N-Methyl-D-Aspartate Receptor-mediated Bidirectional Control of Extracellular Signal-regulated Kinase Activity in Cortical Neuronal Cultures*

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N-Methyl-D-aspartate (NMDA) receptor activation of extracellular-signal regulated kinase (ERK) was examined in primary cortical cultures. Tetrodotoxin, NMDA receptor antagonists, or reduced extracellular calcium (0.1 mM) greatly decreased basal levels of phospho-ERK2, indicating that activity-dependent activation of NMDA receptors maintained a high level of basal ERK2 activation. This activity-dependent activation of phospho-ERK2 was blocked by pertussis toxin and inhibition of calcium/calmodulin-dependent kinase II and phosphatidylinositol 3-kinase but not by inhibition of protein kinase C or cAMP-dependent protein kinase. Addition of a calcium ionophore or 100 μM NMDA decreased phospho-ERK2 in the presence of 1 mM extracellular calcium but enhanced phospho-ERK2 in 0.1 mM extracellular calcium. The reduction in basal phospho-ERK2 by 100 μM NMDA was also reflected as a decrease in phospho-cAMP response element-binding protein. Inhibition of tyrosine phosphatases and serine/threonine phosphatases protein phosphatase 1 (PP1), PP2A, and PP2B did not prevent the inhibitory effect of NMDA. In the presence of tetrodotoxin, NMDA produced a bell-shaped dose-response curve with stimulation of phospho-ERK2 at 10, 25, and 50 μM NMDA and reduced stimulation at 100 μM NMDA. NMDA (50 μM) stimulation of phospho-ERK2 was completely blocked by pertussis toxin and inhibitors of phosphatidylinositol 3-kinase and was partially blocked by a calcium/calmodulin-dependent kinase II inhibitor. These results suggest that NMDA receptors can bidirectionally control ERK signaling.

Mitogen-activated protein kinases constitute a family of serine/threonine kinases, the best understood of which are the extracellular signal-regulated kinases (ERKs); Ref. 1. Ras proteins belong to a superfamily of small GTPases that cycle between inactive GDP-bound states and active GTP-bound states, and represent a point of convergence for the transduction and integration of many extracellular signals that activate mitogen-activated protein kinases (2). Ras-GTP initiates a sequential cascade of events involving recruitment to the membrane and activation of Raf-1, activation of the dual-specificity kinases termed mitogen-activated protein kinase/ERK kinases (MEKs), and finally activation of ERK. Activated ERKs phosphorylate cellular substrates and translocate to the nucleus, where they play an important role in regulating gene transcription (3, 4). In mitotic cells, ERKs constitute a primary effector pathway in controlling cellular proliferation, differentiation, cell cycle regulation, and survival. In brain, recent studies indicate that ERKs play an important role in synaptic plasticity and memory formation (5).

Glutamate is the major excitatory neurotransmitter in the vertebrate brain, and the NMDA subtype of glutamate receptors are among the most widely distributed and abundant receptor-operated ion channels in the central nervous system. In addition to mediating the slow component of glutamate-dependent excitatory postsynaptic currents, NMDA receptors play a vital role in a variety of processes, including neuronal development, synaptic plasticity, learning and memory, and neuronal survival and death (6). NMDA receptor-mediated increases in intracellular calcium have been shown to stimulate ERK signaling, and evidence suggests that NMDA receptor-mediated ERK activation may play an important role in neurotransmission and synaptic plasticity (7, 8). NMDA-dependent hippocampal long-term potentiation is associated with activation of ERK and is blocked by compounds that inhibit the ability of MEK to activate ERK (9, 10). ERK activation has also been shown to be required for hippocampal-dependent associative learning in rats (11), and mice lacking Ras-guanine nucleotide-releasing factor (Ras-GRF) display impaired amygdala-dependent memory consolidation (12).

