Ca^{2+}-Permeable AMPA Receptors Induce Phosphorylation of cAMP Response Element-Binding Protein through a Phosphatidylinositol 3-Kinase-Dependent Stimulation of the Mitogen-Activated Protein Kinase Signaling Cascade in Neurons

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Ca^{2+}-permeable AMPA receptors may play a key role during developmental neuroplasticity, learning and memory, and neuronal loss in a number of neuropathologies. However, the intracellular signaling pathways used by AMPA receptors during such processes are not fully understood. The mitogen-activated protein kinase (MAPK) cascade is an attractive target because it has been shown to be involved in gene expression, synaptic plasticity, and neuronal stress. Using primary cultures of mouse striatal neurons and a phosphospecific MAPK antibody we addressed whether AMPA receptors can activate the MAPK cascade. We found that in the presence of cyclothiazide, AMPA caused a robust and direct (no involvement of NMDA receptors or L-type voltage-sensitive Ca^{2+} channels) Ca^{2+}-dependent activation of MAPK through MAPK kinase (MEK). This activation was blocked by GYKI 53655, a noncompetitive selective antagonist of AMPA receptors. Probing the mechanism of this activation revealed an essential role for phosphatidylinositol 3-kinase (PI 3-kinase) and the involvement of a pertussis toxin (PTX)-sensitive G-protein, a Src family protein tyrosine kinase, and Ca^{2+}/calmodulin-dependent kinase II. Similarly, kainate activated MAPK in a PI 3-kinase-dependent manner. AMPA receptor-evoked neuronal death and arachidonic acid mobilization did not appear to involve signaling through the MAPK pathway. However, AMPA receptor stimulation led to a Ca^{2+}-dependent phosphorylation of the nuclear transcription factor CREB, which could be prevented by inhibitors of MEK or PI 3-kinase. Our results indicate that Ca^{2+}-permeable AMPA receptors transduce signals from the cell surface to the nucleus of neurons through a PI 3-kinase-dependent activation of MAPK. This novel pathway may play a pivotal role in regulating synaptic plasticity in the striatum.

Key words: AMPA; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; CREB; wortmannin; LY 294002; pertussis toxin; G-protein; PP2; cyclothiazide; glutamate; kainate; calcium/calmodulin-dependent kinase II; tyrosine kinase; AMPA toxicity; arachidonic acid; striatum; striatal neurons

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and acts through two classes of receptor: NMDA and non-NMDA [AMPA and kainate (KA)] ionotropic receptors, and G-protein-coupled metabotropic receptors (Hollmann and Heinemann, 1994; Ozawa et al., 1998). Ionotropic glutamate receptors mediate a wide range of physiological processes such as fast excitatory synaptic transmission and neuronal plasticity (Mayer and Westbrook, 1987; Monaghan et al., 1989; Lerma et al., 1997; Ozawa et al., 1998), and their overactivation has been implicated in excitotoxic neuronal loss that occurs during a number of neuropathological conditions (Choi, 1992; Pellegrini-Giampietro et al., 1997; Michaelis, 1998). It appears that extracellular Ca^{2+} influx through glutamate receptor ion channels plays a central role in gene expression associated with synaptic plasticity (Deisseroth et al., 1996; Finkbeiner and Greenberg, 1996; Xia et al., 1996), neuronal development, and survival (Bading et al., 1993, 1995; Ikonomidou et al., 1999) and activity-dependent synaptogenesis (Constantine-Paton and Cline, 1998;

Lin and Constantine-Paton, 1998). There is accumulating evidence suggesting that these processes may require the transduction of signals through the mitogen-activated protein kinase [(MAPK) ERK1 and ERK2] cascade, which classically involves Shc tyrosine phosphorylation, recruitment of the Grb2-Sos complex, and the subsequent sequential activation of Ras, Raf kinase, and MAPK kinase (MEK) (Rosen et al., 1994; Fukunaga and Miyamoto, 1998). NMDA receptor-induced immediate early gene transcription and induction of hippocampal long-term potentiation (LTP) are both thought to involve a Ca^{2+}-dependent activation of the Ras-MAPK pathway in neurons (English and Sweatt, 1996; Finkbeiner and Greenberg, 1996; Xia et al., 1996; English and Sweatt, 1997), however, studies regarding AMPA receptor activation of this cascade are somewhat contradictory. Although AMPA and kainate do not appear to activate MAPK in hippocampal neurons (Kurino et al., 1995), it has been reported that AMPA receptors can stimulate MAPK in cortical neurons through a mechanism that surprisingly requires G-protein $\beta\gamma$ subunits (Wang and Durkin, 1995), which is classically associated with receptors that couple to heterotrimeric G-proteins (Crespo et al., 1994; Luttrell et al., 1997; Gutkind, 1998). Recent studies have shown that phosphatidylinositol 3-kinase (PI 3-kinase) plays a crucial role in $\beta\gamma$ signaling to MAPK at a target upstream of Sos and Ras (Hawes et al., 1996; Lopez-Ilasaca et al., 1997), although

it is not known if this enzyme is required for AMPA receptor signaling to MAPK.

Ca^{2+}-permeable AMPA receptors can mediate NMDA receptor-independent long-term potentiation in the amygdala (Mahanty and Sah, 1998) and hippocampus (Feldmeyer et al., 1999) and probably play an important role in striatal synaptic transmission and plasticity (Lovinger and Tyler, 1996; Williams and Glowinski, 1996). Furthermore, excessive influx of Ca^{2+} through AMPA receptors may contribute to glutamate-induced neuronal cell death associated with cerebral ischemia, seizure activity, and a number of chronic neurodegenerative disorders such as Huntington's disease and amyotrophic lateral sclerosis (Choi, 1995; Pellegrini-Giampietro et al., 1997; Feldmeyer et al., 1999). However, a better understanding of the specific synaptic and intracellular signals that occur during the aforementioned AMPA receptor-mediated physiological and pathological processes is required. A possible intracellular target is MAPK, thus, the aim of this study was to investigate whether striatal AMPA receptors signal to this cascade, and if so, to examine the mechanism of this activation and to address possible functional consequences.

MATERIALS AND METHODS

Chemicals and reagents. (1*S*,3*R*)-ACPD, (*RS*)-AMPA, cyclothiazide, kainic acid, (+)-MK 801, nimodipine, and NMDA were all obtained from Tocris Cookson (Bristol, UK). Anti-CREB (phospho-Ser[133]-specific), Ionomycin, KN-62, LY 294002, PP2, PP3, Ro-31–8220, and wortmannin were from Calbiochem (La Jolla, CA). Genistein and PD 98059 were from Alexis (San Diego, CA). Fura-2 AM was from Molecular Probes (Eugene, OR). Enhanced chemiluminescence reagent (ECL) and [^{3}H]arachidonic acid (200 Ci/mmol) were obtained from Amersham (Little Chalfont, UK). Anti-ACTIVE MAPK polyclonal antibody (pAb) was from Promega (Madison, WI), and ERK1/ERK2 pAb was from Santa Cruz Biotechnology (Santa Cruz, CA). Vectastain Elite ABC Kit and biotinylated goat anti-rabbit IgG were obtained from Vector Laboratories (Burlingame, CA). Bio-Rad protein assay reagent (concentrate) was obtained from Bio-Rad (Hemel Hempstead, UK). Paraformaldehyde (methanol-free) was bought from TAAB Laboratories (Aldermaston, UK). GYKI 53655 was a gift from Eli Lilly (Indianapolis, IN). Bovine serum albumin (fatty acid-free), poly-L-ornithine, and pertussis toxin were from Sigma (Poole, UK). All other reagents were obtained from Sigma or Merck (Poole, UK).

Neuronal cell culture. Primary cultures of mouse striatal neurons were prepared essentially as described previously (El-Etr et al., 1989). Briefly, striata were dissected from 15- to 16-d-old Swiss mouse embryos (NIH, Harlan, UK) and mechanically dissociated using a fire-polished glass Pasteur pipette in PBS (Ca^{2+}- and Mg^{2+}-free) supplemented with glucose (33 mM). Cells were plated into either six- or 24-well Nunc (Roskilde, Denmark) multiwell plates that had been coated previously overnight with 15 μg/ml poly-L-ornithine and then with culture medium supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) for 2 hr. After removal of the final coating solution, cells were seeded (10^6/ml) in a serum-free medium composed of a mixture of DMEM and F-12 nutrient (1:1 v/v) (Life Technologies) supplemented with (in mM) 33 glucose, 2 glutamine, 6.5 sodium bicarbonate, and 5 HEPES buffer, pH 7.4, 100 μg/ml streptomycin, and 60 μg/ml penicillin. A mixture of hormones and salts composed of insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM), and sodium selenate (30 nM) (all from Sigma) was also added to the culture medium. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were used after 6–7 days *in vitro* (DIV) when the majority of cells were neuronal and there were no detectable glial elements.

Immunoblotting. Immunoblot analysis was performed essentially as described previously (Samanta et al., 1998) with minor modifications. Neurons were cultured in six-well plates, placed into a thermostatted water bath at 37°C, and left for 5 min to equilibrate. After this period, the culture medium was removed and replaced with HEPES-buffered incubation medium (HBM) (in mM): NaCl 140, KCl 5, $NaHCO_3$ 5, $MgCl_2 \cdot 6H_2O$ 1.1, Na_2HPO_4 1.2, $CaCl_2$ 1.2, glucose 5.5, and HEPES 20, pH 7.4.

For NMDA agonist treatments and Ca^{2+} dependency studies, $MgCl_2$ and $CaCl_2$ were omitted from the HBM, respectively. Other additions before agonist/depolarization treatments were made as detailed in the legends to the Figures. After 10 min of incubation, fresh HBM containing AMPA (50 μM) (in the presence or absence of 50 μM cyclothiazide), glutamate (100 μM), kainate (100 μM), NMDA (100 μM), (1*S*,3*R*)-ACPD (200 μM), KCl (50 mM), or ionomycin (5 μM) was added to the neurons. After 5 min, the HBM was removed, and the plates were quickly washed with ice-cold PBS, pH 7.4, (Ca^{2+}-free) and placed immediately on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer (200 μl/well) [50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tergitol (type NP-40), 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, 1 μg/ml pepstatin A, 1 mM Na_3VO_4, and 50 mM NaF], left on ice for 10 min, then homogenized with 20 full strokes in a glass-on-glass hand-held homogenizer and centrifuged at $1000 \times g$ for 4 min at 4°C to remove cell debris. The supernatant (crude homogenate) was kept, and protein concentration was determined by the assay of Bradford (1976). Samples were then boiled for 5 min in boiling buffer, giving 62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue in the final sample. Boiled samples (15 μg/lane) were run on 7.5% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membrane (Hybond-C pure; Amersham) by electroblotting. The equal loading and efficient transfer of proteins was confirmed by staining the nitrocellulose with Ponceau Red (Sigma). The nitrocellulose membrane was then incubated in blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl; TBS) containing 5% skimmed milk powder, for 30 min at room temperature on a three-dimensional rocker, followed by two 10 min washes in TBS supplemented with 0.1% (v/v) Tween 20 (TTBS). Blots were then incubated with anti-ACTIVE MAPK pAb (1:12,500 dilution of stock), anti-ERK1/ERK2 pAb (1:750 dilution of stock), or anti-phospho-Ser[133] CREB pAb (1:1500 dilution of stock) in TTBS containing 1% skimmed milk powder (antibody buffer), overnight at room temperature on a three-dimensional rocker. The blots were washed (2 × 10 min) in TTBS and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution of stock) in antibody buffer for 1 hr. Finally, the blots were washed in TTBS (2 × 10 min), rinsed in TBS, and exposed to ECL reagent for 1 min, as described in the Amersham protocol. Excess reagent was removed, and the blots were then exposed to Hyperfilm ECL (Amersham) for 2 min in an autoradiographic cassette and developed. Bands were analyzed using BioImage Intelligent Quantifier software (Ann Arbor, MI) Molecular weights of ERK1/ERK2 and CREB were calculated from comparison with prestained molecular weight markers (MW 27,000–180,000) that were run in parallel with samples.

