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Converging signal on ERK1/2 activity regulates group I mGluR-mediated *Arc* transcription

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Abstract

The expression of *Arc* is tightly coupled to synaptic activities. Recent studies suggested the functional relevance of *Arc* translation in group I metabotropic glutamate receptor (mGluR)-mediated long-term depression. The present study investigated the transcription-dependent changes of *Arc* in response to the activation of group I mGluR by (R, S)-3, 5-dihydroxyphenylglycine (DHPG) in cultured cortical neuron. The increase in *Arc* mRNA did not require *de novo* protein synthesis, indicating that *Arc* is an immediate early gene upon DHPG stimulation. We further examined the major pathways involved in group I mGluR signaling, and found that DHPG-induced *Arc* up-regulation depended on CaMK, PLC, and ERK1/2 activity. Moreover, the activity of NMDA receptors, but not L-type voltage gated calcium channels (L-VGCC), was required for *Arc* transcription. Interestingly, blocking CaMK, PLC, and NMDAR, but not L-VGCC, suppressed DHPG-stimulated ERK1/2 activation. These data suggest the central role of ERK1/2 in group I mGluR-mediated *Arc* transcription.

Keywords

Arc; NMDA receptor; L-type voltage-gated calcium channel; group I metabotropic glutamate receptors; transcription; ERK1/2

Introduction

The expression of immediate early gene *Arc* (activity-regulated cytoskeleton-associated protein) is extremely responsive to synaptic activities [12]. In cultured PC12 cells or cortical neurons, the transcription-dependent increase of *Arc* mRNA can be stimulated by membrane depolarization, NMDA, forskolin, and neurotrophins [24,27,34]. The *in vivo* up-regulation of *Arc* is observed during pentylentetrazole-induced seizure [11], after BDNF-induced [33] and high frequency stimulation (HFS)-induced long-term potentiation (LTP) [17], after novel environment exploration [5], or after avoidance learning [18]. Accumulating evidence also suggests that the activity-dependent transcription of *Arc* may be physiologically relevant to certain brain functions. For example, *Arc* mutant mice show impairments in late phase LTP, NMDA-

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dependent long-term depression (LTD), and consolidation of long-term memories (LTM) [23,25].

Although it is well accepted that ionotropic glutamate receptors regulate *Arc* transcription, the role of metabotropic glutamate receptors (mGluRs) is unknown. Very recently, two independent research groups have demonstrated an interesting correlation between fast dendritic translation of *Arc* and group I mGluR-mediated LTD [21,29]. Mechanistically, the dendritic translation of *Arc* is required for AMPA receptor endocytosis. Although it has been shown that the activation of group I mGluRs stimulates transcription factors (such as CREB and NF- κ B) [14,20] and elevates plasticity-related genes (such as *c-fos* and *erg1*) [10,15], how *Arc* transcription responds to mGluR-mediated intracellular signaling is unknown. This study aims to examine whether the transcription of *Arc* is stimulated by group I mGluR, and to identify regulatory molecules and signaling components.

Materials and methods

Cell culture and treatment

Primary cultures of cortical neurons were obtained from C57BL/6J mice, and maintained as described [34]. DIV (days in vitro) 9 to 12 neurons were treated with the well-characterized selective group I mGluR agonist (R, S)-3, 5-dihydroxyphenylglycine (DHPG) (TOCRIS) at 100 μ M, which is sufficient to trigger ERK1/2 and PLC activation, as well as mTOR-dependent translation and LTD in the CA1 region of the hippocampus [9,15]. A 30min pretreatment with YM 298198 (25nM) or MPEP (10 μ M) was used to block mGluR1 or mGluR5, respectively. To block the activity of CaM kinases (I, II and IV), neurons were pretreated with KN62 (Sigma, at 10 μ M) for 20min before DHPG. Similarly, a 20min pretreatment with U73122 (Calbiochem, at 5 μ M), U0126 (Calbiochem, at 10 μ M), APV (Sigma, at 100 μ M), and nifedipine (Sigma, at 10 μ M) was used to block the activity of PLC, MEK1/2, NMDA receptors, and L-VGCC, respectively.

