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Author Manuscript

J Mol Neurosci. Author manuscript; available in PMC 2011 September 1.

Published in final edited form as:

J Mol Neurosci. 2010 September ; 42(1): 1–8. doi:10.1007/s12031-010-9338-9.

Activation of mGluR5 Induces Rapid and Long-Lasting Protein Kinase D Phosphorylation in Hippocampal Neurons

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Abstract

Metabotropic glutamate receptors (mGluRs), including mGluR5, play a central role in regulating the strength and plasticity of synaptic connections in the brain. However, the signaling pathways that connect mGluRs to their downstream effectors are not yet fully understood. Here, we report that stimulation of mGluR5 in hippocampal cultures and slices results in phosphorylation of protein kinase D (PKD) at the autophosphorylation site Ser-916. This phosphorylation event occurs within 30 s of stimulation, persists for at least 24 h, and is dependent on activation of phospholipase C and protein kinase C. Our data suggest that activation of PKD may represent a novel signaling pathway linking mGluR5 to its downstream targets. These findings have important implications for the study of the molecular mechanisms underlying mGluR-dependent synaptic plasticity.

Keywords

mGluR5; Protein kinase D; DHPG; MPEP; Hippocampus; Primary culture

Introduction

Metabotropic glutamate receptors (mGluRs) are widely expressed throughout the central nervous system (Conn 2003), where they play an important role in synaptic plasticity. Group I mGluRs, comprising mGluR1 and mGluR5, have been implicated in several forms of synaptic plasticity, including long-term depression of synaptic transmission (Oliet et al. 1997; Huber et al. 2000) as well as priming of long-term potentiation (Cohen and Abraham 1996). The signaling pathways linking mGluRs to the molecular effectors of synaptic plasticity are currently under active investigation. Group I mGluRs are G_q-coupled receptors that can signal both through the canonical G_{αq}-phospholipase C (PLC) pathway and through more recently identified G protein-independent mechanisms (Gerber et al. 2007). Signaling pathways that are reported to be activated by group I mGluR stimulation and that have been implicated in mGluR-dependent plasticity include ERK1/2, p38 MAPK, and the PI3K/Akt pathway, although the relative contributions of each of these pathways remain to be determined.

Protein kinase D (PKD) is a serine/threonine kinase that was originally described as an atypical isoform of protein kinase C (PKC), PKC μ (Johannes et al. 1994), but is now considered to belong to the Ca²⁺/calmodulin-dependent kinase superfamily based on

substrate specificity and sequence homology in the catalytic domain (Valverde et al. 1994; Rozengurt et al. 2005). PKD is expressed ubiquitously, and it has been implicated in functions as diverse as regulation of transport from the trans-Golgi network to the plasma membrane, cell motility and adhesion, cell proliferation and apoptosis, nuclear export of histone deacetylases, and oxidative stress (for reviews, see Rozengurt et al. 2005; Avkiran et al. 2008). In neurons, PKD in the Golgi was recently reported to play a role in dendritic trafficking and the establishment of neuronal polarity and dendritic complexity (Yin et al. 2008; Bisbal et al. 2008; Czondor et al. 2009). PKD is activated by binding of diacylglycerol (DAG) and phosphorylation by PKC at Ser-744 and Ser-748 (Iglesias et al. 1998), followed by autophosphorylation at Ser-916, which is believed to correlate with PKD catalytic activity (Matthews et al. 1999). A number of extracellular signals have been shown to induce PKD phosphorylation and activation, including bombesin, bradykinin, vasopressin, platelet-derived growth factor, and insulin-like growth factor (Rozengurt et al. 2005), underlining the importance of this pathway in a variety of cellular functions.

Here, we demonstrate that activation of mGluR5 using the group I mGluR-specific agonist 3,5-dihydroxyphenylglycine (DHPG) results in phosphorylation of PKD at Ser-916 in hippocampal cultures and slices. To our knowledge, this represents the first report of a link between glutamate receptors and PKD, and it raises the possibility that PKD may be one of the pathways important in mGluR-dependent plasticity.

Materials and Methods

Animals

Pregnant E18 Sprague-Dawley rats were obtained from Charles River Laboratories. C57BL/6J mice were bred at MIT. All animals were treated in accordance with NIH and MIT guidelines.

Pharmacological Activators and Inhibitors

The following drugs were used in this study (all from Tocris Biosciences unless stated otherwise): APV, BAPTA-AM, CNQX, (RS)-3,5-dihydroxyphenylglycine (DHPG), genistein, GF109203X, Go6976, LY367385, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), PP2, rapamycin, SB203580, tetrodotoxin (TTX), U0126, U73122, wortmannin, and xestospongin C (Sigma-Aldrich).

