO was used, PBS containing the same concentration of DMSO was used as the control vehicle.

Materials. NMDA, AMPA, (+)-5-methyl-10, 11-dihydro-5H-dibenzo [a,d] cyclohepten-5, 10-imine maleate (MK801), DL-2-amino-5-phosphonovaleric acid (AP-5), 4-(8-methyl-9*H*-1,3-dioxolo[4,5-h][2,3]benzodazepin-5-yl)-benzenamine dihydrochloride (GYKI52466), (*RS*)-3,5-dihydroxyphenylglycine (DHPG), (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), 2-methyl-6-(phenylethynyl)pyridine hydrocholoride (MPEP), 7-(hydroxyimino)cy-clopropa[b]chromen-1*a*-carboxylate ethyl ester (CPCCOet), nifedipine, thapsigargin, and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) were purchased from Tocris Cookson (Ballwin, MO). 1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and 3-[1-3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide (Ro-31–8220) were purchased from Calbiochem (La Jolla, CA). L-Glutamate, 2,5-dimethyl-4-[2-(phenylmethyl)ben-zoyl]-1*H*-pyrrole-3-carboxylic acid methylester (FPL64176), tetrodotoxin (TTX), 2-[1-(3-dimethyl-aminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide (Gö6983), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Fura-2 AM fluorescent Ca^{2+} indicator was purchased from Molecular Probes (Eugene, OR). The membrane-permeable mGluR5 C-terminal Homer-binding (decoy) peptide, Tat-mGluR5ct (YGRKKRRQRRRALTPPSPFR), containing a proline-rich binding motif (PPxxF) (Tu et al., 1998), and its control peptide, Tat-mGluR5mu (YGRKKRRQRRRALTPLSPRR), with a dual point mutation in the binding motif rendering it incapable of binding Homer, were synthesized by Invitrogen. Both peptides gain cell permeability by containing an arginine-enriched cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein (YGRKKRRQRRR) (Schwarze et al., 1999). A Tat control peptide was synthesized, which comprises HIV-1 Tat residues 38–48 (KALGISYGRKK; Tat38–48) outside the transduction domain (Mann and Frankel, 1991).

Statistics. The results are presented as mean ± SEM and were evaluated using a one- or two-way ANOVA, as appropriate, followed by a Bonferroni's (Dunn) comparison of groups using least squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

Results

Activation of group I mGluRs increases ERK1/2 phosphorylation

Activation of group I mGluRs with the selective agonist DHPG increased phosphorylation of ERK1/2 in cultured striatal neurons (Fig. 1*A*). At low concentrations (1 and 3 μM), DHPG did not alter basal levels of pERK1/2. At 10 μM, DHPG significantly increased pERK1/2 levels. A greater increase in pERK1/2 levels was seen at two higher concentrations (30 and 100 μM). No significant changes were seen in cellular levels of ERK after DHPG application at all concentrations surveyed. These data show a dose-dependent increase in ERK1/2 phosphorylation after acti-

vation of DHPG-sensitive group I mGluRs. To evaluate the time course of the pERK1/2 induction, DHPG at 100 μM was added to cultures for different durations. As shown in Figure 1*B*, a rapid and transient increase in ERK1/2 phosphorylation occurred with no changes in ERK1/2 levels. A different time course evaluation was also performed, in which 100 μM DHPG was incubated for 2 min, and cultures were then lysed 3, 8, or 30 min after the termination of DHPG incubation. The data from this study (data not shown) were similar to those obtained by the DHPG incubation at different durations. To define the subcellular distribution of activated pERK1/2, immunofluorescent labeling was performed after DHPG (100 μM; 5 min). Strong pERK1/2 immunostaining was revealed within the nucleus, and weak to moderate staining was revealed in the cytoplasm and neural processes (Fig. 1*C*). In addition, pERK1/2-positive neurons were immunoreactive to the neuron-specific marker MAP2 (Fig. 1*C*), indicating that the ERK1/2 phosphorylation occurred in neuronal cells.

To examine whether NMDA receptors contribute to the DHPG-stimulated ERK1/2 phosphorylation, we evaluated the effect of the NMDA receptor selective antagonists on DHPG action. Pretreatment with the competitive or noncompetitive NMDA receptor antagonists, AP-5 or MK801, respectively, at a dose (50 μM for AP-5 and 1 μM for MK801) sufficient to block the NMDA-induced ERK1/2 phosphorylation, did not change the ERK1/2 phosphorylation induced by DHPG (Fig. 2*A*). Thus, NMDA receptors are insignificant in the DHPG effect. We then evaluated the effect of the AMPA receptor-selective antagonists on the DHPG effect. The AMPA receptor antagonist GYKI52466 (100 μM) did not block the ERK1/2 phosphorylation induced by DHPG, whereas GYKI52466 blocked the AMPA-induced ERK1/2 phosphorylation (Fig. 2*B*). Neither did another AMPA receptor antagonist DNQX at 100 μM, a dose sufficient to block the AMPA phosphorylation of ERK1/2 (data not shown). Thus, like NMDA receptors, AMPA receptors are not involved in the DHPG effect. Finally, L-type voltage-operated Ca^{2+} channels (VOCC) were investigated for their importance for the DHPG effect. We found that the VOCC selective inhibitor nifedipine at 20 μM, which was effective to block the VOCC activator FPL64176-stimulated ERK1/2 phosphorylation, did not alter the hyperphosphorylation of ERK1/2 induced by DHPG (Fig. 2*C*). Thus, the VOCC may not be an important component in the DHPG phosphorylation of ERK1/2.

Cell firing induced by DHPG could cause a release of an assortment of transmitters and modulators in the culture, leading to activation of receptors other than group I mGluRs to account for the observed changes in ERK1/2 phosphorylation. However, this possibility seems less likely because blockade of action potential firing by TTX (1 μM), a Na^+ channel blocker, did not effect the DHPG-induced ERK1/2 phosphorylation (data not shown).