Immunocytochemistry. For immunocytochemical studies, neurons were cultured in 24-well plates and incubated in HBM, pH 7.4, at 37°C, as described for immunoblot analysis. In experiments involving AMPA receptor stimulation, (+)-MK 801 (2 μM) was present in the HBM to block NMDA-type glutamate receptors. Other additions before agonist treatment were made as detailed in the legends to Figures 9 and 10*B*. After 10 min of incubation, fresh HBM containing AMPA (50 μM) (in the presence of 50 μM cyclothiazide) was added to the neurons. After a further 5 min, the HBM was removed, and the plates were quickly rinsed with ice-cold PBS, pH 7.4, (Ca^{2+}-free), and neurons were fixed on ice in PBS containing 4% paraformaldehyde for 1 hr. All other procedures were performed at room temperature. Neurons were permeabilized with 0.2% Triton X-100 made up in PBS containing 1% goat serum (blocking solution) for 1 hr and then incubated with anti-ACTIVE MAPK pAb (1:500 dilution of stock), ERK1/ERK2 pAb (1:200 dilution of stock), or anti-phospho-Ser[133] CREB pAb (1:500 dilution of stock) overnight with constant agitation. After 3 × 10 min washes in PBS, neurons were incubated with biotinylated goat antibody to rabbit IgG (1:200 dilution of stock) for 1 hr, washed 3 × 10 min with PBS, and neuronal staining was localized using the avidin–biotin–peroxidase detection system (Vector Laboratories). Neurons were visualized using a Leica Fluovert microscope.

Intracellular Ca^{2+} measurements. Striatal neurons prepared as described above, were plated into 24-well Nunc multiwell plates containing 13 mm diameter glass coverslips that had been previously coated overnight with 15 μg/ml poly-L-ornithine and then with 2 μg/ml laminin (Life Technologies) for 4 hr. After 6–7 DIV, neurons were loaded with the cell-permeant fluorescent Ca^{2+}-probe fura-2 AM (5 μM) in HBM, pH 7.4, for 45 min at 37°C. Subsequent procedures were performed at room temperature (20–25°C). Coverslips with fura-2-loaded neurons were clamped to a purpose-made recording chamber attached to a Nikon

Diaphot 200 inverted microscope stage and preincubated in HBM, pH 7.4, with or without inhibitors, as detailed in the legend to Table 1. Neurons were stimulated with AMPA (50 μM) or AMPA/cyclothiazide (50 μM/50 μM), and to ensure that AMPA receptor-mediated changes in intracellular Ca^{2+} were being observed in the absence of possible NMDA receptor responses, 1 mM MgCl$_2$ and 2 μM (+)-MK 801 were included in the HBM. Neurons were alternately excited at 340 and 380 nm using a Cairn filter changer. Fluorescence at each excitation wavelength was filtered using a 420 nm barrier filter/400 nm dichroic mirror set and collected using a C4880–81Hamamatsu multiformat CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Data acquisition and analysis was performed using Acquisition Manager (version 3.1) and Lucida Analyze (version 3.51) software from Kinetic Imaging (Liverpool, UK). To improve the signal-to-noise ratio, the collection period during 340 nm excitation (600 msec) was set at three times that used at 380 nm (200 msec). Ratio values in the data presented have not been corrected for this difference. Fluorescence responses from at least six cells in a single field were analyzed on each coverslip. For each experimental condition, cells were analyzed on a minimum of three coverslips from at least two separate striatal cultures. Means ± SEM of the 340 nm/380 nm ratio traces are reported in Figure 3, and means ± SEM of the agonist-induced increases in the ratio values evaluated in Table 1.

Arachidonic acid release. Arachidonic acid release was performed essentially as described previously (Samanta et al., 1998). Neurons, cultured in 24-well plates were incubated overnight with 0.5 μCi/ml [^{3}H]arachidonic acid. Unincorporated label was removed by three successive washes in HBM, pH 7.4, containing fatty acid-free BSA (2 mg/ml; HBM/BSA), and neurons were then preincubated for 10 min in HBM/BSA at 37°C in the presence or absence of enzyme inhibitors, with or without cyclothiazide (50 μM). After removal of the preincubation buffer, neurons were incubated for 15 min in HBM/BSA containing either vehicle or AMPA/cyclothiazide (50 μM/50 μM), at 37°C in the continued presence or absence of enzyme inhibitors, as detailed in the legend to Figure 7. The extracellular medium was removed and centrifuged at 400 × *g* for 5 min to remove dislodged cells and the radioactivity in the supernatant was measured by scintillation counting. Because arachidonic acid is poorly metabolized in primary cultured mouse neurons (Oomagari et al., 1991), the ^{3}H released was assumed to be essentially [^{3}H]arachidonic acid.

Neuronal viability. Experiments were performed using neurons cultured in 24-well plates. The effect of AMPA on neuronal viability was assessed by a colorimetric assay based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue-colored formazan product by mitochondrial succinate dehydrogenase. Additions and agonist treatments were made directly to the neuronal culture medium for 4 hr, as detailed in the legend to Figure 8. Neurons were then washed twice with HBM, pH 7.4, and incubated for 45 min at 37°C in HBM containing MTT (0.5 mg/ml). After this period, the HBM was carefully removed, and the blue formazan product was solubilized in 300 μl of 100% dimethyl sulfoxide. The absorbance of each well was read at 570 nm. Morphological assessment of the effect of AMPA on neuronal viability was made by phase contrast microscopy as detailed in the legend to Figure 8.

Data handling. Immunoblot band intensity, [^{3}H]arachidonic acid release, neurotoxicity, and intracellular Ca^{2+} data were analyzed by unpaired two-tailed Student's *t* tests. Differences were considered to be of statistical significance when *p* < 0.05.

RESULTS

The non-NMDA glutamate receptor desensitization inhibitor cyclothiazide unmasks an AMPA-induced activation of MAPK in cultured striatal neurons

The glutamate receptor agonist AMPA can evoke a range of physiological and neurotoxic processes in striatal neurons (Lovinger and Tyler, 1996; Williams and Glowinski, 1996; Calabresi et al., 1998) by binding to a heterogenous population of postsynaptic AMPA-type glutamate receptors assembled from different combinations of GluR1–GluR4 subunits (Bernard et al., 1997; Kwok et al., 1997). The precise nature of the intracellular mechanisms activated subsequent to AMPA receptor stimulation are not fully understood. Recently, much attention has been focused on the

possible physiological and pathological roles that the MAPK cascade may play at glutamatergic synapses (Fukunaga and Miyamoto, 1998; Murray et al., 1998; Sgambato et al., 1998). Indeed, it has been demonstrated in many neuronal types, including striatal neurons, that stimulation of NMDA-type glutamate receptors causes a Ca^{2+}-dependent activation of MAPK (Xia et al., 1996; Vincent et al., 1998). Although postsynaptic AMPA receptors are classically associated with low Ca^{2+} permeability (Hollmann and Heinemann, 1994) Ca^{2+}-permeable AMPA receptors are present in the striatum (Williams and Glowinski, 1996) and, thus, there may be a population of striatal AMPA receptors that can couple to MAPK in a Ca^{2+}-dependent manner. However, a direct coupling of Ca^{2+}-permeable AMPA receptors to MAPK has not been shown in striatal neurons.

To test whether stimulation of striatal AMPA receptors leads to activation of the ERK1/ERK2 forms of MAPK, immunoblotting of neuronal homogenates with an anti-ACTIVE MAPK pAb, which recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 (p44) and ERK2 (p42) was performed. Treatment of striatal neurons with AMPA (50 μM) for 5 min failed to activate MAPK (Fig. 1*A*, *lane 3*) because there was no significant increase in immunodetectable activated ERK1 and ERK2 (seen as two bands of ~44 and 42 kDa, respectively) above basal levels (Fig. 1*A*, *lane 1*). However, we have previously shown that in cultured striatal neurons, AMPA receptor-mediated responses are rapidly desensitizing (Williams and Glowinski, 1996). To establish whether an apparent lack of activation of MAPK by AMPA in these neurons is caused by rapid AMPA receptor desensitization, striatal neurons were preincubated with the AMPA receptor-specific desensitization inhibitor cyclothiazide (50 μM). In the presence of cyclothiazide, AMPA evoked a large increase in the intensity of the two immunodetectable bands (44 and 42 kDa) compared with basal levels, corresponding to the activated forms of MAPK, ERK1 and ERK2 (Fig. 1*A*, *lane 4*, *B*). Cyclothiazide alone did not activate ERK1 or ERK2 (Fig. 1*A*, *lane 2*), indicating that it was acting specifically at AMPA receptors and was not directly activating MAPK. A set of parallel immunoblots with an antibody that detects total MAPK (active and inactive ERK1/ERK2) were performed to show that the total levels of MAPK were not altered by any of the drug treatments (Fig. 1*A*, *lanes 5-8*). Complementing our results with the anti-ACTIVE MAPK pAb, AMPA/cyclothiazide treatment revealed a doublet of 42 kDa immunoreactivity (seen as a faint slower-migrating band just above the main 42 kDa band) (Fig. 1*A*, *lane 8*), indicative of an AMPA-mediated phosphorylation of p42 MAPK.

