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Metabotropic Glutamate Receptor 1-Induced Upregulation of NMDA Receptor Current: Mediation through the Pyk2/Src-Family Kinase Pathway in Cortical Neurons

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The mechanism underlying the upregulation of NMDA receptor function by group I metabotropic glutamate receptors (mGluRs), including mGluR1 and 5, is not known. Here we show that in cortical neurons, brief selective activation of group I mGluRs with (*S*)-3,5-dihydroxy-phenylglycine (DHPG) induced a Ca^{2+}–calmodulin-dependent activation of Pyk2/CAKβ and the Src-family kinases Src and Fyn that was independent of protein kinase C (PKC). Activation of Pyk2 and Src/Fyn kinases led to increased tyrosine phosphorylation of NMDA receptor subunits 2A and B (NR2A/B) and was blocked by a selective mGluR1 antagonist, 7-(hydroxyamino)cyclopropa[b]chromen-1a-carboxylate ethyl ester, but not an mGluR5 antagonist, 2-methyl-6-(phenylethynyl)pyridine. Functional linkage between mGluR1 activation and NR2A tyrosine phosphorylation through Pyk2 and Src was also demonstrated after expression of these elements in human embryonic kidney 293 cells. Supporting functional consequences, selective activation of mGluR1 by DHPG induced a potentiation of NMDA receptor-mediated currents that was blocked by inhibiting mGluR1 or Src-family kinases. Furthermore, antagonizing calmodulin or mGluR1, but not PKC, reduced the basal tyrosine phosphorylation levels of Pyk2 and Src, suggesting that mGluR1 may control the basal activity of these kinases and thus the tyrosine phosphorylation levels of NMDA receptors.

Key words: NMDA; metabotropic; Pyk2/CAKβ; Src/Fyn; calmodulin; phosphorylation

Cumulative evidence suggests that Src-family activation may play a role in regulating NMDA receptor function and synaptic plasticity. NMDA receptor subunit 2B (NR2B) is the major tyrosine phosphorylated protein in the postsynaptic density fraction (Moon et al., 1994), and its phosphorylation increases during long-term potentiation (LTP) (Rosenblum et al., 1996; Rostas et al., 1996). Src is associated with NMDA receptors, and phosphorylation by Src can upregulate NMDA receptor current (X. M. Yu et al., 1997; Yu and Salter, 1999). Bath application or postsynaptic injection of Src kinase inhibitors blocks the induction of long-term potentiation in CA1 hippocampal neurons (O'Dell et al., 1991; Huang and Hsu, 1999). Furthermore, targeted disruption of the gene coding for Fyn kinase, a member of the Src-family, impairs the induction of LTP (Grant et al., 1992; Kojima et al., 1997). Activated forms of both tyrosine kinases, Src-and Fyn, phosphorylate recombinantly expressed NR2 subunits and upregulate NMDA receptor currents (Kohr and Seeburg, 1996; Zheng et al., 1998). Deletion of the C-terminal domain of NR2A eliminates the potentiation of NR1/NR2A-receptor currents by Src (Kohr and Seeburg, 1996) and impairs synaptic plasticity and contextual memory in mice (Sprengel et al., 1998).

Few studies to date have addressed the signaling mechanisms controlling Src-family kinase activation during glutamatergic neurotransmission. One possible initial signal is the calcium-mediated activation of the proline-rich tyrosine kinase 2 (Pyk2/CAKβ). In neurons, this kinase is stimulated by increased intracellular calcium and also by protein kinase C (PKC) activation (Lev et al., 1995). Activated Pyk2 binds and activates Src-family kinases (Dikic et al., 1996), thus linking increases in intracellular calcium and PKC activity to tyrosine phosphorylation. Both PKC and Src have been implicated in the potentiation of NMDA-mediated currents by G-protein-coupled muscarinic or lysophosphatidic acid receptors in hippocampal neurons (Lu et al., 1999).

Another influence on NMDA receptor function and synaptic plasticity is the G-protein-coupled metabotropic glutamate receptor (mGluR) system. There are at least eight mGluRs (mGluR 1–8), which can be divided into groups I, II, and III on the basis of sequence homology, signal transduction mechanisms, and pharmacological properties (Pin and Duvoisin, 1995). Group I mGluRs, specifically mGluR1α and 5, localize to the periphery of the postsynaptic region (Baude et al., 1993; Lujan et al., 1997) and are coupled to G_q-proteins, mediating increases in inositol phosphates and the subsequent release of calcium from intracellular stores. Activation of group I mGluRs induces pro-excitatory effects, including increased glutamate release from cortical neurons (Strasser et al., 1998), increased neuronal excitability (Anik-sztejn et al., 1991), and upregulation of NMDA-mediated currents in hippocampal and striatal cultures (Fitzjohn et al., 1996). In CA1 pyramidal neurons, activation of group I mGluRs induces several excitatory responses through the activation of Ca^{2+}-dependent

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and independent cationic conductances (Crepel et al., 1994; Guerineau et al., 1995) and inhibition of K^+ currents (Charpak et al., 1990; Guerineau et al., 1994; Luthi et al., 1996). Furthermore, targeted disruption of the genes coding for either mGluR1 or mGluR5 reduces hippocampal LTP and associative learning in mice (Aiba et al., 1994; Conquet et al., 1994; Lu et al., 1997).

We have shown previously that cultured cortical neurons express both mGluR1α and mGluR5 and that selective activation of these mGluRs increased glutamate release and potentiated NMDA-induced neuronal death (Strasser et al., 1998). In this study, we set out to test the hypothesis that the ability of group I mGluRs to upregulate NMDA receptor function might be mediated through Pyk2/CAKβ and Src-family kinases.

Parts of this paper have been published previously in abstract form (Behrens et al., 1999a, 2000).

MATERIALS AND METHODS

Cortical cell cultures

Mixed cultures. Mixed cortical cultures were prepared from Swiss Webster mouse cortices as described previously (Rose et al., 1993). Briefly, astrocyte cultures were prepared from postnatal (day 1–3) Swiss Webster mice and plated at a density of 0.6 hemispheres per plate in 24-well culture plates (Primaria, Falcon) in Eagle's Minimal Essential Media (MEM with Earle's salts, glutamine free; Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 10% horse serum, 20 mM glucose, and 2 mM glutamine. After the astrocyte cultures reached confluency [14–21 d *in vitro* (DIV)], dissociated cortices obtained from fetal mice at 14–16 d gestation were plated onto the previously established glial monolayer. Cultures were kept in MEM supplemented with 5% fetal bovine serum, 5% horse serum, glucose, and glutamine as described above. At 5 DIV, non-neuronal cell division was halted by a 2 d exposure to 10 μM cytosine arabinoside. Mixed cultures were then fed every 3 d with MEM containing 10% horse serum, glucose, and glutamine. Cultures were maintained in a 37°C humidified incubator in a 5% CO_2 atmosphere and used at 14–16 DIV for analysis of the tyrosine phosphorylation of NMDA receptors.

Near-pure neuronal cultures. Dissociated cortical cells were obtained from fetal mice as above but plated in 24-well plates (four to six cortices per plate) coated with poly-D-lysine and laminin. After 3 DIV, cytosine arabinoside was added (10 μM) to halt the growth of non-neuronal cells. Under these conditions <1% of total cells were astrocytes. Cells were used at 9–12 DIV for the study of intracellular signaling cascades.

Dissociated neuronal cultures. Dissociated cortical neurons were prepared as above but plated on poly-D-lysine/laminin-coated coverslips as described (Goslin et al., 1998). After cell attachment, coverslips were placed on top of a preformed cortical astrocyte monolayer that contained N2.1 media with the addition of 5 μM cytosine arabinoside. Cultures were fed every 5 d by replacing one-third of the media with fresh N2.1. Coverslips were used for confocal imaging 14 d after plating (14 DIV).