Primary Hippocampal Cultures

Primary hippocampal cultures were prepared from E18 rat embryos as previously described (Krueger and Nairn 2007). Briefly, hippocampi were isolated in ice-cold dissociation medium and digested for 1 h in 0.01% papain (Worthington). The tissue was triturated, and cells were collected by centrifugation and resuspended in plating medium (neurobasal medium containing B27 supplement, GlutaMax, 1% penicillin/streptomycin, and 10% fetal bovine serum, all from Invitrogen). Cells were plated at high density (250,000 cells per 3.8 cm² well) onto tissue culture plates coated with poly-L-lysine. On the following day, the medium was replaced with neurobasal medium containing all of the above supplements except fetal bovine serum, and cells were maintained in this medium at 37°C, 5% CO₂ for 21 days until use to allow synapse maturation.

Cell Culture Stimulation and Harvest

For stimulation, DHPG (or vehicle control) was added to a final concentration of 100 μM (unless otherwise indicated) and incubated at 37°C, 5% CO₂ for the indicated period of time. For experiments involving time points greater than 5 min after stimulation, the medium containing DHPG was removed after 5 min and replaced with medium without DHPG, and

cells were again incubated at 37°C, 5% CO₂ for the indicated period of time from the onset of stimulation. For experiments involving pre-incubation with inhibitors, the inhibitor (or vehicle control) was added 30 min prior to stimulation, and it remained present throughout the duration of the stimulation. To harvest cells for immunoblotting, plates were washed with ice-cold phosphate buffered saline, and cells were lysed by addition of 50-μl lysis buffer per well (1% SDS, 50 mM Tris, 1 mM EDTA, 1 mM EGTA, pH 7.4, containing freshly added protease and phosphatase inhibitor cocktails (EMD Chemicals) at a dilution of 1:100).

Slice Preparation and Stimulation

Hippocampal slices were prepared from male P26–P30 mice as previously described (Dolen et al. 2007). Briefly, hippocampi were rapidly dissected into ice-cold artificial cerebral spinal fluid (ACSF; in millimolar: NaCl, 124; KCl, 3; NaH₂PO₄, 1.25; NaHCO₃, 26; dextrose, 10; MgCl₂, 1.0; CaCl₂, 2, saturated with 95% O₂ and 5% CO₂). Acute hippocampal slices (500 μm) were prepared using a Stoelting Tissue Slicer and incubated for 4 h in 32.5°C ACSF. For stimulation, slices were incubated in 100 μM DHPG in ACSF for 5 min. They were then either homogenized in Laemmli sample buffer (for analysis of whole tissue homogenate) or processed further to obtain synaptoneurosomes as described below.

Synaptoneurosomes—Synaptoneurosomes were isolated from slices as described (Chen and Bear 2007). Briefly, slices were homogenized, passed through 2×105-μm meshes, then 1×5-μm mesh, and the resulting samples were spun at 1,000×g for 10 min at 4°C. The resulting pellets were then washed, spun at 1,000×g, and resuspended in Laemmli sample buffer containing protease and phosphatase inhibitors.

Immunoblotting

Lysates were processed for immunoblotting as previously described (Krueger and Nairn 2007). Briefly, samples were boiled in Laemmli sample buffer, resolved on 10% SDS-PAGE gels, transferred onto nitrocellulose membranes, and stained for total protein using a Memcode assay (Pierce, Rockford, IL, USA). Membranes were blocked in 5% fat-free milk and then incubated in primary antibody (phospho-PKD Ser-916 or total PKD, both from Cell Signaling Technology), followed by secondary antibody (goat anti-rabbit-IRDye680, Rockland Immunochemicals). Blots were scanned on an Odyssey Infrared Imager (LiCor Biosciences), and the signal intensity for each sample was quantified using the Odyssey 2.0 software. Each sample value was divided by the total protein loading value for the corresponding lane and then normalized to the average sample value of all lanes on the same blot to correct for blot-to-blot variance.

Statistical Analysis

Group comparisons for experiments involving time courses were performed using repeated measures analysis of variance (ANOVA) for time and stimulation. Group comparisons for the DHPG dose–response curve were performed using one-way ANOVA for stimulation. Group comparisons for experiments involving inhibitors were performed using two-way ANOVA for stimulation and inhibitor. Post-hoc analysis was performed using two-tailed Student's *t* tests. Group comparisons for the slice and synaptoneurosome experiments were performed using two-tailed paired Student's *t* tests. All data are expressed as mean ± SEM.