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The abbreviations used are: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; DME, Dulbecco’s modified Eagle’s medium; PDHS, plasma-derived horse serum; PFTX, pertussis toxin; PFTX, tetrodotoxin; PBST, phosphate-buffered saline containing 0.05% Tween 20; CaMKII, calcium/calmodulin-dependent kinase II; PI-3K, phosphatidylinositol 3-kinase; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; CREB, cAMP response element-binding protein; PP, protein phosphatase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; ANOVA, analysis of variance; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; MK-801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclopepten-5,10-imine; AMPA, (S)-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; PD98059, 2-amino-3-(methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminoethyl)hydantoin; MCPG, (S)-α-methyl-4-carboxyphenylglycine; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Go 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-15-methyl-5-oxo-5H-indole[2,3-a]pyrrolo[3,4-c]carbazole; H-89, 2-[(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]-amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride, HCl, AP5, (R)-2-amino-5-phosphonopentanoic acid; NMDA, N-methyl-D-aspartate.
An important aspect of NMDA receptor-mediated increases in intracellular calcium is that different levels of calcium influx stimulate different, and sometimes opposing, responses. For example, low levels of intracellular calcium can preferentially activate phosphatases, leading to long-term depression (13), whereas higher levels of intracellular calcium can activate kinases that support long-term potentiation (14, 15). Similarly, small increases in intracellular calcium can promote neuronal survival (16, 17), whereas large increases lead to cell death (excitotoxicity; Ref. 18). Thus, many calcium-dependent synapt-ic responses may represent a balance between opposing cal- cium-activated processes. In the present study, we demonstrate that NMDA receptor-dependent calcium influx can activate opposing stimulatory and inhibitory pathways that regulate ERK activation in primary cortical cultures. The stimulatory process predominates at lower levels of NMDA receptor activi- tivation, whereas the inhibitory process predominates at higher levels of NMDA receptor activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Female Harlan Sprague Dawley rats were obtained from Charles River (Wilmington, MA), and Dulbecco's modified Eagle's medium (DMEM) and amphotericin B (Fungizone) were purchased from Life Technologies, Inc.; plasma-derived horse serum (PDHS) was purchased from Sigma; trypsin was obtained from Worthington Biochemicals (Freehold, NJ); and penicillin and streptomycin were purchased from Pfizer Inc. (New York, NY). CNQX was purchased from Tocris Neuramin (Essex, United King-dom), and MK-801 and AMPA were from Research Biochemicals Inc. (Natick, MA). Anti-phospho-p44/42 (Thr202,Tyr204), anti-phospho-p44/42 (Tyro204), anti-phospho-p42/44 (Ser217,221), anti-phospho-CAMP response element-binding protein (CREB; Ser133), anti-CREB, and PD98059 were purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Southern Biotechnology Associates (Birmingham, AL), and U0126 was from Promega. Protease inhibitors, ionomycin, MCPG, Go6976, H-89, KN-92, KN-93, calyculin A, and cypermethrine were purchased from Calbiochem; tetrodotoxin was from Sigma; and pertussis tox-in was from List Biological Laboratories (Campbell, CA).

**Preparation of Neuronal Cultures**—Cerebral cortical cultures were prepared from newborn rat pups as described previously (19). In brief, brains were removed and placed in an isotonic salt solution containing 100 units of penicillin G, 100 μg of streptomycin, and 0.25 μg of amphotericin B (Fungizone/ml), pH 7.4. Blood vessels and pia mater were removed, and the tissue was chopped into 2-mm chunks. The brain pieces were then suspended in 25 ml of 0.25% (w/v) trypsin in isotonic salt solution, pH 7.4, and placed in a shaking water bath for 10 min at 37 °C to dissociate the cells. The dissociated cell suspension was then removed and combined with 10 ml of DMEM containing 10% PDHS, and the undisassociated chunks were mixed with 160 μg of DNase and triturated until the cells were dissociated. The dissociated cell suspensions were combined and centrifuged at 1000 g for 10 min, and the resulting pellet was washed with 50 ml of DMEM containing 10% PDHS. Cells were resuspended in DMEM containing 10% PDHS, plated in poly-L-lysine-precoated culture dishes at a density of 4 × 10^6 cells/35-mm dish, and incubated at 37 °C in a humidified incubator with 5% CO₂ and 95% air. On day 3, cells were treated with 10 μM β-cytochrome arabinoside in DMEM containing 10% PDHS. After 2 days of β-cytochrome arabinoside treatment, culture medium was replaced with DMEM containing 10% PDHS, and cultures were grown for an additional 7–9 days before being used in experiments. At this time, cultures consisted of 90% neurons as determined by immunofluorescent staining with anti-β-tubulin. Cultures appeared as many phase-bright cells with characteristic neuronal morphology overlying a small number of flat, phase-dark cells, which had typical astroglial morphology. Each experimen-tal n represents data obtained from neuronal cultures prepared from at least 10 littermates of pups.

**Culture Treatments and Tissue Preparation**—For certain experiments, cortical cultures were serum-deprived for 24 h before use by replacing the serum-containing DMEM with serum-free DMEM. Cortical cultures were washed twice with 1 ml of HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 100 mM glucose, 15 mM pyruvate, and 25 mM HEPES, pH 7.4) and incubated for 10 min in 1 ml of HEPES buffer followed by exposure to agonists (NMDA, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and ionomycin) or tetrodotoxin (TTX) for the indicated times. Antagonists and inhibitors (MK-801, CNQX, PD98059, sodium orthovanadate, Go6976, H-89, KN-92, KN-93, calyculin A, cypermethrin, okadaic acid, and noladinomide) were added 15 min before addition of NMDA. For experiments examining the involvement of phosphoinositide (PTX)-sensitive G-proteins, cultures were preincubated with PTX (1 μg/ml) for 12 h before NMDA exposure. After the indicated exposure times, the HEPES buffer was removed, and the cells were scraped into 100 μl of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA, 2 mM sodium pyruvo-phosphatase, 1 mM activated sodium orthovanadate, 0.2 mM AEBSF, 1 μM aprotinin, 1 mM benzamidine, 10 μM leupeptin, and 10 μg/ml pepstatin). Cells isolated from three dishes were combined, briefly sonicated, and centrifuged at 15,000 × g for 30 min at 4 °C. The resulting supernatant was removed, an aliquot was taken for determination of protein concentration by the bicinchoninic acid assay (Pierce), and the remainder was stored at −80 °C until use.