Activation of MAPK by AMPA/cyclothiazide could also be demonstrated at the single cell level. We first showed, by immunocytochemistry, the presence of the ERK1 and ERK2 forms of MAPK in striatal neurons (see Fig. 9*Ai*). The majority of the neurons were immunopositive for MAPK, with the highest levels of staining found at the neuronal soma, and some weaker staining in the neurites. After closer examination of the cell body, a narrow outer ring of intense staining was visible in the majority of neurons (see Fig. 9*Ai*, *white arrows*), suggesting a cytosolic location for MAPK. Some nuclear staining was also detectable, but to a lesser extent. In a set of control experiments, omission of the primary antibody completely abolished staining for MAPK (see Fig. 9*Aii*). Using the anti-ACTIVE MAPK pAb we showed that, under control conditions, basal levels of activated MAPK are very low (see Fig. 9*Bi*), which may indicate that MAPK activation is tightly regulated in striatal neurons. However, single neurons
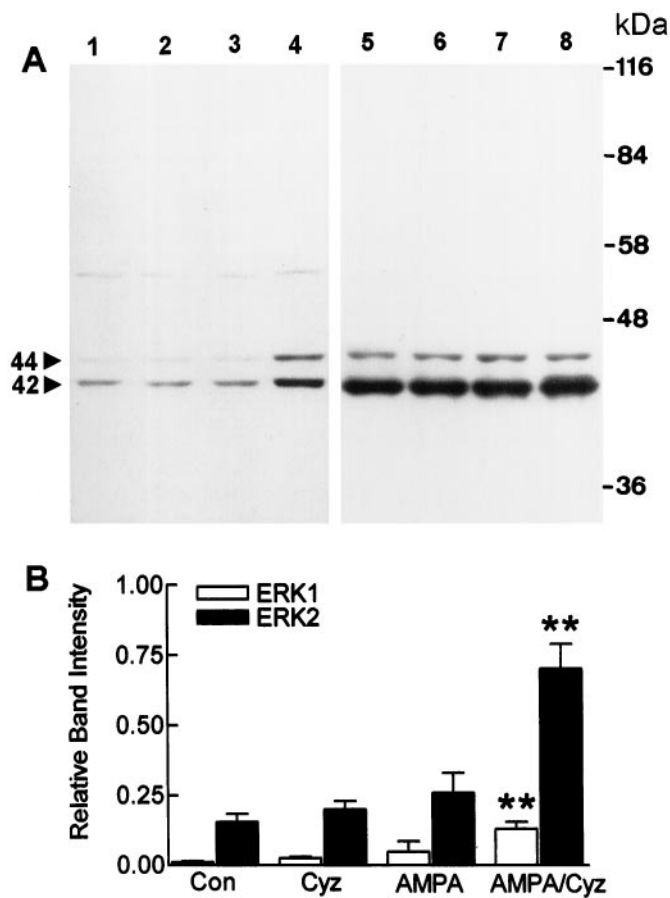
Figure 1. Cyclothiazide unmasks an AMPA-mediated activation of MAPK in striatal neurons. *A*, Crude homogenates (15 μg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lanes 1, 3, 5, 7*) or presence (*lanes 2, 4, 6, 8*) of 50 μM cyclothiazide (*Cyz*), and then exposed for 5 min to vehicle (*lanes 1, 5*), 50 μM Cyz (*lanes 2, 6*), 50 μM AMPA (*lanes 3, 7*), or 50 μM AMPA and 50 μM Cyz (*lanes 4, 8*), were immunoblotted with an antibody that specifically recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 and ERK2 (anti-ACTIVE MAPK pAb) (*lanes 1-4*), or with an antibody that recognizes total levels of ERK1 and ERK2 (anti-ERK1/ERK2 pAb) (*lanes 5-8*). *B*, Data obtained from immunoblot experiments represented in *lanes 1-4* was analyzed using BioImage Intelligent Quantifier software. Each column is the mean ± SEM value of 6–12 independent cultures (*n* = 6–12). *******AMPA/Cyz* was significantly different from control (*Con*) (*p* < 0.001, unpaired two-tailed Student's *t* test).

containing activated MAPK were visible after treatment of cultures with AMPA in the presence of cyclothiazide (see Fig. 9*Bii*, *black arrow*). When neurons were preincubated with the 2,3-benzodiazepine GYKI 53655 (100 μM), a noncompetitive and selective antagonist of AMPA receptors, before application of AMPA/cyclothiazide, no staining for activated MAPK could be detected (see Fig. 9*Biii*). Thus, we have demonstrated, for the first time, an AMPA receptor-mediated activation of MAPK (ERK1/ERK2) in single isolated neurons.

Ca^{2+} influx through AMPA receptors triggers activation of MAPK in striatal neurons

Activation of the MAPK cascade can be a Ca^{2+}-dependent process and in neurons, the activation of ERK1/ERK2 by NMDA is completely dependent on the influx of Ca^{2+} through the receptor (Kurino et al., 1995; Xia et al., 1996), thus, the possibility that our observed stimulation of MAPK by AMPA receptors also

required Ca^{2+} was tested. In Ca^{2+}-containing incubation buffer, glutamate (100 μM) (Fig. 2*A*, *lane 2*), AMPA/cyclothiazide (50 μM/50 μM) (Fig. 2*A*, *lane 3*), and NMDA (100 μM) (data not shown) strongly activated MAPK, as indicated by a large increase in the intensity of the 44 kDa (ERK1) and 42 kDa (ERK2) immunodetectable bands, compared with basal levels (Fig. 2*A*, *lane 1*). However, in the absence of extracellular Ca^{2+}, but the presence of EGTA (200 μM) to chelate any residual Ca^{2+}, all three agonists [glutamate (Fig. 2*A*, *lane 5*), AMPA/cyclothiazide (Fig. 2*A*, *lane 6*), and NMDA (data not shown)] failed to elicit an increase in the 44 kDa (ERK1) and 42 kDa (ERK2) band intensities above basal levels (in the absence of Ca^{2+}) (Fig. 2*A*, *lane 4*). Further evidence that extracellular Ca^{2+} influx is a trigger for MAPK activation in striatal neurons came from the observation that the Ca^{2+} ionophore ionomycin (5 μM), which causes delocalized Ca^{2+} entry after randomly intercalating at sites all over the neuronal plasma membrane, also activated MAPK [(Fig. 2*B*, *lane 3* (ionomycin); *lane 1* (basal)]. It is noticeable that ionomycin treatment did not appear to activate ERK1/ERK2 as efficiently as glutamate (Fig. 2*B*, *lane 2*).

To confirm that AMPA/cyclothiazide-evoked MAPK activation resulted from the influx of Ca^{2+} through AMPA receptors, intracellular Ca^{2+} measurements were performed using fura-2-loaded neurons. AMPA caused a small rise in intracellular Ca^{2+}, which was strongly potentiated (approximately threefold) in the presence of cyclothiazide (Fig. 3). This response was completely blocked by preincubation with the selective AMPA receptor antagonist GYKI 53655 (100 μM) (Fig. 3, Table 1).

AMPA receptor activation of MAPK occurs through MEK and is direct, with no involvement of NMDA receptors and/or L-type voltage-sensitive Ca^{2+} channels
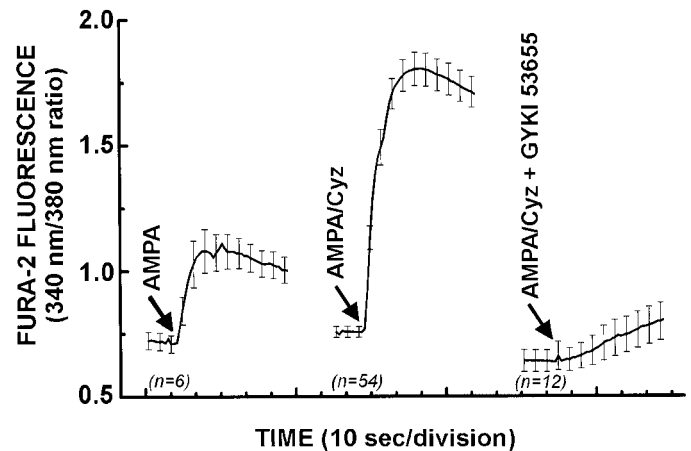
We have shown at the single cell level that GYKI 53655, a selective antagonist at AMPA receptors can block AMPA/cyclothiazide-induced MAPK activation (see Fig. 9*B*). This finding was mirrored in large populations of cultured striatal neurons because the robust activation of ERK1/ERK2 by AMPA/cyclothiazide (Fig. 4*A*, *lane 3*) was completely blocked by preincubation with GYKI 53655 (100 μM) before AMPA/cyclothiazide treatment (Fig. 4*A*, *lane 4*, *C*). The direct upstream activator of MAPK is MAPK kinase (MEK) (Seger and Krebs, 1995). To establish whether or not striatal AMPA receptors activate MAPK via the conventional pathway, i.e., phosphorylation of MAPK by MEK, we performed experiments with PD 98059, a specific inhibitor of the activation of MEK (Alessi et al., 1995). Preincubation of neurons with PD 98059 (50 μM) completely blocked AMPA/cyclothiazide-evoked activation of MAPK (ERK1/ERK2) (Fig. 4*A*, *lane 5*, *C*).

Further pharmacological characterization of the AMPA receptor effect was performed to establish whether AMPA receptors are directly coupled to the MAPK cascade. Ca^{2+} entry through NMDA receptors and/or L-type voltage-sensitive Ca^{2+} channels (VSCC) can activate the MAPK pathway (Rosen et al., 1994; Xia et al., 1996), thus, the ability of AMPA/cyclothiazide to activate MAPK in the presence of the NMDA receptor antagonist (+)-MK 801 (2 μM) or the L-type VSCC blocker nimodipine (5 μM) was examined. The activation of MAPK by AMPA/cyclothiazide (Fig. 4*B*, *lane 2*) was unaltered in the presence of either (+)-MK 801 (Fig. 4*B*, *lane 4*) or nimodipine (Fig. 4*B*, *lane 5*), indicating that AMPA receptor stimulation does not evoke a secondary activation of MAPK from Ca^{2+} entry through NMDA