Results and Discussion

Stimulation of Hippocampal Cultures with DHPG Induces Phosphorylation of PKD

To determine whether activation of group I mGluRs can result in phosphorylation of PKD, primary hippocampal cultures were stimulated with the group I mGluR agonist DHPG, and they were subsequently assessed for PKD phosphorylation by immunoblotting. Since the intention of this study was to investigate signaling pathways that may contribute to mGluR-mediated plasticity, a stimulation paradigm was chosen that was previously shown to induce long-term depression in hippocampal slices (Huber et al. 2001). Specifically, cultures were incubated with 100 μ M DHPG for 5 min, and the DHPG-containing medium was then removed and replaced with DHPG-free medium, followed by a further incubation period that lasted for up to 60 min from the onset of stimulation. This stimulation paradigm resulted in a robust increase in phosphorylation of PKD at the autophosphorylation site Ser-916 (Fig. 1a), which is thought to correlate with PKD catalytic activity (Matthews et al. 1999). A doublet band was observed by immunoblot, corresponding to phosphorylation of the two isoforms PKD1 and PKD2. Since the two bands were affected in a similar manner by DHPG stimulation, they were quantified together to yield an overall measure of PKD phosphorylation. The DHPG-induced PKD phosphorylation was greatest at 5 min, but lasted for at least 60 min after the onset of stimulation (Fig. 1a, b; repeated measures ANOVA for treatment, $p < 0.0001$; treatment \times time interaction, $p < 0.05$). Interestingly, total levels of PKD1 showed a slight but significant decrease at 5 min after DHPG stimulation (Fig. 1a, c; repeated measures ANOVA for treatment, $p < 0.05$; treatment \times time interaction, not significant). Since the magnitude of the decrease was negligible compared to the increase in PKD phosphorylation, it was not further considered in this study.

DHPG-Induced PKD Phosphorylation Is Rapid and Sustained

Since PKD phosphorylation was already elevated to 1,000% of baseline after 5 min of DHPG stimulation, the first time point previously measured, a shorter time course was conducted to further investigate the onset of phosphorylation. To this end, cultures were stimulated for 30 s, 1, 2.5, and 5 min with 100 μ M DHPG. Even after 30 s, PKD phosphorylation levels were increased almost 400%, and they continued to rise across the time course assessed (Fig. 1d; repeated measures ANOVA for treatment, $p < 0.0001$; treatment \times time interaction, $p < 0.0001$). This suggests that the onset of PKD phosphorylation, and hence group I mGluR activation, occurs extremely rapidly in response to DHPG stimulation.

Since PKD phosphorylation was still elevated 60 min after a brief DHPG application, a more extended time course was conducted to investigate how long this phosphorylation would last. Cultures were stimulated with 100 μ M DHPG for 5 min, followed by a DHPG-free period lasting for up to 24 h. As observed previously, PKD phosphorylation was robustly increased to \sim 750% of baseline 1 h after stimulation. By 3 h after stimulation, phosphorylation levels were reduced to \sim 250% of baseline, and they were sustained at this level for up to 24 h (Fig. 1e; repeated measures ANOVA for treatment, $p < 0.0001$; treatment \times time interaction, $p < 0.0001$). These results imply that either group I mGluRs remain active for 24 h after stimulation, possibly due to low levels of DHPG remaining bound to the receptors, or that PKD phosphorylation is maintained independently of mGluR activation.

PKD Phosphorylation Is Highly Sensitive Even to Low Concentrations of DHPG

The DHPG concentration applied in the initial experiments, 100 μ M, was chosen based on previous studies using DHPG to induce mGluR-dependent synaptic plasticity (Huber et al. 2001). However, 50 and 10 μ M are also commonly reported to induce long-term depression

and priming of long-term potentiation, respectively (Huber et al. 2001; Mellentin et al. 2007). To establish a dose–response curve for DHPG-mediated PKD phosphorylation, cultures were stimulated with control, 1, 10, 50, or 100 μM DHPG for 5 min. Even at 1 μM DHPG, a trend toward an increase in PKD phosphorylation was observed, and 10 μM DHPG resulted in close to maximal phosphorylation (Fig. 1f; oneway ANOVA for stimulation, $p < 0.0001$; post-hoc analysis, control vs. 1 μM , $p = 0.081$, control vs. 10, 50, and 50 μM , $p < 0.0001$). These data indicate that PKD phosphorylation is highly sensitive even to low levels of group I mGluR activation. For the sake of consistency, however, all further experiments were performed at the same 100- μM DHPG dose used in the initial characterization.

DHPG-Induced Phosphorylation of PKD Occurs Through mGluR5 but not mGluR1

DHPG is a general agonist of group I mGluRs, including mGluR1 and mGluR5. In order to identify which of these receptors mediates the observed PKD phosphorylation, cultures were pre-incubated with either a specific antagonist of mGluR1 (LY367385, 50 μM) or of mGluR5 (MPEP, 2 μM), and they were then stimulated with DHPG for 5 min. Pre-incubation with the mGluR5 antagonist MPEP completely blocked DHPG-induced PKD phosphorylation (Fig. 2a and Table 1; two-way ANOVA for inhibitor, $p < 0.0001$; inhibitor \times stimulation interaction, $p < 0.0001$; post-hoc analysis MPEP/control vs. MPEP/DHPG, $p = 0.115$). Conversely, pre-incubation with the mGluR1 antagonist LY367385 had no effect of DHPG-induced PKD phosphorylation (Fig. 2b and Table 1; two-way ANOVA for inhibitor, $p = 0.916$; inhibitor \times stimulation interaction, $p = 0.474$). These data suggest that DHPG-induced stimulation of PKD phosphorylation occurs through mGluR5, not mGluR1.