**Gel Electrophoresis and Immunoblotting**—An aliquot of the frozen supernatant was diluted with an equal volume of 2× electrophoresis sample buffer (final concentration, 50 mM Tris-HCl, pH 6.7, 4% w/v glycerol, 4% SDS, 1% 2-mercaptoethanol, and 0.02 mg/ml bromphenol blue) and boiled for 10 min, and 20 μg was separated by size on a 7.5% SDS-polyacrylamide gel using the buffer system of Laemmli (20) and transferred to Immobilon-P polyvinylidene difluoride (Millipore, Bed- ford, MA). Membranes were blocked and transferred (190 g/ml aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Cells isolated from three dishes were combined, briefly sonicated, and centrifuged at 15,000 × g for 30 min at 4 °C. The resulting supernatant was removed, an aliquot was taken for determination of protein concentration by the bicinchoninic acid assay (Pierce), and the remainder was stored at −80 °C until use.

**RESULTS**

**NMDA Receptor Activation of ERK2 Involves CaMKII, PI-3K, and a PTX-Sensitive G-Protein**—NMDA receptor coupling to ERK activation was examined in primary cortical neuronal cultures by measuring changes in levels of phospho-ERK2. Addition of TTX (1 μM) caused a large decrease in the levels of phospho-ERK2 (Fig. 1, A and B), indicating synaptic activity-dependent activation of ERK2 in the cortical cultures. To eliminate this component of ERK activation, cultures were preincubated with 1 μM (TTX) for 1 h before a 5-min exposure to NMDA. In the absence of synaptic activity, the NMDA dose-response curve for stimulation of phospho-ERK2 was bell-shaped (inverted U), with stimulation of phospho-ERK2 at low NMDA concentrations (10 μM and 25 μM) and no stimulation at 100 μM NMDA. Extending the NMDA exposure time from 5 to 60 min yielded a similar bell-shaped dose-response curve (n = 2; data not shown). Fig. 1, C and D, shows that NMDA (50 μM) stimulation of phospho-ERK2 in the presence of TTX was blocked by MK-801 (10 μM), demonstrating that NMDA stimulation of phospho-ERK2 is an NMDA receptor-mediated process. Total ERK levels were unchanged by the above treatments (data not shown).
Fig. 2. The NMDA dose-response curve for stimulation of phospho-ERK2 is bell-shaped in the presence of TTX. Protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2 (p44/42). A and C, representative immunoblots of phospho-ERK1/2 (p44/p42) immunoreactivity. B and D, quantification of phospho-ERK2 by computer-assisted densitometry expressed as optical density. A and B, basal levels of phospho-ERK2 in neuronal cultures were reduced after a 1-h blockade of synaptic activity with TTX (1 μM). Cultures were then exposed to vehicle or NMDA (10–100 μM) for 5 min. The curve was generated by computer interpolation of the data. C and D, NMDA stimulation of phospho-ERK2 in the presence of TTX is blocked by MK-801 (10 μM) added 20 min before NMDA. Values are the means ± S.E.M. of nine (B) and three (D) independent experiments. B, **p < 0.01 versus TTX basal; ††, p < 0.01 versus + TTX basal; †, p < 0.05 versus 10 μM NMDA; ⋆, p < 0.01 versus 25 μM NMDA. D, *, p < 0.01 versus TTX; Ψ, p < 0.01 versus NMDA (ANOVA with Newman-Keuls posthoc test).

Fig. 3. NMDA stimulation of phospho-ERK2 involves CaMKII, PI-3K, and a pertussis-toxin sensitive G-protein. A and B, neuronal cultures were incubated for 1 h in the presence of TTX (1 μM) to eliminate synaptic activity-dependent activation of phospho-ERK1/2, followed by a 5-min stimulation with NMDA (50 μM). Protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2 (p44/42). KN-93 (40 μM) and wortmannin (200 nM) were added 20 min before NMDA. C and D, neuronal cultures were incubated with pertussis toxin (1 μg/ml) 12 h before the 1-h TTX (1 μM) exposure and 5-min NMDA exposure. A and C, representative immunoblot of phospho-ERK1/2 (p44/p42) immunoreactivity. B and D, quantification of phospho-ERK2 by computer-assisted densitometry. Values are expressed as optical density and are the means ± S.E.M. of four or five independent experiments. B, *, p < 0.01 versus respective basal; ⋆, p < 0.05; ⋆⋆, p < 0.01 versus respective NMDA. D, *, p < 0.05 versus TTX (ANOVA with Newman-Keuls posthoc test).
Activation of ERK in response to NMDA receptor-stimulated calcium influx has been previously observed, yet the mechanism(s) underlying this activation process remain unclear. Several signaling messengers, including the tyrosine kinases Src (21) and protein-tyroksine kinase 2 (PYK2) (22), nitric oxide (23), the guanine-nucleotide exchange factor Ras-GRF (24), and the epidermal growth factor receptor (25), have been reported to couple calcium to activation of ERK in a Ras-dependent manner. The next set of experiments examined intracellular messengers involved in coupling NMDA receptor-dependent calcium influx to activation of ERK2 in cortical neuronal cultures. NMDA (50 μM, 5 min) stimulation of phospho-ERK2 was attenuated (61%) by the CaMKII inhibitor KN-93 (40 μM), or the AMPA/kainate receptor antagonist CNQX (100 μM). Protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2 (p44/42). A representative immunoblot of phospho-ERK1/2 (p44/42) immunoreactivity (B, basal; MK, MK-801; CN, CNQX; N, NMDA). B, quantification of phospho-ERK2 by computer-assisted densitometry. Values are expressed as optical density and are the means ± S.E.M. of four independent experiments. *p < 0.01 versus basal (ANOVA with Newman-Keuls posthoc test).