Human embryonic kidney 293 cells. ND-10 cells were grown in MEM 20 mM glucose, 2 mM glutamine, and 10% fetal calf serum. Transfection experiments were performed at 30–50% confluency.

Immunocytochemistry and confocal imaging

For immunocytochemistry, coverslips containing neurons were lifted from the astrocyte monolayer, washed by immersion in PBS, and fixed in ice-cold 4% paraformaldehyde for 30 min. Coverslips were then incubated for 10 min at room temperature in PBS that contained 0.25% Triton X-100. Nonspecific sites were blocked by incubation in PBS containing 10% normal goat serum. For double immunostaining, the coverslips were incubated in 2% normal goat serum containing a 1:1000 dilution of a mouse monoclonal antibody against mGluR1α (PharMingen) and a 1:500 dilution of a rabbit polyclonal antibody against mGluR5 (Chemicon) for 30 min at 37°C. Specific binding was detected using secondary antibodies conjugated to AlexaFluor dyes (594, red, for mGluR5; 488, green, for mGluR1α) (Molecular Probes). Images were collected on a Delta Vision Optical Sectioning Microscope consisting of an Olympus IX-70 microscope equipped with a mercury arc lamp. A photometrics CH 350 cooled CCD camera and a high-precision motorized XYZ stage were used to acquire multiple consecutive optical sections at a 0.2 μm interval for each of the fluorescent probes. A UPAPLO 60× objective was used to collect the images.

cDNA transfections

Human embryonic kidney (HEK) 293 stably expressing NR1 subunits (ND-10) were prepared by cotransfection with pRK5-NR1 (kind gift from H. Monyer, University Hospital for Neurology, Heidelberg, Germany) and pRc/CMV (Invitrogen, Carlsbad, CA) using Lipofectace (Invitrogen). Selection was performed in 500 μg/ml G418, and expression screening was performed by immunological detection of NR1 using specific antibodies (PharMingen, La Jolla, CA). Functional assays were performed by transient transfections with NR2A and detection of NMDA-mediated intracellular calcium rise using fura-2 AM videomicroscopy or electrophysiology. The plasmids used for transient transfections were as follows: pRK5-NR2A (kind gift from Dr. H. Monyer), pcDNA3-mGluR1α, pcDNA3-CADTK (Pyk2, wild type) (kind gift from Dr. H. S. Earp, University of North Carolina, Chapel Hill, NC), pUSEamp-Src (wild type), and pUSEamp-SrcDN (dominant negative) (Upstate Biotechnology, Lake Placid, NY). Transient cotransfections of ND-10 cells were performed using Lipofectamine (12 μl; Invitrogen) and 2 μg of total DNA in a final volume of 1 ml for 2 hr in OPTIMEM (Invitrogen), as recommended by the manufacturer. After transfection, cells were returned to growth media with the addition of *L*(+)-2-amino-5-phosphonopentanoic acid (L-AP5; 500 μM) and (2)-α-methyl-4-carboxyphenylglycine (MCPG; 1 mM) to prevent activation of NMDA and mGluR1 receptors during the expression period. After allowing protein expression for 24 hr, cells were washed three times in HEPES controlled salt solution (HCSS) containing (in mM): NaCl 120, KCl 5.4, MgCl2 0.8, CaCl2 1.8, glucose 15, HEPES 20, pH 7.4, treated in the absence or presence of (*S*)-3,5-dihydroxy-phenylglycine (DHPG) for 5 min, and immediately processed for protein extraction.

Cell treatment and protein extraction

For the study of intracellular signaling cascades, near-pure neuronal cultures were washed three times in HCSS and incubated for 30–45 min at 37°C in the absence or presence of the different drugs and then exposed to DHPG or NMDA. After treatment, cells extracts were prepared as described (Behrens et al., 1999b) in lysis buffer [1% NP-40, 20 mM Tris-Cl, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethyl-sulfonylfluoride (PMSF), 20 μg/ml aprotinin, 20 μg/ml leupeptin] and used for the detection of activated forms of Src, Fyn, and Pyk2 using phosphospecific antibodies (Biosource International, Camarillo, CA). For the study of NMDA receptor tyrosine phosphorylation, cell extracts were prepared from mixed cortical cultures as described previously (Lin et al., 1999). Briefly, cells were treated as above, and treatments were stopped by addition of 50 μl of SDS buffer (1% SDS, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). The resulting extracts were heated at 90°C for 5 min followed by addition of 9 vol of dilution buffer (1% NP40, 1% CHAPS, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Insoluble material was removed by centrifugation at 25,000 × *g* for 30 min, and supernatants were retained for immunoprecipitation with specific antibodies [anti-NR2A and anti-NR2B from either Chemicon (Temecula, CA) or Santa Cruz Biotechnology (Santa Cruz, CA)].

Protein determination

Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Immunoprecipitation

Each protein sample (800–1000 μg) was incubated overnight at 4°C with anti-NR2A or anti-NR2B antibodies (3 μl or 2 μg, depending on the antibody source). For immunoprecipitation of Fyn and Src, 50 μg of total cell lysate obtained as described above was incubated with 1 μg of monoclonal antibodies (anti-Fyn: BD Transduction Laboratories, La Jolla, CA; anti-Src: Upstate Biotechnology). Immunocomplexes were recovered with the aid of either protein G-plus or protein A-agarose. Agarose beads were pelleted by centrifugation and washed three times in 1% NP-40/2 mM orthovanadate in PBS and once in PBS. The immunocomplexes were resuspended in Laemmli's buffer and heated at 90°C for 5 min.

Western blot analysis

For the study of PyK2 and Src-family, 20 μg of protein samples were fractionated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Micron Separations, Westboro, MA) using a semidry electrotransfer system (Novablot, Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% milk in TBS-T buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and were then incubated with phosphospecific antibodies directed to the phosphorylated forms of PyK2 and Src-family (anti-PyK2 ^{y402}, anti-Src^{y418}; Biosource International). For detection of phosphotyrosine, proteins were immunoprecipitated using specific antibodies, separated in 6% SDS gels, and transferred as above. Phosphotyrosine was detected using biotinylated anti-phosphotyrosine antibodies (4G10; Upstate Biotechnology). Protein bands were visualized by chemiluminescence using SuperSignal (Pierce). For immunoprecipitation experiments, blots were subsequently reprobed with specific antibodies directed to Pyk2, Src, Fyn, NR2A, or NR2B (Santa Cruz; 1:1000 for Pyk2, Fyn, and Src, and 1:400 for NR2A or NR2B).

Quantification

The intensity of immunoreactive bands obtained in autoradiographic films was measured with an imaging densitometer (Bio-Rad, Hercules, CA). Phosphorylated levels per protein unit ratios were obtained by dividing the phosphoimmunoreactive densitometry values by those obtained for the respective protein redetection blots.

Electrophysiological recordings of NMDA receptor currents

The glass-bottom 35 mm culture dish containing cortical cultures was placed on the stage of an inverted microscope (Nikon, Tokyo, Japan), and membrane currents were recorded by whole-cell recording using an EPC-9 amplifier (List-Electronic, Germany). Near-spherical cells were chosen for the recording. Recording electrodes of 5–8 MΩ (fire-polished; inner diameter = ~1–1.5 μm) were pulled from Corning Kovar Sealing #7052 glass pipettes (PG52151–4, World Precision Instruments) by a Flaming-Brown micropipette puller (P-80/PC, Sutter Instrument Co.). The offset potential of the recording pipette was routinely corrected to 0 mV after the tip was immersed in the bath medium. This potential was also checked at the end of experiments and corrected if necessary (usually 0–2 mV). Recordings with potential draft of >3 mV were discarded. A gigaseal of 10–50 GΩ was formed before the whole-cell or perforated-patch recording mode was established. For perforated patches, gramicidin D (Sigma, St. Louis, MO) was dissolved in DMSO (10 mg/ml) and freshly diluted to a final concentration of 50 μg/ml in the pipette solution. After gigaohm seal formation, whole-cell configuration was established by application of additional suction; for perforated patch, brief voltage steps of −10 mV were applied to monitor the changes in input resistance and capacitance for 15–20 min before the formation of the perforated patch (Kyrozis and Reichling, 1995; Akaike, 1996). Series resistance compensation was routinely applied during recordings. NMDA current was triggered at a holding potential of −70 mV by 100 μM NMDA plus 0.1 μM glycine delivered by the DAD-12 drug perfusion system (Adams and List). Current signals were digitally sampled at 100 μsec (10 kHz) and filtered by a 3 kHz, three-pole Bessel filter. Current and voltage traces were displayed and stored on a computer using the data acquisition/analysis package, PULSE (HEKA Electronik).