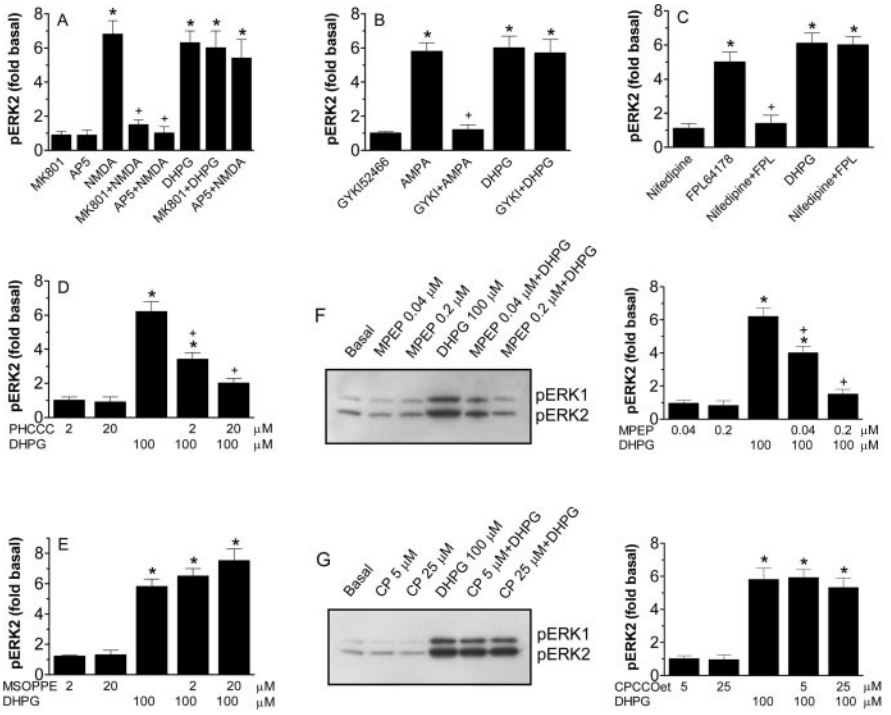

Figure 2. Effects of the antagonists selective for NMDA receptors (AP-5 and MK801; ***A***), AMPA receptors [GYKI52466 (GYKI); ***B***], VOCCs (nifedipine; ***C***), group I mGluRs (PHCCC; ***D***), group II/III mGluRs (MSOPPE; ***E***), mGluR5 (MPEP; ***F***), or mGluR1 [CPCCOet (CP); ***G***] on basal and DHPG-induced ERK1/2 phosphorylation in cultured rat striatal neurons are shown. AP-5 (50 μM), MK801 (1 μM), GYKI52466 (100 μM), nifedipine (20 μM), PHCCC (2 or 20 μM), MSOPPE (2 or 20 μM), MPEP (0.04 or 0.2 μM), or CPCCOet (5 or 25 μM) was incubated 30 min before and during 5 min treatment with NMDA (100 μM), AMPA (100 μM), the VOCC activator FPL64176 (FPL; 20 μM), or DHPG (100 μM). Note that AP-5, MK801, GYKI52466, and nifedipine completely blocked the ERK1/2 phosphorylation induced by the respective agonists. However, these antagonists did not affect the ERK1/2 hyperphosphorylation induced by DHPG. More importantly, MPEP blocked, whereas CPCCOet had no effect on, the DHPG-induced ERK1/2 phosphorylation. ***F***, ***G***, Representative immunoblots are shown left of the quantified data of pERK2. Values are expressed in terms of mean ± SEM ($n = 4–6$). *$p < 0.05$ versus basal levels. $^+p < 0.05$ versus the corresponding agonist.

mGluR5 mediates DHPG-induced ERK1/2 phosphorylation

A series of studies were performed to identify the receptor mechanism mediating the DHPG-induced ERK1/2 phosphorylation. Pretreatment of cultured striatal neurons with the antagonist PHCCC, selective for group I receptors (mGluR1/5), but not (*RS*)-α-methylserine-O-phosphate monophenyl ester (MSOPPE), selective for group II/III receptors (mGluR2/3/4/6/7/8), blocked the DHPG effect (Fig. 2*D*,*E*). The noncompetitive mGluR5 selective antagonist MPEP at low doses of 0.04 and 0.2 μM blocked the ERK1/2 phosphorylation as well (Fig. 2*F*). In contrast, the noncompetitive mGluR1 selective antagonist CPCCOet at high doses of 5 and 25 μM did not significantly alter the ERK1/2 phosphorylation (Fig. 2*G*). The mGluR5 selective agonist CHPG at 1–2 mM induced an increase in pERK1/2 that was comparable with that induced by DHPG (100 μM) and sensitive to MPEP (0.2 μM) but not CPCCOet (25 μM; data not shown). Immunoblots prepared with ERK1/2 antibody showed again that changes in the ERK1/2 phosphorylation were not attributable to changes in total ERK1/2 protein. Together, the above data suggest that selective activation of mGluR5 rather than mGluR1 mediates the DHPG-induced ERK1/2 phosphorylation.

DHPG-induced ERK1/2 phosphorylation is partially dependent on mGluR5-mediated PLCβ1 activation and Ca^{2+} release

The Gαq-coupled mGluR5 activates a predominant downstream effector PLCβ1. Active PLCβ1 in turn increases PI hydrolysis to produce IP_3, which releases Ca^{2+} from intracellular Ca^{2+} stores

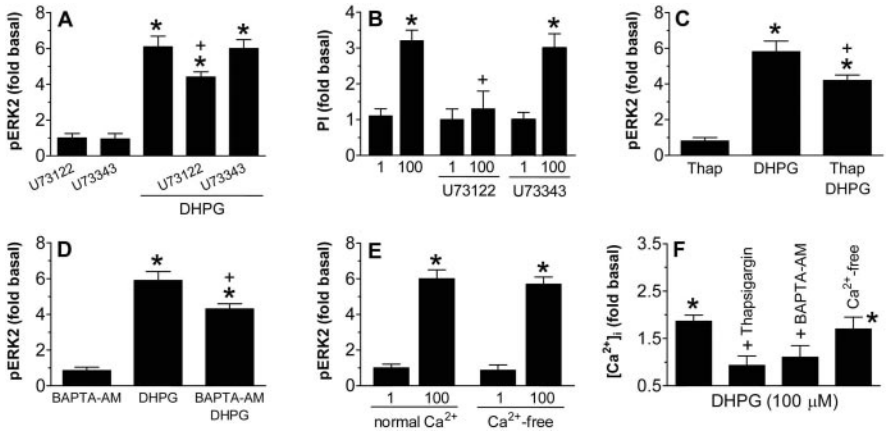

Figure 3. The contribution of the mGluR5-associated signaling pathway to the ERK2 phosphorylation induced by DHPG in cultured rat striatal neurons is shown. ***A***, Pretreatment of striatal neurons with the PLCβ1 inhibitor U73122 partially reduced the ERK1/2 phosphorylation induced by DHPG ($n = 4$). ***B***, U73122, but not U73343, totally blocked the increases in PI hydrolysis induced by DHPG ($n = 3–5$). In ***A*** and ***B***, U73122 or U73343 at 40 μM was incubated 30 min before and during 5 min (***A***) or 0.5–1 min (***B***) treatment with DHPG (100 μM). ***C***, Thapsigargin (Thap) partially reduced the ERK2 phosphorylation induced by DHPG ($n = 4$). ***D***, The Ca^{2+} chelator BAPTA-AM partially inhibited the ERK2 phosphorylation ($n = 5$). ***E***, DHPG (100 μM; 5 min) induced a comparable increase in ERK2 phosphorylation in Ca^{2+}-containing and Ca^{2+}-free media ($n = 4–5$). ***F***, Thapsigargin and BAPTA-AM completely blocked intracellular Ca^{2+} rises induced by DHPG (100 μM). In ***C***, ***D***, and ***F***, thapsigargin (2 μM) or BAPTA-AM (30 μM) was incubated 1 h before and during 5 min treatment with DHPG (100 μM). Values in ***F*** are expressed as mean fold changes of basal levels in terms of the peak amplitude of Ca^{2+} responses measured within 1 min after the start of drug treatment from 16–24 neurons. $^{*}p < 0.05$ versus basal levels. $^{+}p < 0.05$ versus DHPG (100 μM) alone.

