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Activation of Group I Metabotropic Glutamate Receptors Increases Serine Phosphorylation of GluR1 α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid Receptors in the Rat Dorsal Striatum

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Running title: Regulation of GluR1 Phosphorylation by Group I mGluRs

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CaMK, calcium-calmodulin-dependent protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKA, protein kinase A; PKC, protein kinase C; mGluR, metabotropic glutamate receptor; PLC, phospholipase C.

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ABSTRACT

Protein phosphorylation is an important mechanism for the posttranslational modulation of ionotropic glutamate receptors. In this study, we investigated the regulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor GluR1 subunit phosphorylation by the stimulation of group I metabotropic glutamate receptors (mGluRs) in the rat dorsal striatum *in viv*o. Stimulation of group I mGluRs was found to increase GluR1 phosphorylation of serine 831 (Ser831) and serine 845 (Ser845) in phospholipase C (PLC)-coupled Ca^{2+} cascades. Interactions of protein kinases activated by intracellular Ca^{2+} release downstream to PLC modulate the phosphorylation state of GluR1 on Ser831 and Ser845: phosphorylation of GluR1 on Ser831 is upregulated by the protein kinase C (PKC) and calcium-calmodulin-dependent protein kinase (CaMK)/c-Jun N-terminal kinase (JNK) pathways, whereas phosphorylation of GluR1 on Ser845 is upregulated by the protein kinase A (PKA), PKA/extracellular signal-regulated kinase 1/2 (ERK1/2) and PKA/JNK pathways. The phosphorylation state of GluR1 on Ser831 and Ser845 and the activity of protein kinases are further regulated by protein phosphatases. These data suggest that GluR1 phosphorylation of Ser831 and Ser845 via stimulation of group I mGluRs is regulated by the interactions of PLC-coupled protein kinases and protein phosphatases in the dorsal striatum.

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Group I metabotropic glutamate receptors (mGluRs) (mGluR1/5) are densely expressed in the striatum and are co-localized with the majority of either striatonigral or striatopallidal neurons (Kerner et al., 1997; Tallaksen et al., 1998; Testa et al., 1995, 1998). Accumulating evidence indicates that group I mGluRs are dominantly postsynaptic, whereas group II/III mGluRs (mGluR2/3/4/6/7/8) are present in the presynaptic glutamatergic terminals in the dorsal striatum, where they regulate transmitter release (Dev et al., 2000). These mGluR subtypes are heterogeneous in their pharmacology and their connections with intracellular effectors. For instance, stimulation of group I mGluRs increases phosphoinositide (PI) hydrolysis via stimulation of phospholipase C (PLC), which results in the release of intracellular Ca^{2+} from internal stores and activation of protein kinase C (PKC) (Choe and Wang, 2002). Conversely, stimulation of group II/III mGluRs inhibits adenylate cyclase (AC) and cAMP formation (Kim et al., 2007). In addition to the effectors described above, mGluRs have been found to couple to a number of other signaling transducers (Pastorino et al., 2000; Shinomura et al., 2000), and it has been demonstrated that coupling to these second messengers allows mGluRs to regulate diverse physiological and pathological processes in the central nervous system (CNS) (Calabresi et al., 1999; Kalda et al., 2000; Schnabel et al., 1999; Szapiro et al., 2000).

The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor is

composed of four subunits, GluR1-GluR4, which combine to form the various pentameric structures of the receptor in the neurons of the CNS (Borges, 1998; Dingledine et al., 1999). The carboxyl terminal of the GluR1 subunit is functionally and structurally divergent, and the serine residues at positions 831 (Ser831) and 845 (Ser845) are phosphorylated by protein kinases in the neostriatum (Snyder et al., 2000). For instance, Ser831 is phosphorylated by PKC, as well as by calcium-calmodulin-dependent protein kinases (CaMK), whereas phosphorylation of Ser845 is regulated by protein kinase A (PKA) in transfected cells that express GluR1, cultured neurons and hippocampal slices (Banke et al., 2000; Barria et al., 1997; Mammen et al., 1997; Roche et al., 1996; Wang et al., 2006). The phosphorylation sites on GluR2-4 also contribute to the regulatory functions in the brain (Derkach et al., 1999). Similar to protein kinases, PPs have also been found to be important in the regulation of GluR1 AMPA phosphorylation in the forebrain (Snyder et al., 2003). Changes in the phosphorylation state of the receptor, induced by the activation of protein kinases and protein phosphatases (PPs), seem to play an essential role in regulating the functions of the AMPA receptor in the basal forebrain (Snyder et al., 2000, 2003; Yan et al., 1999). For instance, the phosphorylation of AMPA receptor GluR1 subunits revitalizes the conductance of the receptor and thus potentiates rapid excitatory neurotransmission in neurons (Derkach et al., 1999). Taken together, these data suggest that GluR1 AMPA phosphorylation by the

stimulation of mGluRs is regulated by interactions between the protein kinases and PPs in the brain. However, the mechanisms by which the stimulation of group I mGluRs in striatal neurons modulates GluR1 AMPA receptor phosphorylation remain to be elucidated. Intricate understanding of these receptor signaling interactions is required for the understanding of their potential as therapeutic targets. Therefore, this study was conducted to investigate intracellular mechanisms involving phosphorylation of the GluR1 AMPA receptor subunit by group I mGluR stimulation in the dorsal striatum.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (200-250 g) were obtained from Hyo-Chang Science Co. (Daegu, Korea). Rats were individually housed in a controlled environment during all experimental treatments. Food and water were provided *ad libitum* and rats were maintained on a 12 h light/dark cycle. On the day of the experiment, the injections were made in a quiet room to minimize stress to the animals. All animal use procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory

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Animals.

Drugs

The group I mGluR agonist DHPG (100, 250 nmol), the group I mGluR subtype 5 (mGluR5) agonist CHPG (100 nmol), the group I mGluR subtype 1 (mGluR1) antagonist CPCCOEt (5 nmol), the mGluR5 antagonist MPEP (0.5 nmol), the group II/III mGluR antagonist MPPG (100 nmol), the group II mGluR agonist LY379268 (5 nmol), the group III mGluR agonist AP4 (100 nmol), the PLC inhibitor U73122 (20 nmol), the *N*-methyl-D-aspartate (NMDA) antagonists MK801 (2 nmol) and AP5 (2 nmol), the ryanodine-sensitive Ca^{2+} channel blocker dantrolene (2, 20 nmol), the inositol 1, 4, 5-triphosphate (IP_3)-sensitive Ca^{2+} channel blocker xestospongin C (0.001, 0.004 nmol), the PKC inhibitor GF109203X (5, 20 nmol), the CaMK inhibitor KN62 (2, 10, 20 nmol), the mitogen-activated protein kinase kinase (MEK) inhibitor SL327 (75, 150 nmol), the PKA inhibitor KT5720 (2.5, 5 nmol), the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (50, 100, 200 nmol), the PP1/2A inhibitor okadaic acid (0.005, 0.05, 0.5 nmol) or the calcineurin (PP2B) inhibitor cyclosporin A (0.005, 0.05, 0.5 nmol) was dissolved in dimethylsulfoxide (DMSO) and then diluted in artificial cerebrospinal fluid (aCSF) (123 mM NaCl, 0.86 mM $CaCl_2$, 3.0 mM KCl, 0.89 mM $MgCl_2$,

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0.50 mM NaH_2PO_4 and 0.25 mM Na_2HPO_4 aerated with 95 % O_2/5 % CO_2, pH 7.2-7.4). DMSO in combination with aCSF was used as a vehicle control for the respective agents. Xestospongin C was purchased from Calbiochem (Darmstadt, Germany) and the rest of drugs were purchased from Tocris Cookson (Ellisville, MO, USA). The solutions of all drugs were freshly prepared on the day of the experiment and adjusted to a pH of 7.2-7.4 with 1 N NaOH, if necessary. The concentrations of the drugs used were determined based on the results of previous studies (Choe and McGinty, 2000; Choe et al., 2002, 2004; Ghasemzadeh et al., 2003; Mao and Wang, 2000, 2002; Peters and Kalivas, 2006; Shi and McGinty, 2006; Smith et al., 2006), as well as our preliminary study.