**NMDA Receptor Modulation of ERK Signaling Is Bidirectional**—The bell-shaped dose-response curve (Fig. 1) for NMDA stimulation of phospho-ERK2 was an unexpected observation. A bell-shaped dose-response curve for NMDA stimulation of phospho-ERK2 would be predicted if a stimulatory pathway predominated during lower levels of receptor activation and an inhibitory pathway predominated during higher levels of receptor activation. Basal levels of phospho-ERK2 in the cortical cultures were higher than expected in the absence of an added stimulating agent, suggesting high levels of activation by an endogenous mediator. The large decrease in basal phospho-ERK2 in the presence of TTX (Fig. 1) suggests synaptic activity-dependent release of a neurotransmitter that subsequently activates ERK signaling. To determine whether glutamate activation of NMDA receptors played a role in this activity-dependent stimulation of phospho-ERK2, the effects of ionotropic glutamate receptor antagonists on basal phospho-ERK2 were examined (Fig. 3). Incubation with the noncompetitive NMDA antagonist MK-801 (10 μM) for 1 h reduced phospho-ERK2 to barely detectable levels. This inhibitory effect of MK-801 likely resulted from NMDA receptor blockade, because the competitive NMDA antagonist AP5 (50 μM) also reduced basal phospho-ERK2 (n = 2). In contrast, the AMPA/kainate antagonist CNQX (100 μM) did not significantly reduce basal phospho-ERK2. Blockade of metabotropic glutamate receptors with MCPG (1 mM) was also without effect on basal phospho-ERK (data not shown). Total ERK2 levels were not altered by any of the above treatments. These observations suggest that endogenous glutamate release acts at NMDA receptors to maintain high levels of ERK activation. As predicted from the NMDA

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**Fig. 3. Inhibition of basal (synaptic activity-dependent) phospho-ERK2 by either NMDA receptor antagonist or NMDA receptor blockade.** Neuronal cultures were exposed for 1 h to vehicle, NMDA (100 μM), NMDA receptor antagonists MK-801 (10 μM) or AP5 (50 μM), or the AMPA/kainate receptor antagonist CNQX (100 μM). Protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2 (p44/42). A, representative immunoblot of phospho-ERK1/2 (p44/42) immunoreactivity (B, basal; MK, MK-801; CN, CNQX; N, NMDA). B, quantification of phospho-ERK2 by computer-assisted densitometry. Values are expressed as optical density and are the means ± S.E.M. of four independent experiments. *p < 0.01 versus basal (ANOVA with Newman-Keuls posthoc test).

**Fig. 4. Extracellular calcium dependence of NMDA inhibition of phospho-ERK2.** Cultures were incubated with or without NMDA (100 μM) in buffer containing 1.0 mM Ca2+, 0.1 mM Ca2+, or 0.1 mM EGTA for 1 h, and protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2 (p44/42). A, representative immunoblot of phospho-ERK1/2 (p44/42) immunoreactivity. B, quantification of phospho-ERK2 by computer-assisted densitometry. Values are expressed as optical density and are the means ± S.E.M. of four independent experiments. *p < 0.01 versus 1 mM Ca2+ control; †p < 0.05 versus 0.1 mM Ca2+ control (ANOVA with Newman-Keuls posthoc test).
were exposed to the PKC activator TPA (1 μM) and protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK activity-dependent phospho-ERK2 (Fig. 4, A and B). In contrast to the rapid and sustained increase in phospho-ERK2 in response to TPA, NMDA (100 μM) stimulated a time-dependent decrease in phospho-ERK2 (data not shown; n = 5).

