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Short communication

The effect of mGlu5 receptor positive allosteric modulators on signaling molecules in brain slices

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Abstract

Positive allosteric modulators of metabotropic glutamate receptor subtype 5 (mGlu5) have promising therapeutic potential. The effects of selective mGlu5 receptor positive allosteric modulators on signaling molecules in brain slices have not been previously reported. The current study demonstrated that the selective mGlu5 receptor positive allosteric modulator, *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-^{2}H-isoindol-2yl)-methyl] phenyl}-2-hydrobenzamide (CPPHA) potentiated the response to a subthreshold concentration of 3,5-dihydroxy-phenylglycine (DHPG) on extracellular signal-regulated protein kinase (ERK) and cyclic-AMP responsive element-binding protein (CREB) activity, as well as *N*-methyl D-aspartate (NMDA) receptor subunit NR1 phosphorylation in cortical and hippocampal slices. These results suggest that allosteric modulators of mGlu5 receptor could have physiologically significant effects by potentiating the actions of glutamate.

Keywords: Metabotropic glutamate receptor subtype 5; Extracellular signal-regulated protein kinase; Cyclic-AMP responsive element-binding protein; *N*-methyl D-aspartate receptor; *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-^{2}H-isoindol-2yl)-methyl]phenyl}-2-hydrobenzamide

1. Introduction

Positive allosteric modulators of metabotropic glutamate (mGlu) receptors have recently emerged as novel pharmacological targets offering an attractive alternative to the direct activation of mGlu receptors by orthosteric competitive agonists. These types of molecules have been discovered for mGlu1, mGlu2 and more recently for mGlu5 receptors (Knoflach et al., 2001; Johnson et al., 2003; O'Brien et al., 2003, 2004). These positive allosteric modulators offer the potential to increase the efficiency of glutamate transmission during normal stimulation without the risk of inappropriate stimulation of the system. Furthermore, such compounds are more likely to achieve high receptor subtype selectivity by targeting regions of the receptor that are different than these affected by the endogenous ligand. A few potentiators of mGlu5 receptor have been described, including DFB (3,3′-difluorobenzaldazine), and more recently CPPHA (*N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-^{2}H-isoindol-2yl)-methyl] phenyl}-2-hydrobenzamide) (O'Brien et al., 2003, 2004). Both compounds are devoid of direct agonist activity, but can act as selective positive allosteric modulators of competitive agonists of human and rat mGlu5 receptor expressed in Chinese hamster ovary (CHO) cells. DFB potentiates threshold responses to glutamate, quisqualate, and DHPG 3- to 6-fold, with EC_{50} values in the 2–5 μM range. At 10–100 μM, DFB shifts the mGlu5 receptor agonist concentration response curves approximately two fold to the left. CPPHA, a compound from a different structural class, potentiates threshold Ca^{2+} responses to glutamate 6- to 13-fold with EC_{50} values in the 150–400 nM range, and at 10 μM shifts the mGlu5 receptor agonist concentration response curves to glutamate, quisqualate, and DHPG 4- to 9-fold to the left.

In this report, we investigated the effect of DFB and CPPHA on the signaling molecules ERK and CREB in hippocampal and

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cortical slices as well as phosphorylation levels of the NR1 subunit of the NMDA glutamate receptor.

2. Materials and methods

2.1. Materials

All primary antibodies were purchased from Cell Signaling Technology. DFB, DHPG and MPEP were obtained from Tocris. CPPHA was synthesized by the route shown in Scheme 1. Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Biosciences.

2.2. Hippocampal and cortical slice preparations

All experimental protocols were approved by the Wyeth Animal Care and Use Committee and were conducted in accordance with the internationally accepted principles in the care and use of experimental animals. Hippocampal and cortical slices were obtained from 6–8 weeks old male C57BL/6 mice. Slices (300 μm) were placed in Kreb's bicarbonate buffer containing in 125 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 1.25 mM KH_2PO_4. The slices were preincubated in the buffer for 30 min and then were exposed to different compounds of interest for indicated times and snap frozen in liquid nitrogen. The slices were sonicated in boiled homogenization buffer.