Surgery and Intrastriatal Drug Infusion

Rats were anesthetized with 8% chloral hydrate (6 ml/kg, i.p.) and then placed in a Stoelting stereotaxic apparatus. Under aseptic conditions, a 23-gauge stainless steel guide cannula (inner diameter: 0.29 mm, 10 mm in length) was implanted at the coordinates of 1 mm anterior to the bregma, 2.5 mm right to the midline and 4 mm below the surface of the skull. The guide cannula was then sealed with a stainless steel wire of the same length. Rats were then allowed to recover from surgery for 3 days. On the day of the experiment,

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the inner steel wire was replaced with a 30 gauge stainless steel injection cannula (inner diameter: 0.15 mm) with a length of 12.5 mm that protruded 2.5 mm beyond the guide cannula. Throughout the experiments, drugs were infused unilaterally into the central part of the right dorsal striatum in a volume of 1 µl at a rate of 0.2 µl/min in freely moving rats. The progress of the injection was monitored by observing movement of a small air bubble through a length of precalibrated PE-10 tubing inserted between the injection cannula and a 2.5 µl Hamilton microsyringe. After completion of the injection, the injector was left in place for an additional 5 min to reduce any possible backflow of the solution along the injection tract. The physical accuracy of the injection was verified by the reconstruction of microinjection placements (Fig. 1). The possibility of gliosis caused by the implantation of guide cannula and the infusion of drugs dissolved in DMSO/aCSF was also verified using Nissl staining (Data not shown).

Western Immunoblotting

Rats were deeply anesthetized with 8% chloral hydrate and then decapitated at 15 min after the final injection of each drug or vehicle. The brains were then removed, frozen in isopentane at -70˚C, and stored in a deep freezer. Next, sections were serially cut in a

cryostat and the overall dorsal striatum was removed using a steel borer (inner diameter: 2 mm) as described previously (Choe et al., 2004). All tissue samples were then transferred into a microtube containing ice-cold sample buffer (10 mM pH 7.4 Tris-HCl, 5 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA and 1 mM EGTA). The samples were then sonicated for 30 sec on ice and centrifuged at 13,000 rpm for 30 min at 4˚C, after which, the pellet containing mainly nuclei and large debris was discarded. The supernatant was then again centrifuged at 13,000 rpm for 30 min at 4˚C. The supernatant was resolved using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer containing 5% skim milk and then probed with each primary antiserum against GluR1, phosphorylated (p)GluR1-Ser831, pGluR1-Ser845, CaMKII, pCaMKII, extracellular signal-regulated kinase 1/2 (ERK1/2, p44/p42), pERK1/2, JNK (p54/p46), pJNK, PKC, pPKC or β-tubulin at 1:1000 overnight at 4˚C on a shaker. The membrane was then incubated with the appropriate secondary antiserum (KPL, Gaithersburg, MD, USA) at the dilution of 1:1000 for 1 h at room temperature. Antiserum against GluR1 and pGluR1-Ser831 was purchased from Upstate (Lake Placid, NY, USA), pGluR1-Ser845 was obtained from Chemicon International Incorporation (Billerica, MA, USA), CaMKII and pCaMKII was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the rest of the antiserum was

purchased from Cell Signaling Technology (Beverly, MA, USA). Immunoreactive protein bands were detected by enhanced chemiluminescence reagents (ECL) (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA) on X-ray films. The density of immunoblots was measured using an imaging digital camera and NIH Image 1.62 software, and all bands were normalized by β-tubulin for a control of protein loading and expressed as percent control of the unphosphorylated protein levels within each group.

Quantitation of Immunoreactivity

Immunoreactive protein bands on films were semi-quantified using an imaging digital camera and NIH Image 1.62 software. Briefly, the film background was measured and saved as a "blank field" to correct uneven illumination. Next, the upper limit of the density slice option was set to eliminate any background and this value was then used to measure all images. The lower limit was set at the bottom of the LUT scale. The immunoreactive protein bands were then measured using a rectangle that covered the individual band.

Statistics

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Statistical significance of the number of immunoreactive pixels per measured area between groups was determined using a one-way ANOVA on ranked data, followed by a Tukey's HSD (honestly significant difference) test in GraphPad Prism 4 (GraphPad Software Incorporation, San Diego, CA, USA). Data are expressed in terms of mean ± SEM for each group (n = 4-5 per group). A p value < 0.05 was considered to be statistically significant.

Results

Stimulation of Group I mGluRs, but Not Group II/III mGluRs, Increased the Immunoreactivity of pGluR1-Ser831 and Ser845. This study was conducted to determine if stimulation of group I mGluR alters GluR1 phosphorylation of Ser831 and Ser845 in the dorsal striatum. As shown in Fig. 2A, the group I mGluR agonist DHPG significantly increased pGluR1-Ser831 and Ser845 immunoreactivity. Conversely, the mGluR5 antagonist MPEP significantly decreased the immunoreactivity of pGluR1-Ser831 and Ser845 that was induced by DHPG, while the mGluR1 antagonist CPCCOEt decreased only the immunoreactivity that was elevated by DHPG in pGluR1-Ser831 (Fig. 2B). However, the antagonists by themselves did not alter the immunoreactivity of both pGluR1-

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Ser831 and Ser845. Parallel to the experiments, the involvement of group II/III mGluRs in the regulation of GluR1 phosphorylation in the dorsal striatum was examined by intrastriatal infusion of the group II/III mGluR antagonist MPPG, the group II mGluR agonist LY379268 or the group III mGluR agonist AP4. As shown in Fig. 2C and D, neither the group II/III mGluR agonists nor the antagonist had an effect on the immunoreactivity of pGluR1-Ser831 and Ser845 that was elevated by DHPG in the dorsal striatum.

Inhibition of PLC Decreased the Immunoreactivity of pGluR1-Ser831 and Ser845 by DHPG, while Blockade of Intracellular Ca^{2+} Release from Ryanodine-Sensitive Ca^{2+} Channels Decreased only the pGluR1-Ser831 Immunoreactivity that Was Induced by DHPG. Because the immunoreactivity of pGluR1-Ser831 and Ser845 was increased by stimulation of the group I mGluRs in the dorsal striatum, the involvement of PLC in the regulation of GluR1 phosphorylation was investigated. As shown in Fig. 3A, the PLC inhibitor U73122 significantly decreased the immunoreactivity of pGluR1-Ser831, however U73122 partially attenuated the immunoreactivity of Ser845 that was elevated by DHPG when compared with vehicle controls. Because PLC activation can mediate increased influx of Ca^{2+} through NMDA receptors following phosphorylation by PKC or the endoplasmic reticulum (ER) (Choe and Wang, 2002), the involvement of NMDA receptors in

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the regulation of GluR1 phosphorylation by group I mGluR stimulation was investigated by intrastriatal infusion of the NMDA receptor blocker MK801 or AP5 followed by DHPG infusion. The NMDA receptor antagonists by themselves did not alter the immunoreactivity of both pGluR1-Ser831 and Ser845. Neither MK801 nor AP5 altered the immunoreactivity of pGluR1-Ser831 or Ser845 that was induced by DHPG (Fig. 3B). Dantrolene, which blocks the intracellular release of Ca^{2+} from ryanodine-sensitive Ca^{2+} channels in the ER, was infused into the dorsal striatum to investigate the involvement of the receptors in the regulation of GluR1 phosphorylation. As shown in Fig. 3C, only the higher dose of dantrolene decreased the immunoreactivity of pGluR1-Ser831 induced by DHPG, and dantrolene had no effect on the pGluR1-Ser845 immunoreactivity that was induced by DHPG.

