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Two Distinct Signaling Pathways Upregulate NMDA Receptor Responses via Two Distinct Metabotropic Glutamate Receptor Subtypes

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Molecular processes regulating the gain of NMDA receptors modulate diverse physiological and pathological responses in the CNS. Group I metabotropic glutamate receptors (mGluRs), which neighbor NMDA receptors and which can be coactivated by synaptically released glutamate, couple to several different second messenger pathways, each of which could target NMDA receptors. In CA3 pyramidal cells we show that the activation of mGluR1 potentiates NMDA current via a G-protein-independent mechanism involving Src kinase activation. In contrast, mGluR5-mediated enhancement of NMDA current requires G-protein activation, triggering a signaling cascade including protein kinase C and Src. These results indicate that one neurotransmitter, glutamate, can activate two distinct and independent signaling systems to target the same effector. These two pathways are likely to contribute significantly to the highly differentiated control of NMDA receptor function.

Key words: mGluR1; mGluR5; Src tyrosine kinase; PKC; G-protein-independent signaling; potentiation; hippocampus

Glutamatergic signaling via NMDA receptors is essential for CNS function, controlling a wide range of responses from neuronal development to synaptic plasticity. Accordingly, sensitive mechanisms are in place to fine-tune NMDA responses, allowing for the adaptation of gain to ambient requirements. An immediate form of NMDA receptor modulation is mediated by postsynaptic metabotropic glutamate receptors (mGluRs), which frequently neighbor NMDA receptors (Baude et al., 1993; Lujan et al., 1996, 1997). Although interactions between mGluRs and NMDA receptors first were described a decade ago, past studies reached conflicting conclusions as to whether the stimulation of mGluRs potentiates NMDA receptor activity (Aniksztejn et al., 1991; Bleakman et al., 1992; Harvey and Collingridge, 1993) (for review, see Anwyl, 1999; Valenti et al., 2002) or inhibits NMDA responses (Yu et al., 1997; Wang et al., 1998; Zhong et al., 2000; Snyder et al., 2001). There is, however, general agreement that the postsynaptic mGluRs involved in NMDA response modulation belong to Group I, either mGluR1 (Lan et al., 2001; Skeberdis et al., 2001; Heidinger et al., 2002) or mGluR5 (Doherty et al., 1997, 2000; Jia et al., 1998; Awad et al., 2000; Mannaioni et al., 2001; Pisani et al., 2001).

A further unresolved issue is the mechanism underlying the modulation of NMDA receptors by mGluRs. Group I mGluRs are coupled positively via G-proteins to phospholipase C (PLC), leading to the formation of diacylglycerol (DAG) and protein kinase C (PKC) activation and to the production of inositol trisphosphate (IP_3), resulting in the release of Ca^{2+} from intracellular stores (Conn and Pin, 1997). In addition, mGluRs can signal via direct membrane-delimited pathways whereby G-protein subunits may modulate ion channels directly (Swartz and Bean, 1992; Trombley and Westbrook, 1992; McCool et al., 1996; Yu et al., 1997) and via G-protein-independent transduction, resulting in Src tyrosine kinase activation (Heuss et al., 1999). This diversity of second messenger systems, all of which may target NMDA receptors, is suggestive of a complex intracellular network capable of subtle regulation of NMDA receptor function. Several earlier studies presented evidence for a PKC-dependent pathway in the potentiation of NMDA responses by mGluRs (Aniksztejn et al., 1991; Kelso et al., 1992; Pisani et al., 1997; Ugolini et al., 1997; Skeberdis et al., 2001), but other investigators reported a PKC-independent process (Harvey and Collingridge, 1993; Kinney and Slater, 1993; Rahman and Neuman, 1996; Holohean et al., 1999). An established mechanism underlying NMDA receptor upregulation involves tyrosine phosphorylation of the receptor via Src kinase (Salter, 1998), and a signaling cascade involving PLC, PKC, and Src has been shown to target NMDA receptors (Lu et al., 1999; Huang et al., 2001). It is not known, however, whether the activation of mGluRs can initiate this pathway.

Here we systematically assessed the role of the major transduction pathways coupled to Group I mGluRs in regulating NMDA receptors. Experiments were performed in hippocampal CA3 pyramidal cells, which express both subtypes of Group I metabotropic receptors, permitting us to evaluate the effects of selective activation of either mGluR1 or mGluR5 in the same system.

MATERIALS AND METHODS

Slice culture preparation and electrophysiology. Hippocampal slice cultures were prepared from 6-d-old Wistar rats as described previously (Gähwiler et al., 1998) and maintained by using the roller-tube technique. After 2–3 weeks *in vitro* the slice cultures were transferred to a recording chamber with a volume of 1 ml on an upright microscope (Axioscope FS; Zeiss, Oberkochen, Germany). Slices were superfused continuously at a rate of 1–2 ml/min with saline containing (in mM) 137 NaCl, 2.7 KCl, 11.6 $NaHCO_3$, 0.36 NaH_2PO_4, 0.48 $MgCl_2$, 1.8 $CaCl_2$, and 5.6 D-glucose

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plus 0.5 μM tetrodotoxin (TTX) and 10 mg/l phenol red pH-adjusted to 7.4, with an osmolarity of ~310 mOsm and a bath temperature of 29°C. Whole-cell voltage-clamp recordings were obtained from CA3 pyramidal neurons held at −50 mV with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Recording pipettes (2–5 MΩ) were filled with (in mM) 130 K-gluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 10 EGTA, and 4 Mg-ATP (pH-adjusted to 7.3 with KOH; osmolarity ~300 mOsm). In the indicated experiments 10 mM EGTA was replaced with 10 mM BAPTA. Series resistance (6–13 MΩ) and input resistance were monitored regularly. Currents were filtered at 2 kHz and analyzed off-line (pClamp 7; Axon Instruments).

