Age-dependent Participation of Ras-GRF Proteins in Coupling Calcium-permeable AMPA Glutamate Receptors to Ras/Erk Signaling in Cortical Neurons*

Received for publication, November 9, 2005, and in revised form, December 27, 2005 Published, JBC Papers in Press, January 11, 2006, DOI 10.1074/jbc.M512060200

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α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (AMPARs) are ligand-gated sodium channels. Through their ability to mediate the majority of rapid excitatory transmission in the central nervous system, these neurotransmitter receptors have been shown to influence synaptic plasticity. Some of these receptors are also calcium-permeable (CP), and they also have been implicated in regulating synaptic plasticity, particularly in interneurons where their concentration is highest. However, the biochemical pathways emanating from CP-AMPARs that mediate these effects have not been well characterized. In this paper, we show that CP-AMPARs are the predominant AMPAR class responsible for activating the Ras/Erk kinase signaling cascade and the cAMP-response element-binding protein (CREB) transcription factor in the cortex of mature mice. Activation of Ras and Erk, but not CREB, occurs through the calcium/calmodulin regulated Ras-GRF1 and Ras-GRF2 exchange factors, which form AMPA-induced complexes with CP-AMPARs but not calcium-impermeable (CI) AMPARs in vivo. Furthermore, we show that CP-AMPARs are also the major AMPAR type to activate Ras/Erk signaling in pubescent mice; however, at this developmental stage Ras-GRF (guanine nucleotide-releasing factor) proteins are not involved. Finally, in neonatal animals CI-AMPARs, but not CP-AMPARs, are the predominant AMPAR type that activates Ras-Erk signaling and CREB in cortical neurons. This occurs indirectly through activation of L-type voltage-dependent calcium channels, an event that is also Ras-GRF-independent. Thus, Ras/Erk signaling and CREB activity induced by AMPARs occur through age-dependent mechanisms that likely make unique developmentally dependent contributions to synaptic function.

The AMPA2 subtype of glutamate receptors (AMPARs) mediate rapid excitatory transmission in the central nervous system through their sodium permeability (1). The majority of these channels are Ca2+-impermeable (CI-AMPAR). However, Ca2+-permeable forms (CP-AMPAR) also exist, primarily in interneurons (2), although they are present in pyramidal cells as well (3, 4). AMPARs are tetramers composed of various combinations of subunits, GluR1–4. Their cation selectivity is determined by their subunit composition, such that the presence of the GluR2 subunit prevents calcium permeability (2, 5). Synaptic addition of AMPARs contributes to long-term potentiation, whereas the removal of AMPARs from synapses contributes to long-term depression (6).

By virtue of their more promiscuous permeability, CP-AMPARs also have the capacity to influence synaptic plasticity through calcium influx. CP-AMPARs have been shown to contribute to long-term potentiation (7) or long-term depression through aspiny dendrites of γ-aminobutyric acid-containing interneurons, independent of NMDA glutamate receptors (8, 9). A key effector of calcium signaling in neurons that is known to have the potential to influence synaptic plasticity is the Ras/Erk kinase signaling cascade (10, 11). Although AMPA receptors have been documented to activate Erk kinase and CREB transcription factor in some neuronal cell types (12), the mechanism involved is not well understood. One important mediator of calcium signaling to Erk in neurons is the calcium/calmodulin-activated guanine nucleotide exchange factors, Ras-GRF1 and Ras-GRF2. Our previous study on Ras-GRF knock-out mice showed that both proteins mediate NMDAR activation of Ras and Erk in cortical neurons from mature mice but not in neonatal mice, where Ras-GRF levels are very low (13). Moreover, Ras-GRF1 appears to function through direct interaction with the NR2B subunit of the NMDAR (14).

