Cellular/Molecular

Phosphorylation and Feedback Regulation of Metabotropic Glutamate Receptor 1 by Calcium/Calmodulin-Dependent Protein Kinase II

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The metabotropic glutamate receptor 1 (mGluR1) is a $G\alpha_q$-protein-coupled receptor and is distributed in broad regions of the mammalian brain. As a key element in excitatory synaptic transmission, the receptor regulates a wide range of cellular and synaptic activities. In addition to regulating its targets, the receptor itself is believed to be actively regulated by intracellular signals, although underlying mechanisms are essentially unknown. Here we found that a synapse-enriched protein kinase, Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα), directly binds to the intracellular C terminus (CT) of mGluR1a. This binding is augmented by Ca^{2+} *in vitro*. The direct interaction promotes CaMKIIα to phosphorylate mGluR1a at a specific threonine site (T871). In rat striatal neurons, the mGluR1 agonist triggers the receptor-associated phosphoinositide signaling pathway to induce Ca^{2+}-dependent recruitment of CaMKIIα to mGluR1a–CT. This enables the kinase to inhibit the response of the receptor to subsequent agonist exposure. Our data identify an agonist-induced and Ca^{2+}-dependent protein–protein interaction between a synaptic kinase and mGluR1, which constitutes a feedback loop facilitating desensitization of mGluR1a.

Introduction

ʟ-Glutamate, a major neurotransmitter in the mammalian brain, interacts with both ionotropic and metabotropic glutamate receptors (mGluRs) to modulate a variety of cellular and synaptic activities (Traynelis et al., 2010). In the G-protein-coupled mGluR family, eight subtypes of mGluRs (mGluR1–mGluR8) have been cloned so far. Based on distinct pharmacological properties and postreceptor signaling, these mGluR subtypes have been classified into three functional groups (Niswender and Conn, 2010; Traynelis et al., 2010). Group I mGluRs, i.e., mGluR1 and mGluR5 subtypes, have drawn the most attention. As $G\alpha_q$-coupled receptors, mGluR1/5 during activation stimulate phospholipase Cβ1 (PLCβ1) to hydrolyze a lipid signaling molecule, phosphoinositide (PI). This yields diacylglycerol (DAG), an activator of protein kinase C (PKC), and inositol-1,4,5-triphosphate (IP$_3$), which releases Ca^{2+} from internal stores. Released Ca^{2+} ions could then trigger or modulate various downstream signaling pathways. Group I mGluRs are distributed in broad brain areas, including a key basal ganglia structure, the

striatum (Testa et al., 1994; Tallaksen-Greene et al., 1998). As such, these receptors actively regulate normal and abnormal synaptic activities related to motor, neuropsychiatric, neurodegenerative, and cognitive disorders (Traynelis et al., 2010; Nicoletti et al., 2011).

The mGluR1 is the first member of the mGluR family. Like typical G-protein-coupled receptors (GPCRs), mGluR1 is anchored in membranes by seven transmembrane helices. The intracellular C terminus (CT) is particularly large in the long-form splice variant (1a; 359 aa) relative to short-form variants (1b, 1c, and 1d; 57–72 aa). This renders mGluR1a a unique accessibility by various cytosolic binding partners (Enz, 2007, 2012; Fagni, 2012). Through CT interactions, these interacting partners scaffold the receptor at specific subcellular domains and modulate expression and signaling of the receptor (Enz, 2012; Fagni, 2012).