(Nakanishi, 1994; Conn and Pin, 1997). Because mGluR5 mediates the DHPG action (see above), we set out to test the possibility that mGluR5-sensitive PLCβ1 activation and $[Ca^{2+}]_i$ mobilization participate in the transmission of mGluR5 signals to ERK. Using an amino steroid inhibitor of PLCβ1, U73122, we found that this inhibitor at 40 μM reduced the DHPG (100 μM)-induced ERK2 phosphorylation by 32% (Fig. 3*A*). In contrast, an inactive analog of U73122, U73343, did not affect the ERK2 phosphorylation (Fig. 3*A*). Consistent with the ability to inhibit PLCβ1, U73122, but not U73343, totally blocked the DHPG (100 μM)-induced increase in PI hydrolysis (Fig. 3*B*). To test the involvement of Ca^{2+} release, a $[Ca^{2+}]_i$-depleting agent, thapsigargin (2 μM), was added 1 h before DHPG (100 μM; 5 min) to discharge internal Ca^{2+} stores. We found that thapsigargin inhibited the DHPG-induced ERK2 phosphorylation to an extent comparable with that observed after U73122 application (Fig. 3*C*). Similarly, the cell-permeable Ca^{2+} chelators 1,2 bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) (30 μM) (Fig. 3*D*) and calcium green-1/AM (30 μM; data not shown) inhibited the ERK1/2 phosphorylation. In the absence of extracellular Ca^{2+} ions, DHPG preserved its ability to induce robust ERK2 phosphorylation (Fig. 3*E*) and to increase $[Ca^{2+}]_i$ levels as measured by fura-2 fluorescence (Fig. 3*F*). Pretreatment with thapsigargin or BAPTA-AM blocked DHPG-induced $[Ca^{2+}]_i$ rises (Fig. 3*F*). These results support a model that the conventional mGluR5-derived second messenger system (PLCβ1/IP_3/Ca^{2+}) mediates a smaller portion of signals from mGluR5 to ERK1/2. The mGluR5-mediated PLCβ1 activation also increases DCG, an endogenous stimulator of PKC (Nakanishi, 1994; Conn and Pin, 1997). To determine the involvement of the DCG/PKC pathway, we examined the DHPG effect in the presence of PKC inhibitors. We found that the two PKC inhibitors Ro-31-8220 and Gö6983 (1 μM for both inhibitors; 30 min) effectively blocked the PKC activator PMA (0.1 μM; 5 min)-stimulated ERK1/2 phosphorylation, sparing the increases in pERK1/2 levels induced by DHPG (100 μM for 5 min; data not shown). Thus, the DCG/PKC pathway appears to be an insignificant link in transmitting mGluR5/PLCβ1 signals to ERK.

Homer contributes to mGluR5-regulated ERK1/2 phosphorylation

A prominent anchoring protein to mGluR5 is Homer, which binds the long C terminus of mGluR5 (Brakeman et al., 1997; Kato et al., 1998; Tu et al., 1998, 1999). Among three subfamilies of Homer proteins that all bind mGluR5 (Tu et al., 1998), Homer1b/c and Homer2a/b are present at a high and low level, respectively, whereas Homer3 is lacking in striatal neurons *in vivo* (Shiraishi et al., 2004) and in cultures (Ango et al., 2000). To probe roles of Homer proteins in regulating mGluR5 signals, we developed a fusion peptide that could disrupt mGluR5/Homer binding. The peptide and its controls include a membrane-permeable Homer-binding (decoy) peptide Tat-mGluR5ct (Fig. 4*A*,*B*), a mutation peptide Tat-mGluR5mu incapable of binding Homer (Fig. 4*A*), and a Tat38–48 peptide incapable of transducing into cells. We first detected whether Tat-mGluR5ct would transduce into neurons. After bath application of fluorescein-conjugated Tat-mGluR5ct (5 μM), living neurons exhibited intense fluorescence in their cytoplasm and processes, demonstrating intracellular peptide uptake (Fig. 4*C*). In contrast, fluorescein-conjugated Tat38–48 (5 μM) showed no peptide uptake (Fig. 4*C*). Tat-mGluR5ct-fluorescein started to accumulate in neurons 10–15 min after application, peaked in the next 1 h, and remained 3–5 h after washed from the cultures.

Tat-mGluR5ct was designed to disturb mGluR5/Homer binding. To verify its efficacy in this action, effects of the peptide on the coimmunoprecipitation of Homer proteins with mGluR5 were examined in cultured neurons. Incubation with either of the two control peptides, Tat-mGluR5mu and Tat38–48 at 5 μM, did not reduce the coimmunoprecipitation of Homer1b/c and Homer2a/b with mGluR5 (Fig. 4*D*,*E*). However, the coimmunoprecipitation of either Homer1b/c or Homer2a/b with mGluR5 was reduced by Tat-mGluR5ct incubation at 5 μM for 1 h (Fig. 4*D*,*E*). In a reverse coimmunoprecipitation, mGluR5 immunoreactivity was reduced in Homer1b/c precipitates (data not shown). In addition, all three peptides did not affect cellular levels of mGluR5, Homer1b/c, and Homer2a/b proteins. These results indicate that Tat-mGluR5ct possesses the ability to selectively and effectively perturb mGluR5/Homer binding. Although all peptides did not affect mGluR5 expression levels (Fig. 4*D*,*E*), possible impacts of the perturbation of mGluR5/Homer binding on the mGluR5-associated conventional signaling pathway (PI hydrolysis and Ca^{2+} release) and subcellular distribution of mGluR5, Homer1b/c, and the Homer1b/c binding partner Shank were examined. We found that Tat-mGluR5ct treatment (5 μM; 1 h) did not alter PI (Fig. 4*F*) and dynamic Ca^{2+} responses to DHPG (100 μM) (Fig. 4*G*), indicating that the mGluR5-associated PLCβ1/PI/Ca^{2+} pathway is intact after the perturbation of mGluR5/Homer binding. Similarly, Tat-mGluR5ct (5 μM; 1 h) did not cause visible changes in the perikarya and neuritic distribution of mGluR5, Homer1b/c, and Shank (Fig. 5). Neither did Tat-mGluR5mu and Tat38–48 at 5 μM (data not

shown). Thus, the disruption of mGluR5/Homer binding exerted a minimal influence over the morphology of those proteins in cultured neurons.

After the demonstration of the effective perturbation of mGluR5 association with Homer proteins (1b/c and 2a/b) by Tat-mGluR5ct, we then examined effects of the peptide on the DHPG-stimulated ERK1/2 phosphorylation in cultured neurons. We found that Tat-mGluR5ct (5 μM; 1 h) reduced the ERK1/2 phosphorylation induced by 100 μM DHPG by >60% (Fig. 6*A*). On the contrary, the control peptide Tat-mGluR5mu did not alter the ERK1/2 phosphorylation (Fig. 6*B*). These data support the notion that the association of mGluR5 with Homer is required for activating ERK1/2. A separate experiment was conducted to specify effects of the simultaneous inhibition of both Homer and PLCβ1/Ca^{2+} pathways on mGluR5-regulated ERK phosphorylation. Interestingly, whereas Tat-mGluR5ct and U73122/thapsigargin produced the larger and smaller reduction of DHPG-stimulated ERK2 phosphorylation, respectively, application of these agents together almost totally abolished the ERK2 phosphorylation (Fig. 6*C*). This supports an existence of two independent pathways (Homer- and PLCβ1-mediated) to ERK. In addition, Tat-mGluR5ct itself (5 μM; 1 h) had no effect on basal levels of pERK1/2 (Fig. 6*A*), indicating that binding to a Homer by the exogenous Tat-mGluR5ct peptide has no functional consequences in affecting a Homer-linked downstream effector(s), leading to ERK1/2 phosphorylation.