Here we show that the mechanism by which AMPARs activate Ras/Erk signaling is also developmentally regulated in the cortex of mice. Both the subtype of AMPAR used to activate Erk and the mechanism used by specific AMPA receptor subtypes to activate Ras/Erk signaling change during the postnatal development of neurons. Because it has been documented that the consequences of Erk signaling in cells can be influenced by the specific mechanism by which Erk is activated (15), these developmental changes in AMPAR function likely contribute to age-dependent effects of AMPARs on neuronal function.

**MATERIALS AND METHODS**

**Preparation of Cortical Brain Slices**—Cortical brain slices (300 μm thick) were prepared as described previously (16) from mice of different ages. The slices were placed into 6-well plates (2–4 slices/well) with Krebs-Ringer solution (11.1 mM glucose, 1.1 mM MgCl2, 1 mM Na2HPO4, 1.3 mM CaCl2, 25 mM NaHCO3, 120 mM NaCl, 4.7 mM KCl) saturated with 95% O2, 5% CO2 at 25 °C. The brain slices were incubated for 60 min before pharmacological treatment. Stimuli, 100 μM AMPA or 100 μM NMDA (Sigma-Aldrich) were applied in each experiment for different times as indicated in the figures. For the inhibition experiments, inhibitors (1 μM phlanthotoxin–433 tris-trifluoroacetate (PHTx; from Sigma), 100 μM DL-2-α-amino-5-phosphonovaleric acid (APV), or 10 μM nimotoxipine (Sigma) was added as described above 30 min before
treatment of the brain slice cultures in the experiments. At the end of the experiment, cortical slices were lysed rapidly in buffer A (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM NaVO₃), subjected to SDS-PAGE, and immunoblotted with different antibodies as indicated.

Antibodies—Anti-phospho-Erk1/2 antibody, anti-phospho-Ser183-CREB antibody, and anti-CREB antibody were all obtained from Cell Signaling Technology. Anti-GRF1 (C-20) antibody and anti-Erk1 (K-23) antibody were obtained from Santa Cruz Biotechnology. Anti-Ras antibody was obtained from Transduction Laboratories. Anti-GluR1 and GluR2 antibodies were obtained from Chemicon Inc. Immunoblots were quantified by densitometric scans of autoradiographs using NIH Image 1.63 software, making sure that the assay was in the linear range.

Measurement of GDP-bound State of Ras—Ras activation was measured as described previously. Briefly, the slices were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 mM NaCl, 2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM NaVO₃, and 1 mM dithiothreitol. Nucleus-free supernatants containing 150 mg of total protein were affinity-purified using a glutathione S-transferase fusion protein with the Ras-GTP binding domain of c-Raf immobilized on a GST immobilized on a glutathione S-transferase agarose beads (Sigma) by incubation for 1 h at room temperature. The immune complexes were washed four times with lysis buffer and then subjected to SDS-PAGE and immunoblotting with antibodies against GluR1 or GluR2.

RESULTS

CP-AMPARs Activate Ras/Erk Signaling through Ras-GRF1 and Ras-GRF2 in Cortical Neurons in Adult Mouse Brain Slices—To begin to understand how AMPARs lead to Erk and CREB activation in cortical neurons, we first tested the contribution of CP-AMPARs, because calcium activates Erk mitogen-activated protein kinase and CREB in a variety of neuronal cell types. To this end, cortical brain slices from adult mice (postnatal day (p.n.) ≥30) were treated with AMPA (100 μM) for various times, and cell lysates were then assayed for activated Ras and CREB with the appropriate phospho-specific antibodies (Fig. 1). As shown previously (16), AMPA leads to the rapid activation of Erk and CREB (~3-fold), which is sustained for up to 10 min (Fig. 1A) but then rapidly returns to background (see Figs. 2B and 3B). Strikingly, AMPA activation of both Erk and CREB was blocked completely by pretreatment of the brain slices with the CP-AMPAR-specific inhibitor philanthotoxin (17), indicating that CP-AMPARs are the major AMPAR type to couple to Erk and CREB activation in the cortex of adult mice (Fig. 1, A and B).

