α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, but Not N-Methyl-D-aspartate, Activates Mitogen-activated Protein Kinase through G-protein βγ Subunits in Rat Cortical Neurons*

Yizheng Wang and J. on P. Durkin†

From the Cellular Neurobiology Group, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

Glutamate is the major excitatory amino acid in the brains of vertebrates, and its receptors are believed to mediate a wide range of physiological and pathological processes including neurotransmission, plasticity, excitotoxicity, and various forms of neurodegeneration (1). Many of the actions of glutamate are coupled to the influx of extracellular Ca2+ mediated directly or indirectly by NMDA receptors present on neurons (1, 2). More recently, it has been determined that the AMPA glutamate receptor, which normally passes Na+, is also directly Ca2+- permeable (3, 4) and can be an important source of Ca2+ influx in some types of neurons (3–5). Transient changes in intracellular Ca2+ are known to trigger a plethora of cellular responses in neurons including the activation of protein kinases and phosphatases and changes in gene expression (1, 2). The NMDA-mediated entry of Ca2+ has been shown to activate MAPK (6), a serine/threonine kinase known to be important in the transmission of extracellular signals to the nucleus of cells (7–9). While the exact role and impact of NMDA-activated MAPK on neuronal cell growth (12) is unclear, it may be that MAPK serves as a conduit through which NMDA receptor-mediated Ca2+ signals are converted into relevant nuclear responses. By contrast, it is not known if the entry of Ca2+ via AMPA glutamate receptors is coupled to signaling pathways responsible for the activation of MAPK in neurons.

It is becoming increasingly evident that the influx of Ca2+ through different ion channels can elicit distinct cellular responses in neurons (2). For example, the entry of Ca2+ through NMDA receptors and L-type voltage-sensitive calcium channels (VSCCs) have been shown to transmit signals to the nucleus and to regulate gene expression through two distinct signaling pathways (10). It is therefore possible that mechanisms may be present in neurons by which different Ca2+ ion channels can differentially activate MAPK through distinct signaling pathways. In the present study we investigated whether Ca2+ entry through the AMPA glutamate receptor stimulates MAPK in rat cortical neurons, and if so, whether the mechanism of MAPK activation by AMPA is different from that induced by NMDA.

EXPERIMENTAL PROCEDURES

Materials—AMPA and NMDA were purchased from RBI; anti-Ras monoclonal antibody Y13–259 from Oncogene Science; anti-G-protein β subunit from DuPont NEN; and anti-B-Raf kinase and anti-MEK-1 antibodies from Santa Cruz. All other reagents were from Sigma.

Primary Neuron Cultures—Cerebral cortical cultures, prepared from 18-day-old fetal rats (11), were plated on poly-L-lysine-coated 12-well plates (for kinase assays) at 5 × 106 cells/ml in DMEM supplemented with 2 μg/ml L-glutamine, 30 mM glucose, 10% heat-inactivated fetal bovine serum, and 10% horse serum. After 3 days, 15 mg/ml 5-fluorodeoxyuridine and 35 μg/ml uridine were added to inhibit non-neuronal cell growth (12). Greater than 90% of the cells were neurons based on morphology and negative GFAB staining. Cultures were used for experiments 10–13 days after plating. Unless stated, cultures were rinsed twice and placed in DMEM without serum immediately before experiments were initiated. For Na+-free conditions, the cells were incubated in a solution containing 120 mM choline chloride, 5.4 mM KCl, 0.8 mM CaCl2, 15 mM glucose, and 25 mM Tris-HCl, pH 7.4.

MAPK Assay—MAPK activity was measured as described previously (13). Briefly, cultures were rinsed 3 times with cold PBS containing 10 mM NaF and 1 mM PMSF, and the cells were extracted by incubation in Triton X-100 lysis buffer (13) for 30 min at 4°C. Extracts (60 μg of protein) were incubated with protein A-Sepharose beads preabsorbed with preimmune serum for 1 h, and then treated with 100,000 g for 15 min, and then treated with 1 h with an anti-α2 MAPK antibody (14) absorbed to protein A-Sepharose. The immunocomplex formed was collected by centrifugation, washed 4 times with lysis buffer (0.5% Triton X-100), and resuspended in kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, and 10 mM β-nitrophenyl phosphate). MAPK activity in the immunoprecipitate was measured as the extent of incorporation of 32P into MBP (15). Phosphorylated MBP, separated by SDS-PAGE, was visualized by autoradiography or quantitated by the excitation counting of excised bands.