2.3. Immunoblot analysis

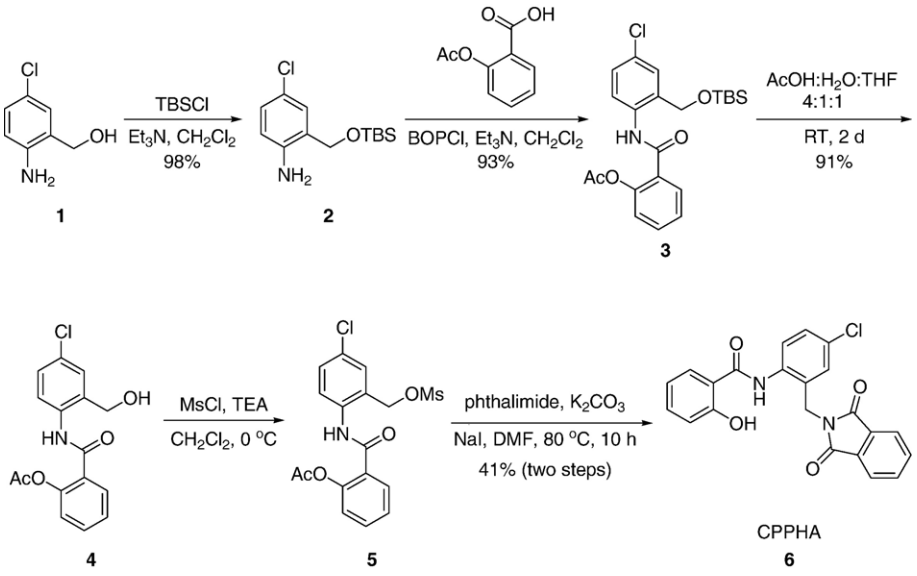
Equivalent amounts of protein for each sample were resolved in 4–12% SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h in Tris-buffered saline (TBS) containing Tween 20 and then incubated with the phosphor-specific antibody of interest [phospho-ERK antibody, 1 : 1000; phospho-CREB Ser-133) antibody, 1 : 1000, phospho-NR1 Ser-897, 1 : 500] overnight at 4 °C followed by incubation with horseradish peroxidase-linked goat anti-rabbit IgG (1 : 10,000) and developed using ECL. The blots then were incubated in stripping buffer (62 mM Tris–HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol). The stripped blots were incubated with antibody-directed against total levels for the respective protein (ERK, antibody, 1 : 1000; CREB antibody, 1 : 1000; NR1 antibody, 1 : 1000). Densitometric analysis of phospho-immunoreactivity and total immunoreactivity for each protein was conducted using the Bio-Rad GS-710 Calibrated Imaging Densitometer and quantified using Quantity One version 4.1.0. Phosphorylated immunoreactivity was normalized to total immunoreactivity. Data were statistically analyzed by one-way ANalysis Of VAriance (ANOVA) followed by Dunnett's multiple comparison tests using GraphPad Prism 4.0 software.

2.4. Neuronal cultures

Rat dissociated hippocampal cell cultures were prepared by previously described methods (Brewer et al., 1993, Brewer and Price, 1996). Briefly, hippocampal tissue was dissected from embryonic day 20 Sprague–Dawley rats, cut into small pieces and incubated for 30 min at 37 °C in solution equilibrated with 95% air / 5% CO_2 and containing (in mM) 166 NaCl, 5.4 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 1.5 $CaCl_2$, 1 $MgSO_4$, 0.5 EDTA, 25 glucose, 1 L-cysteine, and 15–20 U/ml papain. The cells were dispersed by gentle trituration, and the dissociated suspension was collected and spun at 1000 ×*g* for 5 min. The pellet was resuspended in neural basal complete media (Gibco) and plated at 300,000 cells/ml on 8-well poly-D-Lysine/laminin coated culture chambers.