Induction of NMDA currents. NMDA currents were isolated pharmacologically by adding the AMPA/kainate antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX; 40 μM) and the $GABA_A$ receptor antagonist picrotoxin (100 μM). NMDA current amplitudes were measured from baseline holding current to peak. The maximum percentage of potentiation is the average value of at least two NMDA current traces obtained during the peak effect of the agonists and normalized with respect to the average NMDA current measured in the three to five traces immediately preceding the application of agonists (referred to as "baseline"). *I–V* curves of the NMDA response were determined with a ramp protocol from −70 to 0 mV (2 sec duration). The ramp protocol was run before and during NMDA pressure application, and the respective traces were subtracted to obtain the *I–V* curve of the NMDA response in control. Then the same procedure was repeated after the application of DHPG.

To determine whether potentiation of NMDA current by drug treatment was significant, we used the paired Student's *t* test on raw data. When the effects of two treatments were compared on different neurons, the unpaired Student's *t* test was used. For multiple comparisons the percentage of potentiation for each condition was compared by using the one-way ANOVA, followed by Tukey's test. A value of $p < 0.05$ (*) was considered statistically significant and also $p < 0.01$ (**). All numerical data are expressed as the means ± SEM.

Drugs. (*S*)-3,5-dihydroxyphenylglycine (DHPG), (1*S*,3*R*)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD), (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), 7-(hydroxyimino)cyclopropachromen-1a-carboxylateethyl ester (CPCCOEt), (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), 2-methyl-6-(phenylethynyl)pyridine (MPEP), and 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X) were purchased from Tocris Cookson (Bristol, UK). Guanosine 5′-*O*-(2-thiodiphosphate) trilithium salt (GDPβS), NMDA, picrotoxin, 4′,5,7-trihydroxyisoflavone (genistein), and 4′,7-dihydroxyisoflavone (daidzein) were purchased from Sigma (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA), 1-(6-[([17β]-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1*H*-pyrrole-2,5-dione (U-73122), and 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine 1 (PP1) were purchased from Alexis (San Diego, CA). NBQX was obtained from AG Scientific (San Diego, CA) and TTX from Latoxan (Valence, France). 3-((*R*)-2-carboxypiperazin-4yl)-propyl-1-phosphonic acid (CPP) and baclofen were kindly provided by Novartis (Basel, Switzerland). Stock solutions of DHPG, ACPD, GDPβS, NMDA, TTX, and CPP were prepared by dissolving in water (for DHPG and GDPβS freshly prepared every 14 and 3 d, respectively). Stock solutions of CPCCOEt, MPEP, GF109203X, picrotoxin, baclofen, genistein, daidzein, PMA, U-73122, and PP1 were prepared in DMSO. The final concentrations of DMSO used during experiments never exceeded 0.02%, which did not affect NMDA current potentiation ($n = 7$; see also experiments with daidzein). LY367385 stocks were dissolved in 1.1 equivalent NaOH but were diluted 1000× in experiments, resulting in no measurable change in pH.

RESULTS

Activation of group I mGluRs potentiates NMDA current

As observed previously in numerous brain regions, we found that in CA3 pyramidal cells NMDA receptor-mediated currents were enhanced by the concomitant activation of mGluRs (Fig. 1). NMDA currents were induced repetitively in voltage-clamped CA3 pyramidal cells (−50 mV) by applying brief pressure pulses (100–300 msec) to a micropipette filled with NMDA (100 μM) at 40 sec intervals. NMDA responses were blocked completely by the specific antagonist CPP (40 μM; $n = 10$; $p < 0.001$). Positioning the puffer pipette at a distance of ~100 μm from the recorded cell in the absence of a pressure pulse did not alter the holding current. The presence of TTX, NBQX, and picrotoxin in the bath solution prevented the contamination of NMDA currents with synaptic responses. Under these conditions, brief reversible NMDA currents exhibiting minimal variations in amplitude could be induced routinely for 1–2 hr. After a steady baseline of NMDA responses was recorded, the application of the broad-spectrum mGluR agonist ACPD (50 μM for 4 min) or the group I agonist DHPG (10 μM for 2 min) increased the peak amplitude of NMDA currents by 22 ± 4% ($n = 6$; $p < 0.05$ vs baseline) and 38 ± 5% ($n = 23$; $p < 0.001$ vs baseline), respectively. This effect peaked rapidly, within 2 min, but reversal after washout of metabotropic agonists was relatively slow (Fig. 1*C*). Apart from the potentiation of NMDA current, DHPG (10 μM for 2 min) or ACPD (50 μM for 4 min) induced an inward current associated with an increase in input resistance (15 ± 4%, $n = 19$, $p < 0.01$ for DHPG; 13 ± 4%, $n = 7$, $p < 0.05$ for ACPD), as characterized previously (Guérineau et al., 1994). Repeated application of mGluR agonists to the same preparation induced reproducible responses. The potentiation of NMDA current induced by a second application of DHPG (10 μM for 2 min) or ACPD (50 μM