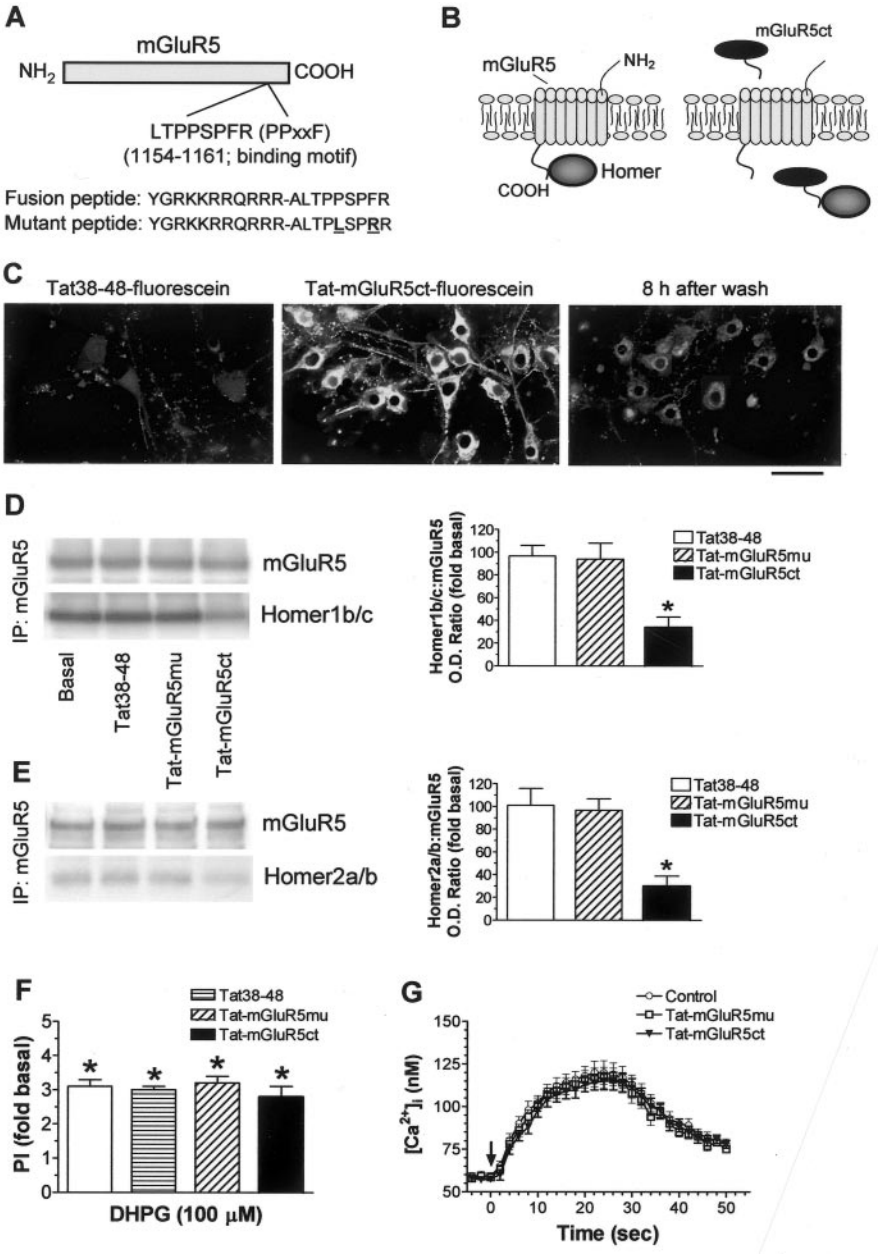

Figure 4. Dissociation of the mGluR5–Homer interaction by Tat peptides. ***A***, A fusion peptide encoding a HIV Tat peptide and Homer binding motif (Tat-mGluR5ct) was synthesized and transduced into cells to perturb the interaction of mGluR5 with Homer proteins. A control peptide was made by a dual amino acid mutation (underlined amino acids) in the Homer binding region. ***B***, Cell-permeable mGluR5ct peptides disrupt mGluR–Homer interactions. ***C***, Confocal images visualizing intracellular accumulation of Tat-mGluR5ct-fluorescein (5 μM) but not Tat38–48-fluorescein (5 μM) 1 h after addition to striatal cultures (representative of 5 experiments). Scale bar, 10 μm. ***D***, ***E***, Coimmunoprecipitation of Homer1b/c (***D***) or Homer2a/b (***E***) with mGluR5 in striatal cultures treated with Tat peptides (5 μM; 1 h) is shown. The coimmunoprecipitation of Homer1b/c and Homer2a/b was significantly reduced by Tat-mGluR5ct but not by Tat38–48 and Tat-mGluR5mu. Left, Representative gels. Right, Means ± SEM of five to six experiments. *$p < 0.05$ versus basal levels. ***F***, Effects of Tat peptides on the increases in PI hydrolysis induced by DHPG (n = 4–5). Each Tat peptide was incubated at 5 μM for 1 h before and during 0.5–1 min treatment with DHPG (100 μM). *$p < 0.05$ versus basal levels. ***G***, Effects of Tat peptides on the intracellular Ca^{2+} rises induced by DHPG. Each Tat peptide was incubated at 5 μM for 1 h before bath application of DHPG (100 μM) for ~1–5 min. An arrow indicates the beginning of DHPG application. Values are expressed as mean ± SEM measured from 15–22 neurons for each group.

Homer1b/c proteins mediate mGluR5 signals to ERK

To substantiate the role of the Homers in forming a signaling pathway from mGluR5 to ERK and to distinguish the relative importance of Homer1b/c and Homer2a/b in this event, an approach using siRNAs was used to selectively reduce the cellular levels of individual Homer1b/c or Homer2a/b transcripts. In a recent effort in this laboratory, a functional RNA interference was proven to exist in cultured striatal neurons based on the findings that cotransfection of the green fluorescent protein (GFP) siRNAs, but not control siRNAs, with a construct expressing GFP depleted expression of GFP (Yang et al., 2004).

To determine whether the Homer1b/c and Homer2a/b siRNAs can selectively reduce cellular levels of their targets, cultures were treated with the siRNAs at 1 μg per well. Whereas control siRNAs did not change total Homer1b/c levels (Fig. 7*A*), Homer1b/c siRNAs markedly reduced Homer1b/c levels (Fig. 7*A*). The Homer1b/c siRNA produced its effect at 16 h, which persisted for 2 d. The suppression was only partial by 4 d after treatment. To control the selectivity of Homer1b/c siRNAs, effects of the siRNAs on cellular levels of Homer2a/b were tested. Homer1b/c siRNAs did not alter immunoreactivity of Homer2a/b (Fig. 7*A*). Similar results were obtained after treat-

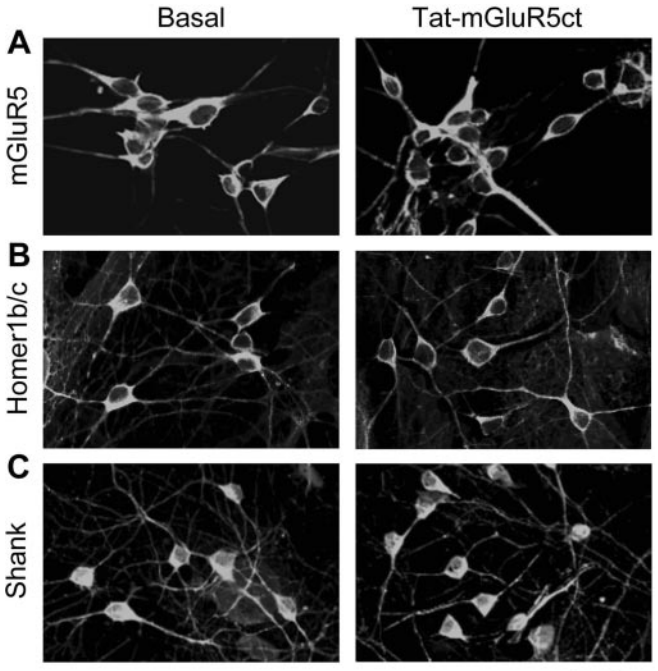

Figure 5. Confocal images showing subcellular distribution of mGluR5 (***A***), Homer1b/c (***B***), and Shank (***C***) immunoreactivity in normal rat striatal cultures and cultures exposed to Tat-mGluR5ct peptides. The peptide was incubated at 5 μM for 1 h before be fixed for immunofluorescent labeling. Note that no significant difference in perikarya and neuritic distribution of mGluR5, Homer1b/c, or Shank was observed between basal and Tat-mGluR5ct-treated cultures. Scale bar, 30 μm.

ment with Homer2a/b siRNAs (Fig. 7*E*). To evaluate the influence of Homer1b/c or Homer2a/b knock-down on mGluR5-mediated PI signaling, effects of these siRNAs on PI hydrolysis were examined. Both basal and DHPG (100 μM)- or CHPG (1 mM)-stimulated PI hydrolysis were not altered in cultures treated with either Homer1b/c (Fig. 7*B*) or Homer2a/b siRNAs (data not shown). Similarly, dynamic Ca^{2+} responses to DHPG (100 μM) measured with fura-2 ratio imaging and perikarya/neuritic distribution of mGluR5 measured with single immunofluorescent labeling in cultured neurons exposed to Homer1b/c or control siRNAs (1 μg per well) were not different from those measured from normal cultures (data not shown). There was no significant difference in cell viability between control and Homer siRNA-treated cultures as detected by the double fluorescein diacetate-propidium iodide staining.