Studies have shown that cAMP response element-mediated gene transcription is a downstream target for ERK signaling (26–29). This pathway appears to involve ERK activation of Rsk2, which translocates to the nucleus and directly activates CREB via phosphorylation of its regulatory Ser133 residue. Using a phospho(Ser133)-CREB-specific antibody, we tested whether the inhibitory effect of NMDA on ERK activation was reflected downstream at the level of CREB. Incubation (1 h) of cortical cultures with the MEK inhibitor U0126 (100 μM) greatly attenuated basal levels of phospho-CREB (Fig. 6) with- out a decrease in total CREB levels (data not shown), demonstrating that basal CREB activation coupled to, and was dependent on, ERK signaling. As was observed with phospho-ERK2, both NMDA (100 μM) and wortmannin (200 nM) significantly reduced basal levels of phospho-CREB. NMDA and wortmannin in combination produced an apparent additive effect to reduce phospho-CREB levels to that observed with U0126.

Lack of Effect of PKA, PKC, and Phosphatase Inhibitors on NMDA Inhibition of ERK Signaling—PKA and PKC have been
shown to regulate ERK activity, and cross-talk between Ras/ERK and these non-mitogen-activated protein kinase signaling pathways may be important for modulation of ERK activity. Regulation of ERK signaling by PKA is complex, and PKA has been shown to both stimulate and inhibit ERK activity (30). Because NMDA receptors can activate PKA via calcium/calmodulin stimulation of adenyl cyclase (31), a potential mechanism for NMDA modulation of ERK signaling is through PKA. To investigate the role of PKA in NMDA receptor modulation of phospho-ERK2 in the cortical cultures, we examined the effects of a selective kinase inhibitor on activity-dependent activation of ERK2 (Fig. 7). In the absence of TTX, inhibition of PKA with H-89 (5 μM) did not alter basal (activity-dependent) levels of phospho-ERK2 and failed to prevent NMDA inhibition of phospho-ERK2, suggesting that PKA does not play a role in NMDA inhibition of phospho-ERK2. Inhibition of PKC with Gö 6976 (1 μM) did not alter phospho-ERK under the above conditions, indicating that PKC also does not play a role in NMDA modulation of ERK signaling. As expected, inhibition of CaMKII with KN-93 (40 μM) and PI-3K with wortmannin (n = 2; data not shown) reduced activity-dependent phospho-ERK2 but did not prevent the inhibitory effect of NMDA.

The next set of experiments examined whether the inhibitory effect of NMDA on phospho-ERK2 resulted from enhanced dephosphorylation by protein phosphatases. In addition to phosphorylation by MEK1/2, dephosphorylation by ERK-specific phosphatases plays an important role in regulating ERK activity, and a potential mechanism in which high levels of NMDA receptor activation could decrease phospho-ERK2 is through stimulation of protein tyrosine phosphatase activity. Therefore, we tested the effect of the tyrosine phosphatase inhibitor sodium orthovanadate on NMDA inhibition of phospho-ERK2. Exposure of cortical cultures to 1 mM sodium orthovanadate for 1 h in the absence of TTX resulted in a significant increase in basal phospho-ERK2 yet did not alter the inhibitory effect of NMDA (Fig. 8, A and B). Thus, NMDA inhibition of phospho-ERK2 does not appear to involve enhanced protein-tyrosine phosphatase activity. In addition, immunoblotting with an antibody that recognized ERK1/2 (Tyr204) when tyrosine 204 is phosphorylated (independent of phospho-threonine 202) gave similar results as the anti-ERK1/2 (p44/42), thus confirming that the inhibitory effect of NMDA is upstream of ERK.

Excessive NMDA receptor activation could inhibit phospho-ERK2 by stimulating serine/threonine phosphatases that act, for example, on CaMKII-phosphorylated substrates. To examine this possibility, the effects of a PP1/PP2A inhibitor (calyculin A) and a PP2B (calcineurin) inhibitor (cypermethrin) on NMDA inhibition of phospho-ERK2 were examined. Exposure of cortical cultures to a combination of calyculin A (200 nM) and cypermethrin (10 nM) for 1 h in the absence of TTX resulted in a large increase in basal phospho-ERK2 (Fig. 9). This increase was also observed in the presence of TTX, indicating that these phosphatases are basally active in the absence of synaptic

**Fig. 6.** CREB phosphorylation in cortical cultures is coupled to ERK signaling and NMDA inhibition of phospho-ERK is reflected as a reduction in phospho-CREB. Neuronal cultures were exposed for 1 h to vehicle, U0126 (100 μM), wortmannin (Wort; 200 nM), NMDA (100 μM), or NMDA plus wortmannin. Protein extracts were analyzed by Western blotting with a phospho-Ser133 CREB-specific antibody. A, representative immunoblot of phospho-CREB immunoreactivity. B, quantification of phospho-CREB by computer-assisted densitometry. Values are expressed as optical density and are the means ± S.E.M. of five independent experiments. Significant difference from basal: *, p < 0.05; **, p < 0.01 versus basal; Ψ, p < 0.05 versus NMDA and NMDA plus Wortmannin (ANOVA with Newman-Keuls posthoc test).