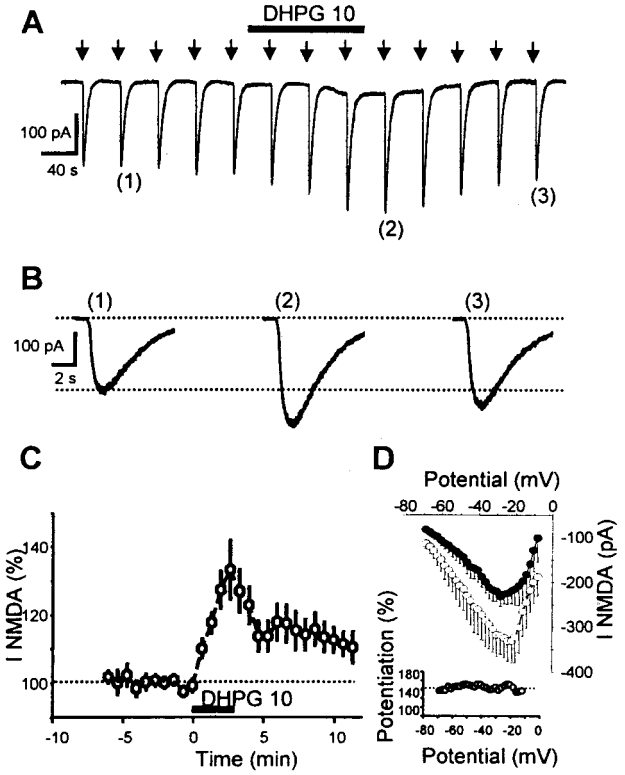

Figure 1. Activation of group I mGluRs potentiates currents mediated by NMDA receptors in CA3 pyramidal cells. *A*, NMDA currents induced by the pressure application of NMDA (100 μM for 200 msec) every 40 sec (*black arrows*) are potentiated by the bath application of the specific group I mGluR agonist DHPG (10 μM for 2 min). The effect is transient and reversible. *B*, Single NMDA current traces from the recording in *A* shown at an expanded time scale before (*1*) during (*2*), and after (*3*) the washout of DHPG. *C*, Average time course of the DHPG-induced potentiation ($n = 23$). *D*, Average current–voltage relationship of NMDA responses obtained with a ramp protocol before (*filled circles*) and after (*open circles*) DHPG application ($n = 5$; mean ± SEM of current is shown every 2 mV). *Inset* shows subtraction of control *I–V* plot from *I–V* plot after the application of DHPG.

for 4 min) corresponded to 93 ± 6% ($n = 4$; $p > 0.05$) and 92 ± 16% ($n = 5$; $p > 0.05$), respectively, of the previous potentiation. The extent of potentiation of the peak NMDA-evoked currents was independent of membrane potential over the range from −70 to −10 mV (Fig. 1*D*).

Potentiation of NMDA current by mGluRs involves tyrosine kinase activation

The application of Src to CA3 pyramidal cells increases NMDA currents (Xiong et al., 1999). Moreover, the activation of mGluRs can lead to Src activation (Fiore et al., 1993; Siciliano et al., 1994; Heuss et al., 1999; Boxall, 2000; Peavy et al., 2001). We therefore tested whether the mGluR-dependent potentiation of NMDA current requires tyrosine kinase activation. We found that the DHPG-induced potentiation of NMDA current (41 ± 6%, $n = 5$; $p < 0.05$ vs baseline) was reduced substantially after the application of the broad-spectrum tyrosine kinase antagonist genistein (30 μM for 15 min; 8 ± 8%; $p > 0.05$ vs baseline and $p < 0.01$ vs DHPG alone) (Fig. 2*A2*). The potentiation of NMDA current was insensitive to the inactive genistein analog daidzein (30 μM for 15 min; 37 ± 14%, $n = 5$; $p < 0.05$ vs baseline) (Fig. 2*A3*). The application of PP1 (25 μM for 15 min), a specific inhibitor of Src kinase, also reduced the DHPG-induced potentiation of NMDA current (58 ± 12% before PP1 treatment, $p < 0.05$ vs baseline compared with 11 ± 6% after PP1 treatment; $n = 5$; $p > 0.05$ vs baseline and $p < 0.01$ vs DHPG alone) (Fig. 2*B*). No effect on NMDA currents was observed when genistein or PP1 was applied alone, suggesting weak background phosphorylation of NMDA receptors in CA3 cells in our preparation.

mGluR1 and mGluR5 mediate NMDA current potentiation

In CA1 pyramidal cells and subthalamic neurons the enhancement of NMDA current by mGluRs is mediated by mGluR5, whereas mGluR1 activation is without effect (Awad et al., 2000; Mannaioni et al., 2001). We found a similar mGluR5-mediated potentiation of NMDA current in CA3 pyramidal cells. mGluR5 was stimulated either by applying the specific agonist CHPG (500 μM for 2 min; $n = 8$) (Fig. 3*A*) or by applying the group I agonist DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR1 antagonist CPCCOEt (50 μM for 10 min; $n = 6$) (Fig. 3*B*). Either approach resulted in a significant potentiation in the amplitude of NMDA current (20 ± 5%, $p < 0.05$ for CHPG vs baseline; 19 ± 5%, $p < 0.05$ for DHPG plus CPCCOEt vs baseline). However, selective activation of mGluR5 resulted in significantly less potentiation than that observed with the coactivation of mGluR1 and mGluR5 with DHPG (DHPG plus CPCCOEt vs DHPG alone; $n = 5$; $p < 0.05$) (Fig. 3*D*). We therefore tested whether the selective activation of mGluR1 also increases NMDA currents. When DHPG (10 μM for 2 min) was applied to cells in the presence of a saturating concentration of the mGluR5 antagonist MPEP (10 μM for 10 min; $n = 5$) (Fig. 3*C*), NMDA current was potentiated significantly (18 ± 4%; $p < 0.05$ vs baseline). Again, selective activation of mGluR1 induced less potentiation than the coactivation of mGluR1 and mGluR5 (DHPG plus MPEP vs DHPG; $n = 5$; $p < 0.01$). Thus both mGluR1 and mGluR5 mediate NMDA current potentiation in CA3 pyramidal cells (Fig. 3*D*). No significant potentiation was detected in the presence of both the mGluR1 and the mGluR5 antagonist (DHPG plus MPEP plus CPCCOEt, 9 ± 4%; $n = 5$; $p > 0.05$) (Fig. 3*D*).