A

B

C
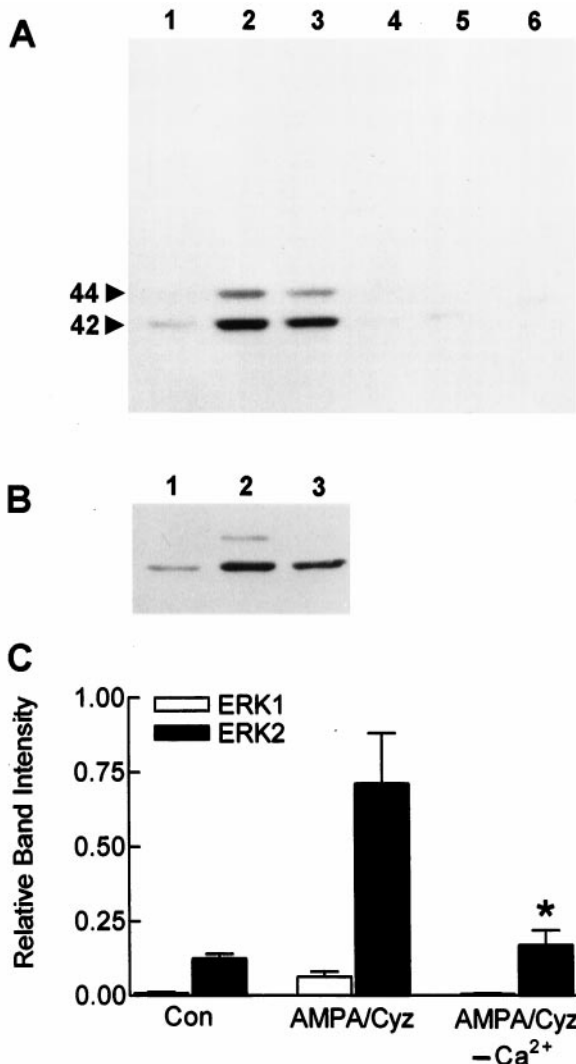
Figure 2. Ca^{2+} dependence of AMPA receptor-evoked MAPK activation in striatal neurons. *A*, Crude homogenates (15 μg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lanes 1, 2, 4, 5*) or presence (*lanes 3, 6*) of 50 μM cyclothiazide (*Cyz*), and then exposed for 5 min to vehicle (*lanes 1, 4*), 100 μM glutamate (*lanes 2, 5*), or 50 μM AMPA and 50 μM Cyz (*lanes 3, 6*) in the presence (*lanes 1-3*) or absence (*lanes 4-6*) of $CaCl_2$ (1 mM), were immunoblotted with an antibody that specifically recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 and ERK2 (anti-ACTIVE MAPK pAb). In experiments in which $CaCl_2$ was omitted from the incubation medium, EGTA (200 μM) was added 1 min before stimulation. *B*, Crude homogenates (15 μg of each), prepared from striatal neurons exposed to vehicle (*lane 1*), 100 μM glutamate (*lane 2*), or 5 μM ionomycin (*lane 3*) for 5 min, were immunoblotted with anti-ACTIVE MAPK pAb. *C*, Data obtained from immunoblot experiments with AMPA/Cyz was analyzed using BioImage Intelligent Quantifier software. Each column is the mean ± SEM value of six independent cultures ($n = 6$). *AMPA/Cyz in the absence of $CaCl_2$ (*AMPA/Cyz–Ca^{2+}*) was significantly different from AMPA/Cyz in the presence of $CaCl_2$ (*AMPA/ Cyz*) ($p < 0.05$, unpaired two-tailed Student's *t* test).

Figure 3. AMPA receptor-evoked increase in intracellular Ca^{2+} is enhanced by cyclothiazide and blocked by GYKI 53655. Fura-2-loaded striatal neurons were exposed to 50 μM AMPA, 50 μM AMPA in the presence of 50 μM cyclothiazide (*AMPA/Cyz*), or AMPA/Cyz in the presence of 100 μM GYKI 53655 added 5 min before stimulation. Fura-2 fluorescence (340 nm/380 nm ratio) was monitored as described in Materials and Methods. Results are means ± SEM of responses from the number of neurons (*n*) shown in parentheses.

vation of neuronal MAPK may arise from the mobilization of intracellular Ca^{2+} stores after stimulation of group I metabotropic glutamate receptors (mGluRs). However, the possibility that AMPA was acting as a ligand at group I mGluRs or that AMPA receptor stimulation was indirectly activating mGluRs was ruled out because, consistent with previous reports (Wang and Durkin, 1995; Vincent et al., 1998), the group I mGluR agonist (1*S*,3*R*)-ACPD (200 μM) failed to activate MAPK (data not shown).

Regulation of AMPA receptor activation of MAPK by a Src family protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II, but not protein kinase C

It is known that MEK needs to be serine phosphorylated for its activation, and a likely kinase for performing this role is Raf (*B*-Raf or c-Raf-1) (Seger and Krebs, 1995). Furthermore, in non-neuronal cells at least, it is known that Raf kinase is itself phosphorylated and consequently activated by one or more upstream kinases, with PKC and the protein tyrosine kinase (PTK) family being the most likely candidates (Seger and Krebs, 1995). The involvement of PKC and/or protein tyrosine phosphorylation in AMPA receptor-evoked activation of MAPK in striatal neurons was tested using kinase inhibitors. Although NMDA receptor-induced activation of neuronal MAPK has been shown to be partially prevented by PKC inhibitors (Kurino et al., 1995; Vincent et al., 1998), preincubation of striatal neurons with the selective PKC inhibitor Ro-31–8220 (5 μM) before AMPA/cyclothiazide treatment did not alter the level of MAPK activation [Fig. 5*A*, *lane 3* vs *lane 2* (AMPA/Cyz), *C*], arguing against an involvement of PKC in AMPA receptor activation of MAPK.

Two families of nonreceptor PTKs, the Src family and the focal adhesion kinase (FAK) family, contain members that are prominently expressed in neurons (Boxall and Lancaster, 1998) and it is believed, although not yet proven, that these Ca^{2+}-dependent enzymes subserve specific physiological roles in the CNS beyond mere housekeeping functions. For example, members of the Src and FAK families have been strongly implicated in controlling

receptors or L-type VSCC. Elevation of the external [K^+] in the incubation buffer to 50 mM, which causes neuronal depolarization and influx of Ca^{2+} through VSCC, did not stimulate MAPK activity above basal levels (data not shown), thus, providing further evidence that L-type VSCC cannot be involved in AMPA receptor activation of MAPK in cultured striatal neurons. Acti-
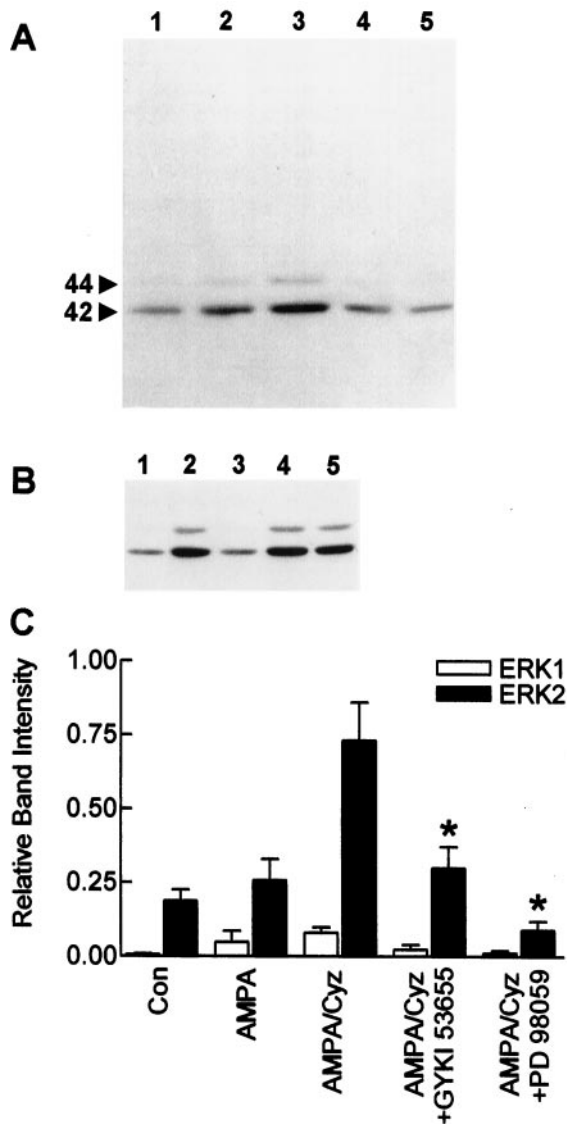
Figure 4. AMPA/cyclothiazide activation of MAPK through MEK is direct and independent of NMDA receptor and/or L-type voltage-sensitive Ca^{2+} channel activation. *A*, Crude homogenates (15 µg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lanes 1, 2*) or presence (*lanes 3-5*) of 50 µM cyclothiazide (*Cyz*), and then exposed for 5 min to vehicle (*lane 1*), 50 µM AMPA (*lane 2*), 50 µM AMPA and 50 µM Cyz (*lane 3*), 50 µM AMPA and 50 µM Cyz in the presence of 100 µM GYKI 53655 added 5 min before AMPA/Cyz (*lane 4*), or 50 µM AMPA and 50 µM Cyz in the presence of 50 µM PD 98059 added 5 min before AMPA/Cyz (*lane 5*), were immunoblotted with an antibody that specifically recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 and ERK2 (anti-ACTIVE MAPK pAb). *B*, Crude homogenates (15 µg of each), prepared from striatal neurons preincubated for 5 min in the absence (1) or presence (*lanes 2-5*) of 50 µM Cyz and then exposed for 5 min to vehicle (*lane 1*), 50 µM AMPA and 50 µM Cyz (*lane 2*), 50 µM AMPA and 50 µM Cyz in the presence of 100 µM GYKI 53655 added 5 min before AMPA/Cyz (*lane 3*), 50 µM AMPA and 50 µM Cyz in the presence of 2 µM (+)-MK 801 added 5 min before AMPA/Cyz (*lane 4*), or 50 µM AMPA and 50 µM Cyz in the presence of 5 µM nimodipine added 5 min before AMPA/Cyz (*lane 5*), were immunoblotted with anti-ACTIVE MAPK pAb. *C*, Data obtained from immunoblot experiments represented in *A* (*lanes 1-5*) was analyzed using BioImage Intelligent Quantifier software. Each column is the mean ± SEM value of five to eight independent cultures (*n* = 5–8). *AMPA/Cyz in the presence of GYKI 53655 or PD 98059 was significantly different from AMPA/Cyz (*p* < 0.05, unpaired two-tailed Student's *t* test).