One modulation of mGluR1a by interacting partners is phosphorylation. In this modification, a given protein kinase interacts with mGluR1a CT and phosphorylates specific residue(s) to regulate mGluR1a (for review, see Mao et al., 2011). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine kinase enriched at synaptic sites (Kennedy et al., 1983; Kelly et al., 1984). This kinase is activated by the binding of Ca^{2+}-activated calmodulin (CaM). Active CaMKII then accesses and phosphorylates its specific substrates, while at the same time it catalyzes intersubunit autophosphorylation (T286 in the α isoform). Autophosphorylation sustains the Ca^{2+}/CaM-independent (autonomous) activity even after the initial Ca^{2+} stimulus subsides (Miller and Kennedy, 1986; Hudmon and Schulman, 2002; Colbran and Brown, 2004; Griffith, 2004), by which CaMKII translates Ca^{2+} transients to the relatively prolonged regulation of its substrates.
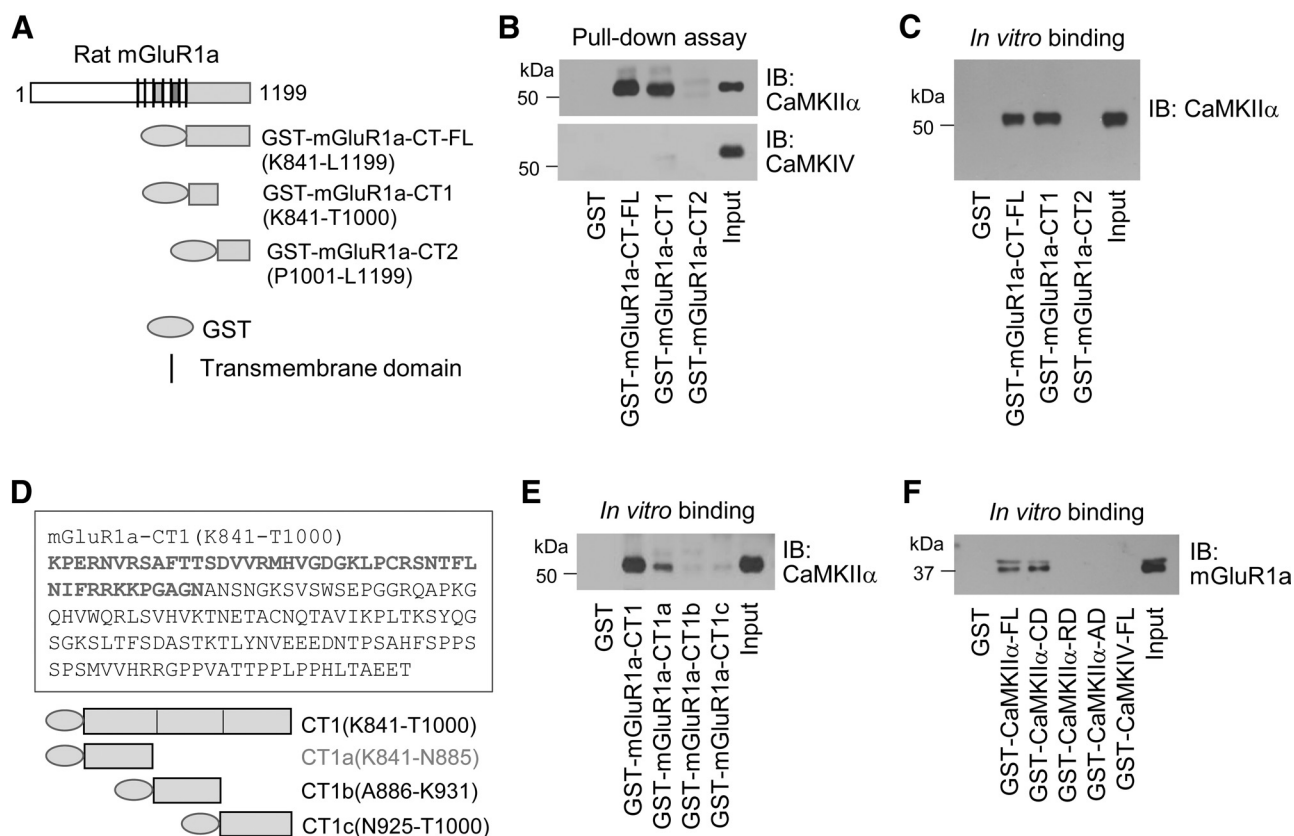
Figure 1. CaMKIIα binds to mGluR1a. **A**, GST-fusion proteins containing FL or fragments of CT of rat mGluR1a. **B**, Pull-down assays with immobilized GST-fusion proteins and rat striatal lysates. Note that mGluR1a–CT–FL and CT1 but not CT2 pulled down CaMKIIα. The input was run alongside the pull-down samples and represents 2% (CaMKIIα) or 4% (CaMKIV) of total protein extract used in the assay. **C**, In vitro binding assays with immobilized GST-fusion proteins and purified CaMKIIα. **D**, GST-fusion proteins containing different parts of mGluR1a–CT1. **E**, In vitro binding assays with immobilized mGluR1a–CT1 fragments and purified CaMKIIα. Note that CT1a, but not CT1b and CT1c, bound to CaMKIIα. **F**, In vitro binding assays with immobilized GST-fusion proteins and purified mGluR1a–CT. GST-fusion proteins contain the CD, RD, or AD of CaMKIIα or CaMKIV. Proteins bound to GST-fusion proteins in either pull-down or binding assays were visualized with immunoblots (IB) using the specific antibodies as indicated. The input was 5% of total CaMKIIα (**C**, **E**) or mGluR1a–CT (**F**) proteins used in in vitro binding assays.

CaMKII interacts with and regulates many synaptic targets (Colbran, 2004), although whether CaMKII interacts with mGluR1a is unknown. Here we found that CaMKIIα directly binds to the proximal CT region of mGluR1a. The binding is Ca^{2+} dependent and results in phosphorylation of mGluR1a at a specific CT site. Agonist activation of mGluR1a in rat striatal neurons triggers a Ca^{2+}-sensitive association of CaMKIIα with mGluR1a and promotes desensitization of the receptor. Together, we have discovered a negative feedback mechanism involving CaMKII–mGluR1a interactions that controls desensitization of mGluR1a.