GTP Loading Assay—Cortical neurons plated in 6-well plates at 5 × 106 cells/ml treated with or without 100 ng/ml PTX for 20 h were metabolically labeled with 0.5 μCi/ml [32P]Pi in PO4-free DMEM for 4 h and then treated with 100 μM AMPA for 2 min. The immunoprecipitation of Ras was as described (15). Briefly, cells were washed twice with cold PBS containing 10 mM NaF and 1 mM PMSF and lysed in 0.5 ml of buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1% Triton X-100) containing 1 μg of the anti-Ras monoclonal antibody Y13–259.
ampA, but Not NMDA, Activates MAP Kinase

After clearing the extracts with a charcoal slurry, protein A-Sepharose coupled with goat anti-rat IgG was added, and the extracts were incubated for 2 h at 4°C. Samples were collected by centrifugation, washed, resuspended in 1 mM KH₂PO₄, and incubated at 85°C for 3 min. Samples were separated by polyethyleneimine cellulose thin layer chromatography and visualized by autoradiography. The percent GTP was calculated as density of GTP/density of GDP.

Detection of the Ras-G-protein βγ-Raf-MEK-1 Complex—Neurons grown on 10-cm dishes (5 × 10⁶/ml) were treated with 100 μM AMPA, 100 μM NMDA, or 50 μM KCl for 3 min. Cultures were rinsed twice with cold PBS containing 10 mM NaF and 1 mM PMSF and 1 ml of extraction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.5% Triton X-100, 1 mM NaF, 20 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM PMSF, 1 μM pepstatin A, and 1.0% bovine serum albumin) containing 20 μg of anti-Ras antibody Y13–259 was added. Cells were scraped and lysed by 20 passages through a 21-gauge needle. A 1:10 v/v charcoal slurry was added and the extracts incubated for 1 h at 4°C. Clarified supernatants, obtained by centrifugation, were incubated with protein A-Sepharose preabsorbed with goat anti-rat IgG overnight at 4°C. The beads were collected, washed three times in buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EGTA, and 0.5% Triton X-100) and once in PBS, and the immunoprecipitate was subjected to SDS-10% PAGE followed by electrophoresis to Immobilon-P (Millipore). Immobilon-P was blocked for 1.5 h with 0.5% bovine serum albumin and 5% powdered milk in PBS, washed twice with PBS, and incubated overnight at 4°C with the antibodies specified in the legends to Figs. 3 and 4. The Immobilon-P was then washed three times with PBS containing 0.1% SDS, incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody for 1.5 h, and washed 5 times with PBS containing 0.1% SDS. The bands were visualized by an Amersham ECL system.