RT-PCR

After DHPG stimulation, the neurons were harvested for total RNA extraction by the Trizol (Invitrogen) method. 0.5 microgram of RNA was reverse transcribed to cDNA using the SuperScript III kit (Invitrogen). The primers used for PCR amplification of *Arc* (26 cycles) are AGACACAGCAGATCCAGCTG and TGGCTTGTCTTCACCTTCAG. The primers AGCCTTTCCTACTACCATTCC and ATTCCGGCACTTGGCTGCAG were used for *c-fos* (24 cycles), and TCCATGACAACCTTGGCATTGTGG and GTTGCTGTTGAAGTCGCAGGAGAC were used for *GAPDH* (19 cycles). PCR products were separated on 1.2% agarose gels, documented by digital imaging, and quantified with Scion Image (Scion Corp., Frederick, MD) software. The value of *Arc* and *c-fos* mRNA level was normalized to that of *GAPDH*.

Western blot analysis

Treated neurons were harvested in sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris-HCl buffer, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β -mercaptoethanol), separated by 10% SDS-PAGE, and transferred to nitrocellulose membrane. The blots were incubated with antibodies against phosphorylated-ERK1/2 (P-ERK1/2) (Cell Signaling, 1:1000), phosphorylated-CREB (P-CREB) (Cell Signaling, 1:1000), or β -actin (Sigma, 1:10,000) overnight at 4°C in PBS with 0.1% Triton X-100 (PBST) and 5% nonfat milk. The membranes were then incubated with horseradish peroxidase-conjugated goat secondary antibodies (1:5,000; Pierce, Rockford, IL) for 1 hr at room temperature. The signal was detected with the ECL system (SuperSignal West Pico; Pierce). The exposed films were

scanned with an Epson flatbed scanner, and quantified by Scion Image software. The value of p-ERK1/2 and p-CREB was normalized to β -actin.

The quantification data were expressed as average $\pm$ SEM for both RT-PCR and Western blots. One-way ANOVA and Student's t-test were used to determine the statistical significance.

Results and Discussion

The activation of metabotropic mGluRs triggers numerous intracellular signaling events through G proteins. Among them, the group I mGluRs, which consist of mGluR1 and mGluR5, are broadly expressed in the forebrain regions. Pharmacological and genetic manipulations have demonstrated their function in anxiety, schizophrenia, stroke, neurodegenerative diseases, and mental retardation [16]. When examined by the cellular models for neuroplasticity, group I mGluRs regulate both LTP and LTD [7,19]. Indeed, stimulation on group I mGluRs leads to the activation of several plasticity-related signaling pathways. In striatum, the group I selective agonist (R, S)-3, 5-dihydroxyphenylglycine (DHPG) stimulates extracellular signal-regulated kinase 1/2 (ERK1/2) and the phosphorylation of transcriptional regulator CREB, followed by *c-fos* transcription [15]. A recent report also demonstrated mGluR1/5-mediated activation of ERK1/2 and CREB, as well as CREB-dependent transcription in the anterior cingulate cortex (ACC) [28]. Here, we show that DHPG stimulated significant phosphorylation of both ERK1/2 and CREB in cultured cortical neurons (Fig. 1A). The activation of ERK1/2 and CREB lasted for at least 1 hr (Fig. 1A).

Molecular characterization of *Arc* promoter have identified several plasticity-related cis-elements, including three serum response elements (SRE) [8,22,27] and a cAMP responsive element (CRE) [8]. Additionally, a novel Zeste-like element and a multi-element containing SARE (Synaptic Activity-Responsive Element) are identified recently [8,22]. Because both CRE- and SRE-mediated transcription may be regulated by ERK1/2, we hypothesized that the group I mGluR-induced ERK1/2 activation may lead to *Arc* transcription. By using RT-PCR analysis, we detected significant increase of *Arc* mRNA in DHPG-stimulated neurons. The transcriptional up-regulation was detected as early as 30min after DHPG stimulation, and lasted for at least 2hrs (Fig. 1B). When neurons were pre-treated with the protein synthesis inhibitor cycloheximide, the up-regulation of *Arc* transcription was intact, indicating that *Arc* behaves as an immediate early gene upon mGlu1/5 stimulation (Fig. 1C). Similar activation profile was observed for another immediate early gene *c-fos* (Fig. 1B, C). To examine the function of mGluR1 and mGluR5, we pretreated neurons with specific antagonists 30 min before DHPG application. The DHPG-induced ERK1/2 phosphorylation was significantly blocked by the mGluR5 inhibitor MPEP, but not by the mGluR1 inhibitor YM 298198 (Fig. 1D). However, blocking either mGluR1 or mGluR5 significantly suppressed DHPG-mediated *Arc* transcription (Fig. 1D). Although the ERK1/2 phosphorylation was intact in YM 298198-treated neurons, mGluR1 may regulate *Arc* transcription through the cAMP pathway. Wang and colleagues have reported that DHPG stimulates cAMP production, and the DHPG-mediated transcription of *Fmr1* depends on both mGluR1 and mGluR5 [28]. The major role of mGluR5 in the activation of ERK/Elk-1 signaling [13] and CREB [14] has been demonstrated in striatal neurons.