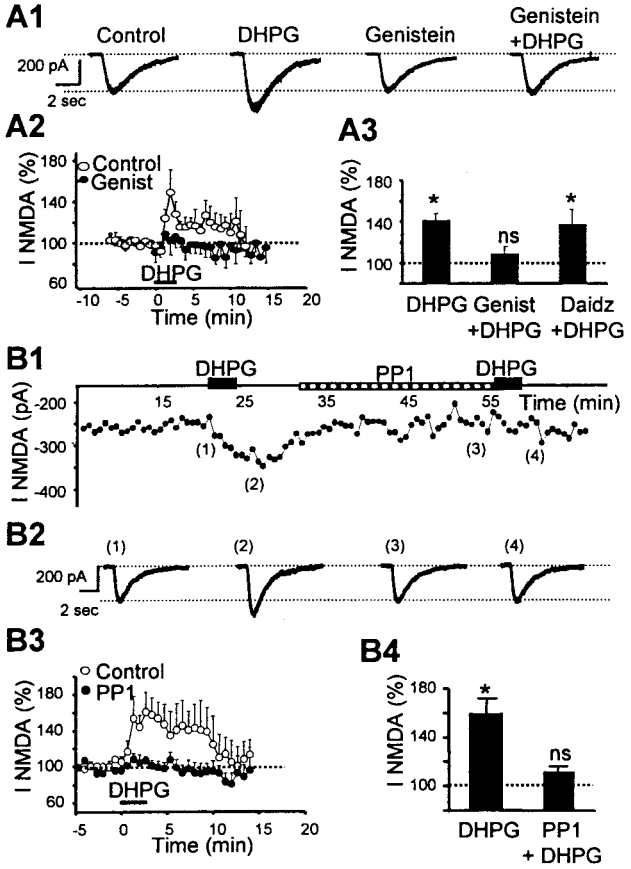

Figure 2. Src is required for mGluR-mediated potentiation of NMDA current. *A*, Genistein, a broad-spectrum blocker of tyrosine kinase, inhibits DHPG-induced potentiation of NMDA current. *A1*, Single traces from the same neuron show that the increase in NMDA current amplitude induced by DHPG (10 μM) is prevented in the presence of genistein (30 μM for 15 min). Genistein alone does not alter NMDA current. *A2*, Averaged results from five cells comparing the effect on NMDA current of 10 μM DHPG alone (*open circles*) and in the presence of genistein (*filled circles*). *A3*, Potentiation of NMDA current by 10 μM DHPG is inhibited by genistein (30 μM; $n = 5$) but not by its inactive analog daidzein (30 μM; $n = 5$); $^*p < 0.05$. *B*, PP1, a specific inhibitor of Src kinase, inhibits the DHPG-induced potentiation of NMDA current. *B1*, Time course of action on peak NMDA current of DHPG (10 μM for 2 min) before and after a 15 min application of 25 μM PP1 in a representative cell. PP1 alone does not alter NMDA current. *B2*, Single NMDA current traces from this cell. *B3*, Averaged results from five cells comparing the effect on NMDA current of DHPG before (*open circles*) and after (*filled circles*) PP1 incubation. *B4*, Pooled data comparing responses to DHPG alone and DHPG in the presence of PP1 ($n = 5$); $^*p < 0.05$. *Dotted lines* indicate baseline or control responses.

G-protein blockade does not prevent mGluR-mediated potentiation of NMDA current

Stimulation of mGluRs can activate Src via a G-protein-independent signaling pathway in CA3 pyramidal cells (Heuss et al., 1999). To determine whether this mechanism contributes to the potentiation of NMDA currents, we examined the response to mGluR activation in cells in which G-protein activity was blocked by intracellular perfusion of GDPβS (1 mM). To establish effective inhibition of G-protein function by GDPβS, we waited until the postsynaptic response to the bath-applied $GABA_B$ agonist baclofen (20 μM for 1 min) was blocked fully in each cell before testing the effects of mGluR activation (124 ± 30 pA just after beginning whole-cell recording vs 5 ± 5 pA after 20–40 min of

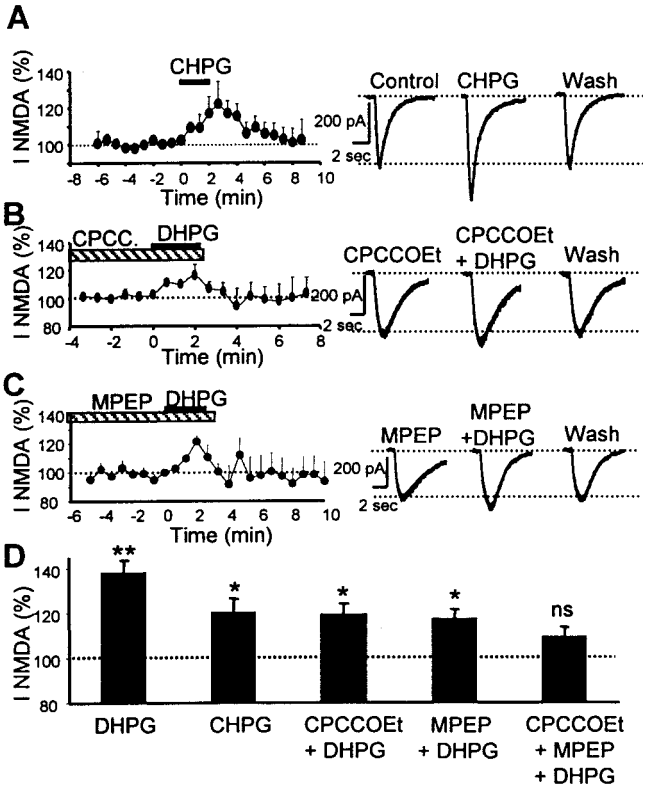

Figure 3. Activation of either mGluR5 or mGluR1 potentiates NMDA current. *A*, Average time course of NMDA current potentiation induced by the mGluR5-specific agonist CHPG (500 μM for 2 min; $n = 8$). Representative traces from one cell are shown (*right*). *B*, Alternatively, mGluR5 was stimulated selectively by the application of DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR1 antagonist CPCCOEt (50 μM for 10 min; $n = 6$). Average time course (*left*) and representative traces (*right*) are shown. *C*, mGluR1 was stimulated selectively by the application of DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR5 antagonist MPEP (10 μM for 10 min). Average time course (*left*) and representative traces (*right*) are shown. *D*, Pooled data indicate that, although the selective activation of either mGluR5 (CHPG, $n = 8$; CPCCOEt plus DHPG, $n = 4$) or mGluR1 (MPEP plus DHPG, $n = 5$) significantly potentiates NMDA current, greater potentiation is observed with the coactivation of mGluR1 and mGluR5 (DHPG, $n = 23$). No significant potentiation is detected in the presence of both mGluR1 and mGluR5 antagonists (CPCCOEt plus MPEP plus DHPG, $n = 5$); $^*p < 0.05$ and $^{**}p < 0.01$.