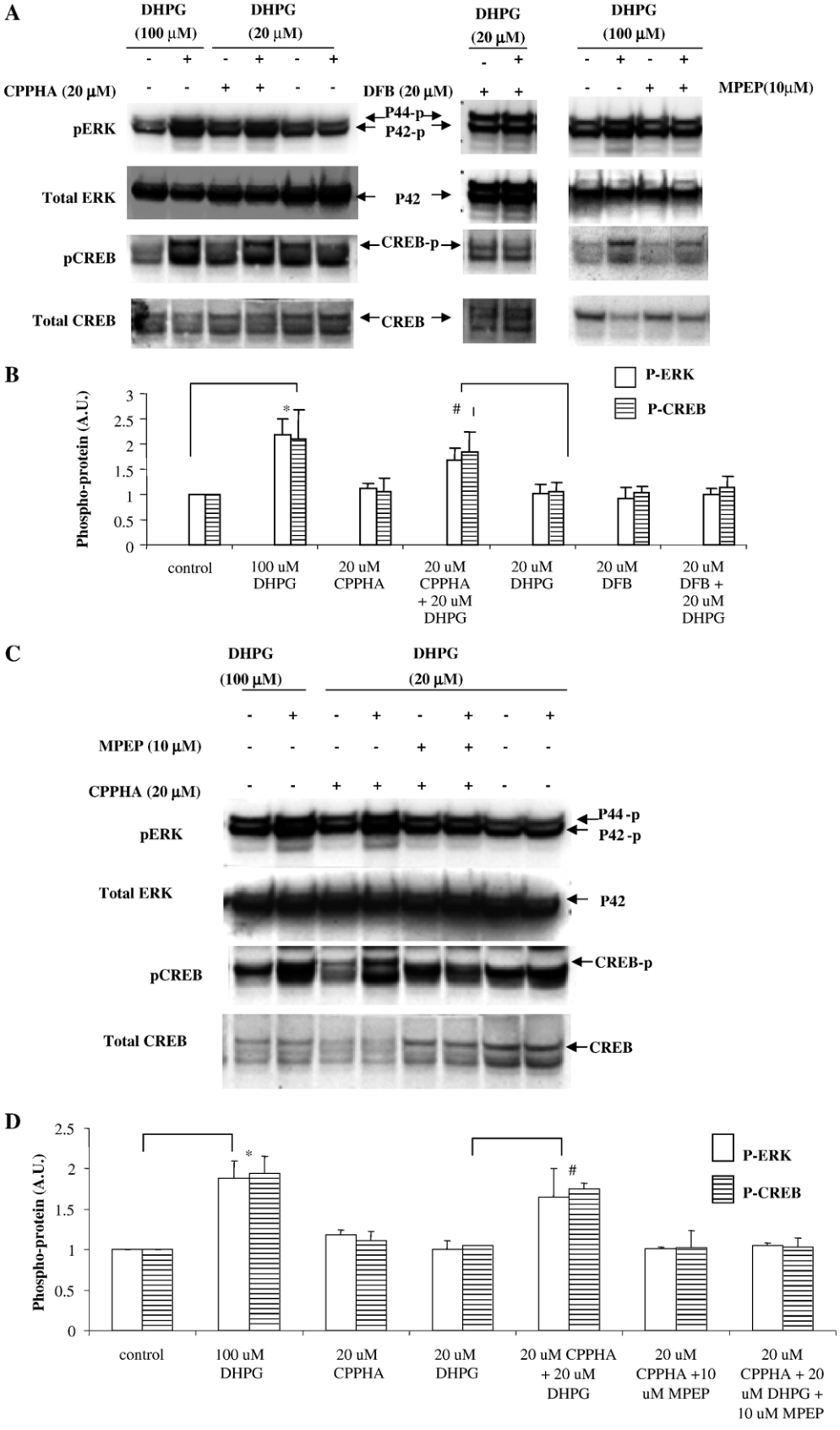
2.5. Immunocytochemistry

Control cultures and cultures treated with either DHPG or CPPHA or CPPHA plus DHPG were immediately placed in ice-



Scheme 1.