The external solution contained (in mM): NaCl 120, KCl 3, $CaCl_2$ 2, HEPES 10, glucose 10, and TTX 0.5 μM. The electrode internal solution contained (in mM): KCl 120, Na_2-ATP 2, BAPTA 0.5, and HEPES 10. Recordings were performed at room temperature (21–22°C) and at pH 7.3, under continuous bath perfusion at ~0.2 ml/min.

Reagents

Unless stated otherwise, all reagents were from Sigma. Metabotropic agonists and antagonists were from Tocris Cookson (Ballwin, MO).

RESULTS

DHPG-mediated activation of group I mGluRs in cortical neuronal cultures increased the tyrosine phosphorylation of Pyk2, Src, and Fyn kinases

Consistent with results obtained for G_q-protein-coupled receptors in non-neuronal cells (Luttrell et al., 1999), selective activation of group I mGluRs by DHPG increased the phosphorylation of the tyrosine kinases Pyk2, Src, and Fyn. Exposure of near-pure cortical neuronal cultures to 100 μM DHPG for 2–10 min increased the phosphorylation of the autocatalytic site of Pyk2 and the Src-family of kinases, tyrosines 402 and 416 (423 in mouse), respectively, as detected using phosphospecific antibodies (Fig. 1*a*,*b*). An increase in the phosphorylation of the autocatalytic site of these kinases is expected to increase its activity (Cooper and MacAuley, 1988; Li et al., 1999). The sequence surrounding the autocatalytic site is highly conserved in the Src-family members, Yes and Fyn; thus the antibody is expected to recognize also the activation of these kinases. Among these three kinases, Src and Fyn are highly expressed in neurons and have been implicated in mechanisms of neuronal plasticity (Grant et al., 1992; Lu et al., 1999). To determine which Src-family member was being activated, we performed immunoprecipitation experiments using antibodies specific for either Src or Fyn and determined by Western blot the phosphorylation state of each kinase using the same phosphospecific antibody as above (pSrc^{Y416}). Five minute exposure to 100 μM DHPG increased the phosphorylation of both Src and Fyn in cortical neurons (Fig. 1*b*).

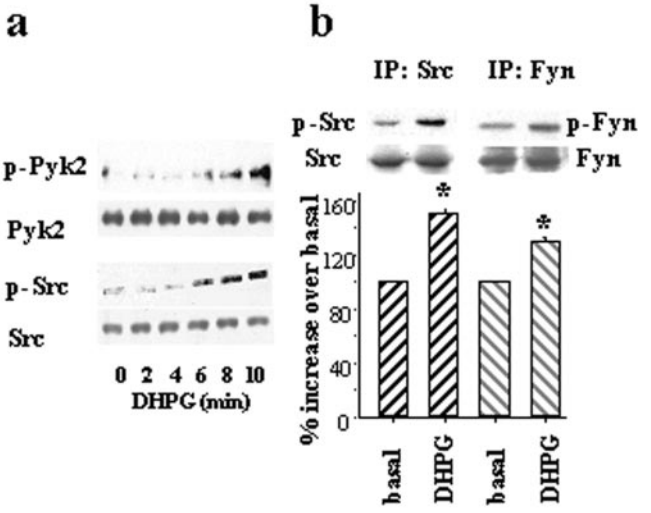

Figure 1. DHPG induced tyrosine phosphorylation of Pyk2, Src, and Fyn kinases in cortical neurons. *a*, Time course. After 12 DIV, near-pure neuronal cultures were exposed to 100 μM DHPG for 2–10 min. The phosphotyrosine content of Pyk2 and the Src-family kinases was determined by Western blot using phosphospecific antibodies against the active forms of the kinases (p-Pyk2: pPyk2$^{(pY402)}$, *top panels*; p-Src: pSrc$^{(pY418)}$, *bottom panels*). Fifteen micrograms of total cell extract were run per lane of 8% SDS-PAGE gels, and the total amount of each specific protein was determined in redetection blots using anti-Pyk2 and anti-Src antibodies (Pyk2 and Src in each blot). *b*, Phosphorylation of Src and Fyn. To analyze whether both Src and Fyn kinases were being activated by DHPG, near-pure cortical neuronal cultures were exposed to 100 μM DHPG for 5 min and immediately processed for protein extraction. Fifty micrograms of total cell lysates were immunoprecipitated using anti-Src or anti-Fyn specific antibodies as described in Materials and Methods. Immunocomplexes were resolved as in *a*, and the phosphorylation content of each kinase was determined by Western blots using the phosphospecific antibody pSrc$^{(pY418)}$. The relative amount of normalized phosphorylation (phosphorylation per protein unit, obtained as the ratio of phosphoprotein/total immunoprecipitated protein) was expressed as a percentage of the control (*basal*, sham wash). Values represent the mean ± SEM obtained for four independent experiments. * indicates statistical difference as compared with basal at $p < 0.05$ by ANOVA.

Activation of group I mGluRs induced the tyrosine phosphorylation of NMDA receptor subunits NR2A/B

The increased phosphorylation of the PyK2, Src, and Fyn kinases prompted us to test the hypothesis that DHPG activation of

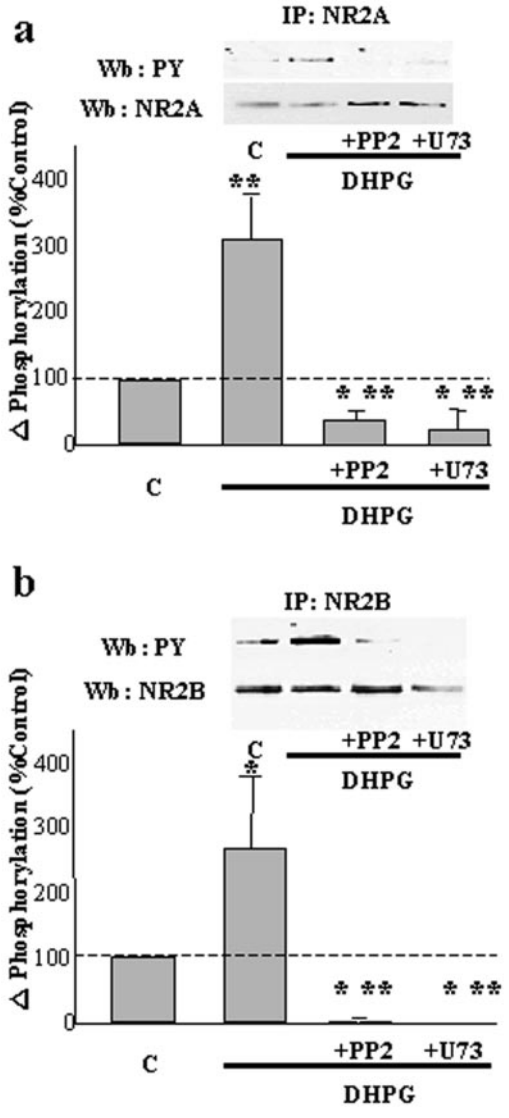

Figure 2. DHPG stimulated the tyrosine phosphorylation of NMDA receptor subunits NR2A/B through activation of the Src-kinase family and PLC in cortical neurons. To analyze the phosphorylation of NR2 subunits, cultures containing neurons and glia were pretreated for 20 min in the absence or presence of the Src-family inhibitor PP2 (1 μM) or the PLC inhibitor U73122 (U73, 20 μM), and then exposed to 100 μM DHPG for 5 min in the presence or absence of the same inhibitors. After protein extraction in 1% SDS, NR2A (*a*) or NR2B (*b*) subunits were immunoprecipitated with specific antibodies (Chemicon) followed by redetection with anti-phosphotyrosine antibody (Wb α-PY: 4G10, Upstate Biotechnology). The immunoreactive bands were quantified by scanning densitometry, and values were calculated as phosphorylation per protein unit (as described in Fig. 1*b* using anti-NR2A or -NR2B antibodies from Santa Cruz Biotechnology). Values represent the mean ± SEM of four to six independent experiments. *C*, Basal, sham wash. * indicates statistical significance as compared with basal, and ** indicates statistical significance as compared with DHPG alone at $p < 0.05$ by ANOVA.