RESULTS AND DISCUSSION

AMPA, NMDA, and KCl-induced depolarization all caused transient ~2.5-fold increases in MAPK activity in primary rat cortical neurons, which peaked 3 min after treatment (Fig. 1A). However, whereas MAPK activity returned to near basal levels within 10 min of NMDA or KCl addition, AMPA-stimulated MAPK declined gradually over a 20–60-min period (Fig. 1A). The stimulation of MAPK by AMPA, NMDA, and KCl, but not epidermal growth factor, was abolished when cortical neurons were incubated in Ca²⁺-free medium (Fig. 1C). However, the increase in MAPK activity by AMPA was unperturbed by incubation in Na⁺-free medium (Fig. 1D), indicating that the influx of Na⁺ and/or Na⁺-induced depolarization through the AMPA receptor was not responsible for MAPK activation. Moreover, while the L-type VSCC blocker, nifedipine (15), abolished the stimulation of MAPK activity mediated by KCl-induced depolarization, it had no effect on the activation of MAPK by either AMPA or NMDA (Fig. 1E). This finding is in agreement with a previous report (6) demonstrating that glutamate and NMDA-stimulated MAPK activity is a Ca²⁺-dependent, VSCC-independent process. Thus, L-type VSCCs appear linked to the depolarization-induced activation of MAPK by KCl, whereas the entry of Ca²⁺ via these channels was not likely a significant factor in MAPK activation by AMPA. The increase in MAPK activity by AMPA was blocked by pretreatment of neurons with the selective AMPA receptor antagonist, CNQX, but not by the NMDA channel blocker, MK801, or MCPG, a metabotropic receptor blocker. Furthermore, trans-(1S,3R)-ACPD, a glutamate metabotropic receptor agonist, did not stimulate MAPK activity (data not shown). Collectively, these results indicate that the influx of Ca²⁺ directly through the AMPA receptor itself was likely responsible for the prolonged activation of MAPK by AMPA in rat cortical neurons.

(EGF) were 100 μM, 100 μM, 50 μM, and 100 ng/ml, respectively. Each cell point is the mean ± S.E. of radioactivity incorporated into myelin basic protein of three separate experiments in triplicate.
MAP kinase activation arises from a protein kinase cascade, which in some cells involves protein kinase C (PKC) (16, 17). However, the down-regulation of PKC (18) in neurons by chronic exposure to the phorbol ester, phorbol 12-myristate 13-acetate (PMA), did not alter AMPA-induced activation of MAPK, although it did ablate MAPK stimulation triggered by acute exposure to phorbol 12-myristate 13-acetate (Fig. 2A). Moreover, the potent PKC inhibitor, calphostin C, had no effect on AMPA-induced MAPK (data not shown). Therefore, the activation of MAPK by AMPA was clearly a PKC-independent process.

It is well established that the Ras protein occupies a pivotal position in the cascade activation of MAPK (19–24). Exposing cortical neurons to AMPA rapidly caused about a 30% increase of Ras activity (Fig. 2, B and C) measured as the ratio of the GTP- to GDP-bound form of the protein. Since Ras-dependent activation of MAPK has been associated with receptors coupled to G-proteins (25–27) and receptors with intrinsic tyrosine kinase (28), we studied the effects of PTX on AMPA-induced MAPK activation. Surprisingly, pretreating cortical neurons with PTX inhibited the increase in MAPK seen with AMPA but not that observed with either NMDA or KCl (Fig. 2D). Moreover, the increase in Ras activity effected by AMPA was sensitive to PTX treatment (Fig. 2, B and C), indicating that the activation of MAPK through the AMPA receptor, but not the NMDA receptor, involved a PTX-sensitive G-protein (presumably a member of the Gi family of proteins) apparently coupled to the AMPA-induced stimulation of Ras in cortical neurons.

Although most G-protein-mediated processes are modulated by activated α subunits, it is now evident that the βγ subunits of G-proteins can also regulate the activity of various effectors (29–33). As shown in Fig. 3A, a G-protein β subunit was found in the immunocomplex precipitated by an anti-Ras antibody in AMPA-challenged neurons but not in control cultures. By contrast, neither NMDA nor KCl stimulation resulted in the coprecipitation of a β subunit with Ras (Fig. 3, B and C). The AMPA-induced association of the β subunit with the Ras immunocomplex was dose-dependent (Fig. 3D) and substantially reduced in cultures subjected to PTX pretreatment (Fig. 3A). As was the case with AMPA-stimulated MAPK, the coprecipitation of the β subunit with Ras was dependent on extracellular Ca²⁺ (Fig. 3C) but not Na⁺ (data not shown). These findings strongly suggest that AMPA, but not NMDA or KCl, induced a dissociation of Gα from βγ dimers and that the subsequent binding of the free βγ subunit to Ras protein, probably through its pleckstrin homology domain (34–36), stimulated a downstream cascade leading to MAPK activation.