cold 4% paraformaldehyde in PBS, pH 7.4, and fixed for 30 min at 4 °C, followed by washing with TBST (TBS plus 0.1% Triton X-100) for 5 min. The cells were blocked with 5% normal goat serum in TBST for 60 min at 4 °C. The cells were incubated with primary antibody [phospho-CREB, (1 : 100)] in 5% BSA in TBST overnight at 4 °C. Cells were washed with TBST and then



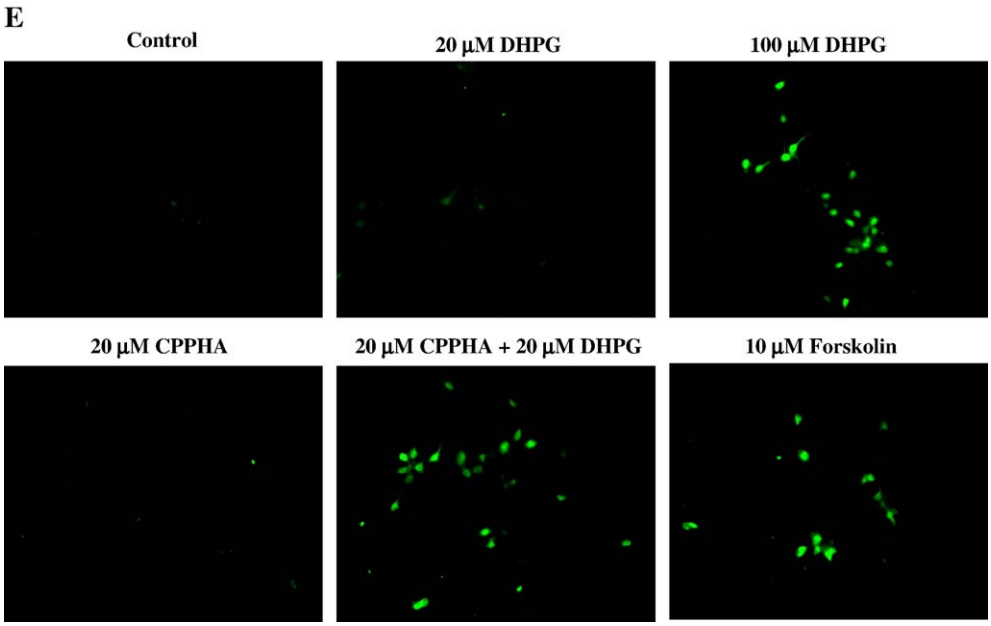


Fig. 1. CPPHA potentiates a subthreshold concentration of DHPG on ERK and CREB activation in cortical and hippocampal slices. (A, B) Cortical slices were treated with DHPG (100 μM) for 5 min as the positive control. The slices were preincubated for 15 min with 20 μM of CPPHA or DFB followed by cotreatment of 20 μM DHPG for 5 min. (C, D) Hippocampal slices were treated with DHPG (100 μM) for 5 min as the positive control. The slices were preincubated for 15 min with 20 μM CPPHA in the presence or the absence of MPEP followed by cotreatment of 20 μM DHPG for 5 min. The levels of phosphorylation in ERK and CREB were assessed via immunoblotting. A (Cortical) and C (Hippocampus), representative immunoblots for phosphorylated ERK (p-ERK), total ERK, phosphorylated CREB (p-CREB), total CREB. B and D, p-ERK and p-CREB immunoreactivity were normalized to total ERK and total CREB immunoactivity, and followed by normalizing to untreated control immunoactivity. Data were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. *Denotes statistical significance compared with control ($p<0.05$, $n=4$); #denotes statistical significance compared with 20 μM of DHPG ($p<0.05$, $n=4$). E, CPPHA potentiates a subthreshold concentration of DHPG effects on ERK activity in primary hippocampal neuronal cultures. Representative immunostaining images were obtained from cultures that were labeled using antibodies specific for phospho-ERK. Control, untreated cells; 20 μM DHPG for 5 min; 100 μM DHPG for 5 min; 20 μM CPPHA for 20 min; 20 μM CPPHA + 20 μM DHPG, cells that were preincubated with 20 μM CPPHA for 20 min and then incubated with 20 μM DHPG for 5 min in the presence of 20 μM CPPHA; 10 μM Forskolin for 5 min (positive control).

incubated with goat anti-rabbit Alexa Fluro@488 (Molecular Probes). Cells were washed with TBS and the coverslips were mounted with Prolong Antifade Mounting reagent (Molecular Probes).