group I mGluRs would increase the tyrosine phosphorylation of NMDA receptors. Cortical neuronal cultures express the NMDA receptor subunits NR1 and NR2A/B (Zhong et al., 1994). DHPG exposure induced a rapid increase in the tyrosine phosphorylation content of both NR2A and 2B subunits (Fig. 2*a*,*b*). The increase in tyrosine phosphorylation of NR2 subunits was blocked by preincubating the cells in the presence of the Src-kinase family inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl pyrazolo[3,4-d]pyrimidine (PP2) (Hanke et al., 1996; Salazar and Rozengurt, 1999) (1 μM), or the phospholipase C (PLC) inhibitor U73122 (20 μM) (Fig. 2). Similar results were obtained when using the tyrosine kinase inhibitor genistein (100 μM) or the specific Src-family inhibitor PP1 (data not shown).

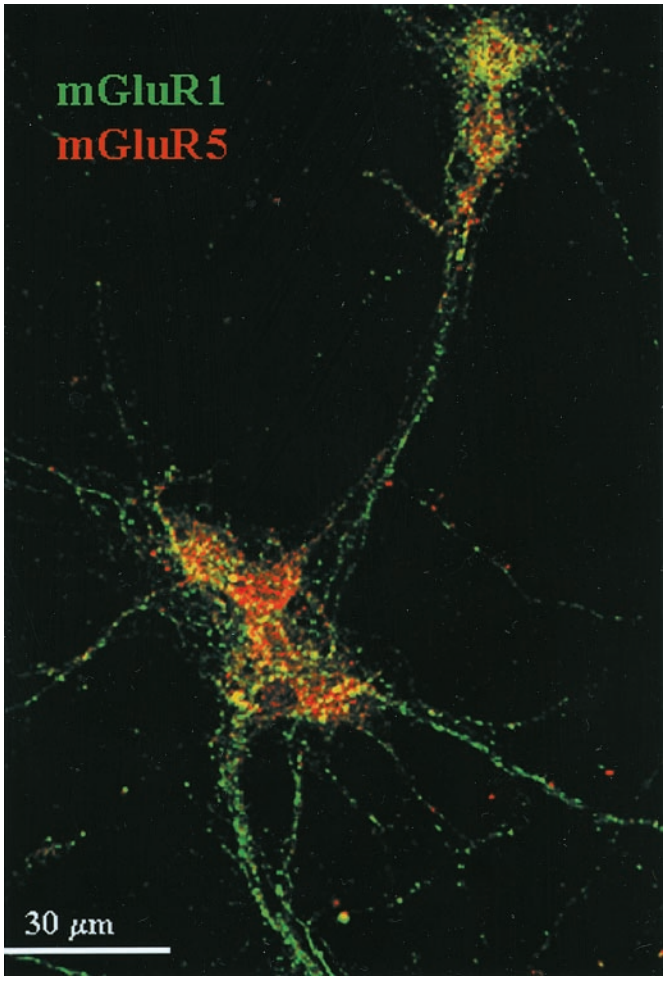

Figure 3. Cultured cortical neurons express both mGluR1 and mGluR5. Dissociated cortical neurons were grown on glass coverslips over a bed of astrocytes. At 14 DIV, the coverslips were lifted from the bed of glia, washed in PBS, and immediately fixed in 4% paraformaldehyde. Detection of mGluR1 and mGluR5 was performed by double immunostaining with specific antibodies [mGluR1: 1:1000 dilution of mouse monoclonal antibody (BD PharMingen); mGluR5: 1:500 dilution of rabbit polyclonal antibody (Chemicon)] and AlexaFluor-conjugated secondary antibodies (anti-mouse AlexaFluor488 and anti-rabbit AlexaFluor594).

DHPG-mediated activation of the Pyk2/Src-family pathway and phosphorylation of NR2A/B occurs through activation of mGluR 1, not mGluR 5

Cultured cortical neurons express both mGluR1 and 5 when analyzed either by Western blots of neuronal membrane fractions (Strasser et al., 1998) or by immunocytochemistry (Fig. 3). However, the distribution pattern for each subunit in dissociated cultures is different: mGluR1α shows higher expression levels in the processes than in the soma, whereas the opposite is true for mGluR5 (Fig. 3). DHPG stimulates both mGluR1 and mGluR5 (EC_{50} 6 and 2 μM, respectively) (Schoepp et al., 1999). To identify which mGluR subtype was responsible for the DHPG-mediated activation of the Pyk2/Src pathway and tyrosine phosphorylation of

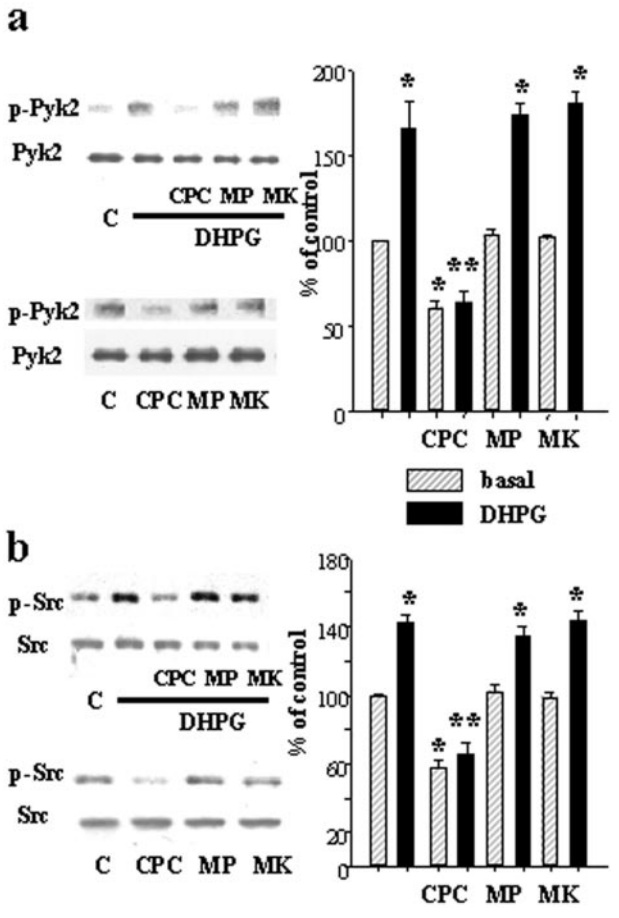

Figure 4. DHPG-mediated activation of the Pyk2/Src/Fyn pathway in cortical neurons occurs through activation of mGluR1. Near-pure cortical neuronal cultures were pretreated for 20 min in the absence (*C*) or presence of the mGluR1 antagonist CPCCOEt (*CPC*, 200 μM), the mGluR5 antagonist MPEP (*MP*, 1 μM), or the NMDA receptor antagonist MK801 (*MK*, 10 μM) and then exposed to vehicle (*basal*) or 100 μM DHPG for 5 min (in the absence or presence of the inhibitors). After treatment, cells were immediately processed for protein extraction. The phosphotyrosine content of Pyk2 (*a*) and the Src-family kinases (*b*) was determined by Western blot using phosphospecific antibodies against the active forms of the kinases (p-Pyk2: pPyk2$^{(pY402)}$, *top panels*; p-Src: pSrc$^{(pY418)}$, *bottom panels*). Fifteen micrograms of total cell extract were run per lane of 8% SDS-PAGE gels, and the total amount of each specific protein was determined in redetection blots using anti-Pyk2 and anti-Src antibodies (Pyk2 and Src in each blot). *Bar graphs* indicate cumulative results obtained from four to six independent experiments. * indicates statistical significance as compared with basal, and ** indicates statistical significance as compared with DHPG alone, at $p < 0.05$ by ANOVA.