**Fig. 7.** NMDA inhibition of basal phospho-ERK2 is not altered by inhibition of PKA and PKC. Neuronal cultures were incubated for 10 min with or without the PKC inhibitor Gö 6976 (1 μM), the PKA inhibitor H-89 (5 μM), or the CaMKII inhibitor KN-93 (40 μM), followed by a 1-h incubation with or without NMDA (100 μM). Protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2 (p44/42). A, representative immunoblot of phospho-ERK1/2 (p44/p42) immunoreactivity. B, quantification of phospho-ERK2 by computer-assisted densitometry. Values are expressed as optical density and are the means ± S.E.M. of six independent experiments. *, p < 0.01 versus respective basal; Ψ, p < 0.01 versus basal control (ANOVA with Newman-Keuls posthoc test).
activity (data not shown). Experiments examining calyculin A and cypermethrin separately (i.e. not in combination) revealed that the enhancing effect was attributable solely to inhibition of PP1/PP2A. Although, in the presence of the calyculin A/cypermethrin combination, NMDA did not reduce phospho-ERK2 to basal control levels, the inhibitory effect of NMDA was evident and paralleled that observed in the absence of the inhibitors. Similar to calyculin A, the PP1/PP2A inhibitor okadaic acid (1.5 μM) enhanced basal phospho-ERK2 but did not alter the NMDA inhibitory effect (data not shown). Thus, although basally active protein phosphatases PP1 and PP2A exert a tonic inhibitory effect on synthetically evoked ERK signaling, it is unlikely that stimulation of PP1, PP2A, PP2B or tyrosine phosphatase activity underlies the inhibitory effect of NMDA on phospho-ERK2.

DISCUSSION

The results of the present study suggest that NMDA receptor coupling to ERK activity in primary cortical neuronal cultures involves activation of opposing stimulatory and inhibitory processes. Differential activation of these processes may reflect different levels of NMDA receptor activity. The stimulatory pathway predominates at lower (physiological) levels of NMDA receptor activity and likely involves calcium stimulation of an unidentified negative feedback pathway. Through the combined effects of stimulatory and inhibitory processes, NMDA receptors may modulate ERK signaling at glutamatergic synapses during both physiological and pathological receptor activation (Fig. 10).

In the present study, we show that inhibitors of PI-3K completely block NMDA stimulation of ERK signaling in cultured cortical neurons. In addition to blocking protein kinase B (PKB) activation, PI-3K inhibitors can also block ERK activation by βγ subunits of heterotrimeric G-proteins (32, 33). Results obtained with PI-3Kγ expression constructs have modified substrate specificity reveal that the protein kinase activity of PI-3Kγ, and not its lipid kinase activity, is required for activation of ERK (34). Thus, bifurcation of the lipid and protein kinase activity of PI-3Kγ may differentially couple to the PKB and ERK pathway, respectively. Although we demonstrate that active PI-3K is required for NMDA stimulation of ERK signaling, the site of action of PI-3K in this pathway is not clear. In some systems, inhibitors of PI-3K block activation of both Ras and ERK (32, 33, 35), and it appears that free βγ subunits can recruit PI-3K and coordinate ERK signaling through an Src-like nonreceptor tyrosine kinase-Shc-Grb2-Ras-Raf signaling cassette (33). In other systems, inhibitors of PI-3K block ERK but not Ras activation (36, 37), indicating that PI-3K is downstream of Ras. Furthermore, although we observed that PI-3K activity is required for NMDA activation of ERK in cultured cortical neurons, it is not clear whether PI-3K is activated on NMDA-induced calcium influx or whether this represents constitutive PI-3K activity. However, the catalytic subunit of PI-3K (p85) has been shown to bind to tyrosine-phosphorylated NMDA receptor NR2 subunits (38). Because binding of the Src homology 2 domain of p85 to phosphotyrosine residues of target proteins increases PI-3K activity (39), NMDA receptors may directly activate PI-3K through protein-protein interactions. In addition, calcium/calmodulin has been reported to activate PI-3K (40), and it has been reported in cultured cerebellar granule neurons that NMDA-stimulated increases in intracellular calcium result in phosphorylation of insulin receptor sub-
NMDA Receptor Modulation of ERK Activation

**FIG. 9.** NMDA inhibition of phospho-ERK2 does not involve stimulation of PPI/P2PA or PP2B activity. Neuronal cultures were incubated for 10 min in the absence or presence of the PP1/PP2A inhibitor calyculin A (200 nm) and the PP2B inhibitor cypermethrin (10 nm), followed by a 1-h incubation with or without NMDA (100 μM). Calyculin A (Cal A) and cypermethrin (Cyp) were added either in combination (A and B) or separately (C). Protein extracts were analyzed by Western blotting with a phosphospecific antibody that only recognizes dually phosphorylated (Thr202/Tyr204) ERK1/2. A, representative immunoblot of phospho-ERK1/2 (p44/p42). B, quantification of phospho-ERK2 by computer-assisted densitometry. Values are expressed as optical density and are the mean ± S.E.M. of three independent experiments. *p < 0.01, versus basal control; **p < 0.01, versus respective basal value (ANOVA with Newman-Keuls posthoc test). C, representative immunoblot of phospho-ERK1/2 (p44/p42) immunoreactivity (n = 2).