activation of the Ras/MAPK signaling pathway (Lev et al., 1995; Finkbeiner and Greenberg, 1996; Hayashi et al., 1999), and it appears that protein tyrosine phosphorylation may be important at several points within the MAPK cascade. To test the involvement of tyrosine phosphorylation in AMPA receptor-evoked activation of MAPK, neurons were pretreated with the broad range tyrosine kinase inhibitor genistein (50 µM) or the Src family-selective tyrosine kinase inhibitor PP2 (10 µM) (Hanke et al., 1996) before AMPA/cyclothiazide. No activation of ERK1/ERK2 by AMPA/cyclothiazide was detected in the presence of either genistein (Fig. 5*A*, *lane 5*, *C*) or PP2 [Fig. 5*B*, *lane 3* vs *lane 2* (AMPA/Cyz)], indicating that stimulation of a Src family protein tyrosine kinase activity is required for AMPA receptor coupling to MAPK. Preincubation of striatal neurons with the pyrazolo[3,4-d]pyrimidine, PP3 (10 µM), a proposed negative control for PP2 (Traxler et al., 1997), did cause some inhibition of AMPA receptor-evoked activation of ERK1/ERK2 (Fig. 5*B*, *lane 4*), however, it was not as great as that caused by PP2.
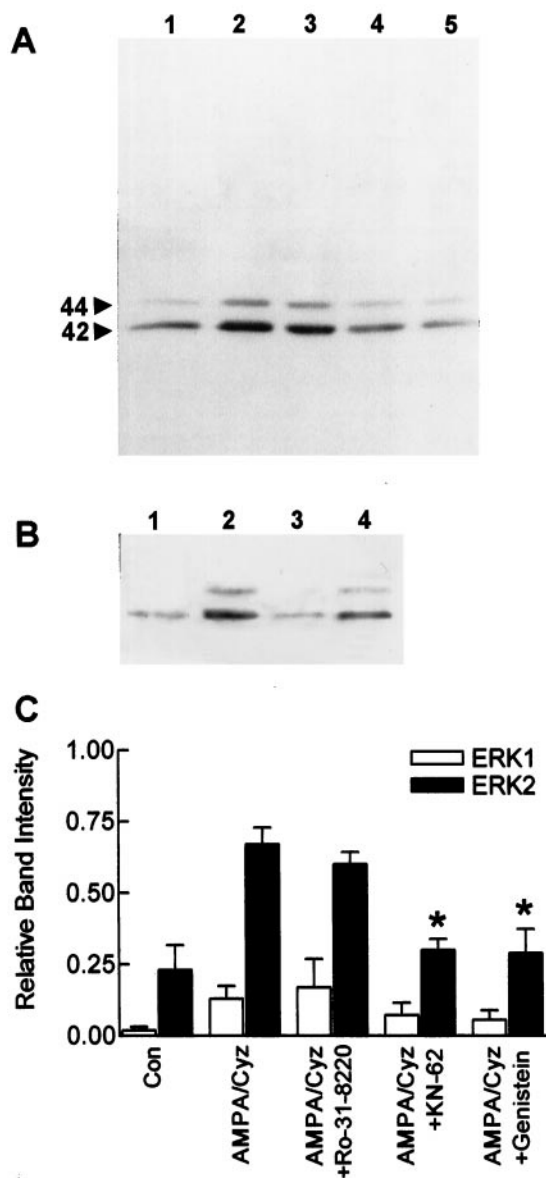
Ca^{2+}/calmodulin-dependent kinase II (CaM-KII), a major protein present at the postsynaptic density region of neurons, is activated following increases in intracellular Ca^{2+} (Hanson and Schulman, 1992), thus, we considered that CaM-KII may play a role in our observed AMPA receptor activation of MAPK. Preincubation of striatal neurons with the selective CaM-KII inhibitor KN-62 (5 µM) before AMPA/cyclothiazide caused a robust inhibition of AMPA receptor-evoked MAPK activation (Fig. 5*A*, *lane 4*, *C*), indicating that CaM-KII may play an important regulatory role in the activation of MAPK by Ca^{2+}-permeable AMPA receptors.

An essential role for a pertussis toxin-sensitive G-protein and phosphatidylinositol 3-kinase (PI 3-kinase) in AMPA receptor coupling to MAPK

Application of AMPA to rat cerebral cortical neurons has been shown to lead to a rapid increase in Ras activity and activation of MAPK (Wang and Durkin, 1995). Ras-dependent activation of MAPK is usually associated with seven transmembrane receptors that couple to heterotrimeric G-proteins (Gutkind, 1998), however, it has been demonstrated in rat cortical neurons that AMPA activates ERK2 (p42) by causing a Ca^{2+}-dependent association of G-protein $\beta\gamma$ subunits, probably G_i, with a Ras, Raf kinase, MEK complex (Wang and Durkin, 1995; Wang et al., 1997). This novel involvement of a heterotrimeric G-protein in ionotropic AMPA receptor signaling was examined. Striatal neurons were pretreated with pertussis toxin (PTX) (1 µg/ml, made up in sterile PBS) or PBS vehicle for 24 hr before experiments with AMPA/cyclothiazide. In agreement with the results of Wang and Durkin (1995), PTX treatment abolished AMPA receptor activation of MAPK [Fig. 6*A*, *lane 3* vs *lane 2* (AMPA/Cyz), *C*), indicating a role for a G_i or G_o-type G-protein in the activation of MAPK by AMPA receptors in striatal neurons.

More recently, additional links between G$\beta\gamma$ subunits and the Ras-MAPK cascade have been identified. Wortmannin, a selective (low nanomolar range) inhibitor of PI 3-kinase has been reported to block G_i-coupled receptor-induced MAPK activation (Hawes et al., 1996), and experiments have indicated that PI 3-kinase is recruited by free G$\beta\gamma$ subunits and coordinates the activation of Src-like nonreceptor PTKs involved in activating the Shc-Grb2-Sos complex (Lopez-Ilasaca et al., 1997; Gutkind, 1998). Furthermore, it has been shown that PI 3-kinase activity can be regulated by Ca^{2+}/calmodulin (Joyal et al., 1997) and therefore, the possibility that PI 3-kinase is required for AMPA

Table 1. AMPA receptor-evoked increases in intracellular Ca^{2+} are insensitive to the protein kinase inhibitors KN-62, PD 98059, and genistein, and the lipid kinase inhibitor wortmannin

Treatments	340 nm/380 nm ratio change (20 sec after stimulation)
AMPA	0.37 ± 0.056 (6)
AMPA/Cyz	1.04 ± 0.071 (54)
AMPA/Cyz + GYKI 53655	0.073 ± 0.017 (12)*
AMPA/Cyz + KN-62	0.96 ± 0.058 (36)
AMPA/Cyz + PD 98059	0.84 ± 0.048 (18)
AMPA/Cyz + genistein	1.00 ± 0.069 (14)
AMPA/Cyz + wortmannin	1.13 ± 0.10 (36)

Fura-2-loaded striatal neurons were preincubated for 5 min in the presence of 5 μM KN-62, 50 μM PD 98059, 50 μM genistein, or 100 nM wortmannin, before the addition of 50 μM AMPA in the presence of 50 μM cyclothiazide (AMPA/Cyz) to evoke Ca^{2+} influx. Fura-2 fluorescence was monitored as described in the Materials and Methods, and increases in the fura-2 fluorescence (340 nm/380 nm ratio) 20 sec after the addition of agonist are shown. Responses to AMPA (50 μM) alone and to AMPA/Cyz (50 μM/50 μM) in the presence of 100 μM GYKI 53655 (added 5 min before AMPA/Cyz) are included for comparison. Results are means $\pm$ SEM of responses from the number of neurons (*n*) measured.

*AMPA/Cyz + GYKI 53655 was significantly different from AMPA/Cyz ($p <$ 0.001, unpaired two-tailed Student's *t* test). No significant differences were found between AMPA/Cyz and AMPA/Cyz in the presence of any of the kinase inhibitors tested.
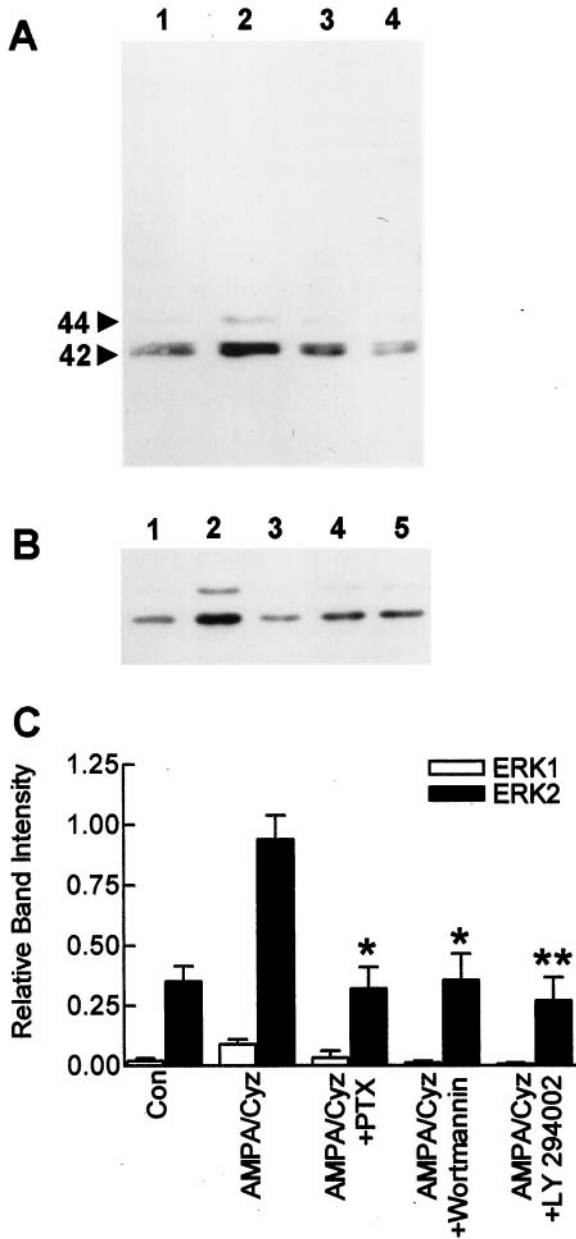
receptor-mediated MAPK signaling was examined. Pretreatment of striatal neurons with wortmannin (100 nM) (Fig. 6*A*, *lane 4*, *C*) or the highly selective PI 3-kinase inhibitor LY 294002 (50 μM) (Fig. 6*C*) completely blocked the activation of ERK1/ERK2 MAPK by AMPA/cyclothiazide. This is the first report that AMPA receptor coupling to MAPK is dependent on PI 3-kinase.

Complementing the AMPA/cyclothiazide data, in a set of additional experiments we showed that the nondesensitizing AMPA receptor agonist KA (100 μM) also activated MAPK, exclusively through AMPA receptors (no involvement of kainate receptors was indicated by a complete sensitivity of the KA effect to GYKI 53655), in a PTX- and wortmannin-sensitive manner (Fig. 6*B*).

We considered it possible that the kinase inhibitors KN-62, PD 98059, genistein and wortmannin blocked AMPA/cyclothiazide-evoked MAPK activation by inhibiting the entry of Ca^{2+} through AMPA receptors. To address this, intracellular Ca^{2+} measurements were made using fura-2-loaded neurons. AMPA/cyclothiazide-evoked increases in cytosolic Ca^{2+} were not altered by any of the kinase inhibitors tested (Table 1), and, thus, it seems likely that CaM-KII, MEK, tyrosine kinase, and PI 3-kinase activities all have sites of action at points downstream of Ca^{2+} influx.

Is cytosolic phospholipase A_2 (cPLA$_2$) a putative downstream target for ERK1/ERK2 MAP kinases in striatal neurons?