Blockade of Intracellular Ca^{2+} Release from IP_3-Sensitive Ca^{2+} Channels Decreased the pGluR1-Ser845 Immunoreactivity that Was Elevated by CHPG. In order to identify if the intracellular Ca^{2+} regulates the phosphorylation of GluR1-Ser845, xestospongin C, which blocks the intracellular release of Ca^{2+} from IP_3-sensitive Ca^{2+} channels in the ER, was infused into the dorsal striatum. Xestospongin C decreased the immunoreactivity of pGluR1-Ser831, but not Ser845, elevated by DHPG (Fig. 3D). However, xestospongin C decreased the immunoreactivity of pGluR1-Ser845 elevated by the mGluR5 agonist CHPG

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in a dose-dependent manner (Fig. 3E).

Inhibition of PKC or CaMK Decreased pGluR1-Ser831 Immunoreactivity Induced by DHPG, while Inhibition of MEK or PKA Decreased pGluR1-Ser845 Immunoreactivity. Because dantrolene decreased the level of pGluR1-Ser831 immunoreactivity after DHPG stimulation, the involvement of Ca^{2+}-dependent protein kinases in the regulation of GluR1 phosphorylation by DHPG was investigated. As shown in Fig. 4A and B, the PKC inhibitor GF109203X and the CaMK inhibitor KN62 significantly decreased the DHPG-induced immunoreactivity of pGluR1-Ser831, but not that of pGluR1-Ser845. Conversely, the MEK inhibitor SL327 and the PKA inhibitor KT5720 significantly decreased the amount of pGluR1-Ser845 immunoreactivity that was elevated by DHPG (Fig. 4C and D). In addition, only the higher dose of the JNK inhibitor SP600125 decreased the immunoreactivity of both pGluR1-Ser831 and Ser845 induced by DHPG in the dorsal striatum (Fig. 4E).

Inhibition of CaMK and PKA Decreased only the Immunoreactivity of pJNK, and pERK1/2 and pJNK, respectively, by DHPG. This study was conducted to determine the

sequence of protein kinases downstream to PLC in the regulation of GluR1 phosphorylation in the dorsal striatum. As shown in Fig. 5A, the PKC inhibitor GF109203X did not alter the immunoreactivity of pCaMKII, pERK1/2 or pJNK that was elevated by DHPG stimulation. However, the CaMK inhibitor KN62 significantly decreased the immunoreactivity of pJNK, but not that of pERK1/2 or pPKC that was elevated by DHPG stimulation (Fig. 5B). Similar to the previous experiment, the PKA inhibitor KT5720 significantly decreased the immunoreactivity of pERK1/2 and pJNK elevated by DHPG, but not that of pCaMKII and pPKC in the dorsal striatum (Fig. 5C).

Inhibition of PP1/2A, but Not PP2B, Synergistically Increased the Immunoreactivity of pGluR1-Ser831 and Ser845 by DHPG. This study was conducted to investigate the involvement of PPs in the regulation of the GluR1 phosphorylation by group I mGluR stimulation in the dorsal striatum. As shown in Fig. 6A and B, treatment with the PP1/2A inhibitor okadaic acid alone significantly increased the immunoreactivity of pGluR1-Ser831 and Ser845 which was synergistically increased by DHPG infusion. Similarly, treatment with the PP2B inhibitor cyclosporin A alone also increased the immunoreactivity of pGluR1-Ser831 and Ser845 (Fig. 6C). In contrast to treatment with okadaic acid, the increased immunoreactivity of pGluR1-Ser831 and Ser845 by cyclosporin

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A was not altered by DHPG (Fig. 6D). Alteration of the immunoreactivity of pGluR1-Ser831 was more sensitive to cyclosporin A than okadaic acid, whereas the opposite was true for the alteration of pGluR1-Ser845 immunoreactivity.

Inhibition of PP1/2A or PP2B Alone Increased the Immunoreactivity of pCaMKII, pERK1/2, pJNK and pPKC in a Dose-Dependent Manner. Since PPs dephosphorylate pGluR1-Ser831 and Ser845, this study was conducted to investigate the effects of PP inhibition on the activity of protein kinases in the dorsal striatum. As shown in Fig. 7A, treatment with the PP1/2A inhibitor okadaic acid alone significantly increased the immunoreactivity of pCaMKII, pERK1/2, pJNK and pPKC in a dose-dependent manner. Similarly, treatment with the PP2B inhibitor cyclosporin A alone also increased the immunoreactivity of the phosphorylated protein kinases (Fig. 7B).

Discussion

Regulation of GluR1 AMPA receptor subunit phosphorylation on Ser831 and Ser845 by the stimulation of mGluRs was investigated in the rat dorsal striatum *in vivo*. The present data suggest that the phosphorylation of AMPA receptor GluR1 subunits at Ser831 is subject

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to the upregulation by repeated cocaine exposure. Complex signaling integrations among glutamate receptors, Ca^{2+} channels, protein kinases, and protein phosphatases participate in this upregulation. The present data demonstrate that interactions of protein kinases downstream to PLC upregulate GluR1 phosphorylation via group I mGluR stimulation. Phosphorylation of GluR1-Ser831 is upregulated by Ca^{2+}-dependent PKC/CaMK, while phosphorylation of GluR1-Ser845 by PKA. The phosphorylation state of GluR1 and protein kinases is further regulated by PPs. These data suggest that GluR1 phosphorylation is subject to the upregulation by the stimulation of group I mGluRs. Complex signaling integrations among glutamate receptors, Ca^{2+} channels, protein kinases, and PPs participate in this upregulation.

The results of this study show that stimulation of group I mGluRs significantly increases the immunoreactivity of pGluR1-Ser831 and Ser845 in the dorsal striatum. Previous studies demonstrate that the immunoreactivity of GluR1 and pGluR1-Ser831 is co-expressed in gamma-aminobutyric acid (GABA)ergic neurons in the rat caudate putamen (Ba et al., 2006) and GluR1 neurons in the striatum are more common in projecting to the external pallidum (GPe) than internal pallidum (GPi) (Deng et al., 2007). In addition, GluR1 is also expressed in parvalbumin-containing interneurons in the striatum (Ba et al., 2006). These data suggest that stimulation of group I mGluRs phosphorylates GluR1 AMPA receptors on Ser831 and Ser845 in the striatopallidal GABAergic neurons and parvalbumin-containing

interneurons.