CP-AMPARs could conceivably activate Erk indirectly through depolarization-induced activation of L-type voltage-sensitive calcium channels or NMDA glutamate receptors in these brain slices, because these two calcium channels have previously been shown to activate Erk in some neuronal preparations (27). However, neither the NMDAR inhibitor APV nor the L-type channel inhibitor nimodipine had inhibitory effects similar to those of philanthotoxin, consistent with the conclusion that the CP- but not CI-AMPARs activate Erk in these preparations (Fig. 1A).

Both Ras-GRF1 and Ras-GRF2 are activators of Ras, and thus its effector protein, Erk, and both Ras-GRF1 and Ras-GRF2 are activated in cells by elevated calcium levels through calmodulin binding to their IQ motifs (18, 19). Both proteins are also expressed preferentially in central nervous system neurons, so we examined whether they mediate CP-AMPAR activation of Erk in cortical brain slices from adult mice. To this end, AMPA was tested for the ability to activate Ras and Erk in brain slices from control and Ras-GRF knock-out mice (p.n. ≥30) were stimulated for various times with 100 μM AMPA, and then cell lysates were probed with phospho-specific Ras or total Ras antibodies (A) or with phospho-CREB or total CREB antibodies (B). In some cases the samples were pretreated with philanthotoxin (PhTx), APV, or nimodipine (Nim) for 30 min before stimulation. The results are representative of two experiments, each performed in duplicate.

FIGURE 1. AMPA activation of Erk and CREB in cortical brain slices from mature mice is mediated by CP-AMPARs. Cortical brain slices from adult mice (p.n. ≥30) were stimulated for various times with 100 μM AMPA, and then cell lysates were probed with phospho-specific Ras or total Ras antibodies (A) or with phospho-CREB or total CREB antibodies (B). In some cases the samples were pretreated with philanthotoxin (PhTx), APV, or nimodipine (Nim) for 30 min before stimulation. The results are representative of two experiments, each performed in duplicate.

FIGURE 2. CP-AMPARs activate Ras and Erk signaling through Ras-GRF proteins in cortical brain slices from mature mice. Cortical brain slices from control (WT, wild type) or double Ras-GRF knock-out mice (p.n. ≥30) were for 30 min before stimulation. The results are representative of two experiments, each performed in duplicate for panels A and C and at least five times for panel B.
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Figure 3. Developmentally dependent mechanisms mediate AMPA activation of Erk in cortical brain slices. Brain slices from wild type (A and C) or double Ras-GRF knock-out mice (B and D) at either age p.n. 20 (A and B) or p.n. 6 (C and D) were treated as described in the legends for Figs. 1 and 2. Cell lysates were prepared and assayed for either active Erk or active CREB. The results are representative of experiments performed three times, each in duplicate. c, control; Nim, nimodipine; PhTx, philanthotoxin; WT, wild type.

shown), indicating that both Ras-GRF1 and Ras-GRF2 couple CP-AMPARs to Ras and Erk in cortical neurons from adult mice.

Interestingly, although CREB is regulated by Erk upon stimulation of a number of receptors (10), this was not the case for CP-AMPARs, because AMPA activation of CREB was normal in double Ras-GRF knock-out mice in which Erk activation was blocked (Fig. 2C). Nevertheless, AMPA activation of CREB was completely blocked by philanthotoxin (Fig. 1B), indicating that CP-AMPARs activate this transcription factor by a mechanism independent of both Ras-GRFs and Erk (Fig. 2B). These findings also show that CP-AMPARs are in fact functional in neurons lacking Ras-GRFs, although they lose the ability to activate Ras/Erk signaling.

Overall these findings show that CP-AMPARs are the dominant class of AMPARs responsible for activating Ras/Erk signaling in adult cortical neurons. These results also show that they function through both Ras-GRF1 and Ras-GRF2 exchange factors.