We have previously shown that stimulation of Ca^{2+}-permeable cyclothiazide-sensitive AMPA receptors leads to a release of arachidonic acid (AA) from striatal neurons (Williams and Glowinski, 1996). This release can be prevented by removing extracellular Ca^{2+} or by preincubation with the selective cPLA$_2$ inhibitor AACOCF$_3$ before AMPA receptor stimulation (M. S. Perkinton and R. J. Williams, unpublished observations), suggesting that striatal AMPA receptors mobilize AA through the activation and Ca^{2+}-dependent translocation of cPLA$_2$. In non-neuronal cells, MAP kinases phosphorylate cPLA$_2$ on Ser^{505} and/or Ser^{727}, and it has been proposed that this may be crucial for agonist-induced

Figure 5. Involvement of a Src family protein tyrosine kinase and CaM-KII, but not protein kinase C, in AMPA receptor-evoked activation of MAPK. *A*, Crude homogenates (15 μg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lane 1*) or presence (*lanes 2-5*) of 50 μM cyclothiazide (*Cyz*) and then exposed for 5 min to vehicle (*lane 1*), 50 μM AMPA and 50 μM Cyz (*lane 2*), 50 μM AMPA and 50 μM Cyz in the presence of 5 μM Ro-31–8220 added 5 min before AMPA/Cyz (*lane 3*), 50 μM AMPA and 50 μM Cyz in the presence of 5 μM KN-62 added 5 min before AMPA/Cyz (*lane 4*), or 50 μM AMPA and 50 μM Cyz in the presence of 50 μM genistein added 5 min before AMPA/Cyz (*lane 5*), were immunoblotted with an antibody that specifically recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 and ERK2 (anti-ACTIVE MAPK pAb). *B*, Crude homogenates (15 μg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lane 1*) or presence (*lanes 2-4*) of 50 μM Cyz, and then exposed for 5 min to vehicle (*lane 1*), 50 μM AMPA and 50 μM Cyz (*lane 2*), or 50 μM AMPA and 50 μM Cyz in the presence of either 10 μM PP2 (*lane 3*) or 10 μM PP3 (*lane 4*) both added 5 min before AMPA/Cyz, were immunoblotted with anti-ACTIVE MAPK pAb. *C*, Data obtained from immunoblot experiments represented in *A* (*lanes 1-5*) was analyzed using BioImage Intelligent Quantifier software. Each column is the mean $\pm$ SEM value of four to six independent cultures (*n* = 4–6). *AMPA/Cyz in the presence of KN-62 or genistein was significantly different from AMPA/Cyz ($p < 0.05$, unpaired two-tailed Student's *t* test).

Figure 6. Essential role for PI 3-kinase and a G_i/G_o-type G-protein in the activation of MAPK by Ca^{2+}-permeable AMPA receptors. *A*, Crude homogenates (15 μg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lane 1*) or presence (*lanes 2-4*) of 50 μM cyclothiazide (*Cyz*) and then exposed for 5 min to vehicle (*lane 1*), 50 μM AMPA and 50 μM Cyz (*lane 2*), 50 μM AMPA and 50 μM Cyz in the presence of 1 μg/ml pertussis toxin (PTX) added 24 hr before AMPA/Cyz (*lane 3*), or 50 μM AMPA and 50 μM Cyz in the presence of either 100 nM wortmannin (*lane 4*) or 50 μM LY 294002 (*C*) both added 5 min before AMPA/Cyz, were immunoblotted with an antibody that specifically recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 and ERK2 (anti-ACTIVE MAPK pAb). *B*, Crude homogenates (15 μg of each), prepared from striatal neurons exposed for 5 min to vehicle (*lane 1*), 100 μM KA (*lane 2*), 100 μM KA in the presence of 100 μM GYKI 53655 added 5 min before KA (*lane 3*), 100 μM KA in the presence of 1 μg/ml PTX added 24 hr before KA (*lane 4*), or 100 μM KA in the presence of 100 nM wortmannin added 5 min before KA (*lane 5*), were immunoblotted with anti-ACTIVE MAPK pAb. *C*, Data obtained from immunoblot experiments represented in *A* (*lanes 1-4*) was analyzed using BioImage Intelligent Quantifier software. Each column is the mean $\pm$ SEM value of five to six
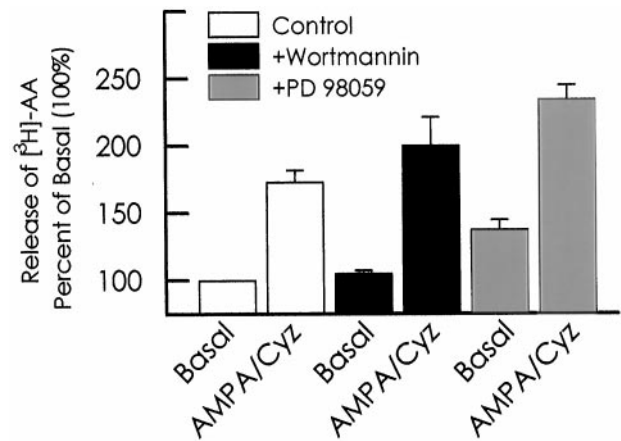
Figure 7. AMPA receptor-evoked mobilization of arachidonic acid in striatal neurons is independent of the MAPK cascade. Striatal neurons were preincubated for 10 min in HEPES-buffered medium containing fatty acid-free BSA (2 mg/ml; HBM/BSA) in the presence or absence of enzyme inhibitors (100 nM wortmannin or 50 μM PD 98059), with or without 50 μM cyclothiazide (*Cyz*) and then incubated for 15 min in HBM/BSA containing either vehicle (*Basal*), or 50 μM AMPA and 50 μM Cyz (*AMPA/Cyz*) in the continued presence or absence of enzyme inhibitors as shown. Both AMPA and Cyz alone failed to release [³H]AA (data not shown). Results are expressed as the percent release of [³H]AA relative to basal values, which was defined as 100%. Data are the mean $\pm$ SEM values of three to five independent cultures ($n = 3–5$), each performed in triplicate.

activation of $cPLA_2$ (Lin et al., 1993; Borsch-Haubold et al., 1998). Thus, it is possible that one of the functional consequences of AMPA receptor-mediated activation of MAPK in striatal neurons is the phosphorylation and activation of $cPLA_2$ leading to AA mobilization. To examine this possibility, AMPA receptor-evoked [³H]AA release from striatal neurons was assayed in the absence or presence of inhibitors of MAPK activity. In control conditions, treatment of neurons with AMPA/cyclothiazide (50 μM/50 μM) produced a 175 $\pm$ 8.5% release of [³H]AA above basal levels (Fig. 7). Preincubation of neurons with the MEK inhibitor PD 98059 (50 μM) or the PI 3-kinase inhibitor wortmannin (100 nM), both of which completely inhibit AMPA receptor-evoked activation of the ERK1 and ERK2 forms of MAPK (Figs. 4*A*, 6*A*, respectively), had no significant effect on AMPA receptor-evoked [³H]AA release (Fig. 7; AMPA/cyclothiazide + wortmannin, 195 $\pm$ 20% of paired basal; AMPA/cyclothiazide + PD 98059, 196 $\pm$ 6% of paired basal). These data strongly suggest that ERK1/ERK2 do not regulate agonist-evoked AA mobilization in cultured striatal neurons, although it should be noted that PD 98059 increased basal [³H]AA levels by 30–40% through an as yet unknown mechanism (Fig. 7).

## Inhibition of PI 3-kinase or MAPK activity does not modulate chronic AMPA receptor-evoked neurotoxicity in striatal neurons

Activation of MAPK may be important during oxidative and excitotoxic stimuli (Guyton et al., 1996; Murray et al., 1998; Samanta et al., 1998), thus, we considered it possible that our

←

independent cultures ($n = 5–6$). *AMPA/Cyz in the presence of PTX or wortmannin was significantly different from AMPA/Cyz ($p < 0.05$, unpaired two-tailed Student's *t* test) and **AMPA/Cyz in the presence of LY 294002 was significantly different from AMPA/Cyz ($p < 0.001$, unpaired two-tailed Student's *t* test).

A

B
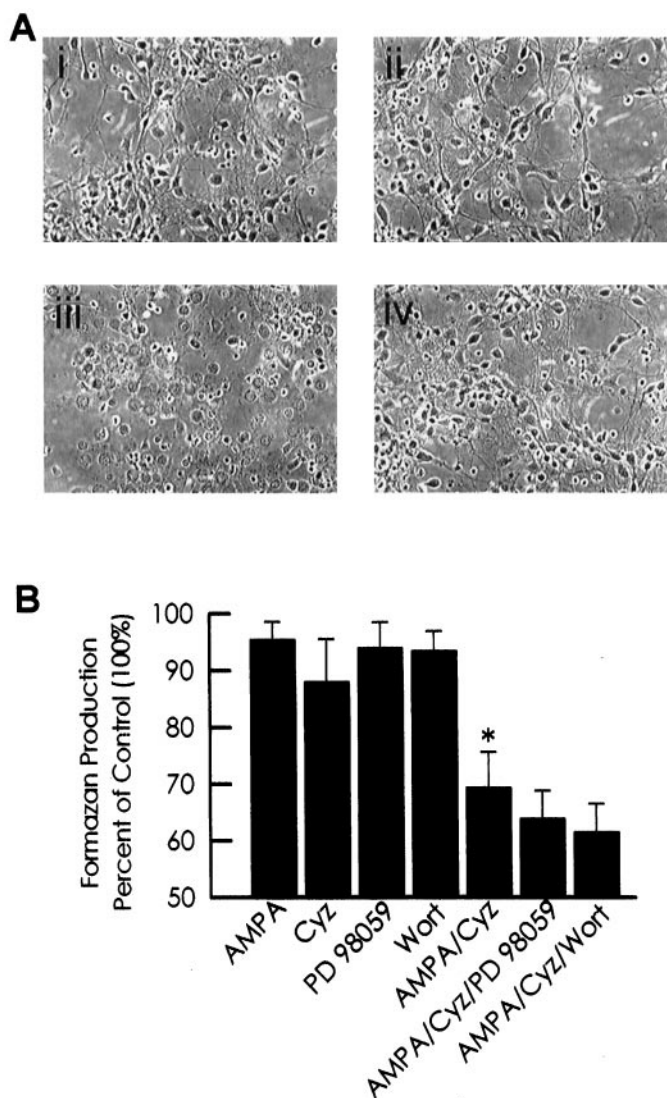
Figure 8. Onset of Ca^{2+}-permeable AMPA receptor-induced neurotoxicity is neither reversed nor accelerated by inhibition of MAPK/PI 3-kinase activity. *A*, Striatal neurons were exposed to vehicle (*i*), 50 μM AMPA (*ii*), 50 μM AMPA in the presence of 50 μM cyclothiazide (*Cyz*) (*iii*), or 50 μM AMPA and 50 μM Cyz in the presence of 100 μM GYKI 53655 added 5 min before AMPA/Cyz (*iv*) for 4 hr and then photographed under phase contrast microscopy (320× magnification). *B*, Striatal neurons were exposed to vehicle or AMPA, with or without Cyz (as described above), in the presence or absence of 50 μM PD 98059 or 100 nM wortmannin (*Wort*) which were added 5 min before treatments. After 4 hr exposure, the culture medium was removed and MTT added as described in Materials and Methods. Results are expressed as the percent of formazan production relative to control, which was defined as 100%. Each column is the mean ± SEM value of four independent cultures (*n* = 4), each performed in triplicate. *AMPA/Cyz was significantly different from AMPA ($p < 0.05$, unpaired two-tailed Student's *t* test).