Blockade of mGluR5 decreases the immunoreactivity of pGluR1-Ser831 and Ser845, whereas blockade of mGluR1 decreases only pGluR1-Ser831 immunoreactivity in the dorsal striatum. Consistent with our findings, the results of a previous study show that the group I mGluR agonist DHPG increases GluR1 phosphorylation of Ser831 and Ser845 in hippocampal slices (Delgado and O'Dell, 2005). Our data also show that antagonists for mGluR1, mGluR5 and NMDA receptors by themselves did not alter the immunoreactivity of pGluR1-Ser831 and Ser845. These data suggest that basal release of glutamate in the dorsal striatum does not affect on the alteration of GluR1-Ser831 and Ser845 phosphorylation. Furthermore, group I mGluRs are positively coupled to GluR1 phosphorylation in which serine phosphorylation at 831 and 845 is independently regulated by stimulation of the group I mGluR subtypes mGluR1 and mGluR5, with stimulation of mGluR1 being required for GluR1 phosphorylation of serine 831, and mGluR5 being required for GluR1 phosphorylation of both serine 831 and 845 in the dorsal striatum. The results of this study show that neither blockade nor stimulation of group II/III mGluRs had an effect on the immunoreactivity of pGluR1-Ser831 and Ser845 induced by DHPG stimulation in the dorsal striatum. These data suggest that stimulation of group II/III mGluRs is not necessary for postsynaptic GluR1 phosphorylation. However, blocking mGluRII/III in the glutamatergic

terminals would influence GluR1 phosphorylation by enhancing glutamate release (Wang et al., 2005). These findings suggest that GluR1 phosphorylation of Ser831 and Ser845 is specifically regulated by the stimulation of group I mGluR subtypes in the dorsal striatum.

Stimulation of group I mGluRs activates PLC, which hydrolyzes PI to IP_3 and diacylglycerol and results in the release of Ca^{2+} from the ER as well as increased influx of Ca^{2+} through NMDA receptors following phosphorylation by PKC (Choe and Wang, 2002). The results of this study demonstrate that inhibition of PLC with U73122 completely decreases the immunoreactivity of pGluR1-Ser831, while inhibition of PLC partially attenuates the immunoreactivity of pGluR1-Ser845 elevated by DHPG stimulation. These data suggest that U73122 is not able to fully attenuate PLC-mediated phosphorylation of Ser845 at the applied concentration. Blockade of intracellular Ca^{2+} release only decreases the pGluR1-Ser831 immunoreactivity induced by DHPG infusion in the dorsal striatum. However, blockade of NMDA receptors does not affect the alterations of pGluR1 immunoreactivity induced by DHPG, suggesting that GluR1-Ser831 phosphorylation in response to group I mGluR stimulation in the dorsal striatum is triggered by the PLC-mediated intracellular Ca^{2+}-cascades.

Increased levels of Ca^{2+} as a result of stimulation of ryanodine-sensitive Ca^{2+} channels in the ER can regulate GluR1 phosphorylation by activating Ca^{2+}-dependent protein kinases.

The results of this study demonstrate that inhibition of either PKC or CaMK decreases the pGluR1-Ser831 immunoreactivity induced by DHPG stimulation in the dorsal striatum, whereas inhibition of either MEK or PKA decreases pGluR1-Ser845 immunoreactivity. Previous studies have shown that PKC is activated by the stimulation of group I mGluRs in primary striatal cultures (Paolillo et al., 1998), and that either PKC or CaMK can regulate GluR1 phosphorylation of serine 831 in transfected cells, cultured neurons and hippocampal slices (Barria et al., 1997; Mammen et al., 1997; Roche et al., 1996). The results of this study also demonstrate that inhibition of PKC does not alter the immunoreactivity of all markers induced by DHPG stimulation, but that inhibition of CaMK decreases only the pJNK immunoreactivity in the dorsal striatum. These findings suggest that GluR1 phosphorylation of serine 831 as a result of stimulation of the group I mGluRs is upregulated by PLC-coupled PKC/CaMK, in which PKC and CaMK/JNK independently regulate GluR1-Ser831 phosphorylation in the dorsal striatum.

Although phosphorylation of GluR1-Ser845 is regulated by the stimulation of dopamine D1 (D1) receptor-dependent PKA in neostriatal slices (Snyder et al., 2000), D1 receptor stimulation does not appear to be required for phosphorylation of the GluR1-Ser845 to occur because increased levels of Ca^{2+} induced by the stimulation of group I mGluRs are able to upregulate GluR1 phosphorylation of serine 845. In this study, the blockade of intracellular

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Ca^{2+} release from IP_3 receptors attenuates an increase in mGluR5-stimulated phosphorylation of GluR1-Ser845, while mGluR1/5 does not. Several studies have shown that group I mGluR stimulation leads to increased cAMP levels in brain slices (Wang and Johnson, 1995) and increased PKA activity in the cortex of rats (Bandrowski et al., 2001). These data suggest that mGluR5 modulates Ca^{2+}-dependent cAMP/PKA pathways that are known to have profound effects on pGluR1-Ser845. The results of this study also demonstrate that inhibition of PKA decreases the immunoreactivity of pERK1/2 and pJNK that is elevated by DHPG stimulation. Similarly, stimulation of PKA with 8-bromo-cAMP increases the immunoreactivity of pERK1/2 in the rat dorsal striatum (Choe and McGinty, 2000). Taken together, these findings suggest that, at least, the phosphorylation of GluR1-Ser845 is regulated by the activation of Ca^{2+}-dependent PKA in the dorsal striatum, in which PKA regulates both JNK and ERK1/2.

Similar to protein kinases, PPs regulate a variety of neuronal activity in the dorsal striatum (Wang et al., 2006). The results of this study demonstrate that inhibition of PP1/2A or PP2B alone increases the immunoreactivity of pGluR1-Ser831 and Ser845 in the dorsal striatum. Moreover, inhibition of PP1/2A, but not PP2B synergistically increases the pGluR1-Ser845 immunoreactivity induced by DHPG stimulation in the dorsal striatum. In addition, inhibition of PP1/2A or PP2B alone increases the immunoreactivity of pCaMKII,

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pERK1/2, pJNK and pPKC, suggesting that the basal activity of PPs in striatal neurons is high enough to dephosphorylate basal GluR1-Ser831 and Ser845 phosphorylation and phosphorylated protein kinases. These data also suggest that PPs are most likely activated by increased Ca^{2+} levels via group I mGluR stimulation, and that this has a greater impact on the regulation of the phosphorylation state of GluR1 by dephosphorylating pGluR1-Ser845 than pGluR1-Ser831 as discussed above.

In summary, the cellular mechanisms underlying group I mGluR-mediated GluR1 AMPA receptor subunit phosphorylation of serine residues 831 and 845 was investigated in the rat dorsal striatum *in vivo*. As shown in Fig. 8, stimulation of group I mGluRs increases the phosphorylation of GluR1-Ser831 and Ser845 by PLC-coupled Ca^{2+} cascades. Interactions of protein kinases downstream to PLC regulate the phosphorylation state of GluR1 on Ser831 and Ser845 via stimulation of the group I mGluRs, with phosphorylation of GluR1-Ser831 being upregulated by PKC/CaMK pathways in which PKC and CaMK/JNK independently regulate GluR1-Ser831 phosphorylation. However, phosphorylation of GluR1-Ser845 is regulated by the stimulation of PKA in which PKA regulates both JNK and ERK1/2. In addition, the phosphorylation state of GluR1 and protein kinase is further regulated by PPs, which is believed to alter the functions of AMPA receptor in striatal neurons.