If a G-protein dependent activation of Ras is an early step in the AMPA-induced stimulation of MAPK, then the downstream involvement of Raf kinase could be anticipated (37–40). Since B-raf transcripts are found at their highest levels in brain (41) and B-Raf, rather than Raf-1, is responsible for nerve growth factor-stimulated MEK-1 activation in PC12 cells (42), we determined whether stimulating cortical neurons with AMPA caused the coprecipitation of B-Raf with Ras. As shown in Fig.

**Fig. 2.** AMPA activates Ras and MAPK through a PTX-dependent but PKC-independent pathway. A, neurons were treated with 100 nM PMA (TPA) for 20 h and stimulated with 100 μM AMPA or 100 nM PMA for 3 min. MAPK activity was then determined (25). Results represent the mean ± S.E. of three separate determinations in triplicate. B, untreated controls or cultures treated with 100 ng/ml PTX for 20 h were labeled with [32P]H3PO4 for 4 h and then stimulated with 100 μM AMPA for 2 min. Ras was immunoprecipitated from cell lysates, and bound guanine nucleotides were separated by thin layer chromatography (11). C, densitometric determinations of GTP/(GTP + GDP) ratios from the autoradiograph presented in B. The results are representative of six separate determinations. D, untreated controls or cultures treated with 100 ng/ml PTX for 20 h were left unstimulated or were stimulated with 100 μM AMPA, 100 μM NMDA, or 50 mM KCl for 3 min before MAPK activity was determined. Results are representative of the mean ± S.E. of three experiments in triplicate. MBP, myelin basic protein.
G-protein subunit and Raf kinase. MEK acts immediately downstream of Raf kinase, and upon its phosphorylation and activation, it phosphorylates and activates MAPK (43, 44). As was observed with the β subunit, the coprecipitation of p95B-raf with Ras was dependent on extracellular Ca2+ entry (2). It is reasonable to suggest that neurons could use these different paths to MAPK activation to generate divergence in the downstream signals and cellular responses to MAPK activity. Elucidating the signaling pathways specifically coupled to the entry of Ca2+ through AMPA, NMDA, VSCCs, and other ionotropic channels will facilitate our understanding of the role these receptors play in neurons under both physiological and pathological conditions.

Fig. 3. AMPA, but not NMDA or KCl, causes the association of a G-protein β subunit with Ras complex. Following the treatments indicated below, Ras immunoprecipitates were subjected to SDS–10% PAGE, electrophoresed, and probed with an anti-G-protein β subunit antibody as described (16). The blots were then stripped and reprobed with an anti-Ras antibody (16). A, untreated control or cultures treated with 100 ng/ml PTX for 20 h were stimulated with 100 μM AMPA for 3 min. B, cultures were left unstimulated (lane C) or were stimulated with 100 μM AMPA (lane A), 100 μM NMDA (lane N), or 50 mM KCl (lane K) for 3 min. C, the effect of extracellular Ca2+ on the coprecipitation of G-protein β subunit with Ras. Cultures were incubated in DMEM with or without 2.4 mM Ca2+ for 15 min and then stimulated with the indicated doses of AMPA for 3 min.

4A, while both the p68 and p95 forms of Raf kinase were detected in the immunocomplex precipitated by the anti-Ras antibody, only the levels of p95B-raf kinase were increased after exposing neurons to AMPA. Moreover, the p95B-raf kinase that coprecipitated with Ras in AMPA-challenged neurons exhibited a slow gel mobility shift, an indicator of Raf kinase activation (38). As was observed with the β subunit, the coprecipitation of p95B-raf with Ras was dependent on extracellular Ca2+ entry (Fig. 4B). We next determined whether MEK-1 (MAP kinase/ERK-activating kinase) was present in the complex containing G-protein β subunit and Raf kinase. MEK acts immediately downstream of Raf kinase, and upon its phosphorylation and activation, it phosphorylates and activates MAPK (43, 44). As demonstrated in Fig. 4C, while there was no indication of an increased association of MEK-1 with the Ras complex precipitated from AMPA-stimulated cells, an AMPA dose-dependent activation of MEK-1 was observed, measured as a slow gel mobility shift of the protein. Both the binding of MEK-1 to the Ras complex and its subsequent activation by AMPA were found to be dependent on extracellular Ca2+ (data not shown).