Using the same Homer1b/c and Homer2a/b siRNAs (1 μg per well; 24 h), the ability of DHPG to phosphorylate ERK1/2 was tested. Control siRNAs did not affect DHPG (100 μM)-induced ERK1/2 phosphorylation (Fig. 7*C*). In contrast, Homer1b/c siRNAs reduced the ERK1/2 phosphorylation (Fig. 7*C*). At day 4, DHPG (100 μM) primarily resumed its ability to increase pERK1/2 levels, indicating the reversible nature of the Homer1b/c siRNA effect. Both control and Homer1b/c siRNAs did not alter total ERK1/2 proteins (Fig. 7*C*). When Homer1b/c siRNAs were coincubated with U73122 and thapsigargin, DHPG no longer induced a significant increase in ERK2 phosphorylation (Fig. 7*D*). Unlike Homer1b/c siRNAs, Homer2a/b siRNAs (1 μg per well) did not change the DHPG-stimulated ERK2 phosphorylation (Fig. 7*F*). In cultures treated with Homer1b/c siRNAs (1 μg per well), NMDA (50–100 μM; 5 min) increased ERK1/2 phosphorylation similar to that in cultures treated with control siRNAs (data not shown). These results support that Homer1b/c proteins are essential elements of the post-mGluR5 signaling complex mediating mGluR5 signals to ERK1/2.

Physiological roles of mGluR5-sensitive ERK1/2 signaling in regulating gene expression

One of important roles that active ERK1/2 play is the regulation of gene expression via phosphorylating nuclear transcription factors (Wang et al., 2004). We therefore tested the response of a closely related nuclear factor target, Elk-1 (Sgambato et al., 1998), and a more common factor, CREB, to the active mGluR5/ERK1/2 pathway. In addition, an immediate early gene c-*fos* was tested as a reporter of inducible gene expression downstream to Elk-1 and CREB to reveal a final output of gene expression. We found that DHPG (100 μM) induced a rapid and transient increase in phosphorylation of Elk-1 (Fig. 8*A*) and CREB (Fig. 8*B*) and induction of c-Fos expression (Fig. 8C). All three events kinetically corresponded well with each other and with the dynamic ERK1/2 phosphorylation specified in Figure 1*B*, which fits into a sequential model: ERK1/2 is first activated, which leads to Elk-1/CREB phosphorylation followed by c-Fos induction. In contrast to the elevated pElk-1 and pCREB levels, cellular levels of Elk-1 and CREB were not altered by DHPG (Fig. 8*A*,*B*).

To determine whether ERK1/2 mediate the Elk-1/CREB phosphorylation and c-Fos expression, a mitogen-activated protein kinase kinase (MEK) selective inhibitor, U0126, was used to inhibit the ERK1/2 pathway in response to its stimulation. The inhibition was confirmed by

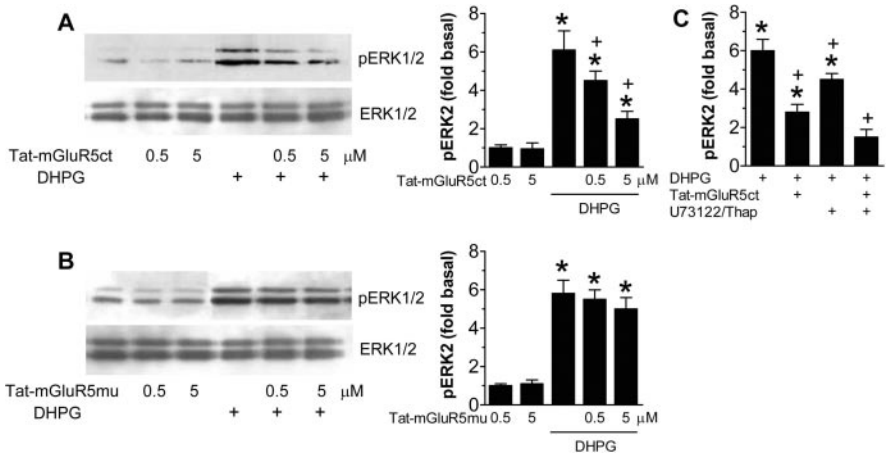

Figure 6. Influence of the dissociation of mGluR5–Homer interaction by Tat peptides on DHPG-induced ERK1/2 phosphorylation in cultured rat striatal neurons. ***A***, ***B***, Effects of Tat-mGluR5ct (***A***) and Tat-mGluR5mu (***B***) on ERK1/2 phosphorylation induced by DHPG. The Tat peptides (0.5 or 5 μM) were incubated 1 h before and during 5 min treatment with DHPG (100 μM). Note that Tat-mGluR5ct, but not Tat-mGluR5mu, reduced the DHPG-induced ERK1/2 phosphorylation. Representative immunoblots are shown left of the quantified data of pERK2 (mean ± SEM; $n = 5–6$). *$p < 0.05$ versus basal levels. $^{+}p < 0.05$ versus DHPG alone. ***C***, Effects of coincubation of Tat-mGluR5ct, U73122, and thapsigargin (Thap) on DHPG-induced ERK2 phosphorylation. Tat-mGluR5ct (5 μM), U73122 (40 μM), and thapsigargin (2 μM) were incubated 1 h before and during 5 min treatment with DHPG (100 μM). In the presence of all three agents, DHPG no longer induced a significant increase in ERK2 phosphorylation. *$p <$ 0.05 versus basal levels. $^{+}p < 0.05$ versus DHPG alone.

complete blockade of the DHPG-induced ERK1/2 phosphorylation by 1 μM of U0126 (Fig. 9*A*). Consistent with the ability to block the ERK1/2 phosphorylation, U0126 blocked the DHPG (100 μM)-induced Elk-1 and CREB phosphorylation (Fig. 9*B*,*C*). U0126 also mostly reduced the c-Fos response to DHPG (Fig. 9*D*). No significant differences were found in total ERK1/2, Elk-1, and CREB proteins after the U0126 treatment (Fig. 9*A–C*). These results suggest that the MEK-mediated ERK1/2 activation leads to the subsequent phosphorylation of the two transcription factors and c-Fos expression after mGluR5 activation.

Two independent pathways (PLCβ1/Ca^{2+} vs Homer1b/c) mediate mGluR5 signals to ERK based on the above data. To evaluate the importance of ERK activated via different pathways for regulating gene expression, mGluR5-regulated Elk-1/CREB phosphorylation and c-Fos expression were analyzed under the condition in which one pathway was inhibited, whereas another was intact. When the Homer1b/c pathway was disrupted by Tat-mGluR5ct, DHPG induced no changes in Elk-1 (Fig. 10*A*) and CREB (Fig. 10*B*) phosphorylation and c-Fos expression (Fig. 10*C*). Interestingly, the same results were also seen when the PLCβ1/Ca^{2+} pathway was inhibited by U73122 and thapsigargin or when the two pathways were inhibited simultaneously by the Tat peptide and the inhibitor agents (Fig. 10). These data indicate that the activation of ERK by both pathways is corequired to elevate pERK1/2 to a level sufficient to facilitate gene expression.