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Footnotes

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Legends for Figures

Fig. 1. A schematic diagram (panel A) and a striatal section (panel B) illustrating reconstruction of microinjection placements, a circle punched out for Western immmnoblot analysis and a microinjection tract (arrows). CPu, caudate-putamen; NAc, nucleus accumbens; cc, corpus collosum; LSN, lateral septal nuclei; AC, anterior commissure; LV, lateral ventricle. Bar represents 0.5 mm.

Fig. 2. Regulation of pGluR1-Ser831 and Ser845 via the stimulation of group I mGluRs in the dorsal striatum. The group I mGluR agonist DHPG increases the immunoreactivity of pGluR1-Ser831 and Ser845 when compared with vehicle controls (A). However, the mGluR5 antagonist MPEP decreases the immunoreactivity of pGluR1-Ser831 and Ser845 induced by DHPG stimulation, whereas the mGluR1 antagonist CPCCOEt decreases only the pGluR1-Ser831 immunoreactivity (B). In addition, the group II/III mGluR antagonist MPPG (C) or the group II/III mGluR agonist LY379268 or AP4 (D) does not alter the immunoreactivity of pGluR1-Ser831 and Ser845 induced by DHPG stimulation. $^{*}p < 0.05$ vs. vehicle groups; $^{\#}p < 0.05$ vs. DHPG groups.

Fig. 3. Involvement of PLC-coupled Ca^{2+} cascades in the regulation of GluR1 phosphorylation in response to the stimulation of group I mGluRs in the dorsal striatum. The PLC inhibitor U73122 decreases the immunoreactivity of pGluR1-Ser831 and Ser845

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induced by DHPG stimulation when compared with vehicle controls (A). The NMDA receptor blocker MK801 or AP5 does not alter the immunoreactivity of both pGluR1-Ser831 and Ser845 that is induced by DHPG (B). The higher dose of dantrolene decreases the immunoreactivity of pGluR1-Ser831, but not pGluR1-Ser845, that is elevated by DHPG stimulation (C). The IP_3-sensitive Ca^{2+} channel blocker xestospongin C decreases the immunoreactivity of pGluR1-Ser831, but not pGluR1-Ser845, elevated by DHPG (D). However, xestospongin C decreased the immunoreactivity of pGluR1-Ser845 elevated by the mGluR5 agonist CHPG in a dose-dependent manner (E). $^{*}p < 0.05$ vs. vehicle groups; $^{\#}p < 0.05$ vs. DHPG or CHPG groups.

Fig. 4. Involvement of protein kinases in the regulation of GluR1 phosphorylation by the stimulation of group I mGluRs in the dorsal striatum. The PKC inhibitor GF109203X (A) or the CaMK inhibitor KN62 (B) decreases pGluR1-Ser831, but not pGluR1-Ser845, immunoreactivity induced by DHPG stimulation. The MEK inhibitor SL327 (C) or the PKA inhibitor KT5720 (D) also decreases pGluR1-Ser845 immunoreactivity induced by DHPG stimulation, but only the higher dose of the JNK inhibitor SP600125 (E) decreases the immunoreactivity of both pGluR1-Ser831 and Ser845. $^{*}p < 0.05$ vs. vehicle groups; $^{\#}p < 0.05$ vs. DHPG groups.

Fig. 5. Determination of the interactions of protein kinases downstream to PLC in the

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regulation of GluR1 phosphorylation in response to the stimulation of group I mGluRs in the dorsal striatum. The PKC inhibitor GF109203X does not alter the immunoreactivity of pCaMKII, pERK1/2 or pJNK induced by DHPG stimulation (A). The CaMK inhibitor KN62 also does not alter the immunoreactivity of pERK1/2 or pPKC induced by DHPG stimulation, except for pJNK immunoreactivity (B). The PKA inhibitor KT5720 decreases the immunoreactivity of pERK1/2 and pJNK induced by DHPG stimulation, but not that of pCaMKII and pPKC (C). $*p < 0.05$ vs. vehicle groups; $^{\#}p < 0.05$ vs. DHPG groups.

Fig. 6. Involvement of PPs in the regulation of GluR1 phosphorylation by group I mGluR stimulation in the dorsal striatum. The PP1/2A inhibitor okadaic acid alone (A) increases the immunoreactivity of pGluR1-Ser831 and Ser845, which is synergistically increased by DHPG (B). The PP2B inhibitor cyclosporin A alone also increases the immunoreactivity of pGluR1-Ser831 and Ser845 (C). However, increased immunoreactivity of pGluR1-Ser831 and Ser845 in response to PP2B is not altered by DHPG (D). $*p < 0.05$ vs. vehicle groups; $**p < 0.05$ vs. DHPG groups.

Fig. 7. The effect of PP inhibition on the phosphorylation of protein kinases in the dorsal striatum. The PP1/2A inhibitor okadaic acid alone increases the immunoreactivity of pCaMKII, pERK1/2, pJNK and pPKC in a dose-dependent manner (A). Similarly, the PP2B inhibitor cyclosporin A alone also increases the immunoreactivity of the

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phosphorylated protein kinases (B). *$p < 0.05$ vs. vehicle groups.

Fig. 8. A postulated diagram illustrating the intracellular mechanisms underlying group I mGluR-regulated GluR1 AMPA receptor phosphorylation of serine residues 831 and 845 in the dorsal striatum. Stimulation of mGluR1/5 increases the phosphorylation of GluR1-Ser831 via PLC-coupled Ca^{2+} cascades. Activation of Ca^{2+}-dependent PKC and CaMK/JNK regulates GluR1-Ser831 phosphorylation (A). Similarly, mGluR5 stimulation also increases the phosphorylation of GluR1-Ser845 via PLC-coupled Ca^{2+} cascades. Activation of PKA by increased Ca^{2+} levels in turn regulates JNK and ERK pathways that lead to GluR1-Ser845 phosphorylation or directly regulates GluR1 phosphorylation (B). A large box (in gray) represents deactivation of protein kinase activities by PPs. $[Ca^{2+}]i$, intracellular calcium concentration.

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Fig. 1.

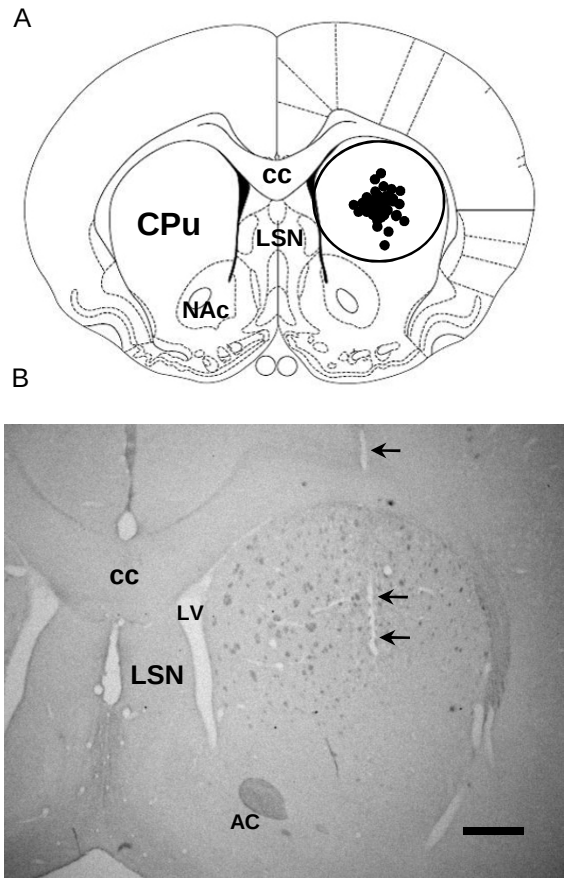

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Fig. 2.