Ras-GTP (42). Our findings suggest that the CaMKII inhibitor KN-93 attenuated NMDA-stimulated phospho-ERK2 in a non-specific manner. A similar bell-shaped curve for NMDA stimulation of phospho-ERK2 in the presence of TTX, with maximal stimulation at 50 μM NMDA and only slight activation at 100 μM NMDA. The bell shape of the curve did not reflect a temporal and transient response, because a similar bell-shaped curve was observed after both 5 and 15 min of NMDA exposure. Furthermore, in the absence of TTX, 100 μM NMDA decreased basal phospho-ERK2 levels, with no stimulation observed at any time point examined (15 s–1 h). These observations may indicate the presence of a critical period in which NMDA receptor activation is more sensitive to inhibition by CaMKII inhibitors.

**FIG. 10.** Diagram of proposed pathways for NMDA receptor regulation of ERK activation in cortical cultures. Increased intracellular calcium through NMDA receptors can stimulate ERK activity via an Src-like nonreceptor tyrosine kinase-Pi-3K-G bg signaling cassette (1). A separate calcium-activated pathway that involves SynGAP may also regulate Ras activation (2). In the absence of calcium, constitutively active SynGAP may maintain Ras in an inactive state by enhancing the endogenous GTPase activity of Ras (42). NMDA receptor activation leads to calcium influx and activation of CaMKII, which phosphorylates and inactivates SynGAP, resulting in increased levels of active Ras. A similar CaMKII- and Pi-3K/G bg-dependent activation pathway(s) has been shown to couple AMPA receptor-mediated calcium influx to ERK signaling in striatal neuronal cultures (44). Control of Ras-GTPase activity via Ca2+/CaMKII/SynGAP could also gate ERK signaling through receptor tyrosine kinases (RTK) and G-protein receptor-coupled receptors (GPCR). Finally, pathological increases in intracellular Ca2+ during NMDA receptor hyperactivity may activate an inhibitory pathway (3). Although the effectors that transduce this inhibitory pathway are yet to be defined, it could function to turn off ERK activation in an inhibitory pathway (4). Expression of NMDA receptor subunits in striatal neurons found that calcium-permeable AMPA receptors (pretreated with cyclothiazide to prevent desensitization) also couple to ERK activation through a similar Pi-3K-CaMKII-G bg signaling pathway for calcium-induced ERK activation. However, it should be noted that the present study does not directly demonstrate involvement of SynGAP in NMDA regulation of ERK signaling.

An unexpected finding in the present study was a bell-shaped curve for NMDA stimulation of phospho-ERK2 in the presence of TTX, with maximal stimulation at 50 μM NMDA and only slight activation at 100 μM NMDA. The bell shape of the curve did not reflect a temporal and transient response, because a similar bell-shaped curve was observed after both 5 and 60 min of NMDA exposure. Furthermore, in the absence of TTX, 100 μM NMDA decreased basal phospho-ERK2 levels, with no stimulation observed at any time point examined (15 s–1 h). These observations may indicate the presence of a critical period in which NMDA receptor activation is more sensitive to inhibition by CaMKII inhibitors.
calcium-activated inhibitory pathway that can attenuate Ras/ERK signaling. Hyperfunctioning of Ras signaling is well known to alter normal cell function, and studies have suggested that differences in the activation kinetics of ERK may be important in determining the appropriate cellular response (45). For example, nerve growth factor stimulation of ERK activity in PC-12 cells is sustained for several hours and results in growth arrest and differentiation, whereas epidermal growth factor stimulation of ERK activity is transient (usually peaking in 5–10 min) and stimulates growth and proliferation (46, 47). Thus, control of NMDA receptor-mediated ERK activity within an optimal range may be important in determining the correct downstream response. In addition, the inhibitory pathway could function to block ERK signaling during neurotoxic activation of NMDA receptors. Although this may seem counterintuitive, once the apoptotic signal is turned on by pathological activation of NMDA receptors, as is well known to occur in response to 100 μM NMDA, it would be counterproductive for survival signals to remain active. For example, enhanced CREB phosphorylation has been reported to be critical for neuronal survival and to protect against neurotoxicity (48–50), and our observation of reduced phospho-CREB is in agreement with this suggestion. Thus, inhibition of ERK signaling may play an active role in NMDA receptor-mediated cell death (both apoptotic and necrotic). Support for this comes from evidence showing that activation of the ERK pathway can protect against proapoptotic signals in some cell types. Constitutive activation of ERK signaling in PC-12 cells can inhibit apoptosis, whereas withdrawal of nerve growth factor leads to inhibition of ERK signaling and apoptosis (51). Similarly, ERK signaling can protect against FAS-stimulated apoptosis in Jurkat cells and can protect against tumor necrosis factor α-induced apoptosis in L929 cells (52). NMDA receptor-linked survival and apoptotic cell death are both critical during brain development, and NMDA concentration-dependent bidirectional control of ERK signaling may correlate with observations that both underactivation and overactivation of NMDA receptors lead to neuronal cell death in the developing brain (53–55).