NR2A/B, we took advantage of subtype-selective antagonists for mGluR1, 7-(hydroxyamino)cyclopropa[*b*]chromen-1a-carboxylateethylester (CPCCOEt; 200 μM) (IC_{50} 36 μM), and for mGluR5, 2-methyl-6-(phenylethynyl)-pyridine (MPEP; 1–2 μM) (IC_{50} 34 nM) (Annoura et al., 1996; Gasparini et al., 1999; Litschig et al., 1999). Activation of both Pyk2 and Src/Fyn by DHPG was blocked by a 20 min preincubation with CPCCOEt, but neither MPEP nor the NMDA antagonist MK-801 had any effect (Fig. 4*a*,*b*, *top left panels* and *bar graphs*). CPCCOEt also reduced the basal level of phosphorylation of Pyk2 and Src/Fyn (Fig. 4*a*,*b*, *bottom left panels* and *bar graphs*). Furthermore, DHPG enhancement of NR2A/B phosphorylation was also blocked by a 20 min preincubation with CPCCOEt but not MPEP (Fig. 5*a*,*b*), whereas no effects were observed with the NMDA receptor antagonist

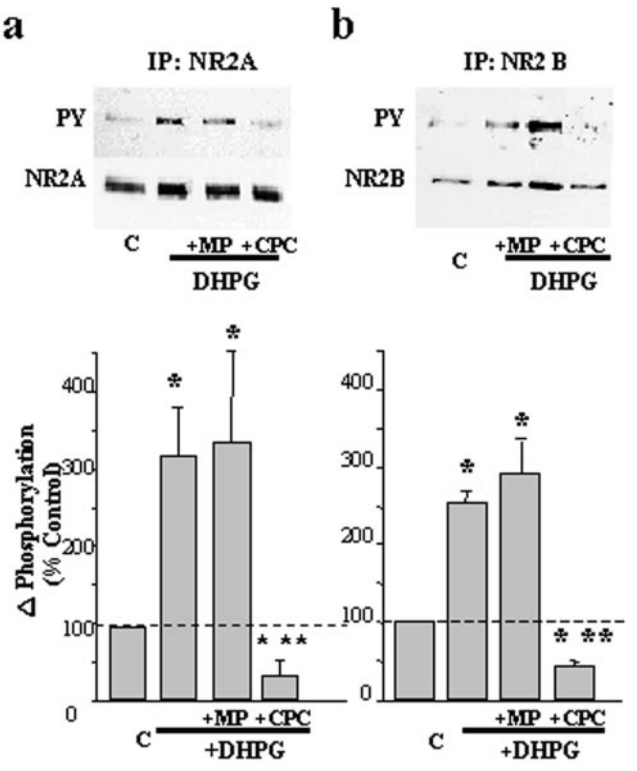

Figure 5. The tyrosine phosphorylation of NR2A/B subunits by DHPG was mediated by activation of mGluR1 in cortical neurons. Cultures were pretreated for 20 min in the absence or presence of the mGluR1 antagonist CPCCOEt (*CPC*, 200 μM) or the mGluR5 antagonist MPEP (1 μM) and then exposed to 100 μM DHPG for 5 min (in the absence or presence of the inhibitors). After protein extraction in 1% SDS, NR2A (*a*) or NR2B (*b*) subunits were immunoprecipitated with specific antibodies followed by redetection with anti-phosphotyrosine antibody (Wb α-PY). The immunoreactive bands were quantified by scanning densitometry (*bottom panels*) and expressed as in Figure 2. Values represent mean ± SEM obtained for three to six independent experiments. * indicates statistical significance as compared with basal, and ** indicates statistical significance as compared with DHPG alone at $p < 0.05$ by ANOVA.

MK801 (10 μM) or the PKC inhibitor GF 109203X (5 μM) (data not shown). As a control for MPEP effectiveness, concentrations as low as 0.1 μM MPEP reduced the ability of the selective mGluR5 agonist 2-chloro-5-hydroxyphenylglycine (Doherty et al., 1997) to raise intracellular calcium in cortical neurons, as determined by fura-2 AM videomicroscopy (data not shown).

Involvement of mGluR1 was confirmed by the use of other selective mGluR1 antagonists, (*R*,*S*)2-methyl-4-carboxyphenylglycine (200 μM), and *N*-phenyl-7-(hydroxyamino)cyclopropa[b]chromen-1a-carboxamide (100 μM) (Annoura et al., 1996) (data not shown). Lack of mGluR5 contribution was confirmed by the use of another selective mGluR5 antagonist, 2-methyl-6-(2-phenylethynyl)pyridine (5 μM) (Varney et al., 1999) (data not shown).

Activation of mGluR1 increased the tyrosine phosphorylation of NR2A in HEK293 cells

We next set out to determine whether mGluR1 activation would increase tyrosine phosphorylation of NR2A when reconstituted in a cell line system. HEK293 cells, while expressing Src, express low levels of Pyk2 (Della Rocca et al., 1997) and have low constitutive levels of tyrosine phosphorylation (Holmes et al., 1997), making them a favorable system in which to analyze signaling mechanisms leading to tyrosine phosphorylation. This

system was used previously in studies demonstrating an increase in NMDA receptor function by recombinant Src or Fyn kinases (Kohr and Seeburg, 1996; Zheng et al., 1998; Tezuka et al., 1999; Xiong et al., 1999). In these studies, the upregulation of recombinant NMDA receptor function was obtained either by introducing high levels of recombinant kinases in the patch pipette or by co-transfection with the active forms of the kinases.

We performed transient transfection experiments in HEK293 cells stably expressing the NR1 subunit of the NMDA receptor (clone ND-10). A small increase in the tyrosine phosphorylation content of NR2A was observed after ND-10 cells transiently transfected with NR2A and mGluR1α expression plasmids (1 μg each) were exposed to 100 μM DHPG for 15 min in the presence of the tyrosine phosphatase inhibitor orthovanadate (Fig. 6*a*).

To allow detection of NR2A phosphorylation in the absence of orthovanadate, we increased the levels of Pyk2 and Src by additional co-transfection with expression plasmids (0.3 μg, respectively). After transient co-transfection with NR2A/mGluR1α/Pyk2/Src plasmids, exposure to DHPG induced the activation of both Pyk2 and Src, as assessed by detection with phosphospecific antibodies (Fig. 6*b*, *left panels*), as well as increased phosphorylation of NR2A (Fig. 6*c*). The DHPG-mediated activation of Pyk2 and Src was observed only when cells were co-transfected with expression plasmids for both kinases, remaining at control levels when ND-10 cells were transfected with only one of the kinase expression plasmids (Fig. 6*b*, *middle* and *right panels*). To confirm the involvement of Pyk2 and Src activation in mediating the phosphorylation of NR2A on stimulation of mGluR1, similar transfection experiments were performed in which either the kinase-deficient form of Pyk2 (Pyk2KD, 0.3 μg) or the dominant-negative form of Src plasmids (pUSEamp-SrcDN, 0.3 μg) was substituted. Under these conditions, no DHPG-dependent increase in tyrosine phosphorylation of NR2A was obtained (Fig. 6*c*).