GDPβS diffusion; $n = 6$; $p < 0.001$) (Fig. 4*A*). In addition, GDPβS prevented the increase in input resistance induced by DHPG (1 ± 2%, $n = 6$ vs 15 ± 4% in control, $n = 19$; $p < 0.05$; data not shown), further indicating that the class of G-protein associated with group I mGluRs was blocked effectively. Under conditions of G-protein blockade, DHPG (10 μM for 2 min) still potentiated NMDA current (29 ± 5%, $n = 6$; $p < 0.01$ vs baseline) (Fig. 4*B*). Moreover, this G-protein-independent increase in NMDA current was blocked by the Src inhibitor PP1 (25 μM for 20 min; −1 ± 4%, $n = 5$; $p > 0.05$ vs baseline) (Fig. 4*F*).

mGluR1, but not mGluR5, can potentiate NMDA current via a G-protein-independent mechanism

To assess whether both group I mGluRs can signal via G-protein-independent mechanisms, we repeated the above experiments but selectively stimulated either mGluR1 or mGluR5. In GDPβS-treated cells in which baclofen no longer induced a response, the application of the mGluR5-specific agonist CHPG (500 μM for 2

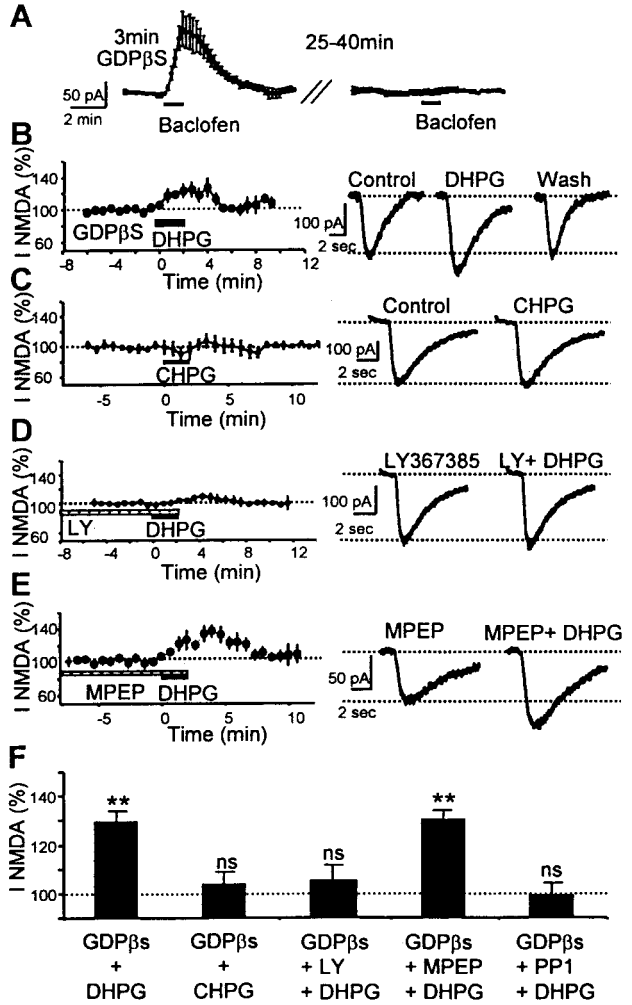

Figure 4. G-protein-independent potentiation of NMDA current is mediated by mGluR1 but not by mGluR5. *A*, Within 3 min of establishing the whole-cell configuration with a patch pipette containing 1 mM GDPβS, the application of baclofen (20 μM for 1 min) induces an outward K^+ current. Having allowed 20–40 min for GDPβS to diffuse into the cell, reapplication of baclofen no longer produces a response, indicating a blockade of the G-protein function ($n = 6$). *B*, After the baclofen response is blocked completely, DHPG (10 μM for 2 min) still potentiates NMDA current ($n = 6$). Representative traces from one cell are shown on the *right*. *C*, Average time course of NMDA current in six GDPβS-treated cells exposed to the mGluR5-specific agonist CHPG (500 μM for 2 min), indicating a lack of potentiation. Representative traces from one cell are shown on the *right*. *D*, Average time course of NMDA current in five GDPβS-treated cells in which mGluR5 activation is obtained by applying DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR1 antagonist LY367385 (50 μM for 10 min), again showing that NMDA current is not potentiated. Representative traces from one cell are shown on the *right*. *E*, Average time course of NMDA current in five GDPβS-treated cells in which mGluR1 is activated selectively by applying DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR5 antagonist MPEP (10 μM for 10 min), showing marked potentiation. Representative traces from one cell are shown on the *right*. *F*, Pooled data for GDPβS-treated cells showing that the activation of mGluR1 plus mGluR5 with DHPG ($n = 6$) significantly potentiates NMDA current. Selective activation of mGluR5 with CHPG ($n = 6$) or LY367385 plus DHPG ($n = 5$) does not potentiate NMDA current, whereas the selective activation of mGluR1 (MPEP plus DHPG, $n = 5$) potentiates NMDA current to a similar degree, as does the coactivation of mGluR1 plus mGluR5 with DHPG. DHPG-induced potentiation is blocked completely by the Src inhibitor PP1 (25 μM for 20 min; $n = 5$); $^{**}p < 0.01$.