CP-AMPARs Activate Ras/Erk Signaling in a Ras-GRF-independent Manner in Neurons from Brains of Pubescent Mice—The expression levels of both Ras-GRF1 and Ras-GRF2 are developmentally regulated such that they are very low in neonatal animals (13). These levels rise with postnatal development and are highest in the adult brain. Thus, we tested whether the mechanism of AMPA activation of Erk is age-dependent. Fig. 3 shows that AMPA stimulation leads to Erk activation in brain slices from pubescent mice (p.n. 20) (Fig. 3A) to a level similar to that found in the brain slices of mice from older mice (p.n. 30, see Fig. 1A). Moreover, AMPA activation of Erk was still completely blocked by philanthotoxin, but not by APV or nimodipine, indicating that like neurons from adult animals, CP-AMPAR receptors (but not CI-AMPAR receptors, NMDA receptors, or L-type voltage-dependent calcium channels) mediate the majority of AMPA receptor activation of Erk activation in pubescent mice. However, AMPA signaling to Erk in brain slices from pubescent double Ras-GRF knock-out mice was normal (Fig. 3B). Thus, CP-AMPARs signal to Erk in pubescent mice but through a mechanism that is distinct from that found in older mice (p.n. ≥30).

CI-AMPARs Activate Ras/Erk Signaling through L-Type Channel Activation in Neonatal Cortical Neurons—Finally, brain slices from neonatal animals (p.n. 6) were investigated. The magnitude of Erk and CREB activation by AMPA stimulation was similar to that found in brain slices from adult and pubescent mice (Fig. 3C, compare with Fig. 1A). However, in these neonatal samples, Erk and CREB activation by AMPA was not blocked by the CP-AMPAR inhibitor philanthotoxin (Fig. 3C), implying that CI-AMPARs were involved instead. Because CI-AMPARs depolarize neurons, we tested the possibility that Erk and CREB activation by these receptors is indirect through L-type voltage-sensitive calcium channels. This hypothesis was correct, because pretreatment of neonatal brain slices with nimodipine, an L-type channel blocker, completely suppressed AMPA activation of Erk and CREB (Fig. 3C). In contrast, APV, an NMDAR blocker, had no effect (Fig. 3C). Moreover, AMPA activation of Erk was normal in double Ras-GRF knock-out mice (Fig. 3D). Because cortical neurons from this age can be purified and cultured in vitro, they were tested, and the results were found to be indistinguishable from those obtained from brains slices (data not shown). Thus, in contrast to samples from older mice, CI-AMPARs are the predominant class of AMPARs connected to Ras/Erk and CREB signaling in neonatal cortical neurons. However, as in pubescent mice, Ras-GRF proteins are not involved (Fig. 3D).

Ras-GRF1 and Ras-GRF2 Form Complexes with CP- but Not CI-AMPARs in Vivo—To begin to understand how Ras-GRF proteins couple to CP-AMPARs in neurons from adult mice, complex formation between Ras-GRF proteins and AMPARs was probed via co-immunoprecipitation experiments. In particular, brain slices from adult mice were exposed to AMPA or buffer for 5 min, and lysates were then immunoprecipitated with anti-GRF antibodies and immunoblotted with anti-GluR antibodies. Because available Ras-GRF antibodies are not totally specific for an individual Ras-GRF family member, single Ras-GRF knock-out mice were used to investigate individual Ras-GRF family member interactions (Fig. 4A). For example, Ras-GRF1 was investigated (Fig. 4A, lanes 1 and 2) by using tissue from Ras-GRF1 knock-out mice (ras-grf1−/−). The results demonstrate the formation of an AMPA-induced complex between Ras-GRF1 and the GluR1 subunit of AMPARs in brain slices from mature mice (Fig. 4A, first two lanes, top three rows). Similar results were obtained with Ras-GRF1 knock-out mice (ras-grf1−/−), showing AMPA-induced complex between Ras-GRF2 and GluR1 (Fig. 4A, second two lanes, top 3 rows).