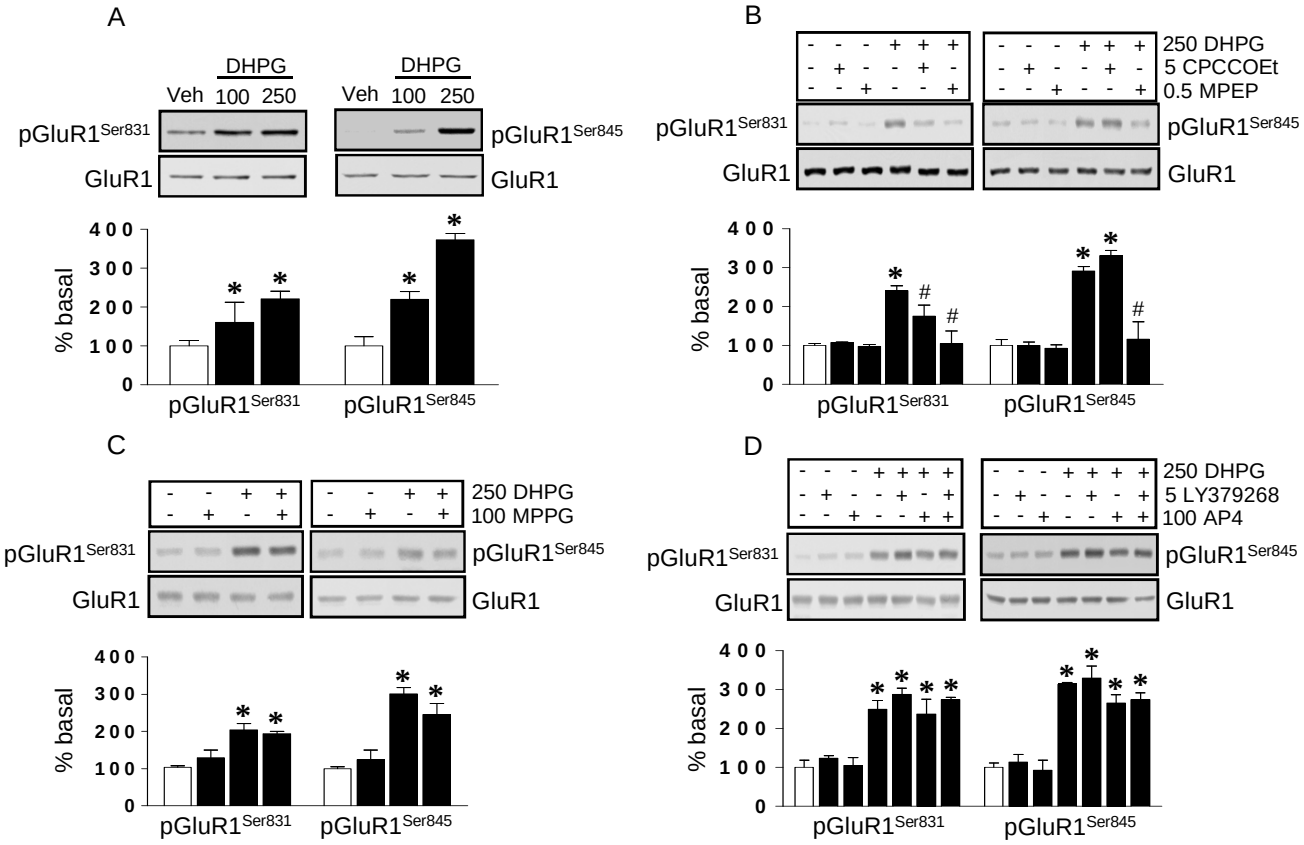

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Fig. 3.

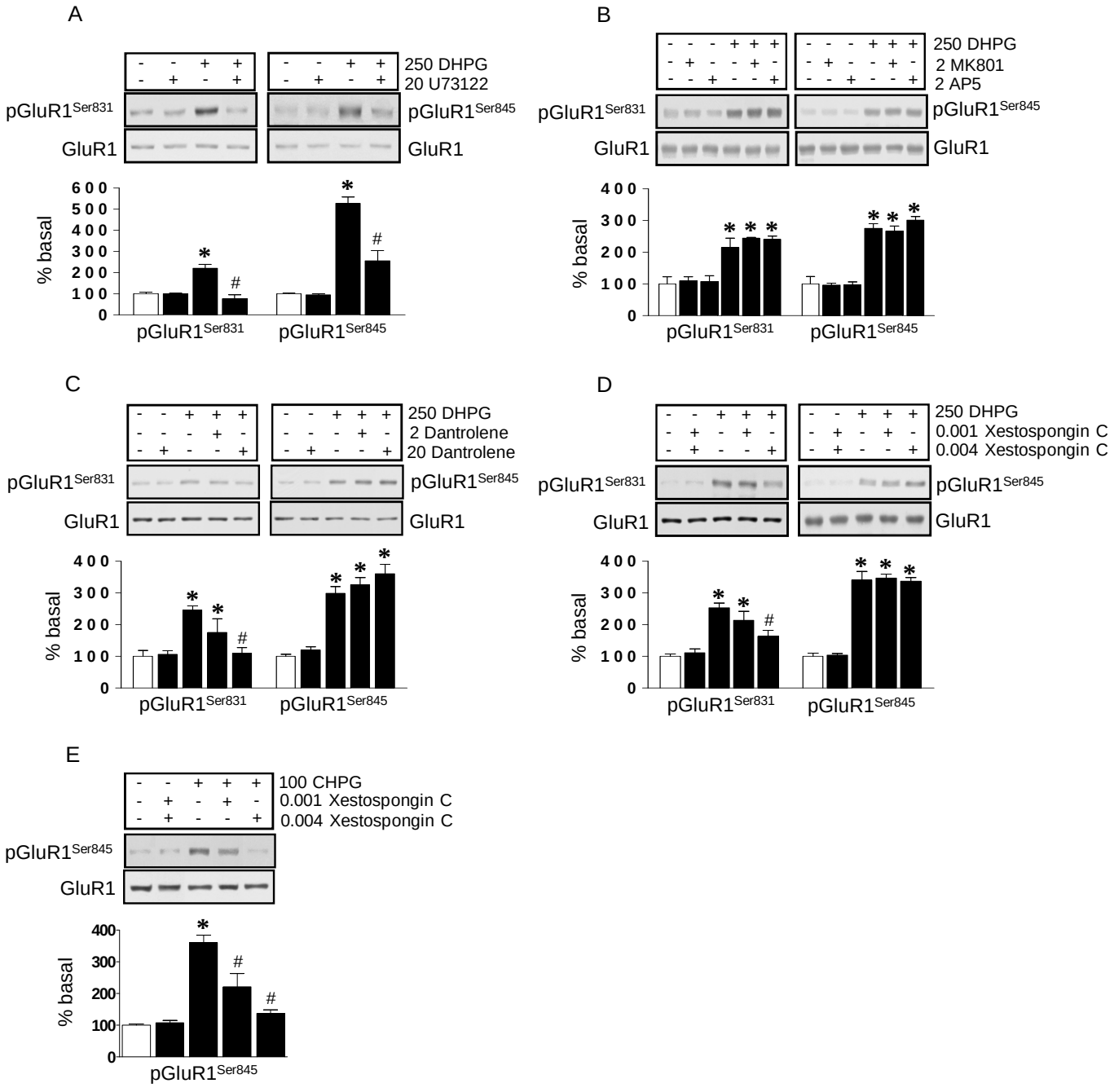

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Fig. 4.