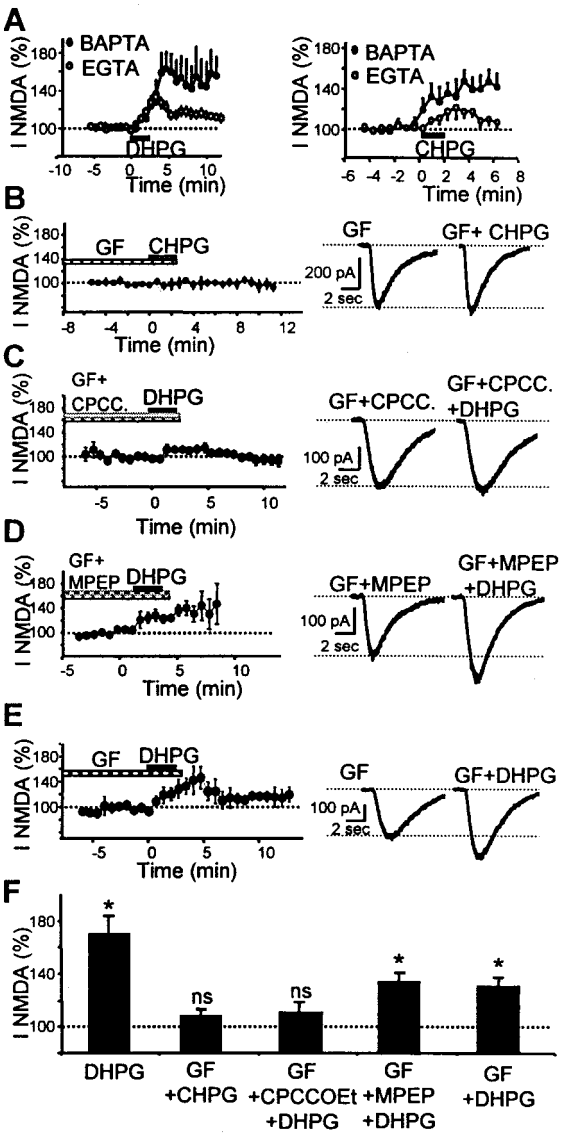

Figure 5. Blocking PKC activation prevents NMDA current potentiation by mGluR5 but not by mGluR1. For all experiments with the PKC inhibitor, GDPβS was not present and BAPTA (10 mM) was used in the pipette solution to minimize the Ca^{2+}-dependent inhibition of NMDA current. *A*, *Left*, Average time course of NMDA current potentiation induced by DHPG (10 μM for 2 min) either with EGTA (*open circles*; 10 mM; $n = 23$) or with BAPTA (*filled circles*; 10 mM; $n = 6$) in the recording pipette. *A*, *Right*, Data using the same protocol but with the mGluR5-specific agonist CHPG (500 μM for 2 min) either with EGTA (10 mM; $n = 8$) or with BAPTA (10 mM; $n = 7$) in the recording pipette. *B*, Inhibition of PKC with the specific inhibitor GF109203X (2 μM for 20 min) prevents NMDA current potentiation in response to the mGluR5-specific agonist CHPG (500 μM for 2 min; $n = 6$). Representative traces from one cell are shown on the *right*. *C*, Similarly, inhibition of PKC prevents NMDA current potentiation in response to mGluR5 activation by the application of DHPG (10 μM for 2 min; $n = 4$) in the presence of a saturating concentration of the mGluR1 antagonist CPCCOEt (50 μM for 10 min). Representative traces from one cell are shown on the *right*. *D*, In contrast, the selective activation of mGluR1 by the application of DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR5 antagonist MPEP (10 μM for 10 min) still potentiates NMDA current under conditions in which PKC is blocked ($n = 5$). Representative traces from one cell are shown on the *right*. *E*, Average time course of NMDA current potentiation induced by DHPG (10 μM for 2 min; $n = 5$) in the presence of the PKC inhibitor. Representative traces from one cell are shown on the *right*. *F*, Pooled data showing that, after the inhibition of PKC, the selective activation of mGluR5 (CHPG, $n = 6$; or LY367385 min) failed to potentiate NMDA current ($3 \pm 5\%$, $n = 6$; $p > 0.05$ vs baseline) (Fig. 4*C*). The same lack of effect was observed when mGluR5 was stimulated by applying DHPG (10 μM for 2 min) in the presence of a saturating concentration of the mGluR1 antagonist LY367385 (50 μM for 10 min) ($5 \pm 6\%$, $n = 5$; $p > 0.05$ vs baseline) (Fig. 4*D*). Blocking G-protein activity did not, however, prevent the potentiation of NMDA current by selective activation of mGluR1 (10 μM DHPG for 2 min in the presence of a saturating concentration of the mGluR5 antagonist 10 μM MPEP for 10 min; $29 \pm 3\%$, $n = 5$; $p < 0.01$ vs baseline and $p > 0.05$ vs GDPβS plus DHPG alone) (Fig. 4*E*). Thus the potentiation of NMDA current by mGluR5, but not by mGluR1, absolutely requires G-protein activation. The potentiation of NMDA current induced by selective mGluR1 activation after G-protein blockade was significantly greater than that induced by mGluR1 activation in control [$18 \pm 4\%$, $n = 5$ in presence of GTP (Fig. 3) compared with $29 \pm 3\%$, $n = 5$ in presence of GDPβS; $p < 0.05$ (Fig. 4)], suggesting that a G-protein-dependent process also may antagonize mGluR-mediated potentiation of NMDA receptor function (see Discussion).