Importantly, neither Ras-GRF1 nor Ras-GRF2 was detected in a complex with the GluR2 subunit of AMPARs before or after AMPA stimulation of adult brain slices, despite the abundance of Ras-GRF proteins and GluR2 in cell lysates from mature brain slices (Fig. 4A, bottom three rows). This finding is consistent with the fact that Ras-GRFs are calcium-activated proteins and would not be expected to couple to AMPARs containing GluR2 subunits, which are known to prevent calcium influx in response to glutamate (2).

Finally, because we found that Ras-GRFs do not couple AMPARs to Erk in the cortex of juvenile mice, complex formation between Ras-GRF1 and GluR1 was tested at these ages. As expected complex formation was not detected in brain slices from neonatal (p.n. 0) or pubescent
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FIGURE 4. Ras-GRF proteins bind to GluR1 but not GluR2 containing AMPARs in an AMPA- and age-dependent manner. A, control and AMPA-stimulated cortical brain slices from mature (p.n. >30) Ras-GRF2 knock-out mice (to visualize just Ras-GRF1 (left two lanes)) and Ras-GRF1 knock-out mice (to visualize just Ras-GRF2 (right two lanes)) were lysed and then immunoprecipitated with Ras-GRF antibodies. The immunoprecipitates (IP) were then immunoblotted (IB) with either GluR1 antibodies (upper group) or GluR2 antibodies (lower group). Cell lysates from each experiment were probed for Ras-GRF and GluR proteins. B, experiments were performed as described in A except that brain slices from earlier ages were used.

(p.n. 10 and 20) brain slices (Fig. 4B). This is despite the fact that CP-AMPARs are abundant in pyramidal cortical neurons from young animals (4). This fact is not represented by the immunoblots of GluR1 shown in Fig. 4, because this figure represents GluR1 contained in both CI- and CP-AMPARs, whereas only a small percentage of GluR1 is associated with CP-AMPARs.

DISCUSSION

The results of experiments described in this paper show striking and unanticipated changes in the mechanisms underlying AMPA-type glutamate receptors activation of the Ras/Erk signaling cascade and the CREB transcription factor during postnatal development of the cortex of the mouse brain (see Fig. 5). These developmental changes are likely to have important effects on how Erk regulates neuronal function, because it has been documented in other cell systems that the consequence of Erk signaling in cells can be influenced not only by its magnitude but also by the specific mechanism used for its activation (15).

We observed changes in both of the subclasses of AMPARs involved and changes in the mechanism by which a specific subclass of AMPARs activates these signaling molecules. CP-AMPARs are at their highest levels during early postnatal development of cortical pyramidal cells (4). Nevertheless, they do not contribute significantly to AMPA-induced Erk activation or CREB regulation, because philanthotoxin, a specific inhibitor of CP-AMPARs, failed to block AMPA-induced Erk or CREB activation in brain slices from day 6 mice (or in cultured cortical neurons from neonatal mice). Instead, at this developmental stage CI-AMPARs appear to mediate this process. This occurs indirectly through depolarization-induced activation of L-type voltage-sensitive calcium channels, because AMPA activation of both Erk and CREB were completely blocked by the L-type channel inhibitor nimodipine.

In cortical brain slices from day 20 and older mice, CP-AMPARs but not CI-AMPARs are the predominant contributors of Erk activation, because the CP-AMPAR inhibitor philanthotoxin completely inhibited AMPA activation of Erk, whereas neither nimodipine nor the NMDAR inhibitor APV had any effect. The mechanisms underlying the developmentally dependent coupling of CP-AMPARs to Erk in the face of decreasing levels of CP-AMPARs (at least in pyramidal cells) (4) remains to be investigated. However, the developmentally dependent uncoupling of CI-AMPARs from Erk appeared to be due to L-type calcium channel uncoupling from Erk regulation, because nimodipine was effective in blocking AMPAR activation in neonatal neurons but not in neurons from older mice. Because this developmental change in L-type channel function has not been reported before, we confirmed this idea directly by testing the effects of the L-type channel agonist Bay-K-8644 and found that, as predicted, Erk activation was activated in brain slices from neonatal mice but not from mice more than 20 days old (data not shown). Overall, these finding show that the responsibility for Ras/Erk regulation by AMPARs switches from CI-AMPARs to CP-AMPARs in the neonatal period to CP-AMPARs in the pubescent period and afterward.