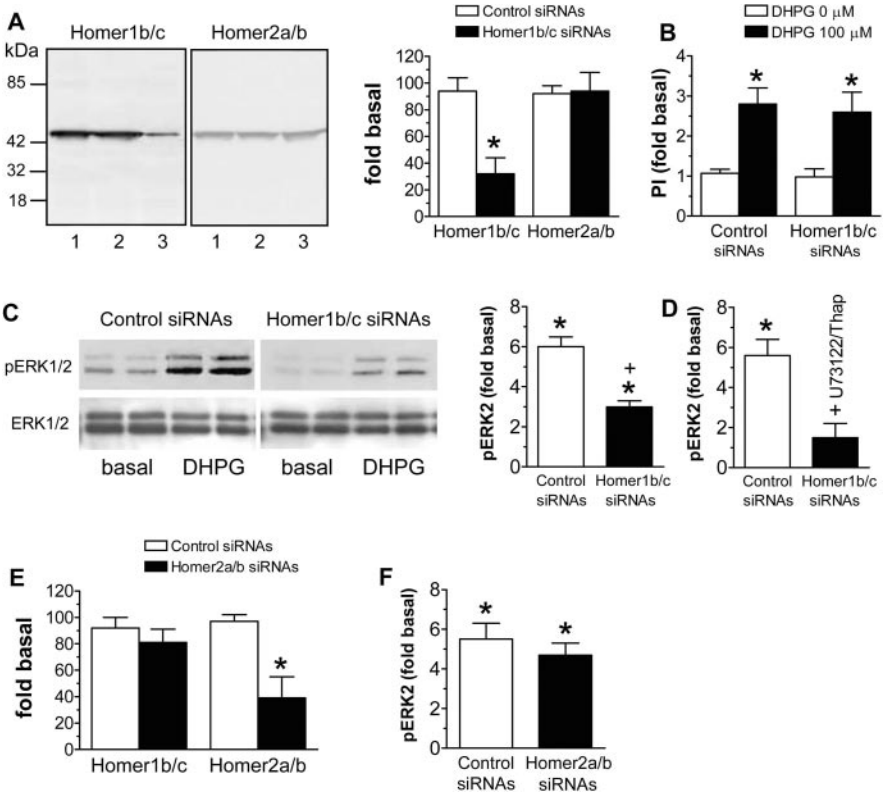

Figure 7. Effects of reduced Homer1b/c or Homer2a/b proteins on ERK2 phosphorylation induced by DHPG in cultured rat striatal neurons. ***A***, Homer1b/c siRNAs, but not control siRNAs, selectively reduced cellular Homer1b/c, but not Homer2a/b, protein levels. Representative immunoblots are shown left of the quantified data (mean ± SEM; $n = 6$). Lane 1, Basal; lane 2, control siRNAs; and lane 3, Homer1b/c siRNAs. ***B***, Control and Homer1b/c siRNAs did not affect basal and DHPG-stimulated PI hydrolysis (mean ± SEM; $n = 5$). ***C***, Homer1b/c siRNAs reduced the DHPG-induced ERK1/2 phosphorylation. Representative immunoblots are shown left of the quantified data (mean ± SEM; $n = 5–6$). ***D***, DHPG did not induce a significant increase in ERK2 phosphorylation in the presence of U73122 and thapsigargin (Thap) in Homer1b/c siRNA-treated cultures. ***E***, Homer2a/b siRNAs, but not control siRNAs, selectively reduced cellular Homer2a/b, but not Homer1b/c, protein levels (mean ± SEM; $n = 5$). ***F***, DHPG induced an indistinguishable increase in ERK2 phosphorylation in control siRNA- and Homer2a/b siRNA-treated cultures (mean ± SEM; $n = 4$). Control or Homer siRNAs were incubated for 2–4 h at 1 μg per well on a 24-well plate, and cultures were lysed 24 h later for detecting Homer protein levels (***A***, ***E***) or effects of DHPG (100 μM; 0.5–1 min) on PI hydrolysis (***B***). In ***C***, ***D***, and ***F***, 24 h after treatment with siRNAs (1 μg per well; 2–4 h), effects of DHPG (100 μM; 5 min) alone or in combination with U73122 (40 μM) and thapsigargin (2 μM) on pERK2 levels were examined. $^*p < 0.05$ versus basal levels. $^+p < 0.05$ versus DHPG in the presence of control siRNAs.

Discussion

The present study investigated signaling mechanisms involved in mediating group I mGluR signals to ERK in cultured neurons. We found that selective activation of mGluR5 activated ERK. The ERK activation is partially mediated by the mGluR5-associated conventional PLCβ1/IP_3/Ca^{2+} pathway. More interestingly, the mGluR5-regulated ERK activation primarily relies on a Ca^{2+}-independent mechanism involving synaptic adaptor Homer1b/c proteins that have a direct association with the C terminus of mGluR5. Furthermore, ERK activated by both pathways can amount up to a high level in the nucleus to phosphorylate the two transcription factors, Elk-1 and CREB, resulting in c-Fos expression. These results reveal the two coordinated signaling mechanisms imperative for processing the mGluR5-mediated synapse-to-nucleus communication for the facilitatory regulation of transcription.

Although group I mGluRs are found to increase ERK phosphorylation in cultured glia (Schinkmann et al., 2000; Peavy et al., 2001) and a heterologous expression system [Chinese hamster ovary (CHO) cell lines] with transfected mGluR1 or mGluR5 (Ferraguti et al., 1999; Thandi et al., 2002), the role of mGluR1/5 in such an event and detailed signaling mechanisms are poorly understood in neurons. In the CHO cells with transfected mGluR5, the agonist-induced ERK1/2 phosphorylation was insensitive to the manipulations of depleting intracellular or extracellular Ca^{2+} ions (Thandi et al., 2002), indicating a Ca^{2+}-independent mechanism involved in the event. In neurons (this study), unlike the CHO cells, a Ca^{2+}-dependent mechanism derived from Ca^{2+} release via activating the conventional PLCβ1/IP_3 pathway contributes to the mGluR5-regulated ERK1/2 phosphorylation, although this pathway appears to mediate only a small portion of signals. Extracellular Ca^{2+} ions, on the other hand, are not important because the removal of extracellular Ca^{2+} ions and blockade of major ion channels (NMDA and AMPA receptors and VOCCs) did not affect DHPG-stimulated ERK1/2 phosphorylation. Nevertheless, the participation of Ca^{2+} signals by Ca^{2+} release in neurons provides a common signaling network node (or hub) for signaling interactions, which is advantageous to coordinate appropriate signaling responses to various cellular stimuli.

An important finding in this study is the identification of Homer1b/c proteins in forming a major signaling pathway for linking mGluR5 to ERK1/2. Homer1b/c proteins are expressed