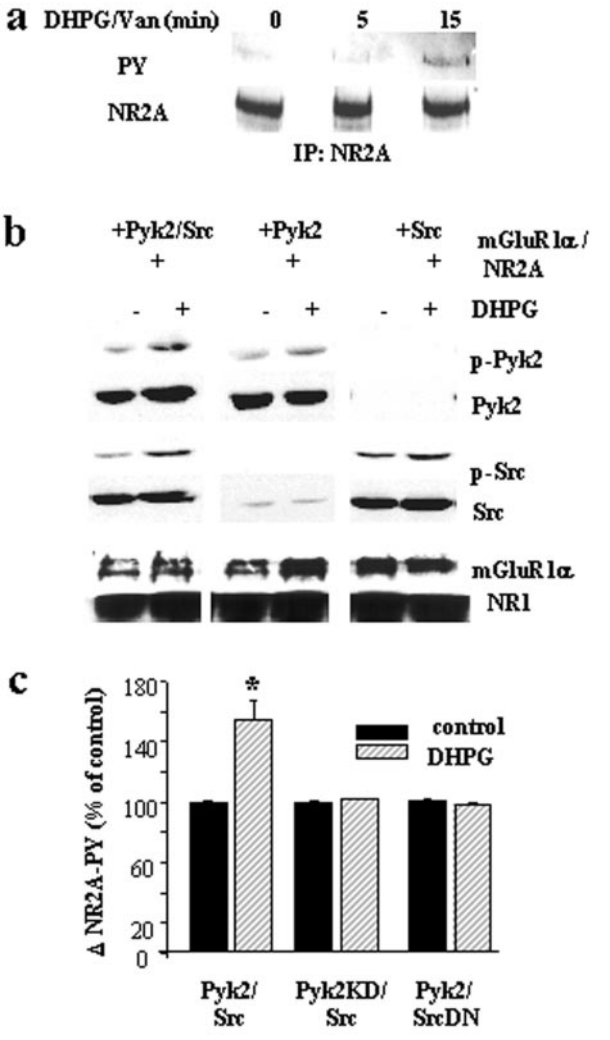

Figure 6. Activation of mGluR 1 induces the tyrosine phosphorylation of NR2A through activation of Pyk2 and Src kinases in HEK293 cells. HEK293 cells stably expressing NR1 (clone ND-10) were transiently transfected with either NR2A or mGluR1α and treated in the absence or presence of DHPG for the indicated times in the presence of orthovanadate (*a*) or transfected with NR2A/mGluR1α/Pyk2/Src and treated in the absence or presence of DHPG for 5 min (*b*, *c*). After treatment, cultures were immediately processed for protein extraction and Western blotting (*b*) or immunoprecipitation with specific NR2A antibodies, and phosphotyrosine levels were detected with anti-pTyr antibody (4G10) (*a*, *c*). *a*, Western blotting using anti-phosphotyrosine antibodies (αPY: 4G10, *top panel*) or NR2A-specific antibody (*bottom panel*). *b*, Extracts from ND-10 cells transfected with NR2A/mGluR1α/Pyk2/Src (+*Pyk2*/*Src*), NR2A/mGluR1α/Pyk2 (+*Pyk2*), or NR2A/mGluR1α/Src (+*Src*) were processed for Western blotting using either anti-Pyk2 antibodies (either phospho-Pyk2: p-Pyk2, or anti-Pyk2) or anti-Src (either phospho-Src: p-Src, or anti-Src). Expression levels of mGluR1α and NR1 are shown as mGluR1a and NR1. *c*, *Bar graph* depicting the percentage increase in tyrosine phosphorylation of NR2A after treatment in the absence or presence of DHPG in ND-10 cells transfected with NR2A/mGluR1α/Pyk2/Src (*Pyk2*/*Src*), with NR2A/mGluR1α/Src and the kinase deficient form of Pyk2 (*Pyk2KD*/*Src*), or with NR2A/mGluR1α/Pyk2 and the dominant negative form of Src (*Pyk2*/*SrcDN*). Results plotted in *c* are cumulative of three independent experiments. * indicates statistical significance as compared with control at $p < 0.05$ by ANOVA.

Activation of mGluR1 increased NMDA-mediated currents

To establish that the observed increase in NR2A/B phosphorylation corresponded to enhancement of NMDA receptor function in cortical neurons, we analyzed the effects of selective mGluR1 activation on NMDA-induced currents in the whole-cell and perforated-patch configuration.

When mGluR1 was selectively stimulated by application of DHPG in the presence of the mGluR5 antagonist MPEP, NMDA receptor responses were slowly increased, reaching statistical significance only after 15 min (Fig. 7). MPEP by itself had no effect on current when co-applied with NMDA (mean NMDA steady-state current before MPEP: 615 ± 170 pA; after 3 min MPEP: 593 ± 180 pA; $n = 3$ neurons). Selective activation of mGluR1 affected both peak and steady-state NMDA currents (Fig. 7*a*). When currents were analyzed in the whole-cell configuration, some, but not all, cells studied showed an increased NMDA current response after a 2 sec application of NMDA in the presence of 100 μM DHPG + 5 μM MPEP (data not shown). When currents were analyzed in the perforated–patch configuration, this variability was still present after 6 min but slowly developed into a statistically significant increase in NMDA currents (Fig. 7*b*). Fifteen minute preincubation with either the Src-kinase inhibitor PP2 (5 μM) or the mGluR1 antagonist CPCCOEt (200 μM) reduced the basal NMDA currents (mean NMDA steady-state current: 531.1 ± 244, $n = 10$; after 15 min preincubation with PP2: 196.4 ± 164, $n = 5$; after 15 min preincubation with CPCCOEt: 217 ± 38, $n = 4$) and completely prevented a DHPG-mediated increase in NMDA currents (Fig. 7). Furthermore, as occurred with the activation of the Pyk2/Src/Fyn pathway and tyrosine phosphorylation of NR2 A/B, prolonged exposure to either CPCCOEt or PP2 reduced NMDA-

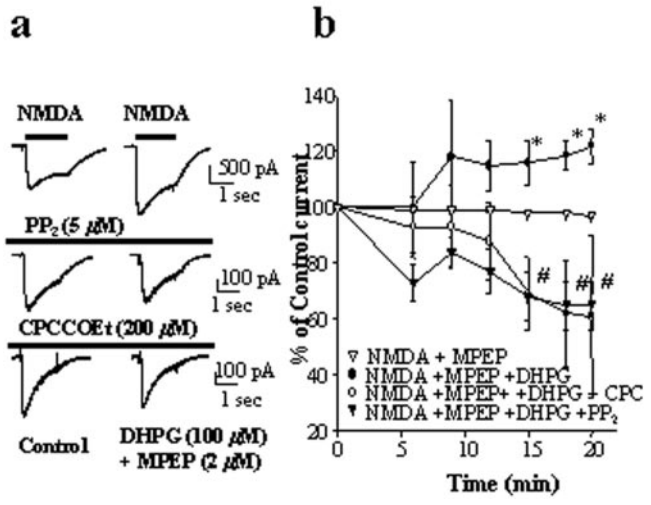

Figure 7. Selective activation of mGluR1 increased NMDA receptor current in cortical neurons. NMDA-induced currents in cortical neurons were recorded using the perforated-patch configuration. After obtaining stable recording of NMDA currents, DHPG (100 μM) plus the mGluR5 antagonist MPEP (2 μM) was applied, and NMDA currents were recorded for the next 20 min. *a*, Raw traces depicting the effect of selective activation of mGluR1 before and after 15 min exposure to DHPG/MPEP in the absence (*top traces*) or presence of the Src-family inhibitor PP2 (5 μM) (*middle traces*) or the mGluR1 antagonist CPCCOEt (200 μM) (*bottom traces*). *b*, Cumulative data obtained for the effects of selective activation of mGluR1 with DHPG/MPEP on NMDA steady-state currents in the absence or presence of the Src-family inhibitor PP2 (5 μM) or the mGluR1 antagonist CPCCOEt (200 μM). *Symbols* indicate the following: NMDA currents in the absence (▽) or presence of DHPG/MPEP (●), in the presence of DHPG/MPEP and 200 μM CPCCOEt (○), or in the presence of DHPG/MPEP and 5 μM PP2 (▼). Neurons were preincubated with the inhibitors for 15 min. * and # indicate statistical significance as compared with time 0 (before DHPG) at $p < 0.05$ by ANOVA; $n = 5–8$ neurons per condition.