Finally, the mechanism by which CP-AMPARs activate Ras/Erk signaling changes during postnatal development, specifically between p.n. 20 and 30. This conclusion was reached by studying brain slices from mice lacking Ras-GRF proteins. In samples from mice 30 days or older, AMPA stimulation of Ras and Erk was completely blocked in double knock-out mice but not in single knock-out mice, indicating that both Ras-GRF family members couple CP-AMPARs to Ras/Erk signaling in mature mice. Additional support for this idea derived from experiments in brain slices demonstrating AMPA-induced complexes between Ras-GRF proteins and CP-AMPARs but not CI-AMPARs. In contrast, brain slices from 20 day-old double knock-out mice showed no defect in AMPA activation of Erk and no detectable AMPA/Ras-GRF complexes. Thus CP-AMPARs switch from a Ras-GRF independent mechanism of Ras activation to a Ras-GRF dependent mechanism between p.n. 20 and 30 in the brain cortex of mice.
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Ras-GRF proteins have also been shown to couple NMDA-type glutamate receptors to Ras/Erk signaling in the neurons of the central nervous system (13, 14). We showed that the mechanism underlying this phenomenon is also developmentally dependent, such that in neonatal neurons NMDAR functions independently of Ras-GRFs, but upon postnatal development corticospinal neurons becomes Ras-GRF dependent (13). Interestingly, Ras-GRFs become capable of mediating NMDAR activation of Erk by postnatal day 20 (13), whereas we show here that Ras-GRFs do not couple CP-AMPARs to Ras and Erk mitogen-activated protein kinase until a later age (p.n. 30). One possible explanation for this difference is that CP-AMPARs are enriched in interneurons, and their developmental pattern is likely different from the NMDAR-containing pyramidal cells, which comprise the major neuron type in the cortex. Another potentially interesting difference between NMDAR and AMPAR signaling is the mechanism of CREB regulation. Although NMDARs maintain CREB activation through Ras-GRFs as shown in brain slices from mature mice, AMPARs do this independently of Ras-GRFs.

Both CI-AMPARs and CP-AMPARs have been implicated in the regulation of synaptic plasticity (7, 9, 20). Regulation of AMPAR levels on the cell surface, and thus fast excitatory neurotransmission, is a key mechanism used by neurons to modulate synaptic strength (21, 22). As characterized in this paper and elsewhere, AMPARs also have the capacity to activate the Ras/Erk signaling cascade and the CREB transcription factor (12), both of which have also been implicated in the regulation of synaptic plasticity (10, 23). We document here that the mechanism used by neurons to modulate synaptic strength (21, 22). As characterized in this paper and elsewhere, AMPARs also have the capacity to activate the Ras/Erk signaling cascade and the CREB transcription factor (12), both of which have also been implicated in the regulation of synaptic plasticity (10, 23). We document here that the mechanism used by neurons to modulate synaptic strength and to regulate Ras/Erk and CREB changes between day 6 and day 20 of postnatal development of the cortex; CI-AMPARs and L-type channels decrease in this ability, whereas CP-AMPARs increase in ability. Thus, it is likely that their specific contributions to the regulation of synaptic function also change during this period.

Moreover, we found that CP-AMPARs switch their mechanism of Ras and Erk activation from a Ras-GRF-independent to a Ras-GRF-dependent pathway after puberty. This finding implies that the contribution of CP-AMPARs to synaptic plasticity is also altered during this later phase of brain development, and a growing body of evidence supports the idea that specific GEFs not only activate GTPases but also participate in the selection of specific effector proteins for activation by the GTPase (24–26).

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