The function of ERK1/2 has been implicated in Ca^{2+} - and BDNF-mediated *Arc* transcription [33,34]. Here, we found that inhibition of MEK activity by U0126 significantly suppressed the DHPG-induced ERK1/2 phosphorylation (Fig. 2B), as well as the transcriptional up-regulation of both *Arc* and *c-fos* (Fig. 2A). Interestingly, partial elevation of p-CREB, which may be supported by CaM kinases [32], remained in neurons treated with U0126 (Fig. 2B).

Because the function of Ca^{2+} -stimulated signaling has been strongly implicated in both ERK1/2 activation and *Arc* transcription [27], we further examined the role of PLC and CaM kinases, both of which are coupled to mGluR1/5 activation [15]. When neurons were pre-treated with the PLC inhibitor U73122, the DHPG-induced *Arc* up-regulation was significantly blocked (Fig. 2A). Next, we used KN62 to inhibit CaM kinases I/II/IV, and observed significant blockage of *Arc* transcription in DHPG-stimulated neurons (Fig. 2A). Although the promoters of *c-fos* and *Arc* share some common cis-elements (such as SRE), it appeared that they are differentially regulated. The DHPG-induced *c-fos* up-regulation was only blocked by U73122, but not by KN62 (Fig. 2A). Interestingly, inhibition of PLC and CaM Kinases significantly blocked both p-ERK1/2 and p-CREB activation (Fig. 2B). These data suggest that mGluR1/5-mediated activation of PLC and CaMK may converge on ERK1/2, and regulate *Arc* transcription.

The regulatory effects of PLC activity on ERK1/2 and *Arc* expression implicate a role of intracellular Ca^{2+} in the mGluR1/5 signaling [7,15]. We next examined how extracellular Ca^{2+} could regulate *Arc* transcription. At the post-synaptic sites, activation of mGluR1/5 may modulate the function of NMDA receptors and L-VGCC, both of which are strongly implicated in regulating plasticity-related genes [31]. Specifically, DHPG stimulates the phosphorylation of NR1 at both S896 and S897 in the neostriatum [4], which may, in turn, facilitate Ca^{2+} influx through the NMDA channels. The activation of group I mGluR1 also facilitates L-VGCC [3]. Indeed, pharmacological blockage of L-VGCC dampens the DHPH-induced LTD in the spinal cord [6], and the transcription of *Fmr1* gene in the ACC [28]. To block the channel activity of NMDA receptors and L-VGCC, we pre-incubated neurons with APV and nifedipine, respectively. Interestingly, inhibition of NMDARs suppressed DHPG-induced up-regulation of both *Arc* and *c-fos* (Fig. 3). In contrast, inhibition of L-VGCC by nifedipine did not block DHPG-induced up-regulation of *Arc* and *c-fos* (Fig. 3). Consistent with our hypothesis on the role of ERK1/2 as the key regulator, inhibition of NMDAR, but not L-VGCC, suppressed the DHPG-induced ERK1/2 phosphorylation (Fig. 3). These results demonstrated an interesting function of channel-specific influx of extracellular Ca^{2+} on mGluR1/5-mediated signaling and *Arc* expression.