Role of the PLC → DAG → PKC pathway in mGluR-mediated potentiation of NMDA current

Having established that mGluR1 activation can potentiate NMDA current via a G-protein-independent pathway, we addressed the mechanism underlying G-protein-dependent potentiation via mGluR5. Previous work in CA1 pyramidal cells has demonstrated that the G-protein-dependent activation of a pathway involving the sequential activation of PLC → DAG → PKC → Pyk2/CAKβ → Src induces tyrosine phosphorylation of NMDA receptors, resulting in their functional potentiation (Lu et al., 1999; Huang et al., 2001). To obtain evidence for the activation of a similar pathway in CA3 cells, we examined the effects of specifically blocking PKC, but without blocking G-proteins. To facilitate the interpretation of our results involving the PLC → DAG → PKC pathway, we tried to minimize the contribution from the PLC → IP_3 → Ca^{2+} pathway by performing all of the subsequent experiments with BAPTA (10 mM) in the recording pipette solution (Adler et al., 1991). Intracellular BAPTA resulted in significantly greater potentiation of NMDA current in response to DHPG (10 μM for 2 min) than with EGTA as the intracellular Ca^{2+} buffer ($73 \pm 13\%$, $n = 6$; $p < 0.05$ vs EGTA solution) (Fig. 5*A*). Furthermore, the greater potentiation with intracellular BAPTA was more apparent with the activation of mGluR1 than mGluR5 [10 μM MPEP plus 10 μM DHPG potentiated by $41 \pm 13\%$, $n = 5$ with BAPTA vs $18 \pm 4\%$, $n = 5$ with EGTA; $p < 0.05$; (data not shown); CHPG potentiated by $39 \pm 10\%$, $n = 7$ with BAPTA vs $20 \pm 5\%$, $n = 8$ with EGTA; $p > 0.05$ (Fig. 5*A*)].

The addition of the PKC inhibitor GF109203X (2 μM for 20 min) to the bath prevented NMDA current potentiation in response to selective mGluR5 activation ($7 \pm 6\%$, $n = 6$; $p > 0.05$ vs baseline for CHPG 500 μM; $10 \pm 8\%$, $n = 4$; $p > 0.05$ vs baseline for 10 μM DHPG plus 50 μM CPCCOEt) (Fig. 5*B,C*). In contrast, potentiation of NMDA current induced by the selective activation of mGluR1 (10 μM DHPG for 2 min plus 10 μM MPEP

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plus DHPG, $n = 4$) does not potentiate NMDA current, whereas the selective activation of mGluR1 (MPEP plus DHPG, $n = 5$) induces a potentiation comparable with that seen with the coactivation of mGluR1 plus mGluR5. Note that NMDA current potentiation induced by the coactivation of mGluR1 plus mGluR5 after PKC inhibition is reduced but still significant ($n = 5$); $^*p < 0.05$.

for 10 min) was not blocked after PKC inhibition with GF109203X (33 ± 6%, $n = 5$; $p < 0.05$ vs baseline) (Fig. 5*D*). We observed that, after the selective activation of mGluR1 (DHPG plus MPEP) in presence of BAPTA, the maximal degree of potentiation with or without GF109203X was not significantly different ($p > 0.05$).

Similarly, the potentiation of NMDA current with the activation of mGluR1 plus mGluR5 with DHPG (10 μM for 2 min) persisted in the presence of GF109203X (10 μM for 2 min; 30 ± 7%, $n = 5$; $p < 0.05$ vs baseline) (Fig. 5*E*). Control experiments showed that GF109203X completely blocked the potentiation of NMDA currents induced by the bath application of PMA (250 nM for 10 min), a PKC activator (28 ± 3%, $n = 5$, $p < 0.05$ vs baseline for PMA alone; 5 ± 3%, $n = 4$, $p > 0.05$ vs baseline for PMA plus GF109203X; data not shown).

DISCUSSION

Our results show that in CA3 pyramidal cells the activation of either postsynaptic mGluR1 or mGluR5 leads to potentiation of NMDA receptor current via a Src-dependent mechanism. The mGluR5-dependent potentiation of NMDA current is mediated exclusively via G-protein- and PKC-dependent activation of Src, whereas mGluR1-dependent potentiation can occur via G-protein- and PKC-independent activation of Src. The upregulation of NMDA responses by Src is well established, involving the phosphorylation of receptor tyrosine residues, which increases channel open probability (Salter, 1998; Ali and Salter, 2001). This mode of NMDA receptor enhancement can be triggered by muscarinic or lysophosphatidic acid receptors (G-protein-coupled receptors) or by receptors for leptin, EphB, or BDNF (Levine et al., 1998; Lu et al., 1999; Shanley et al., 2001; Takasu et al., 2002). Because mGluRs also have been shown to activate Src (Fiore et al., 1993; Siciliano et al., 1994; Heuss et al., 1999; Boxall, 2000; Peavy et al., 2001), the potentiation of NMDA responses via this mechanism was not unexpected.

We have found that either mGluR1 or mGluR5 can mediate NMDA current enhancement in CA3 pyramidal cells. In contrast, studies in various other brain areas have identified mGluR5 as uniquely responsible for NMDA receptor potentiation (Doherty et al., 1997, 2000; Jia et al., 1998; Awad et al., 2000; Mannaioni et al., 2001; Pisani et al., 2001). We interpret these findings as a reflection of low mGluR1 expression, the presence of alternative splice variants of mGluRs, or of a low incidence of colocalization of mGluR1 and NMDA receptors in these other cell types. In CA3 pyramidal cells immunohistochemical studies have revealed postsynaptic localization of both mGluR1 and mGluR5 (Shigemoto et al., 1997), and in *Xenopus* oocytes coexpressing mGluR1 and NMDA receptors metabotropic agonists do potentiate NMDA responses (Lan et al., 2001; Skeberdis et al., 2001). A recent study now has revealed mGluR1-mediated potentiation of NMDA current in cortical neurons (Heidinger et al., 2002).

Our results suggest that synaptic NMDA currents are potentiated by activation of group I mGluRs. As shown previously, however, experiments to test this hypothesis are confounded by the strong presynaptic depression of neurotransmitter release that follows the activation of group I mGluRs (Gereau and Conn, 1995; Manzoni and Bockaert, 1995; Rodriguez-Moreno et al., 1998; Fitzjohn et al., 2001; Mannaioni et al., 2001; Watabe et al., 2002).