2.6. Slice preparation for electrophysiology

NMDA-evoked current was recorded from visually IR-camera identified CA1 pyramidal neurons in a standard whole-cell recording mode. NMDA was delivered locally to the recording site using a fast local perfusion technique (Veselovsky et al., 1996). Electrodes were pulled from borosilicate glass capillaries (TW150F; World Precision Instruments, Sarasota, FL). Pipette resistances ranged between 2 and 5 MΩ when filled with the intracellular solution (in mM): 70 K-Gluconate, 70 CsCl, 1 $CaCl_2$, 5 Mg-ATP, 5 EGTA, 10 HEPES, and 1 QX314, pH adjusted to 7.4. The extracellular solution for voltage-clamp experiments was ACSF with addition of 0.5 μM tetrodotoxin (Sigma) to suppress action potentials. Data were collected using a MultiClamp 700A amplifier and digitized at 1 kHz using DigiData 1322A and pClamp9.0 software (all from Axon Instruments). Data were analyzed using ClampFit9.0 (Axon Instruments). Experiments were performed at 33 °C.

3. Results

3.1. The effect of DFB and CPPHA on phosphorylation of ERK and CREB

The extracellular signal-regulated protein kinase (ERK) and the transcription factor cyclic-AMP-responsive element-binding protein (CREB) are perhaps the most intensively studied molecules in the field of synaptic plasticity. Phosphorylation of ERK1/2 on Thr202/Tyr204 leads to activation of this kinase to mediate an array of various signalling pathways that control neuronal plasticity (Thomas and Huganir, 2004). Similarly, phosphorylation of CREB at Ser133 leads to recruitment of other transcription machinery of CREs to regulate gene transcription, which is an important step in the induction of gene expression that is essential to learning and memory (Carlezon et al., 2005). The effect of DFB and CPPHA on a subthreshold concentration of DHPG for the phosphorylation of ERK and CREB was assessed in mouse hippocampal and cortical slices using phosphorylation state-specific antibodies. Preincubation of cortical and hippocampal slices with CPPHA (20 μM) alone had no effect on basal levels, but potentiated the DHPG (20 μM)-induced increase in phosphorylation of ERK and CREB, approaching

levels seen with 100 μM DHPG alone. Treatment of slices with DHPG (20 μM) alone did not increase ERK or CREB phosphorylation. In contrast, DFB (20 μM) caused little potentiation effect (Fig. 1A and B). The potentiating effects of CPPHA with DHPG could be blocked by the mGlu5 receptor selective antagonist MPEP (Fig.1C and D). In parallel with the brain slices, we also analyzed the effect of CPPHA in rat primary hippocampal cultures using an immunocytochemistry approach. Consistent with brain slice observations, we found that CPPHA can potentiate a subthreshold concentration (20 μM) of DHPG on CREB phosphorylation in primary hippocampal cultures (Fig. 1E).