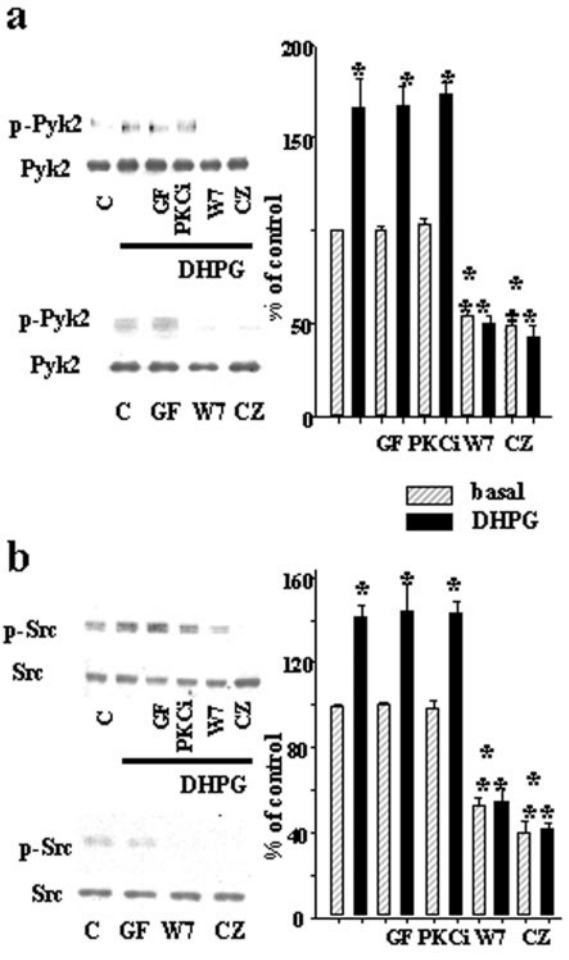

Figure 8. DHPG-mediated activation of the Pyk2/Src/Fyn pathway in cortical neurons was blocked by calmodulin antagonists but not PKC inhibitors. Cultures were preincubated for 20 min in the absence or presence of PKC inhibitors, GF109203X (*GF*, 5 μM) or the myristoylated inhibitor peptide (19–27) (*PKCi*, 25 μM); calmodulin antagonists, calmidazolium chloride (*CZ*, 30 μM) or W7 (100 μM), and then exposed to either vehicle (*basal*) or 100 μM DHPG for 5 min in the presence or absence of the inhibitors. Detection and analysis of p-Pyk2 (*a*) and p-Src/Fyn (*b*) was performed as in Figure 4. *Bar graphs* represent cumulative data of five independent experiments. * indicates statistical significance as compared with basal, and ** indicates statistical significance as compared with DHPG alone at $p < 0.05$ by ANOVA.

mediated currents below their initial level (Fig. 7*b*). CPCCOEt by itself had no direct effect when co-applied with NMDA (mean NMDA steady-state current before CPCCOEt: 240 ± 74 pA; after 3 min CPCCOEt: 280 ± 87 pA; $n = 5$ neurons).

Mechanism of activation of the Pyk2/Src pathway in cortical neurons

Activation of Pyk2 in neurons was shown to depend indirectly on increases in intracellular calcium, possibly through PKC activation (Lev et al., 1995; Lu et al., 1999). However, in non-neuronal systems, activation of Pyk2 can also occur through a Ca^{2+}–calmodulin-dependent pathway (Della Rocca et al., 1997). The lack of effects of the PKC inhibitor GF109203X on tyrosine phosphorylation of NR2A/B prompted us to study the possibility of calmodulin dependence in the activation of the Pyk2/Src/Fyn pathway by DHPG in cortical neurons. Although exposure of near-pure neuronal cultures to the PKC activator phorbol-12-myristate-13-acetate (PMA, 1 μM) induced a marked increase in Pyk2 phosphorylation that was inhibited by the specific PKC inhibitor GF109203X (Pyk2 phosphorylation after PMA was 250 ± 10% of control; after PMA + GF109203X it was 100 ± 13% of control), neither this inhibitor nor the myristoylated inhibitor peptide (19–27) had any effect on either the basal phosphorylation level or the DHPG-mediated phosphorylation of Pyk2 or Src/Fyn (Fig. 8*a*,*b*). On the other hand, pre-exposure to the calmodulin antagonists calmidazolium chloride (30 μM), W7 (100 μM), or ophiobolin (30 μM) (data not shown) prevented the effects of DHPG on Pyk2 and Src/Fyn phosphorylation (Fig. 8*a*,*b*). As occurred with the mGluR1 antagonist CPCCOEt (Fig. 4), calmodulin antagonists were able to decrease the basal phosphorylation levels of Pyk2 and Src/Fyn kinases (Fig. 8*a*,*b*, *bottom panels*).

DISCUSSION

The main findings of the present study are that (1) selective activation of group I mGluRs stimulated tyrosine phosphorylation of NR2A/B subunits in cortical neurons; (2) this stimulation was mediated specifically by mGluR1 and an intracellular cascade involving PLC, calmodulin, Pyk2, and the Src-family kinases (Src/Fyn); (3) the mGluR1-mediated tyrosine phosphorylation of NMDA receptors had functional consequences, increasing NMDA receptor-mediated current; and (4) the tonic activity of mGluR1 regulated the basal activity of Pyk2 and Src/Fyn and thus may control the basal tyrosine phosphorylation of NMDA receptors in cortical neurons.

The cross-linking of group I mGluRs and NMDA receptors through the synaptic protein Shank provides a structural basis for the functional coupling observed here. Shank proteins are associated with the NMDA receptor/PSD-95 complex and appear to be recruited to excitatory synapses by virtue of their interaction with GKAP (Naisbitt et al., 1999), a synaptic protein that binds to the guanylate kinase domain of PSD-95 (Kim et al., 1997; Naisbitt et al., 1997). In addition, Shank contains domains that interact with Homer, a neuronal protein that selectively binds to the C terminus of group I mGluRs and IP_3 receptors (Tu et al., 1999). The Homer–Shank interaction promotes clustering of group I mGluRs and may explain the perisynaptic localization of these metabotropic glutamate receptors (Baude et al., 1993; Lujan et al., 1997). Thus, Shank may be a molecular bridge linking the NMDA receptor complex with Homer and its associated proteins, the group I mGluRs and IP_3 receptors, permitting biochemical linkage between group I mGluRs and NMDA receptors (Sala et al., 2001). Consistent with this view, mGluR1 and mGluR5 were implicated in the regulation of the locomotor network output in lamprey spinal cord neurons through a postsynaptic interaction with NMDA receptors (Krieger et al., 2000).