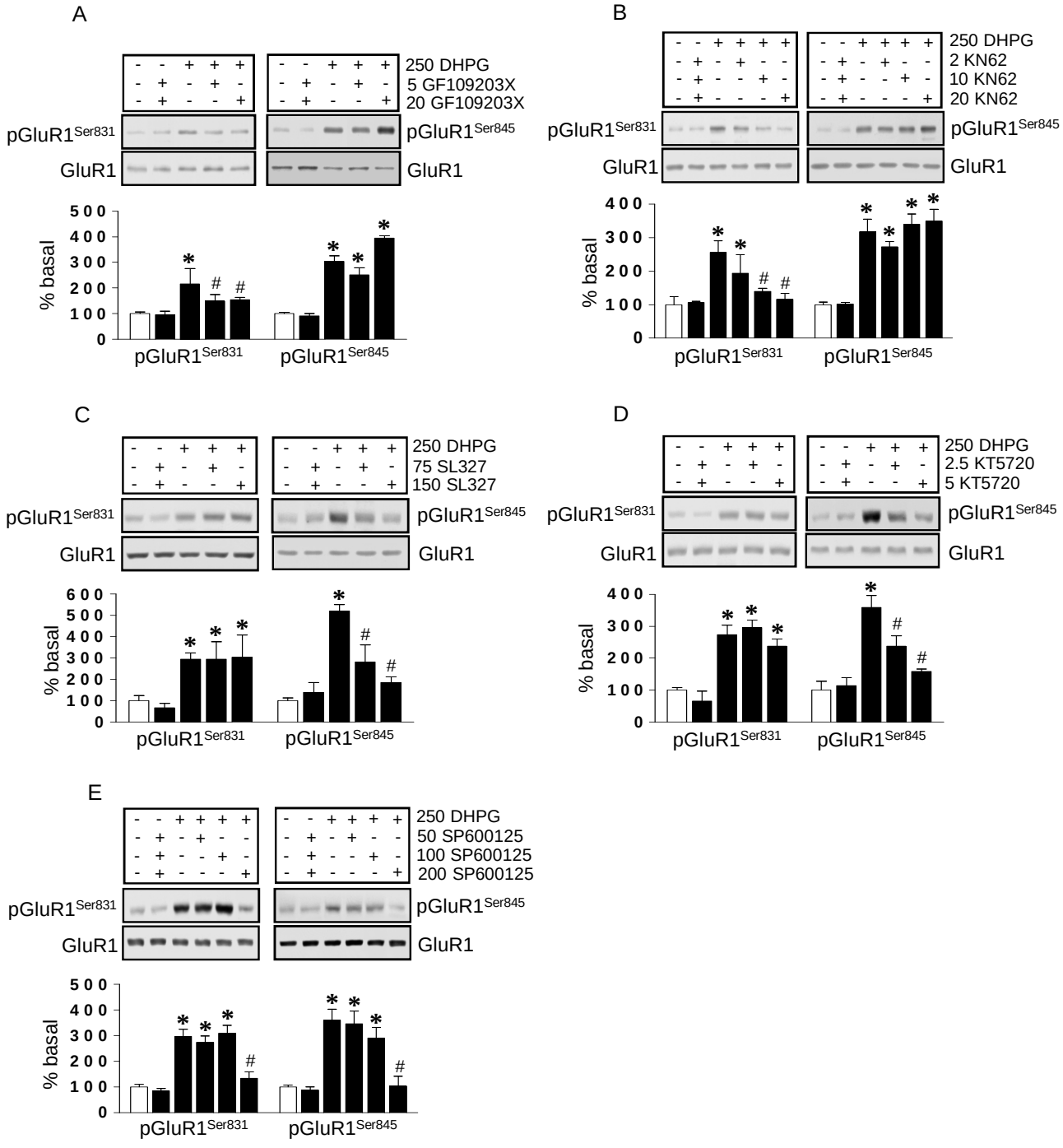

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Fig. 5.

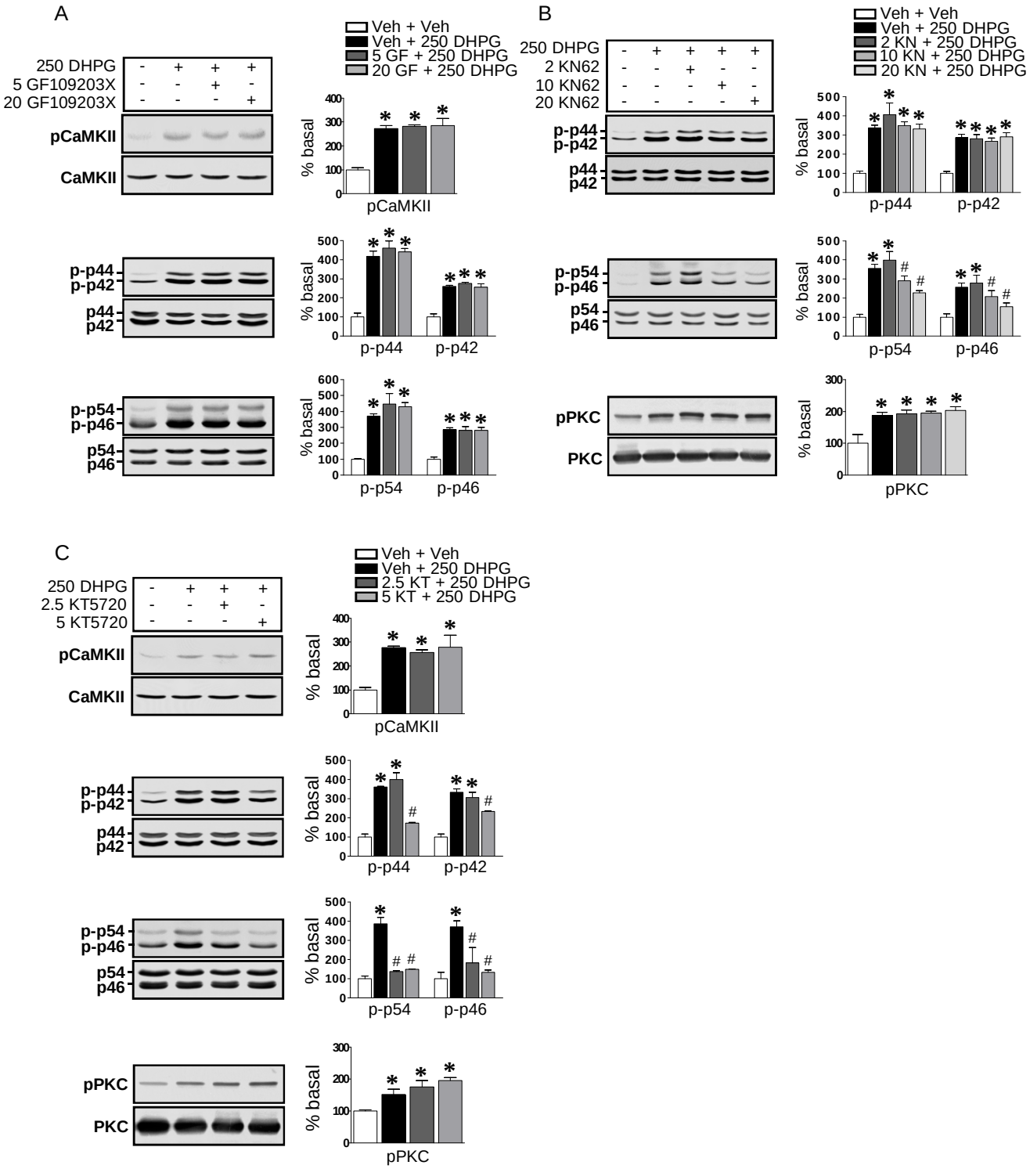

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Fig. 6.