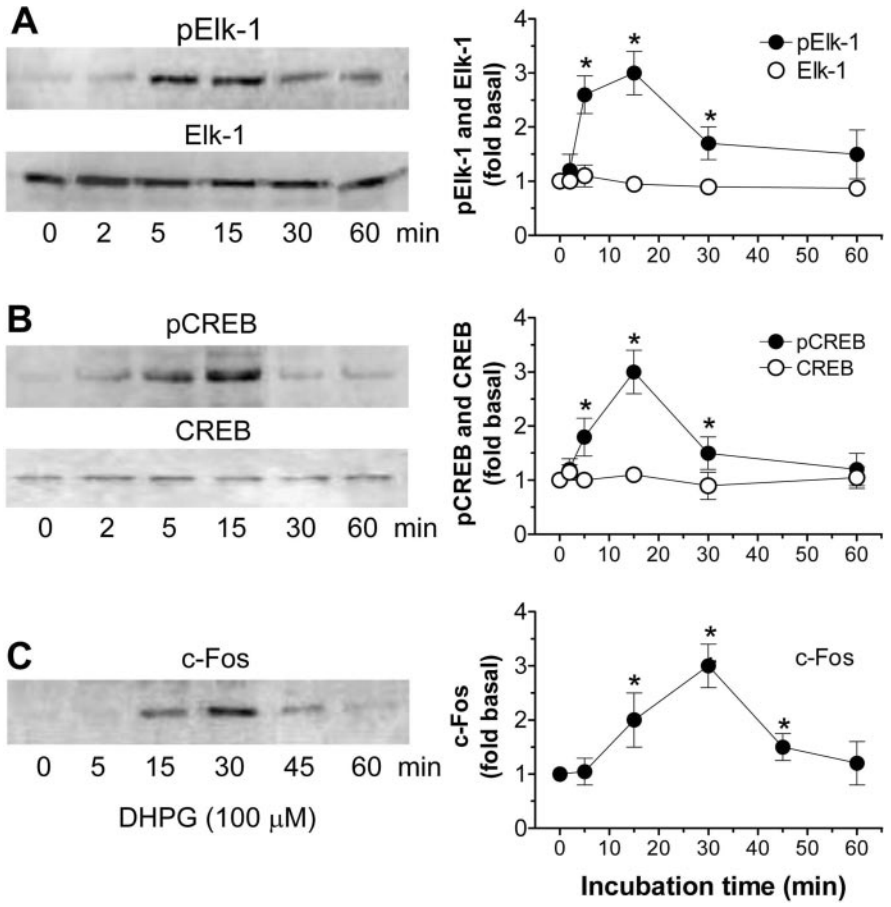

Figure 8. Effects of activation of mGluR5 with DHPG on Elk-1 and CREB phosphorylation and c-Fos expression in cultured rat striatal neurons. DHPG induced a rapid and transient increase in Elk-1 (***A***) and CREB (***B***) phosphorylation followed by an increase in c-Fos protein levels (***C***). Total Elk-1 and CREB proteins remained unchanged throughout the time course (***A***, ***B***). DHPG was incubated at 100 μM for different durations. Representative immunoblots are shown left of the quantified data (mean ± SEM; $n = 4–5$). *$p < 0.05$ versus basal levels.

constitutively in rat striatal neurons at a high level (Ango et al., 2000; Shiraishi et al., 2004) and are preferentially colocalized with mGluR5 (Xiao et al., 1998). The C-terminal coiled-coil domain of the proteins promotes the formation of homomeric multivalent complexes, such as Homer dimers and tetramers (Brakeman et al., 1997; Xiao et al., 1998), whereas the highly conserved N-terminal EVH1 domain forms heteromeric binding to a proline-rich motif in the C terminus of mGluR5 (Gertler et al., 1996; Kato et al., 1998; Tu et al., 1998) as well as to other synaptic and cytoplasmic proteins. In the area of PSD, Homer1b/c proteins are enriched in both the central and lateral regions (Xiao et al., 1998). The mGluR5 is also present at a higher concentration at the lateral PSD margin (Baude et al., 1993; Nusser et al., 1994; Lujan et al., 1997). Such a unique ultrastructural arrangement allows Homer1b/c to form a physical tether linking membrane mGluR5 with other PSD proteins. These linkages may perform a signaling function to transduce mGluR5 signals to ERK cascades. Indeed, the disruption of mGluR5/Homer1b/c binding by a cell permeable Tat-fusion peptide reduced the mGluR5 agonist-induced ERK1/2 phosphorylation (this study). Similar results were also seen after the selective inhibition of Homer1b/c synthesis with siRNAs. These results support a notion that mGluR5 needs to physically couple to Homer1b/c to transmit signals to ERK. Homer2a/b proteins also bind the C terminus of mGluR5. However, basal levels of Homer2a/b are normally much lower than those of Homer1b/c and mGluR5, and siRNA knock-down of the proteins did not affect the mGluR5 agonist-induced ERK1/2 phosphorylation. Thus, Homer2a/b proteins, in contrast to Homer1b/c, are less likely involved in organizing the mGluR5-ERK coupling.

Homer binds the proline-rich motif in IP_3 receptors (Tu et al., 1998) and the putative Homer ligand motif present in ryanodine receptors (Feng et al., 2002; Hwang et al., 2003). Thus, Homer proteins, by directly linking to these receptors on internal Ca^{2+} stores, may significantly influence group I mGluR-sensitive Ca^{2+} signaling. Indeed, transfection of the noncrosslinking form of Homer proteins (*Homer1a*) increased the latency of mGluR1- and mGluR5-mediated Ca^{2+} rises in cultured rat cerebellar neurons with native mGluR1 or transfected mGluR5 (Tu et al., 1998; Ango et al., 2002). The delay of the Ca^{2+} responses was interpreted as a lack of direct interaction between mGluR1/5–Homer1a complex and IP_3 receptors (Tu et al., 1998; Ango et al., 2002). Overexpression of *Homer1a* also caused a decrease in the amplitude of mGluR1-mediated Ca^{2+} responses in cultured rat cerebellar Purkinje cells (Tu et al., 1998) and an increase in the amplitude of mGluR5-mediated Ca^{2+} responses in cultured rat cerebellar granule cells (Ango et al., 2002). This discrepancy of *Homer1a* in regulating the amplitude of Ca^{2+} responses may result from the nature of the Ca^{2+} signaling in different subpopulations of cerebellar neurons (Ango et al., 2002). As to the long form of Homer proteins with crosslinking (dimerization) capacity, overexpression of Homer1b did not alter native mGluR1-mediated Ca^{2+} responses in cerebellar Purkinje cells (Tu et al., 1998), whereas coexpression of Homer1b reduced mGluR5-mediated Ca^{2+} responses in cerebellar granule cells (Ango et al., 2002). No data were available before this study in respect of the effect of downregulated expression of endogenous Homer proteins on mGluR1/5-mediated Ca^{2+} responses in neurons. In this study, the two novel approaches (Tat-fusion peptides and siRNAs) were developed to perturb the mGluR5/Homer association or inhibit cellular synthesis of Homer1b/c. Both approaches were found to have a minimal influence over the mGluR5 agonist-stimulated Ca^{2+} release. Thus, the mGluR5 function in regulating intracellular Ca^{2+} mobilization is primarily preserved after the use of either approach. In support of this, mGluR5-mediated PI hydrolysis remained unchanged in the cultures treated with Tat-fusion peptides or Homer1b/c siRNAs (this study), and the signaling from the Gαq-protein-coupled receptor to Ca^{2+} release through PLCβ/IP_3 was intact in pancreatic acinar cells of the Homer1 knock-out mouse (Yuan et al., 2003; Shin et al., 2003). Nevertheless, because the mGluR5-regulated ERK phosphorylation is primarily Ca^{2+} independent in nature, the Homer1b/c-controlled Ca^{2+} signal, if there is any, may not play a significant role in the event.