The enhancement of NMDA receptor currents by G-protein-coupled muscarinic receptors has been linked to the sequential activation of PKC and Src and the consequent tyrosine phosphorylation of NR2 subunits (Lu et al., 1999). In hippocampal neurons, activation of mGluR5, not mGluR1, was recently shown to be responsible for the upregulation of NMDA currents (Mannaioni et al., 2001) by a mechanism probably mediated by PKC (Bruno et al., 2001), and Pyk2 was shown as a key tyrosine kinase in the induction of hippocampal LTP (Huang et al., 2001). PKC can activate neuronal Pyk2 (Lev et al., 1995), and phorbol ester-mediated activation of PKC can induce the tyrosine phosphorylation of NMDA receptors in hippocampal neurons (Grosshans and Browning, 2001). Taken together, these results suggest that in hippocampal neurons, activation of mGluR5, not mGluR1, may upregulate NMDA receptor currents through a PKC-mediated activation of the Pyk2/Src kinase pathway.

In cortical neurons, however, inhibition of PKC had no effect on either basal or DHPG-mediated activation of the Pyk2/Src/Fyn pathway, nor did it have an effect in NR2 phosphorylation (this study). Thus, we conclude that if there is a PKC-mediated regulation of the Pyk2/Src/Fyn pathway in mGluR1-induced phosphorylation of NMDA receptors in cortical neurons, such involvement is not via classical or novel family members (targets for the inhibitors used), but rather through atypical members insensitive to these inhibitors (Toullec et al., 1991; Martiny-Baron et al., 1993). Alternatively, other molecules besides PKC may be responsible for the mGluR1-mediated activation of Pyk2 observed in cortical neurons. The strong suppression of both basal and DHPG-mediated phosphorylation of Pyk2 and Src/Fyn observed with calmodulin inhibitors suggests that the mGluR1-induced activation of the Pyk2 Src/Fyn pathway in cortical neurons is mediated preferentially through a calcium–calmodulin-dependent mechanism. Both mGluR1 and mGluR5 can interact with calmodulin in a calcium-dependent manner (Minakami et al., 1997; Ishikawa et al., 1999), and recent study of Chinese hamster ovary cells stably expressing mGluR1α showed that stimulation of mGluR1 induces the activation of focal adhesion kinase through a calmodulin-dependent mechanism independent of PKC (Shinohara et al., 2001).

Upregulation of NMDA-receptor function by tyrosine phosphorylation is well established, although the exact mechanisms are not precisely known. Application of recombinant Src kinase increases whole-cell currents through NMDA receptors, whereas application of a purified protein tyrosine phosphatase decreases these currents (Yu and Salter, 1999). The increase in NMDA channel activity caused by tyrosine phosphorylation was suggested to reflect enhanced gating of existing receptors, rather than recruitment of new receptors (Salter, 1998), and Src-mediated phosphorylation of recombinant NR2A/B was proposed to relieve the voltage-independent Zn^{2+} inhibition of NMDA receptors (Zheng et al., 1998; Xiong et al., 1999; Vissel et al., 2001), although these results were not reproduced in native receptors (Xiong et al., 1999). However, sequence analysis shows that there are at least 25 tyrosine residues susceptible to phosphorylation in NR2A and NR2B, and their individual effects on NMDA receptor function are just beginning to be elucidated (Zheng et al., 1998; Cheung and Gurd, 2001; Nakazawa et al., 2001; Roche et al., 2001; Vissel et al., 2001). It is possible then that tyrosine phosphorylation of NMDA receptors may play a role other than direct regulation of NMDA currents. Recently, a different mechanism of regulation of NMDA receptor function by tyrosine phosphorylation was proposed. Tyrosine phosphorylation of NR2 subunits prevented the downregulation of recombinant NR1/2A receptor (Vissel et al., 2001) and prevented the calpain-mediated truncation of the C-terminal domains of NMDA receptors in synaptic membranes (Bi et al., 2000). Taken together, the above results suggest that tyrosine phosphorylation of NR2 subunits may not only induce the direct upregulation of NMDA receptor current but may also control receptor recycling, thus supporting a major role for the Pyk2/Src/Fyn pathway in the stability and function of postsynaptic NMDA receptors. Recent findings showing that activation of mGluR1α causes a rapid increase in the number of functional NMDA receptors when expressed in *Xenopus* oocytes (Lan et al., 2001), and present results showing that mGluR1 antagonists reduce the basal activity of the Pyk2/Src/Fyn pathway and bring the tyrosine phosphorylation levels and currents of NMDA receptors below their initial level, suggest the interesting possibility that mGluR1, by controlling the basal tyrosine phosphorylation levels of NR2 subunits, may also regulate receptor recycling as described by Vissel and collaborators (2001) and thus may increase the number of functional NMDA receptors at the synapse in cortical neurons.

Postsynaptic mGluR1 and mGluR5 may have different effects on NMDA receptor function. As noted above, a general pro-excitatory role of group I mGluRs is well accepted, although some opposite effects have been observed (for review, see Nicoletti et al., 1999). In particular, activation of postsynaptic group I mGluRs induced an immediate, membrane-delimited downregulation of NMDA-mediated currents in cortical neurons (S. P. Yu et al., 1997) that appears to be preferentially mediated by mGluR5 (S.-P. Yu, M. M. Behrens, and D. W. Choi, unpublished observations). However, this membrane-delimited effect of mGluR5 was not observed when currents were analyzed in the perforated-patch recording setting, suggesting the involvement of soluble factors in the mGluR1-mediated upregulation of NMDA currents. In the whole-cell recording setting, these soluble components are washed out, thus allowing the observation of the membrane-delimited modulation of NMDA currents by mGluR5. What is then the physiological consequence of this membrane-delimited modulation of NMDA receptors by mGluR5? One possibility is that although both mGluR1α and mGluR5 can localize to the perisynaptic region and thus to the vicinity of NMDA receptors, they do not do so in the same synapse, and the tyrosine phosphorylation effects of mGluR1 activation are ob-

served only in those synapses containing mGluR1α and NMDA receptors and not in those containing mGluR5. Indeed, the differential distribution of mGluR1α and mGluR5 observed in dissociated cortical neurons (Fig. 3) would give support to this hypothesis. An alternative possibility would be that mGluR1α and mGluR5 do colocalize to the same synapses, with the net effect on NMDA receptors varying as a function of factors such as time and activity in other relevant signaling pathways affecting Src-family kinases or NMDA receptor phosphorylation.

Recent data obtained in hippocampal CA1 pyramidal neurons also suggest differential effects for mGluR1 and mGluR5 (Mannaioni et al., 2001). Using an approach similar to the one used in the present study, those investigators concluded that mGluR1α mediated the DHPG-induced increases in intracellular calcium, whereas mGluR5 mediated the DHPG-induced suppression of the Ca^{2+}-activated potassium current (I_{AHP}) and potentiation of NMDA receptor current. The suggestion that mGluR1α and mGluR5 might regulate NMDA receptors differently in CA1 pyramidal neurons versus cortical neurons is intriguing and most likely explained by differential access to signaling cascades. Both mGluR1α and mGluR5 are able to interact with the anchoring protein Homer, which through the protein Shank produces the link to NMDA receptors (Sala et al., 2001). A plausible explanation then to the differential effects of group I mGluRs on NMDA receptors is that mGluR1α and mGluR5 have other partners besides Homer that allow the biochemical coupling of either mGluR1α or mGluR5 to the tyrosine phosphorylation of NMDA receptors. In mass spectroscopy analysis of the NMDA receptor complex, as many as 70 signaling proteins were associated with NMDA receptors at postsynaptic densities, including the components of the biochemical network described in this work, i.e., mGluR1, PyK2, Src, and Fyn (Husi et al., 2000; Walikonis et al., 2000). The presence or absence of specific signaling components at individual synapses might convey specificity to NMDA receptor regulation by mGluRs, as well as to the cascades of enzymatic reactions that carry the NMDA receptor-mediated signal into the interior of the cell (Kennedy, 2000). Localization might also convey regional specificity to signaling cascades; for example, mGluR1-induced activation of intracellular signaling pathways in cortical neurons appears primarily in dendrites (M. M. Behrens and T. Bartfai, unpublished observations).

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