What is the functional relevance of *Arc* expression upon group I mGluR activation? Two recent reports have suggested that the translational control of *Arc* in dendrites is required for DHPG-induced hippocampal CA1 LTD and AMPAR endocytosis [21,29]. The translation of newly induced *Arc* mRNA is also required for HFS-stimulated LTP in the dentate gyrus [17]. Although mGluR-LTD is considered to be dependent of new protein synthesis and independent of transcription, the function of *Arc* transcription is conceivable. It appears that the full-scale DHPG-induced *Arc* translation also depends on new transcription. When neurons are co-treated with DHPG and transcription inhibitor, a lower increase in *Arc* protein expression is observed [29]. A genome-wide analysis demonstrates the transcription of SRE-regulated genes, as well as increase in SRF binding activity, after the induction of mGluR-LTD in the hippocampus [10]. Because almost every mRNA transcript has a definite half-life, it is suggested that the translation templates (i.e., mRNAs) need to be replenished. Interestingly, the reported time course of mGluR-LTD rarely extends to over 60min after induction. It would be interesting to determine the effects of transcription inhibitor on mGluR-LTD at a much later time point.

Alternatively, the transcriptional up-regulation of *Arc* may be counteracting to LTD in brain regions other than hippocampus. For example, correlated to the role of mGluR1/5 in pain-related fear memory [26] and LTD [30], DHPG stimulation results in CREB-dependent transcription of *Fmr1* gene in the ACC [28]. Interestingly, the induction of immediate early genes (such as *c-fos* and *NGFI-A*) after amputation suppressed the mGluR-dependent LTD in the ACC [30].

In summary, we show, for the first time, that the up-regulation of *Arc* mRNA was stimulated by mGluR1/5 activation. The regulatory pathways involved in both intracellular and extracellular Ca^{2+} converge on ERK1/2 and modulated *Arc* expression. Because the induction of *Arc* transcription and mGluR1/5 function are both implicated in certain aspects of plasticity, neurological disease, and neurodevelopment [1,2], future experiments should address the causal role of group I mGluR-mediated *Arc* transcription in these brain functions.

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Figure 1.

Activation of group I mGluRs leads to significant elevation of *Arc* mRNA. Cortical neurons were stimulated by DHPG. The cells were harvested 30min, 1hr, or 2hr after the treatment. A. Western blot analysis shows that DHPG stimulates significant activation of ERK1/2 and CREB. B. Semi-quantitative RT-PCR shows that DHPG stimulates significant elevation of *Arc* and *c-fos* mRNA. C. DHPG-induced elevation of *Arc* and *c-fos* mRNA is independent of *de novo* protein synthesis. The neurons were pre-treated with a translation inhibitor, cycloheximide at 1μg/ml, for 20min before DHPG. One hour after DHPG application, cells were harvest and subjected to RT-PCR. D. Neurons were pre-treated for 30min with either YM 298198 (an mGluR1 antagonist) or MPEP (an mGluR5 antagonist) or both before DHPG

application. Samples were collected 1 hr after DHPG. Phosphorylation of ERK1/2 and *Arc* mRNA level was determined by Western blots and RT-PCR, respectively. Representative images are in the upper panels. The quantifications, with data from at least 4 independent experiments, are in the lower panels. *: $p < 0.05$ when compared to control non-treated neurons. #: $p < 0.05$ when compared to DHPG-treated neurons.

Figure 2.

ERK1/2 activation is the common signaling pathway to regulate group I mGluR-mediated *Arc* transcription. Cortical neurons were pre-treated with inhibitors for CaMK (KN62), PLC (U73122), and MEK (U0126) before DHPG application. Samples were analyzed with RT-PCR (A) and Western blots (B) 1 hr after DHPG treatment. Representative images are in the upper panels. The quantifications, with data from at least 4 independent experiments, are in the lower panels. *: $p < 0.05$ when compared to control non-treated neurons. #: $p < 0.05$ when compared to DHPG-treated neurons.

Figure 3.

NMDA receptors, but not L-VGCC, regulate *Arc* transcription and ERK1/2 activation in DHPG-stimulated neurons. Cortical neurons were pre-treated with the NMDAR antagonist APV or the L-VGCC antagonist nifedipine before DHPG application. Samples were analyzed with RT-PCR (for *Arc* and *c-fos*) or Western blot (for pERK1/2) 1hr after DHPG treatment. Representative images are in the upper panels. The quantifications, with data from at least 4 independent experiments, are in the lower panels. *: $p < 0.05$ when compared to control non-treated neurons. #: $p < 0.05$ when compared to DHPG-treated neurons.