Signal transduction pathways

Earlier investigations into the transduction mechanisms underlying mGluR-mediated upregulation of NMDA receptor function have focused primarily on the possible involvement of the PLC/PKC pathway. Approximately one-half of these studies concluded that PKC activation is required for potentiation (Aniksztejn et al., 1991; Kelso et al., 1992; Pisani et al., 1997; Ugolini et al., 1997; Skeberdis et al., 2001), whereas others found no indication for PKC involvement (Harvey and Collingridge, 1993; Kinney and Slater, 1993; Rahman and Neuman, 1996; Holohean et al., 1999). Our data provide a reasonable resolution for this discrepancy by showing that both a PLC/PKC-dependent pathway as well as a G-protein- and PKC-independent pathway can lead to Src-mediated potentiation of NMDA current. Our results are consistent with those from a recent study suggesting that mGluR5 signals via PKC to enhance NMDA-mediated responses (Jia et al., 1998). A detailed characterization of this transduction pathway in CA1 pyramidal cells has provided the following activation sequence: G-protein → PLC → DAG → PKC → Pyk2/CAKβ → Src → NMDA receptors (Lu et al., 1999; Huang et al., 2001).

Signal transduction via mGluR1 appears to be more complex. We have shown previously in CA3 pyramidal cells that the same population of synaptic mGluR1s, activated by the stimulation of mossy fibers, signals via divergent pathways, one G-protein-dependent and the other G-protein-independent (Heuss et al., 1999). Although both pathways could be involved in the potentiation of NMDA current by mGluR1, our data show that the G-protein-independent mechanism predominates. Thus both G-protein inhibition as well as PKC blockade (see also Heidinger et al., 2002) did not prevent mGluR1-mediated potentiation.

The mechanism underlying the G-protein-independent activation of Src by mGluR1 is not known. In other systems G-protein-independent signaling by metabotropic receptors can initiate the binding of the adapter protein arrestin to the activated receptor, resulting in the recruitment of Src (Hall et al., 1999). Arrestin has been shown to bind to mGluR1a (Dale et al., 2001; Mundell et al., 2001), but whether this association leads to Src activation remains to be determined.

Modulation by Ca^{2+}

An unexpected observation in our study was that selective activation of mGluR1 resulted in significantly greater potentiation of NMDA current after G-protein blockade than under control conditions. Thus the activation of mGluRs appears to induce a concomitant G-protein-dependent inhibition of NMDA current. Both mGluR1 and mGluR5 mediate a G-protein-dependent release of intracellular calcium stores (Valenti et al., 2002). Moreover, NMDA receptor function is inhibited by a rise in intracellular calcium (Mayer and Westbrook, 1985; Legendre et al., 1993; Lieberman and Mody, 1994; Rosenmund et al., 1995). Hence it is likely that the G-protein-dependent release of intracellular calcium via mGluR activation will depress NMDA responses. Our experimental protocol leads to an increase in intracellular Ca^{2+} concentration via two mechanisms: (1) Ca^{2+} influx through the repetitively activated NMDA receptor channels and (2) Ca^{2+} release from IP_3-sensitive stores in response to G-protein-dependent activation of PLC via mGluR stimulation. Further evidence for our hypothesis is provided by the observation that increasing the DHPG concentration to 50 μM (3 min) in the presence of 3 mM extracellular calcium with low intracellular calcium buffering suppresses the potentiation (data not shown), whereas accelerating intracellular Ca^{2+} buffering by including

BAPTA in the recording pipette substantially enhances the potentiation of NMDA current and delays recovery of mGluR-induced potentiation (Fig. 5*A*). Small differences in experimental conditions affecting Ca^{2+} homeostasis therefore may account for the conflicting observations with respect to mGluR-mediated effects on NMDA receptor function. Indeed, studies in which relatively high levels of intracellular Ca^{2+} would be expected, either because of low Ca^{2+} buffering or because of increased IP_3 production induced by high mGluR agonist concentrations, report mGluR-mediated reduction of NMDA current (Wang et al., 1998; Zhong et al., 2000; Snyder et al., 2001). Similarly, in our preparation high concentrations of DHPG (100 μM; our unpublished data) depressed NMDA current. However, an increase in intracellular Ca^{2+} also will enhance PKC activity (Nishizuka, 1988), which would potentiate NMDA responses via the Src pathway. The net result of Ca^{2+} on NMDA responses is thus difficult to predict and is likely to depend on the extent and compartmental localization of the intracellular Ca^{2+} rise. In addition to a Ca^{2+}-dependent reduction in NMDA current, it should be pointed out that direct membrane-delimited inhibition via G-proteins also may lead to NMDA current inhibition (Yu et al., 1997).

Conclusion

Why should a neuron possess two parallel pathways to modulate NMDA receptor function, one G-protein-dependent and one G-protein-independent? G-protein function is controlled through a complex interplay of regulatory molecules, permitting both enhanced transduction as well as functional uncoupling of G-proteins from cognate receptors (Alagarsamy et al., 2001). A receptor system, which includes the added option of G-protein-independent signaling, will provide a cell with greater flexibility in responding to stimuli under a wide range of physiological and pathophysiological conditions. Not only could certain responses be maintained when G-protein-dependent pathways have been shut off, but more focused activation of specific proteins would be ensured. For example, in the case of mGluRs selective G-protein-independent signaling would allow for the activation of Src without the concomitant triggering of transduction cascades associated with PLC, PKC, or IP_3.

Our finding that the coactivation of mGluR1 and mGluR5 induced larger responses than the activation of either subtype alone suggests that the two receptor subtypes target different populations of NMDA receptors (Salter, 2001). Thus mGluR1 receptors, which in CA3 cells can be activated synaptically (Heuss et al., 1999), may modulate synaptic NMDA receptors, whereas mGluR5 receptors in these cells might be activated primarily by glutamate spillover and preferentially target extrasynaptic NMDA receptors.

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