DHPG-Induced Phosphorylation of PKD Is Dependent on Phospholipase C Activation

One question arising from the above observations concerns the signaling pathways linking mGluR5 stimulation to PKD phosphorylation. Group I mGluRs are G-protein coupled receptors that can signal through a canonical G_{α_q} -phospholipase C β (PLC β) signaling pathway, but also through more recently identified alternative pathways (Gerber et al. 2007). To determine whether PLC β is required for DHPG-induced PKD phosphorylation, cultures were pre-incubated with a PLC inhibitor (U73122, 50 μM), followed by DHPG stimulation for 5 min. Pre-incubation with the PLC inhibitor resulted in a significant reduction in DHPG-induced PKD phosphorylation (Fig. 2c and Table 1; two-way ANOVA for inhibitor, $p < 0.0001$; inhibitor \times stimulation interaction, $p < 0.0001$), although a small DHPG-induced increase was still detected in the presence of the inhibitor (post-hoc analysis U73122/control vs. U73122/DHPG, $p < 0.0001$). This may be due either to incomplete inhibition of PLC β by U73122, or to the activation of alternative signaling pathways that bypass the G_{α_q} -PLC β pathway.

DHPG-Induced Phosphorylation of PKD Is Dependent on PKC but not Calcium Signaling

Activation of PLC β results in production of the second messengers IP $_3$ and DAG, which in turn lead to release of calcium and activation of PKC. To test whether either calcium release or PKC activation were required for DHPG-stimulated PKD phosphorylation, cultures were pre-incubated with a calcium chelator (BAPTA-AM, 10 μM), an inhibitor of IP $_3$ R-mediated calcium release (xestospongin C, 1 μM), or one of two PKC inhibitors (GF109203X and Go6976, 10 μM each). Of these, the PKC inhibitor GF109203X most potently reduced DHPG-induced PKD phosphorylation (Fig. 2d and Table 1; two-way ANOVA for inhibitor, $p < 0.0001$; inhibitor \times stimulation interaction, $p < 0.0001$), although as with the PLC inhibitor, a small DHPG-induced increase was still detected (post-hoc analysis GF109203X/control vs. GF109203X/DHPG, $p < 0.01$). The PKC inhibitor Go6976 also significantly, but not completely, reduced DHPG-induced PKD phosphorylation (Table 1; two-way ANOVA for inhibitor, $p < 0.0001$; inhibitor \times stimulation interaction, $p < 0.0001$; post-hoc analysis Go6976/

control vs. Go6976/DHPG, $p < 0.0001$). By contrast, neither the calcium chelator BAPTA-AM nor the IP₃R inhibitor xestospongine C decreased DHPG-induced PKD phosphorylation. In fact, there was a slight increase in PKD phosphorylation in the presence of BAPTA-AM (Table 1; two-way ANOVA for inhibitor, $p < 0.05$; inhibitor \times stimulation interaction, $p = 0.203$), whereas xestospongine C had no effect (Table 1; two-way ANOVA for inhibitor, $p = 0.547$; inhibitor \times stimulation interaction, $p = 0.116$). These data suggest that activation of PKC, but not calcium release, is required for DHPG-induced PKD phosphorylation.

DHPG-Induced Phosphorylation of PKD Is Modulated by ERK1/2 and Possibly p38 MAPK Pathways

It is possible that the slight increase in PKD phosphorylation remaining in the presence of PLC and PKC inhibitors may occur indirectly through other pathways activated by mGluR5 stimulation. A number of pathways have been reported to lie downstream of group I mGluRs, including the MEK/ERK1/2 pathway, the p38 MAPK pathway, the PI3K/Akt/mTOR pathway, and Src and other tyrosine kinases (Gerber et al. 2007). To test this hypothesis, cultures were pre-incubated with inhibitors to each of these targets prior to DHPG stimulation (Table 1). None of the inhibitors significantly reduced DHPG-induced PKD phosphorylation. This suggests that the remaining PKD phosphorylation is not due to activation of any of these individual pathways (although we cannot rule out that a combination of pathways may be involved). Interestingly, however, the MEK inhibitor U0126 (10 μ M) caused a significant enhancement of DHPG-induced PKD phosphorylation, implying that the MEK/ERK1/2 pathway modulates PKD phosphorylation through a feedback mechanism following group I mGluR stimulation (two-way ANOVA for inhibitor, $p < 0.05$; inhibitor \times stimulation interaction, $p < 0.05$; post-hoc analysis control/DHPG vs. U0126/DHPG, $p < 0.05$). Similarly, a trend towards an increase was observed in the presence of the p38 MAPK inhibitor SB203580 (10 μ M, two-way ANOVA for inhibitor, $p = 0.297$; inhibitor \times stimulation interaction, $p = 0.087$; post-hoc analysis control/DHPG vs. SB203580/DHPG, $p < 0.05$). In contrast, the PI3K inhibitor wortmannin (1 μ M), the mTOR inhibitor rapamycin (1 μ M), the Src inhibitor PP2 (25 μ M), and the general tyrosine kinase inhibitor genistein (100 μ M) had no significant effect.