The results of this study are consistent with the hypothesis that the transmembrane influx of Ca2+ through AMPA recep-
AMPA, but Not NMDA, Activates MAP Kinase

22787

15. Rosen, L., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994) Neuron 12, 1207–1221
16. L'Allemain, G., Sturgill, T. M., and Weber, M. J. (1991) Mol. Cell. Biol. 11, 1002–1008
17. Johnson, G. L., and Vaillancourt, R. R. (1994) Curr. Opin. Cell Biol. 6, 230–238
18. Chida, N., Kato, N., and Kuroki, T. (1986) J. Biol. Chem. 261, 13013–13018
19. Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S., and Brugge, J. S. (1992) Cell 68, 1031–1040
20. Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) Cell 68, 1041–1050
21. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
22. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolffman, A. (1993) Science 260, 1658–1661
23. Minden, A., Lin, A., McMahon, M., Lange-Carte, C., Derijard, B., Davis, R. J., and Karin, M. (1994) Science 266, 1719–1723
24. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
25. Gardner, A. M., Vaillancourt, R. R., and Johnson, G. L. (1993) J. Biol. Chem. 268, 17896–17901
26. Winitz, S., Russell, M., Qian, N.-X., Gardner, A., Dwyer, L., and Johnson, G. L. (1993) J. Biol. Chem. 268, 19196–19199
27. Albias, J., van Corven, E. J., Horst, P. L., Milligan, G., and Moolenaar, W. H. (1993) J. Biol. Chem. 268, 22235–22238
28. Blumer, K. J., and Johnson, G. L. (1994) Trends Biochem. Sci. 19, 236–240
29. Tang, W. J., and Gilman, A. G. (1991) Science 254, 1500–1503
30. Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R., and Bourne, H. R. (1992) Nature 356, 159–161
31. Neer, E. J. (1995) Cell 80, 249–257
32. Crespo, P., Xu, N., Simonds, W. F., and Gutkin, J. S. (1994) Nature 369, 418–420
33. Koch, W. J., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12706–12710
34. Lefkowitz, R. J. (1993) Cell 74, 409–412
35. Pavson, T. (1995) Nature 373, 537–540
36. Inglesio, J., Koch, W. J., Touhara, K., and Lefkowitz, R. J. (1995) Trends Biochem. Sci. 20, 151–155
37. Kyriakis, J. M., App, H., Zhang, X.-F., Banerje, P., Brautigan, D. L., Rapp, U. F., and Avruch, J. (1992) Nature 358, 417–421
38. Daum, G., Eisenmann-Tappe, I., Fries, H.-W., Trammel, J., and Rapp, U. R. (1994) Trends Biochem. Sci. 19, 474–480
39. Leevers, S. L., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
40. Stokoe, D., MacDonald, S. G., Gadwallade, K., Symons, M., and Hancock, J. F. (1994) Science 264, 1463–1467
41. Storm, S. M., Cleveland, L., and Rapp, R. U. (1990) Oncogene 5, 345–351
42. Jaiswal, R. K., Moodie, A., Wolfman, A., and Landreth, G. E. (1994) Mol. Cell. Biol. 14, 6944–6953
43. Crews, C., Alessandrini, A. A., and Erikson, R. L. (1992) Science 258, 478–480
44. Crews, C., Alessandrini, A. A., and Erikson, R. J. (1992) Cell Growth & Differ. 3, 135–142
α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, but Not N'-Methyl-D-aspartate, Activates Mitogen-activated Protein Kinase through G-protein β γ Subunits in Rat Cortical Neurons
Yizheng Wang and Jon P. Durkin

J. Biol. Chem. 1995, 270:22783-22787.
doi: 10.1074/jbc.270.39.22783

Access the most updated version of this article at http://www.jbc.org/content/270/39/22783

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 20 of which can be accessed free at http://www.jbc.org/content/270/39/22783.full.html#ref-list-1