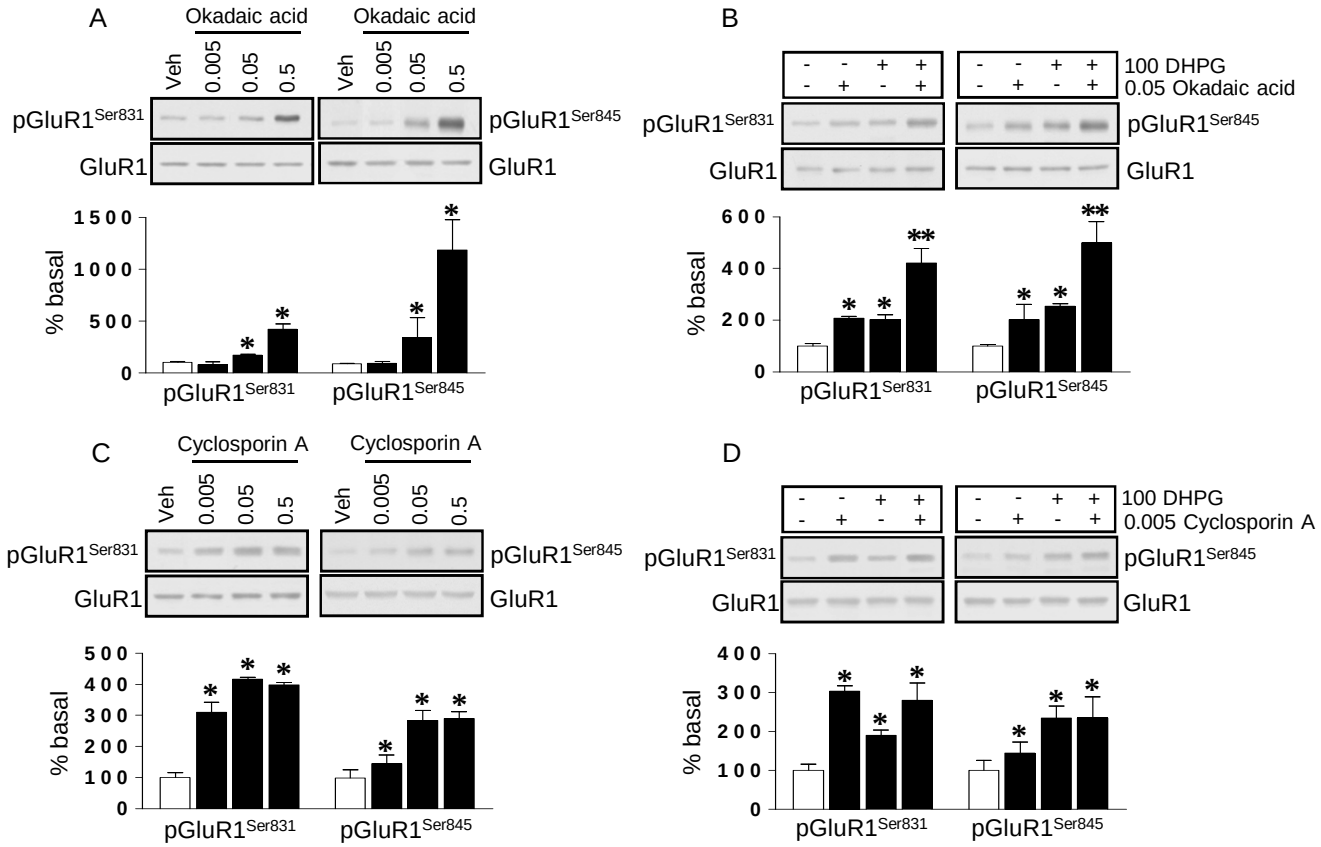

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Fig. 7.

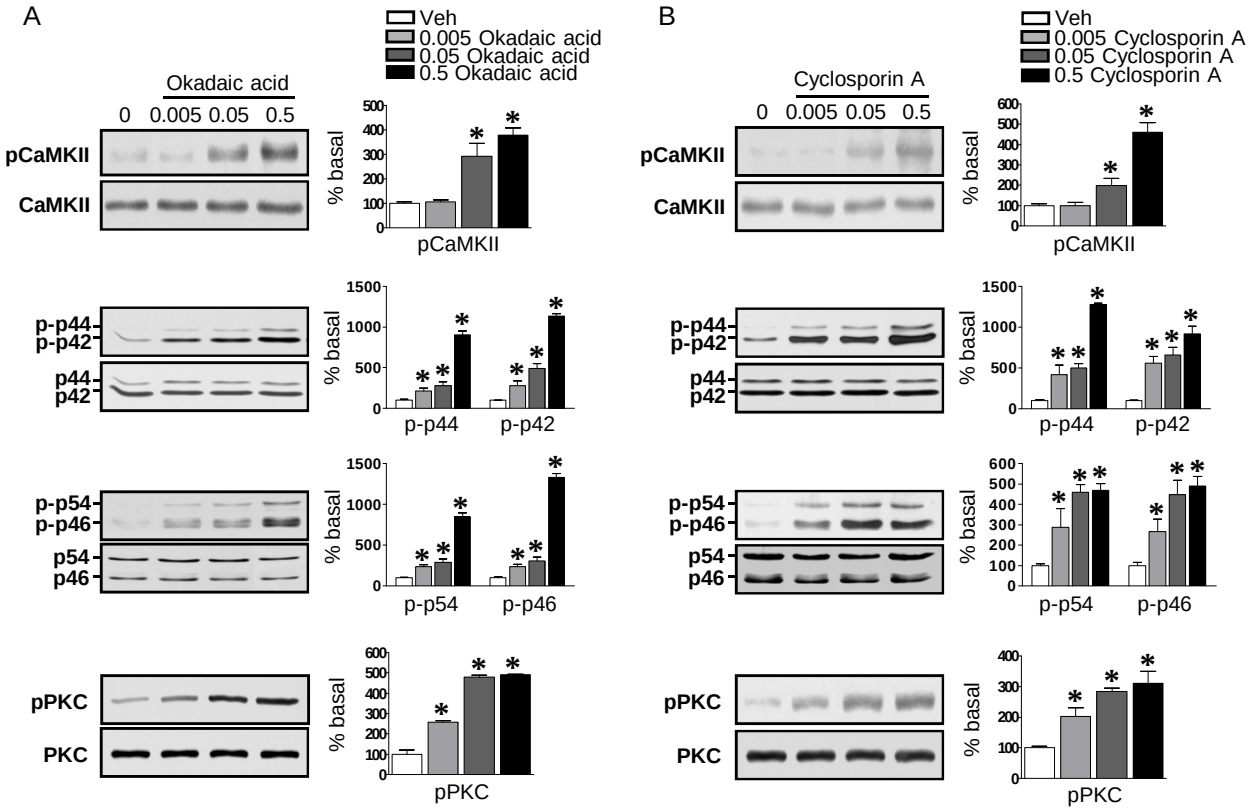

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Fig. 8.