DHPG-Induced PKD Phosphorylation Is not Dependent on Network Activity or Activation of Ionotropic Glutamate Receptors

One consequence of the activation of mGluR5 is an increase in neuronal excitability and network activity (Netzeband et al. 1997; Ireland and Abraham 2002), and it is possible that PKD phosphorylation is an indirect result of this increase in network activity in the hippocampal culture, rather than being caused directly by PLC β and PKC activation. To investigate this possibility, cultures were pre-incubated with an inhibitor of the sodium channels required for the generation of action potentials (TTX, 1 μ M), an inhibitor of AMPA/kainate-type ionotropic glutamate receptors (CNQX, 50 μ M), or an inhibitor of the NMDA-type ionotropic glutamate receptors (APV, 100 μ M), all of which are known to contribute to neuronal network activity. However, no significant effect on DHPG-induced PKD phosphorylation was observed with any of these inhibitors (Table 1), suggesting that PKD phosphorylation is likely to be a direct consequence of PLC β and PKC activation, rather than an indirect effect of increased network activity.

In Hippocampal Slices, mGluR-Induced PKD Phosphorylation May Occur Specifically at Synapses

To determine whether DHPG-induced PKD phosphorylation is a phenomenon specific to dissociated neurons, or whether it also occurs in more structurally intact preparations, PKD phosphorylation was assessed in acute hippocampal slices. Slices were stimulated for 5 min with 100 μ M DHPG, and PKD phosphorylation levels were subsequently analyzed in whole

tissue homogenate and in synaptoneurosome, a preparation used to isolate the synaptic component of the signal. Interestingly, unlike in dissociated cultures, no increase in PKD phosphorylation was observed at the whole homogenate level (Fig. 3a; Student's *t* test, not significant). However, PKD phosphorylation was significantly increased in synaptoneurosome following DHPG stimulation, albeit at a substantially lower level than that observed in dissociated cultures (Fig. 3b; Student's *t* test, $p < 0.05$). These data suggest that group I mGluR stimulation of PKD phosphorylation is not unique to dissociated neurons, but that in an intact network, this phosphorylation may occur specifically in the synaptic compartment and may be restricted in magnitude by the local cellular environment.

Conclusions

In this study, we report for the first time that stimulation of group I mGluRs in hippocampal neurons results in phosphorylation of PKD at a site thought to correlate with PKD catalytic activity. Together, our data provide an important basis for further investigation into the role of PKD in mGluR5-mediated plasticity. Given the known cellular functions of PKD, there are several potential mechanisms by which PKD may effect the molecular alterations induced by mGluR5 activation. PKD is a regulator of polarized membrane trafficking, and trafficking of glutamate receptors to and from the plasma membrane is a central element of synaptic plasticity (Malenka and Bear 2004). In addition, PKD plays a role in regulating transcription through its effect on histone deacetylases and stimulation of mGluRs results in transcriptional activation (Gerber et al. 2007) that could potentially be enhanced by concomitant PKD-mediated alterations in chromatin structure. Detailed studies will be necessary to identify how these and other potential mechanisms may link mGluR5-induced PKD phosphorylation to synaptic plasticity or related mGluR-mediated cellular functions.

Acknowledgments

We would like to thank Kathleen Oram, Zachary Cohen, Erik Sklar, and Suzanne Meagher for excellent technical and administrative assistance. This work was supported by grants from HHMI, FRAXA, NIMH, NICHD, and the Simons Foundation.