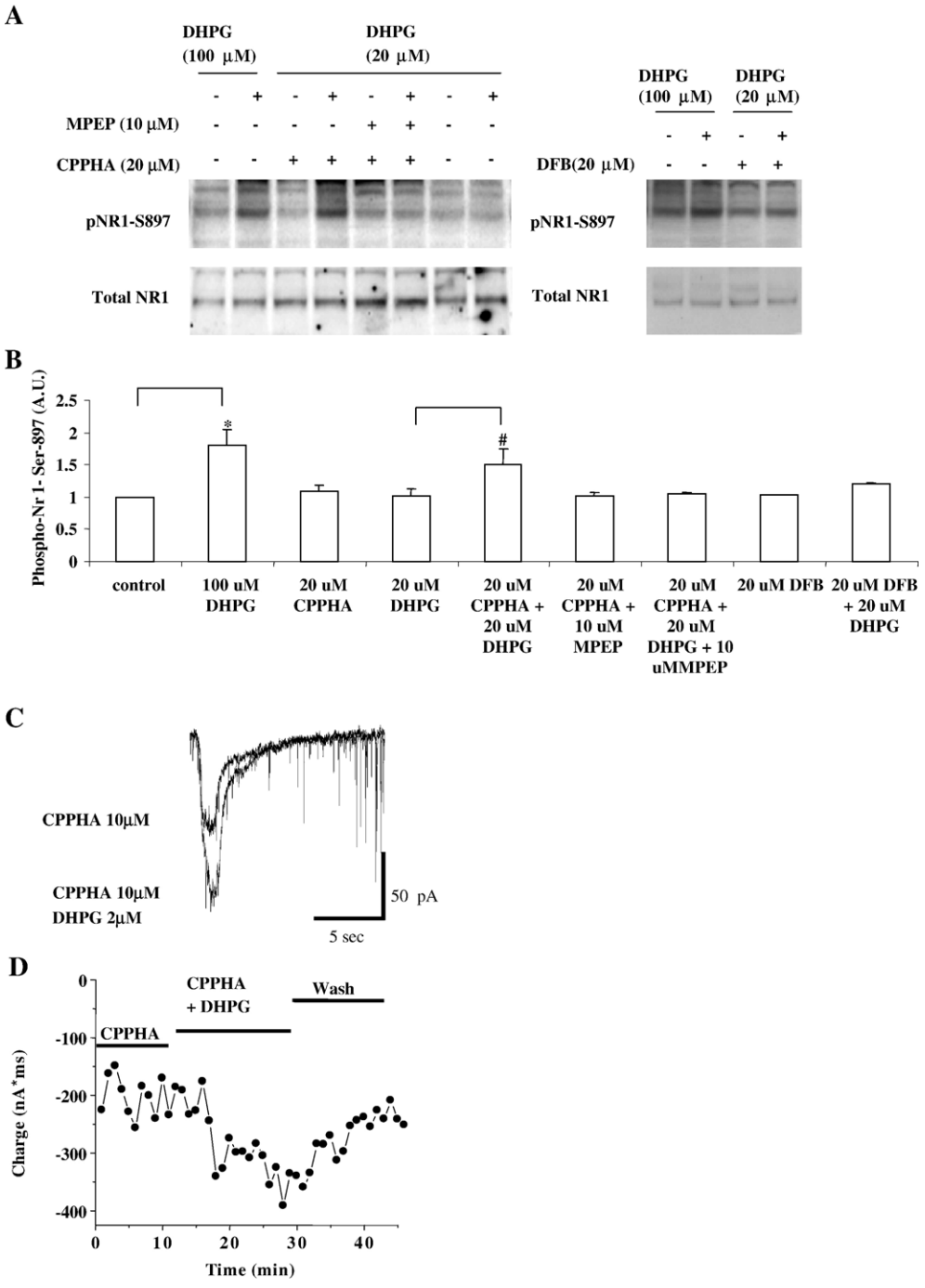


Fig. 2. A, representative immunoblot for phosphorylated NR1-Ser897 (pNR1-S897), total NR1. B, pNR1-S897 immunoreactivity was normalized to total NR1 imunoactivity, followed by normalizing to untreated control immunoactivity. Data were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. *Denotes statistical significance compared with control ($p<0.05$, $n=4$); #denotes statistical significance compared with 20 μM DHPG ($p<0.05$, $n=4$). (C, D), CPPHA enhances NMDA-evoked currents in CA1 pyramidal neurons. C, Representative recordings showing NMDA-evoked current in the presence of 10 μM CPPHA and its potentiation after co-application of 2 μM DHPG. Holding potential was −60 mV. Currents were elicited by a 1-s long pulses (60 s inter-pulse interval) of 100 μM NMDA directly to the recording site. Nine out of 13 neurons showed response. D, Graph showing the time course of DHPG-induced potentiation of NMDA-evoked current in the presence of CPPHA. Drug concentrations are the same as shown in C.