observed AMPA receptor-evoked activation of MAPK through PI 3-kinase could represent an early neurotoxic or survival signal that might be significant during AMPA/cyclothiazide-induced excitotoxicity. Application of AMPA (50 μM) to striatal neurons for 4 hr produced no visible morphological effects [(Fig. 8*Aii* vs *Ai* (control)], and the capacity of striatal neurons to metabolize the tetrazolium salt MTT into formazan using mitochondrial succinate dehydrogenase, a marker for neuronal viability, was unaltered (Fig. 8*B*). However, in the presence of cyclothiazide (50

μM; which alone had no deleterious effects), AMPA induced profound morphological changes to striatal neurons, including a loss of neurites and cell body integrity (Fig. 8*Aiii*). Furthermore, the ability of neurons to metabolize MTT into formazan was significantly reduced (Fig. 8*B*). Both of these effects were completely blocked by preincubation with the AMPA receptor antagonist GYKI 53655 (100 μM) (Fig. 8*Aiv*; data not shown), demonstrating that toxicity was mediated through cyclothiazide-sensitive AMPA receptors as previously proposed (May and Robison, 1993; Cebers et al., 1997). Chronic exposure (4 hr) of striatal neurons to either the MEK inhibitor PD 98059 (50 μM) or the PI 3-kinase inhibitor wortmannin (100 nM) had no effect on neuronal morphology (data not shown) or MTT turnover (Fig. 8*B*) and neither accelerated nor attenuated the neurotoxic actions of AMPA/cyclothiazide (Fig. 8*B*). These data suggest that these kinases do not play a role in neurotoxicity evoked by chronic AMPA receptor stimulation in striatal neurons.

AMPA receptors induce phosphorylation of the nuclear transcription factor CREB through a MAPK-dependent mechanism in striatal neurons

It has been reported that glutamate can induce phosphorylation and activation of the nuclear transcription factor CREB on the regulatory site Ser^{133} in different brain regions (Ginty et al., 1993; Xia et al., 1996; Obrietan et al., 1998; Sgambato et al., 1998; Vanhoutte et al., 1999), and it is thought that CREB activation may be an important step in processes underlying long-term memory (Frank and Greenberg, 1994; Deisseroth et al., 1996; Ginty, 1997). Although NMDA-type glutamate receptors have been implicated in Ca^{2+}-dependent activation of CREB (Ginty et al., 1993; Xia et al., 1996; Deisseroth et al., 1996), it is not known whether CREB is also a downstream target for AMPA receptor-activated signaling pathways. We tested whether activation of Ca^{2+}-permeable AMPA receptors leads to stimulation of CREB in cultured striatal neurons, using an antibody that detects CREB when it is phosphorylated on the regulatory site Ser^{133} (anti-pCREB). Immunoblotting of neuronal homogenates, containing the nuclear fraction, and single cell immunocytochemistry with anti-pCREB showed that AMPA/cyclothiazide (50 μM/50 μM) induced a robust phosphorylation of CREB on Ser^{133} in the nucleus of striatal neurons (Fig. 10*A, lane 2, Bii*). This effect was entirely dependent on the presence of extracellular Ca^{2+} (Fig. 10*A, lane 3*), was blocked by the AMPA receptor antagonist GYKI 53655 (100 μM) (Fig. 10*Biii*), and was strongly inhibited by PD 98059 (50 μM) and wortmannin (100 nM) (Fig. 10*A, lanes 4* and *5*, respectively), indicating that stimulation of CREB by AMPA receptors is dependent on a PI 3-kinase-sensitive activation of the MAPK cascade.

DISCUSSION

There is some discrepancy in the literature with regard to the ability of AMPA receptors to activate the MAPK cascade. It has been reported that AMPA receptors can couple to the MAPK pathway in cortical neurons (Wang and Durkin, 1995), whereas in hippocampal neurons AMPA and kainate do not appear to activate MAPK (Kurino et al., 1995). In the latter study, a lack of AMPA effects could be caused by either rapid receptor desensitization or reduced Ca^{2+} permeability resulting from the presence of Q/R-edited GluR2 subunits in the native receptor complexes: Ca^{2+} influx through glutamate receptor channels is thought to be the trigger for MAPK activation (Xia et al., 1996). Although it has been reported that AMPA and NMDA induce
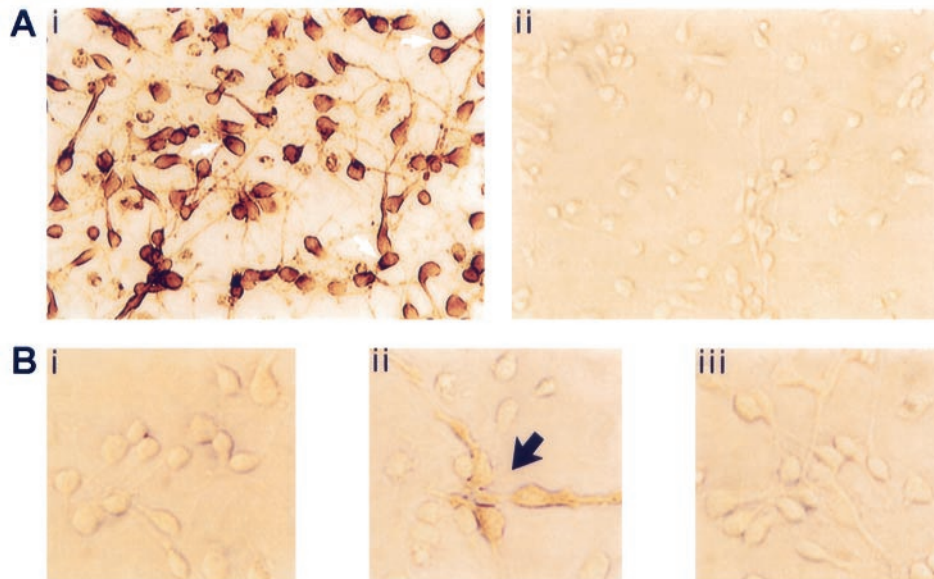
Figure 9. Localization of MAPK and activation by AMPA receptor stimulation in cultured striatal neurons. *A,* Striatal neurons were immunostained with an antibody to the MAPK enzymes ERK1 and ERK2 (anti-ERK1/ERK2 pAb) (*i*), or without primary antibody (*ii*), as described in Materials and Methods. *White arrows* indicate intense cytosolic staining of ERK1 and ERK2. *B,* Striatal neurons were either unstimulated (*i*) or, after incubation with 50 μM cyclothiazide for 5 min, stimulated with 50 μM AMPA (in the presence of 50 μM cyclothiazide) for 5 min in the absence (*ii*) or presence (*iii*) of 100 μM GYKI 53655 added 5 min before stimulation. After stimulation, neurons were immunostained with an antibody that specifically recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 and ERK2 (anti-ACTIVE MAPK pAb) as described in Materials and Methods. *Black arrow* indicates neurons that are immunopositive for anti-ACTIVE MAPK pAb.
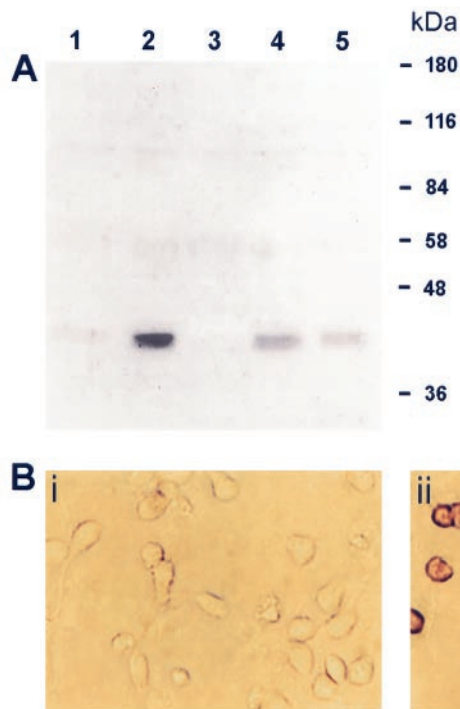
Figure 10. Involvement of the MAPK cascade in AMPA receptor-induced phosphorylation of the nuclear transcription factor CREB in cultured striatal neurons. *A,* Crude homogenates (15 μg of each), prepared from striatal neurons preincubated for 5 min in the absence (*lane 1*) or presence (*lanes 2-5*) of 50 μM cyclothiazide (*Cyz*) and then exposed for 5 min to vehicle (*lane 1*), 50 μM AMPA and 50 μM Cyz (*lane 2*), 50 μM AMPA and 50 μM Cyz in the absence of CaCl$_2$ (1 mM) (*lane 3*), or 50 μM AMPA and 50 μM Cyz in the presence of either 50 μM PD 98059 (*lane 4*) or 100 nM wortmannin (*lane 5*) both added 5 min before stimulation, were immunoblotted with an antibody that detects CREB when phosphorylated at Ser[133] (anti-phospho-Ser[133] CREB pAb). In experiments in which CaCl$_2$ was omitted from the incubation medium, EGTA (200 μM) was added 1 min before stimulation. *B,* Striatal neurons were either unstimulated (*i*) or, after incubation with 50 μM cyclothiazide for 5 min, treated with 50 μM AMPA (in the presence of 50 μM cyclothiazide) for 5 min in the absence (*ii*) or presence (*iii*) of 100 μM GYKI 53655 added 5 min before stimulation. Neurons were immunostained with an anti-phospho-Ser[133] CREB pAb as described in Materials and Methods.

comparable increases in MAPK activity in cultured striatal neurons (Vincent et al., 1998), a direct Ca^{2+}-permeable AMPA receptor coupling to MAPK cannot be concluded because of a lack of characterization of the AMPA response along with no antagonist data. Furthermore, this increase in MAPK activity with AMPA is somewhat surprising, as it has previously been shown that AMPA evokes either very weak or no functional responses in striatal neurons (Tence et al., 1995; Williams and

Glowinski, 1996) unless rapid receptor desensitization is blocked with cyclothiazide (Petitet et al., 1995; Williams and Glowinski, 1996). Similarly, in this study we show that AMPA alone does not significantly activate MAPK. However, a robust AMPA receptor-evoked activation of ERK1 and ERK2 was unmasked with cyclothiazide. Intracellular Ca^{2+} measurements revealed that AMPA produced only a small rise in cytosolic Ca^{2+}, which may explain our observed lack of activation of MAPK with AMPA alone. By contrast, in the presence of cyclothiazide, a large threefold increase in AMPA-evoked rises in cytosolic Ca^{2+} was observed, and this enhanced response probably underlies the unmasking of AMPA receptor-evoked MAPK stimulation. Further pharmacological characterization of AMPA/cyclothiazide-evoked stimulation of ERK1/ERK2 demonstrated that activation resulted from the direct influx of Ca^{2+} through the AMPA receptor itself with no secondary facilitatory involvement of either NMDA receptors or L-type voltage-sensitive Ca^{2+} channels.