As noted above, high concentrations of NMDA are well known to induce excitotoxic cell death, and we cannot exclude the possibility that decreased phospho-ERK reflected a non-specific effect in dying neurons. However, a characteristic feature of NMDA-induced neuronal death is that it is a delayed event and does not occur until several hours after NMDA receptor activation. We did not observe any lactate dehydrogenase leakage during the 1-h NMDA exposure, and visual inspection of the cultures showed no morphological signs of cell death during this period. In addition, in these same cortical cultures, we have previously shown that both 50 and 100 μM NMDA are excitotoxic, producing ~75 and ~100% neuronal death, respectively, after 24 h (56). In contrast, these two concentrations of NMDA had very different effects on phospho-ERK, and it is unlikely that a non-specific effect of excitotoxicity would manifest within the first 5 min of NMDA stimulation to produce such a dramatically different effect.

It is well known that phosphatase activity plays a role in regulating the phosphorylation state of a phosphoprotein, and the best-understood means for deactivation of ERK kinases is through dephosphorylation of the threonine and tyrosine residues of the TEY regulatory motif. Protein phosphatases known to dephosphorylate ERK include the serine/threonine PP2A (57) and a family of inducible tyrosine/threonine phosphatase with specificity and selectivity for the TEY motif of ERK (58). It is unlikely that these phosphatases are involved in the inhibitory effect of NMDA on ERK, because inhibition of tyrosine phosphatase and PP1/PP2A activity did not alter the inhibitory effect. It is also unlikely that PP2B (calcineurin) plays a role, because its inhibition also failed to prevent NMDA inhibition of phospho-ERK. The large increase in activity-dependent phospho-ERK in response to PP1/PP2A inhibition, indicating that PP1 or PP2A, or both, exert tonic inhibitory control on activity-dependent ERK signaling in our cortical cultures, may confound interpretation of these results. In addition, an involvement of serine/threonine phosphatases other than PP1, PP2A, and PP2B cannot be ruled out. Finally, the failure of a PKA and PKC inhibitor to alter the NMDA response argues against these kinases playing a role in NMDA-receptor modulation of ERK activation. Although we were unable to determine the mechanism for how strong NMDA receptor activation leads to inhibition of ERK signaling, it is likely to occur upstream of ERK, because a similar inhibition of phospho-MEK was observed. This also suggests that strong NMDA receptor activation reduced phospho-ERK levels by turning off its activation and not by enhancing inactivation (dephosphorylation). Interestingly, cross-talk between PKB and ERK signaling has recently been demonstrated at the level of Raf (59, 60). PKB was shown to phosphorylate Raf on Ser259, resulting in inhibition of ERK activation and a shift in the cellular response in a human breast cancer cell line from cell cycle arrest to proliferation (60), and we are currently investigating whether PKB, acting as a negative feedback regulator of Raf, plays a role in NMDA inhibition of ERK activation in cortical cultures.

Activity-dependent CREB phosphorylation was found to be dependent on ERK signaling in our cortical cultures. Similar to inhibition of ERK phosphorylation, strong NMDA receptor activation also inhibited CREB phosphorylation. NMDA concentration-dependent bidirectional control of CREB phosphorylation by NMDA receptors has also been recently reported in hippocampal cultures, where low receptor activation (20 μM NMDA) produced sustained CREB phosphorylation, and strong receptor activation (100 μM NMDA) produced sustained dephosphorylation (61). The inhibitory effect of NMDA was found to be developmentally regulated and occurred only in mature cultures (>14 days in vitro). However, the decrease in phospho-CREB was not associated with decreased phospho-ERK1/2 and appeared to be caused by activation of PP1. Thus, although both studies demonstrate sustained decreases of phospho-CREB after strong NMDA receptor stimulation, the differences in the observations may indicate the presence of multiple inhibitory mechanisms and possibly brain regional differences. Interestingly, we observed that NMDA and wortmannin produced an apparent additive inhibitory effect on phospho-CREB. Although we did not examine the role of PP1 in inhibition of phospho-CREB, the additive effect could represent a combination of partial ERK inhibition and activation of PP1.

In summary, the current study adds to the growing body of evidence revealing the complexity and dynamic nature of NMDA receptor coupling to ERK signaling in brain. Our data suggest that NMDA receptor-stimulated calcium influx can both activate and inhibit ERK signaling in cultured cortical neurons (Fig. 10). The stimulatory pathway is activated by low to moderate NMDA receptor activity, is tonically activated by synaptic activity, and is dependent on PI-3K activity and, to a lesser degree, CaMKII. Finally, we provide evidence of an unidentified inhibitory pathway that down-regulates ERK signaling in response to excessive NMDA receptor activation.

REFERENCES
N-Methyl d-Aspartate Receptor-mediated Bidirectional Control of Extracellular Signal-regulated Kinase Activity in Cortical Neuronal Cultures
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