Abbreviations

ACSF	Artificial cerebrospinal fluid
ANOVA	Analysis of variance
DAG	Diacylglycerol
DHPG	3,5-Dihydroxyphenylglycine
mGluR	Metabotropic glutamate receptor
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C

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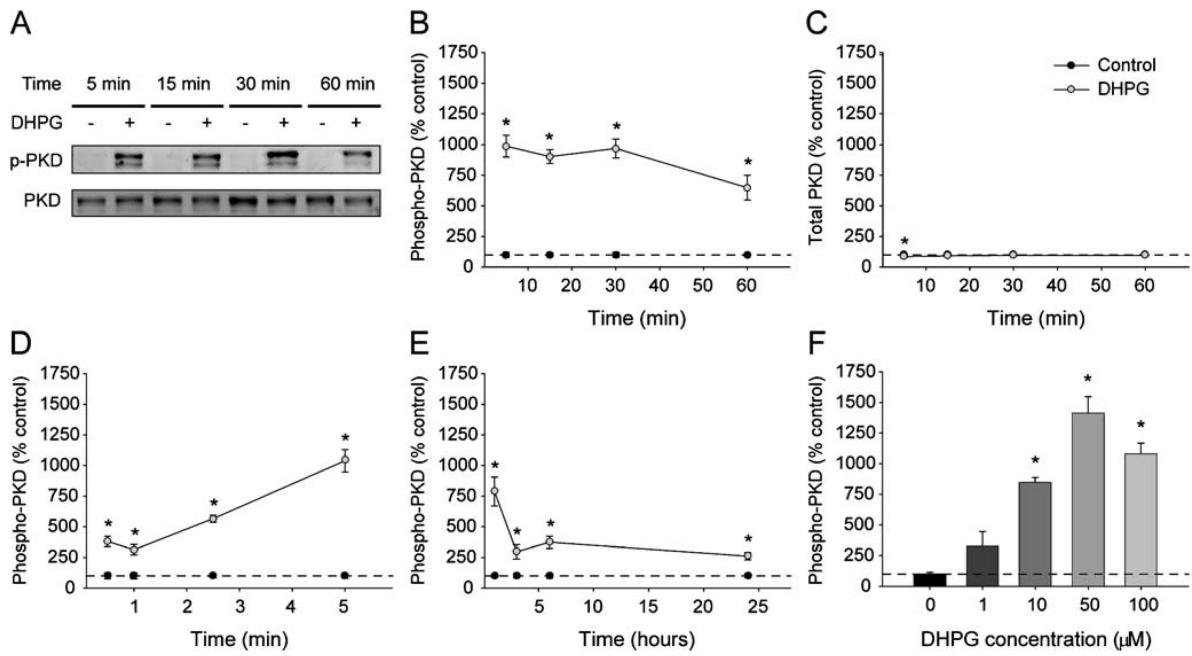


Figure 1.

DHPG stimulation induces phosphorylation of PKD that is rapid and long lasting. Hippocampal neurons were stimulated with DHPG for 5 min, followed by incubation with DHPG-free medium for the indicated periods of time. **a** Immunoblot of phospho-PKD (Ser-916) and total PKD at 5, 15, 30, and 60 min after onset of stimulation with 100 μM DHPG. **b** Quantification of PKD phosphorylation at 5, 15, 30, and 60 min after onset of stimulation with 100 μM DHPG (gray circles) or control (black circles)(n=8). **c** Quantification of total PKD levels at 5, 15, 30, and 60 min after onset of stimulation with DHPG or control. **d** Quantification of PKD phosphorylation at 0.5, 1, 2.5, and 5 min after onset of stimulation with 100 μM DHPG (gray circles) or control (black circles)(n=8). **e** Quantification of PKD phosphorylation at 1, 3, 6, and 24 h after onset of stimulation with 100 μM DHPG (gray circles) or control (black circles)(n=10). **f** Quantification of PKD phosphorylation at 5 min after stimulation with control, 1, 10, 50, or 100 μM DHPG (n=7). All data are expressed as percent control at each time point. The symbol *asterisk* indicates a significant difference between control- and DHPG-treated samples. *Error bars* indicate SEM