AMPA receptors appear to activate the MAPK cascade in cortical neurons through a novel mechanism involving the $\beta\gamma$ subunits of PTX-sensitive G-proteins (Wang and Durkin, 1995). Our finding that AMPA receptor-evoked activation of MAPK in striatal neurons was sensitive to PTX supports the concept that ionotropic receptors can transduce signals through G_i/G_o-type G-proteins (Wang et al., 1997; Rodriguez-Moreno and Lerma, 1998). Recently, seven-transmembrane receptors coupled to inhibitory G-proteins have been shown to signal to MAPK in non-neuronal cells through a mechanism requiring the recruitment of PI 3-kinase (Hawes et al., 1996; Lopez-Ilasaca et al., 1997; Gutkind, 1998). Interestingly, distinct from the well established phosphotyrosine-dependent activation of PI 3-kinase, rises in cytosolic Ca^{2+} can activate PI 3-kinase in chinese hamster ovary cells by inducing a direct association of calmodulin with both SH2 domains of the regulatory subunit (p85) of the kinase (Joyal et al., 1997). We, therefore, considered that PI 3-kinase could be an important intermediate in our observed G_i/G_o- and Ca^{2+}-dependent activation of MAPK by AMPA receptors. Indeed, AMPA receptor activation of MAPK was completely inhibited by the PI 3-kinase inhibitors wortmannin and LY 294002, indicating an essential role for PI 3-kinase in AMPA receptor-stimulated ERK1 and ERK2 activation in striatal neurons. This is the first report of an involvement of PI 3-kinase in the regulation of postsynaptic AMPA receptor signaling in the CNS. MAPK activation was not modified by the PKC inhibitor Ro-31–8220, which is consistent with previous reports in non-neuronal cells that signaling to MAPK via $G\beta\gamma$ subunits and PI-3 kinase is independent of PKC (Luttrell et al., 1997). Thus, although the specific protein–protein interactions that lead to activation of the Ras-MAPK pathway by AMPA receptors are not currently known, it seems reasonable to propose that AMPA receptor-evoked rises in cytosolic Ca^{2+} may trigger activation of PI 3-kinase and then recruitment of the lipid kinase to the MAPK cascade may, as is the case with seven-transmembrane G_i/G_o-type G-protein linked receptors, be orchestrated by free $G\beta\gamma$ subunits. The specific exchange factors regulating Ras activity after AMPA receptor stimulation also remain to be determined. An involvement of the neuron-specific guanine nucleotide exchange factor, Ras-GRF, seems plausible because it has recently been demonstrated that Ras-GRF can be activated in response to increases in intracellular Ca^{2+} (Farnsworth et al., 1995; Finkbeiner and Greenberg, 1996) and/or free G-protein $\beta\gamma$ subunits that induce

phosphorylation of Ras-GRF by as yet unknown kinases (Mattingly and Macara, 1996).

However, Ca^{2+}/calmodulin-dependent activation of Ras-GRF does not appear to involve PTKs (Farnsworth et al., 1995), thus, our results indicating that tyrosine phosphorylation may be an important step in AMPA receptor activation of MAP kinase suggests that additional Ca^{2+}-dependent routes to Ras may be activated. It has been proposed that PI 3-kinase can coordinate the activation of Src-like nonreceptor PTKs (Lopez-Ilasaca et al., 1997; Gutkind, 1998). Furthermore, the Src and FAK families of PTKs have been strongly implicated in controlling Ca^{2+}-dependent activation of the Ras/MAPK signaling pathway, however, it is not clear whether members of these two families act independently or together to coordinate association of the Grb2-Sos complex, which leads ultimately to the sequential activation of Ras, Raf kinase, and MEK (Lev et al., 1995; Finkbeiner and Greenberg, 1996). We found that AMPA receptor-evoked activation of MAPK was completely blocked in the presence of the tyrosine kinase inhibitors genistein and PP2. It has recently been reported that PP2 selectively inhibits tyrosine kinases of the Src family, namely Src, Fyn, and Lck (all of which are present in the brain), while having negligible inhibitory activity against Jak2 or Zap PTKs (Hanke et al., 1996). Our results with PP2 agree with previous work demonstrating that Src-like protein tyrosine kinases regulate Ca^{2+}-dependent activation of the MAPK cascade (Rusanescu et al., 1995; Finkbeiner and Greenberg, 1996) and synaptic plasticity involving AMPA receptor activation (Boxall and Lancaster, 1998; Hayashi et al., 1999). Whether or not PYK2, a member of the FAK family that has been shown to be important in Ca^{2+}-dependent activation of the MAPK cascade (Lev et al., 1995), is involved in neuronal AMPA receptor signaling remains to be determined.

CaM-KII is enriched at the postsynaptic density of glutamatergic synapses where one of its major roles is thought to be in the regulation of glutamate receptor responses (Fukunaga et al., 1992). It has been shown that CaM-KII can phosphorylate AMPA receptor subunits (Mammen et al., 1997), resulting in enhanced receptor currents (McGlade-McCulloh et al., 1993; Tan et al., 1994), and this has been implicated in the strengthening of postsynaptic responses associated with synaptic plasticity (Barria et al., 1997). We found that selective inhibition of CaM-KII activity with KN-62 substantially reduced AMPA/cyclothiazide-evoked activation of MAPK without altering Ca^{2+} influx through the receptor. These data indicate that CaM-KII can be a positive modulator of AMPA receptor signaling but that in the presence of cyclothiazide the kinase probably regulates AMPA receptor-mediated MAPK activation at a point downstream of Ca^{2+} entry.

Our observation that ionomycin, which evokes a diffuse rise in bulk cytosolic Ca^{2+} concentration, caused a significantly smaller activation of MAPK in comparison with that resulting from glutamate receptor stimulation, supports the hypothesis that MAPK requires high localized Ca^{2+} rises for its activation in neurons (Finkbeiner and Greenberg, 1996, 1997). It seems likely therefore, that the initial Ca^{2+}-sensing components of the MAPK cascade are in close proximity to the Ca^{2+}-permeable AMPA receptors located at the plasma membrane.

One functional consequence of Ca^{2+}-permeable AMPA receptor activation is a release of the signaling molecule AA from neurons (Williams and Glowinski, 1996), which has been implicated in a range of physiological and pathological processes in the CNS (Piomelli, 1994). A likely candidate for mobilizing AA is

Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$), which can be phosphorylated and activated by MAP kinases (Lin et al., 1993), therefore, it seems likely that AMPA receptor-evoked activation of MAPK could be involved in the release of AA from striatal neurons. However, neither the MEK inhibitor PD 98059 nor the PI 3-kinase inhibitor wortmannin altered AMPA receptor-evoked AA mobilization, suggesting that ERK1 and ERK2 are not involved in Ca^{2+}-dependent AA release from striatal neurons. Another MAPK such as stress-activated protein kinase, which is known to phosphorylate $cPLA_2$ on Ser^{505} and Ser^{727}, may perform this role (Borsch-Haubold et al., 1998).

There are conflicting reports as to whether MAPK is a neurotoxic or a survival signal during oxidative stress and excitotoxic injury (Guyton et al., 1996; Murray et al., 1998; Samanta et al., 1998). It is known that stimulation of Ca^{2+}-permeable AMPA receptors causes neuronal loss (May and Robison, 1993; Cebers et al., 1997; Pellegrini-Giampietro et al., 1997), therefore, it is possible that activation of MAPK could be either an excitotoxic or survival signal triggered by AMPA receptor-mediated Ca^{2+} overload. We observed that prolonged stimulation of Ca^{2+}-permeable AMPA receptors induced neurotoxic effects in striatal neurons, however, no acceleration of, or protection against, AMPA receptor-evoked neurotoxicity could be detected when the MAPK pathway was inhibited either at the level of PI 3-kinase or MEK. These results suggest that MAPK does not modulate AMPA receptor-mediated neuronal loss and possibly subserves some other functional role in striatal neurons.

Downstream targets for receptor-activated intracellular signaling pathways include a wide range of transcription factors that coordinate changes in the expression of a vast array of genes (Hill and Treisman, 1995). NMDA glutamate receptors have been shown to cause Ca^{2+}-dependent stimulation of CREB (Ginty et al., 1993; Xia et al., 1996), however, the ability of AMPA receptors to do so has not been previously addressed. We now report for the first time that AMPA receptor stimulation can also lead to Ca^{2+}-dependent phosphorylation of CREB. It has been reported that CREB is phosphorylated on Ser^{133} and activated by $Ca^{2+}/$calmodulin-dependent protein kinases in neurons (Sheng et al., 1990, 1991; Dash et al., 1991; Deisseroth et al., 1998). More recently, it has been demonstrated that Ca^{2+}-dependent phosphorylation of CREB on Ser^{133} can occur via the ERK-Rsk signaling cascade following their nuclear translocation, and this ERK-dependent activation may be critically involved in cAMP response element (CRE)-mediated changes in gene transcription associated with processes underlying learning and memory (Xing et al., 1996; Impey et al., 1998). Furthermore, glutamate-induced stimulation of CREB has been shown to be dependent on the MAPK cascade (Obrietan et al., 1998; Sgambato et al., 1998; Vanhoutte et al., 1999). We observed negligible Ser^{133}-phosphorylated CREB after treatment of striatal neurons with AMPA/cyclothiazide in the presence of either PD 98059 or wortmannin and, thus, conclude that Ca^{2+}-permeable AMPA receptors phosphorylate CREB on Ser^{133} via the PI 3-kinase-dependent MAPK cascade. CREB lacks consensus ERK phosphorylation sites, and elucidation of the CREB kinase responsible for AMPA receptor-induced CREB activation is presently unknown, however, the ERK-activated kinases Rsk1, Rsk2, and Rsk3 are likely candidates (Impey et al., 1998).

In summary we have shown that Ca^{2+}-permeable AMPA receptors can couple to the MAPK pathway through a PTX-sensitive G-protein in striatal neurons. Activation of this pathway is completely dependent on the recruitment of PI 3-kinase to the cascade, and together with the need for a Src-like tyrosine kinase and CaM-KII activity, a putative model can be predicted whereby these Ca^{2+}-dependent kinases provide a mechanism for the rapid, input-specific, and highly localized translation of increases in intracellular Ca^{2+} induced by glutamate activation of AMPA receptors into activation of the nuclear transcription factor CREB. This pathway may play a pivotal role in coordinating gene transcription associated with short-term and/or long-term changes in synaptic plasticity in the striatum.

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