3.2. The effect of DFB and CPPHA on phosphorylation of NMDA receptor subunit NR1 Ser-897

One of the major functions of the mGlu5 receptor is the direct modulation of NMDA receptor activity which allows for the fine tuning of glutamatergic transmission both in vitro and in vivo (Awad et al., 2000; Mannaioni et al., 2001; Heidinger et al., 2002). It has been shown in two independent brain samples from schizophrenia patients, that there is a significant decrease in phosphorylation levels at serine 897 of the NMDA receptor (NR1) subunit (Emamian et al., 2004). Together with the report that antipsychotics increase phosphorylation of NR1 at S897 in vivo, these data suggest that insufficient phosphorylation at Ser-897 may contribute to the neuronal pathology underlying schizophrenia (Leveque et al., 2000). The effects of DFB and CPPHA on NR1 Ser-897 phosphorylation were evaluated in slices. Similar to studies with ERK and CREB, slices preincubated with CPPHA (20 μM) alone did not alter the basal level of NR1, but potentiated the DHPG (20 μM)-induced phosphorylation of NR1 Ser-897, approaching levels seen with DHPG at 100 μM alone (Fig. 2A and B). The effects were blocked by the mGlu5 receptor selective antagonist MPEP, suggesting CPPHA's potentiation effect on NR1-Ser897 is mediated by the mGlu5 receptor. DFB had no effect on basal or DHPG-induced phosphorylation of NR1 Ser-897. To further characterize the effect of CPPHA on NMDA receptor activity, we also performed whole cell voltage-clamp recordings from CA1 pyramidal cells in rat hippocampal slices on NMDA receptor currents. Preincubation of slices with 10 μM of CPPHA, which alone had no effect, potentiated a subthreshold concentration of DHPG (2 μM) effect on NMDA-evoked currents (Fig. 2C and D).

4. Discussion

In brain slices, we found that CPPHA can potentiate the effects of DHPG on ERK and CREB activity and on NMDA NR1 Ser-897 phosphorylation. Also, in primary rat hippocampal cultures, we found that CPPHA can potentiate the CREB phosphorylation. There were previous reports that activation of mGluR5 results in increase in phosphorylation of ERK and CREB in vivo and in cultured neurons or CHO cells expressing mGluR5 (Choe and Wang, 2001; Mao and Wang, 2003). Our data support the notion that CPPHA is a mGlu5 receptor positive allosteric modulator in native systems, suggesting that regulation of mGlu5 receptor activity by a positive allosteric modulator can have physiological significance. DFB showed a lesser potentiation than CPPHA on these signaling molecules. This is consistent with the fact that in our recombinant rat mGlu5 receptor expressing HEK 293 cells lines, DFB has a more modest effect on competitive agonist responses, increasing calcium mobilization response only about 2–3 fold. In contrast, CPPHA increases the effect of a threshold concentration of agonist by approximately 7 fold (data not shown). This is also consistent with a previous report showing that DFB has much weaker effects on calcium mobilization response than that of CPPHA (O'Brien et al., 2003). In addition, we performed whole cell voltage-clamp recordings from CA1 pyramidal cells in rat hippocampal slices to test the effect of CPPHA on NMDA receptor currents. We found that CPPHA can potentiate NMDA evoked current in hippocampal slices, which is consistent with data reported by O'Brien et al. (2003).

In conclusion, using a native system such as brain slices or primary cultures, we have demonstrated that positive allosteric modulators of mGlu5 receptor can alter major signaling molecules known to be important in modulating synaptic transmission and neuronal plasticity. The ability to increase the efficiency of glutamate transmission during normal stimulation conditions is an advantage of positive allosteric modulators. The result of this synaptic modulation may have useful physiological implications in conditions where there are postulated deficiencies in glutamatergic function such as schizophrenia or mild cognitive impairment. Recently, another novel and more potent mGlu5 receptor positive allosteric modulator CDPPB has been reported to have antipsychotic-like effects in rat behavioral models (Kinney et al., 2005). As more mGlu5 receptor positive allosteric modulators are identified, the currently described native systems can be employed to study the functional characteristics of these new compounds.

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