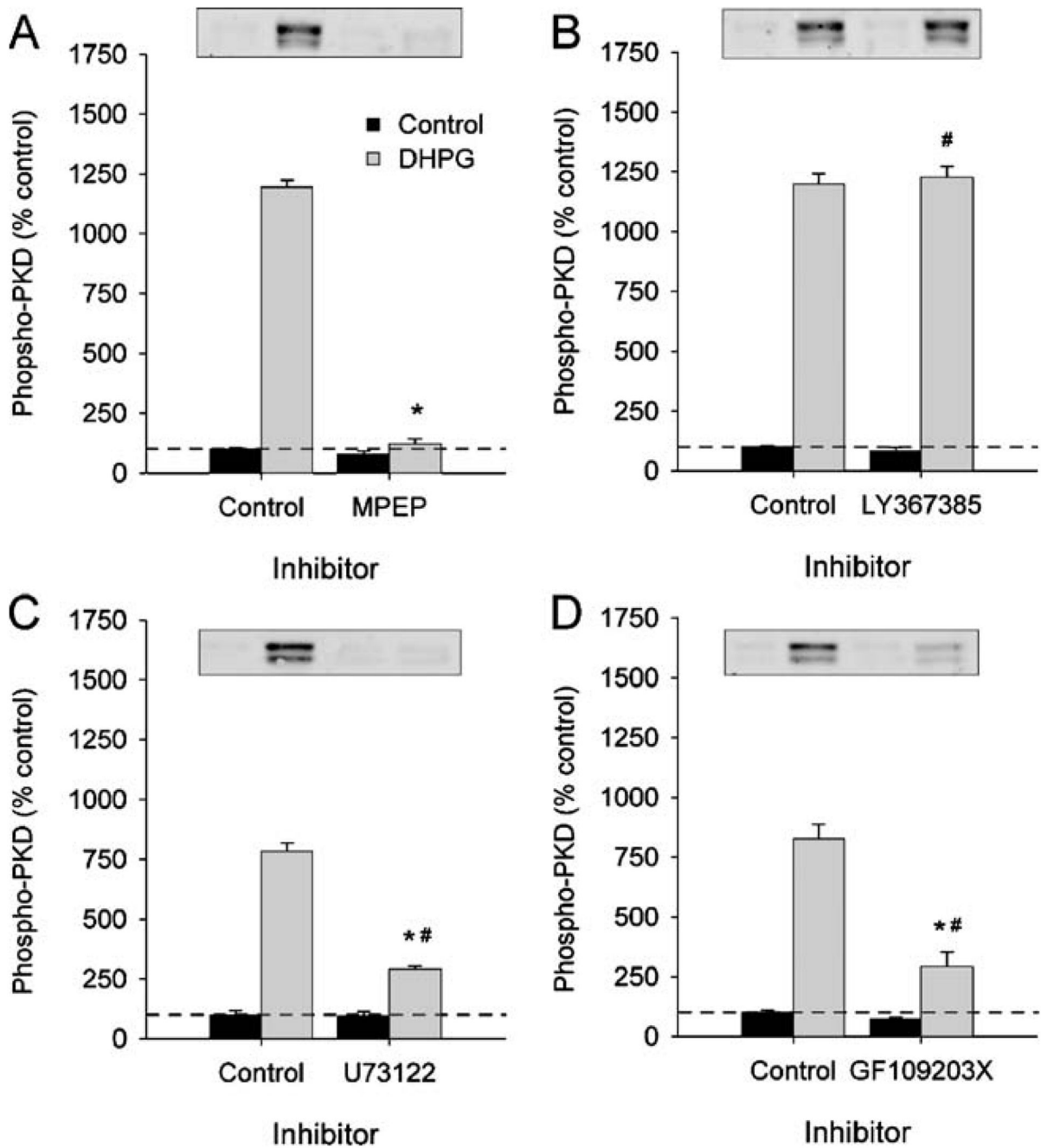


Figure 2. DHPG-induced PKD phosphorylation is dependent on activation of mGluR5, PLC, and PKC, but not mGluR1. Hippocampal neurons were pre-incubated for 30 min with inhibitors of a mGluR5 (MPEP, $n=7$), **b** mGluR1 (LY367385, $n=7$), **c** PLC (U73122, $n=6$), and **d** PKC (GF109203X, $n=7$), followed by stimulation with 100 μ M DHPG for 5 min. *Black bars* represent control-treated samples, and *gray bars* represent DHPG-treated samples. All data are expressed as percent control/control (exact values are reported in Table 1). The symbol *asterisk* indicates a significant difference between control/DHPG- and inhibitor/DHPG-treated samples. The *number sign* indicates a significant difference between inhibitor/control- and inhibitor/DHPG-treated samples. *Error bars* indicate SEM