Neither disruption of the Homer1b/c-mGluR5 binding by Tat

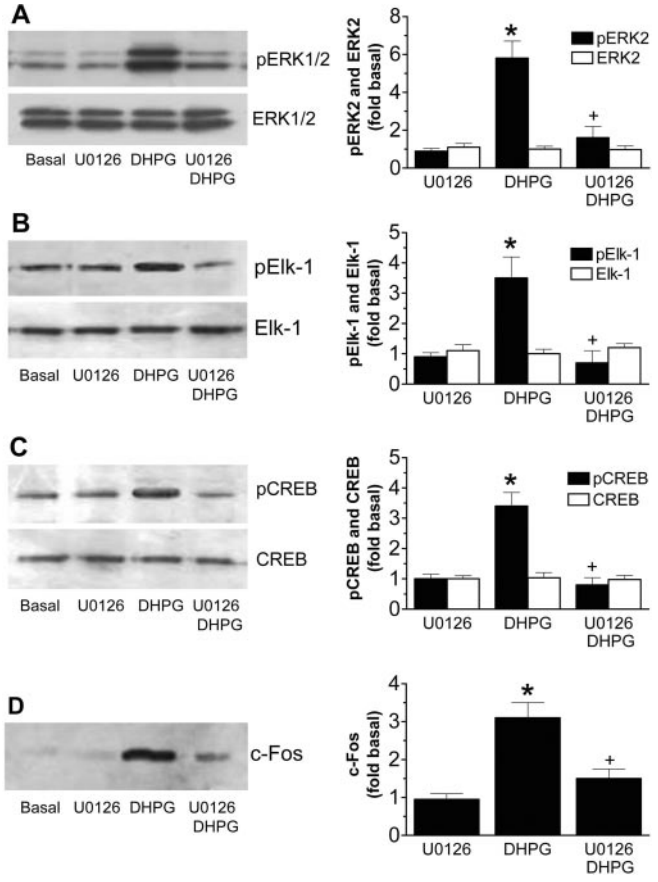

Figure 9. Effects of the MEK inhibitor U0126 on DHPG-induced phosphorylation of ERK1/2, Elk-1, and CREB and c-Fos expression in cultured rat striatal neurons are shown. U0126 blocked the DHPG-induced phosphorylation of ERK1/2 (***A***), Elk-1 (***B***), and CREB (***C***) and c-Fos expression (***D***), without altering cellular levels of total ERK1/2, Elk-1, and CREB proteins. U0126 (1 μM) was incubated 30 min before and during treatment with DHPG (100 μM) for 5 min (pERK1/2), 15 min (pElk-1 and pCREB), or 30 (c-Fos) min. Representative immunoblots are shown left to the quantified data (mean ± SEM; $n = 4–5$). *$p < 0.05$ versus basal levels. $^{+}p < 0.05$ versus DHPG alone.

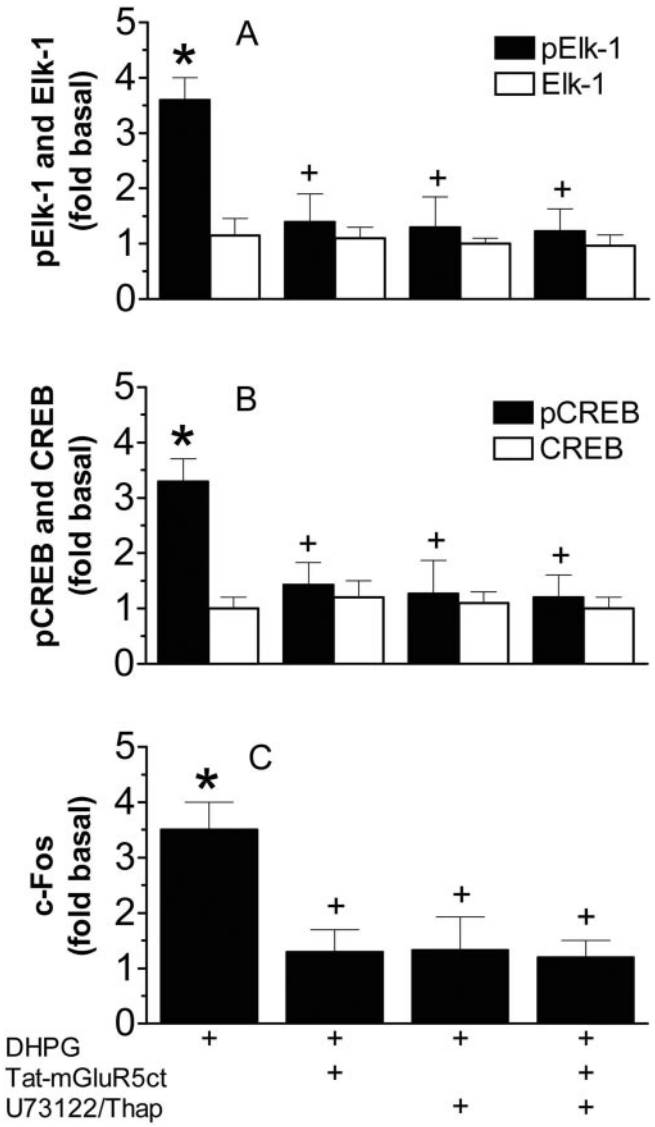

Figure 10. Effects of the Tat peptide and U73122/thapsigargin (Thap) on DHPG-stimulated phosphorylation of Elk-1 (***A***) and CREB (***B***) and c-Fos expression (***C***) in cultured rat striatal neurons are shown. The DHPG-stimulated Elk-1/CREB phosphorylation and c-Fos expression were blocked to a similar extent by Tat-mGluR5ct, U73122/thapsigargin, or both. Tat-mGluR5ct (1 μg per well) was incubated for 2–4 h, and the effect of DHPG (100 μM; 5 min) was tested 24 h later. U73122 (40 μM) and thapsigargin (2 μM) were incubated for 1 h before and during 5 min incubation of DHPG (100 μM). Values are expressed as mean ± SEM ($n = 4–5$). *$p < 0.05$ versus basal levels. $^{+}p < 0.05$ versus DHPG alone.

peptides nor reduced cellular levels of Homer1b/c by siRNAs altered basal levels of mGluR5 expression (this study). Similarly, antisense inhibition of Homer1b/c did not affect constitutive expression of mGluR5 and other Homer binding partners (IP_3 receptors and Shank proteins) in the rat striatum *in vivo* (Ghasemzadeh et al., 2003). Moreover, Tat peptides and Homer1b/c siRNAs had a minimal effect on the subcellular distribution of mGluR5 in cultured striatal neurons (this study). In knock-out mice, Shin et al. (2003) also found that deletion of each or all Homer proteins (Homer1, 2, and 3) did not affect localization or expression of any isoform of IP_3 receptors in the pancreatic acinar cells. Thus, Homer1b/c alone may have a limited control over the trafficking or stabilization of mGluR5 and other key binding partners in matured neurons. Instead, Homer1b/c may play a significant role in crosslinking the receptor with various submembranous proteins to coordinate a sophisticated postreceptor signaling response.

One of noticeable roles that active ERK plays is to regulate gene expression. After activation, the cytoplasmic ERK translocates into the nuclear compartment to perform such a function (Kim and Kahn, 1997; Davis et al., 2000). There are a number of various extracellular signals that can regulate gene expression through the ERK superhighway. In this study, a high level of pERK1/2 was seen in the nucleus after mGluR5 stimulation, indicating a potential role in regulating gene expression. In the additional attempt of clarifying this potential, we found that the mGluR5 agonist-induced ERK phosphorylation kinetically corresponded well with increased phosphorylation of two transcription factors (Elk-1 and CREB) and c-Fos expression. Moreover, selective inhibition of the PLCβ1/IP_3/Ca^{2+} pathway abolished the Elk-1/CREB phosphorylation and c-Fos induction. Thus, the Ca^{2+}-dependent ERK signals are essential for linking mGluR5 to transcription (Vanhoutte et al., 1999; Zanassi et al., 2001; Mao and Wang, 2003b,c). Interestingly, the Elk-1/CREB phosphorylation and c-Fos induction were also abolished after the inhibition of the specific part of pERK1/2 that was activated by the Homer1b/c-mediated Ca^{2+}-independent pathway. Apparently, both the Ca^{2+}-dependent and -independent pathways are neces-

sary for elevating active ERK to a level sufficient to affect gene expression. The corequirement of Ca^{2+}-dependent and -independent ERK activation allows interactions of the mGluR5 signaling pathway with many other Ca^{2+}-dependent and -independent signaling effectors to accurately regulate transcription in response to glutamate signals at various amplitudes and patterns.

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