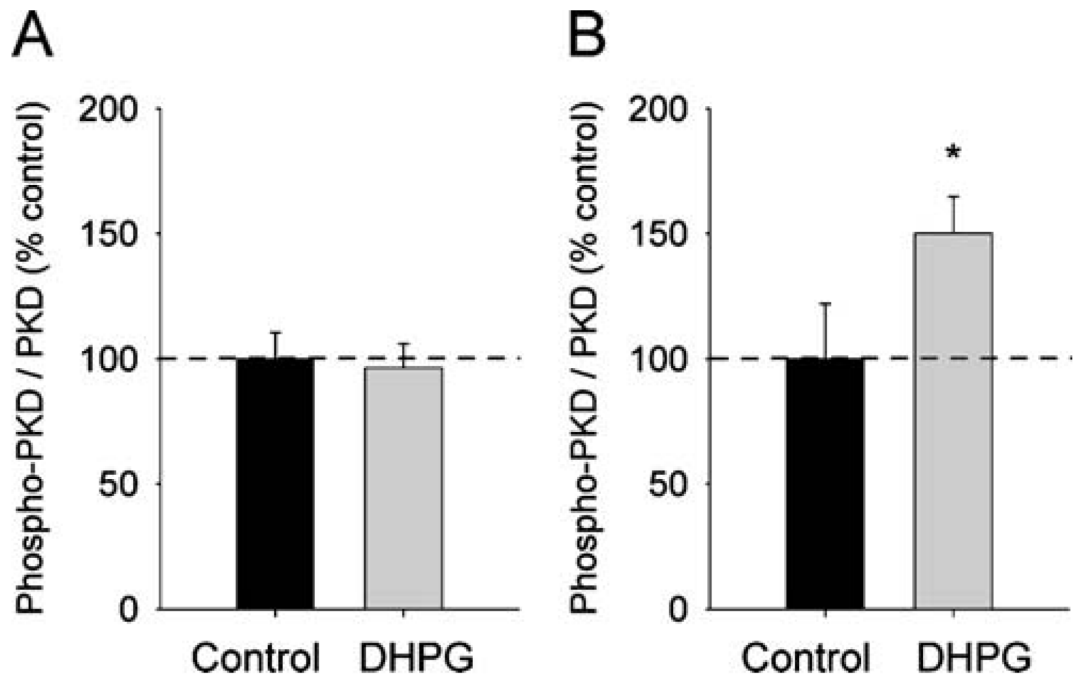


Figure 3.

DHPG-induced PKD phosphorylation in hippocampal slices is restricted to the synaptic compartment. Hippocampal slices were stimulated with 100 μ M DHPG for 5 min, then analyzed for PKD phosphorylation in **a** whole tissue slices ($n=6$) and **b** synaptoneurosomes ($n=6$). *Black bars* represent control-treated samples; *gray bars* represent DHPG-treated samples. All data are expressed as percent control. The symbol *asterisk* indicates a significant difference between control- and DHPG-treated samples. *Error bars* indicate SEM

Table 1

Summary data for inhibitor experiments

Inhibitor	Target	Concentration (µM)	n	Phospho-PKD levels			Statistics		
				Control/control	Control/DHPG	Inhibitor/Control	Inhibitor/DHPG	ANOVA	Post-hoc
MPEP	mGluR5	2	7	100±5.3	1193.2±29.7	77.0±12.8	120.3±22.1	1, 2, 3	1
LY367385	mGluR1	50	7	100±4.8	1198.6±43.4	80.2±16.9	1226.3±44.3	1	2
U73122	PLC	50	6	100±15.0	783.8±34.2	93.8±19.0	291.0±12.9	1, 2, 3	1, 2
GFI09203X	PKC	10	7	100±10.4	825.1±61.5	70.6±8.5	291.5±61.3	1, 2, 3	1, 2
G66976	PKC	10	7	100±12.0	804.7±26.1	86.3±9.6	552.5±21.9	1, 2, 3	1, 2
BAPTA-AM	Calcium	10	5	100±34.1	1286.2±72.1	164.0±48.0	1539.9±108.4	1, 2	2
Xestosopogin C	IP ₃ R	1	5	100±33.8	1291.4±99.6	168.0±40.9	1143.3±64.6	1	2
U0126	MEK1/2	10	7	100±12.9	888.5±26.6	116.3±16.9	1121.2±93.1	1, 2, 3	1, 2
SB203580	p38 MARK	10	7	100±12.9	888.5±26.6	79.1±15.7	978.6±26.3	1	1, 2
Wortmannin	PI3K	1	7	100±12.9	888.5±26.6	73.1±23.6	828.6±44.0	1	2
Rapamycin	mTOR	1	5	100±16.5	992.9±156.1	92.7±14.0	918.3±73.5	1	2
PP2	Src	25	5	100±12.6	623.8±43.4	83.2±20.4	636.9±36.8	1	2
Genistein	Tyr kinases	100	5	100±12.6	623.8±43.4	83.0±30.4	695.9±62.5	1	2
TTX	Na ⁺ channel	1	5	100±24.6	1297.2±67.0	151.9±18.9	1156.2±101.5	1	2
CNQX	AMPA	50	5	100±24.6	1297.2±67.0	106.9±25.8	1341.9±137.4	1	2
APV	NMDAR	100	5	100±24.6	1297.2±67.0	116.2±9.0	1369.6±37.4	1	2

Hippocampal neurons were pre-incubated for 30 min with inhibitors as indicated, followed by stimulation with 100 µM DHPG for 5 min. All data are expressed as percent control/control. For the ANOVA statistics, "1" indicates a significant main effect of stimulation, "2" indicates a significant main effect of inhibitor, and "3" indicates a significant stimulation×inhibitor interaction. For the post-hoc tests, "1" indicates a significant difference between control/DHPG- and inhibitor/DHPG-treated samples, and "2" indicates a significant difference between inhibitor/control- and inhibitor/DHPG-treated samples