Materials and Methods

Animals. Adult male Wistar rats weighting 200–350 g (Charles River) were individually housed at 23°C and humidity of 50 ± 10% with food and water available *ad libitum*. The animal room was on a 12 h light/dark cycle with lights on at 7:00 A.M. All animal use procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee.

Cloning, expression, and purification of glutathione S-transferase fusion proteins. Glutathione S-transferase (GST)-fusion proteins containing full-length (FL) or truncated proteins of interest were synthesized as described previously (Liu et al., 2009; Guo et al., 2010). Briefly, the cDNA fragments encoding the mGluR1a–CT–FL(K841–L1199), mGluR1a–CT1(K841–T1000), mGluR1a–CT2(P1001–L1199), mGluR1a–CT1a(K841–N885), mGluR1a–CT1b(A886–K931), mGluR1a–CT1c(N925–T1000), mGluR1a intracellular loop 1 [mGluR1a–IL1(R618–E629)], CaMKIIα catalytic domain [CaMKIIα–CD(L91–S272)], CaMKIIα regulatory domain

[CaMKIIα–RD(H273–S314)], CaMKIIα association domain [CaMKIIα–AD(G315–H478)], or GluA1–CT(E809–L889) were generated by PCR amplification from FL cDNA clones. These fragments were subcloned into BamHI–EcoRI sites of the pGEX4T-3 plasmid (GE Healthcare). Initiation methionine residues and stop codons were incorporated where appropriate. To confirm appropriate splice fusion, all constructs were sequenced. GST-fusion proteins were expressed in *Escherichia coli* BL21 cells (GE Healthcare) and purified from bacterial lysates as described by the manufacturer. GST- or His-tagged CaMKIIα–FL(M1–H478) and GST-tagged CaMKIV–FL(M1–Y473) were expressed and purified via a baculovirus/Sf9 insect cell expression system.

Western blot analyses. Western blots were performed as described previously (Guo et al., 2010). Briefly, proteins were separated on SDS Nu-PAGE Bis-Tris 4–12% gels (Invitrogen) and were transferred to polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies usually at 1:1000 overnight at 4°C. This was followed by an incubation of secondary antibodies (1:2000). Immunoblots were developed with the enhanced chemiluminescence reagent (GE Healthcare).

Affinity purification (pull-down) assay. Solubilized striatal extracts (50–100 μg of proteins) were diluted with 1× PBS/1% Triton X-100 and incubated with 50% (v/v) slurry of glutathione-Sepharose 4B beads (GE Healthcare) saturated with GST alone or with a GST-fusion protein (5–10 μg) at 4°C for 2 h. Beads were washed four times with 1× PBS/1% Triton X-100. Bound proteins were eluted with 4× lithium dodecyl sulfate (LDS) loading buffer, resolved by SDS-PAGE, and immunoblotted with a specific antibody.

In vitro *binding assay.* His-tagged CaMKIIα (~57 kDa, 17 ng) was equilibrated to binding buffer (200 mM NaCl, 0.2% Triton X-100, 0.1

mg/ml BSA, and 50 mM Tris, pH 7.5) with or without 0.5 mM $CaCl_2$, 1 μM CaM, or 1 mM EGTA as indicated. Binding reactions were initiated by adding purified GST-fusion proteins and were remained at 4°C for 2–3 h. GST-fusion proteins were precipitated using 100 μl of 10% glutathione-Sepharose 4B beads. The precipitate was washed three times with binding buffer. Bound proteins were eluted with 4× LDS loading buffer, resolved by SDS-PAGE, and immunoblotted with a specific antibody.

Coimmunoprecipitation. Rats were anesthetized and decapitated. Brains were removed, and coronal sections were cut. The striatum was removed and homogenized on ice in the homogenization buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, a protease inhibitor cocktail (Thermo Fisher Scientific), and a phosphatase inhibitor cocktail (Thermo Fisher Scientific). Homogenates were centrifuged at $760 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $10,000 \times g$ at 4°C for 30 min to obtain P2 pellets (synaptosomal fraction). P2 pellets were solubilized in the homogenization buffer containing 1% sodium deoxycholate for 1 h at 4°C. Solubilized proteins (150 μg) were incubated with a rabbit antibody against CaMKIIα or mGluR1a. The complex was precipitated with 50% protein A or G agarose/Sepharose bead slurry (GE Healthcare). Proteins were separated on Novex 4–12% gels and probed with a mouse antibody against CaMKIIα or mGluR1a. HRP-conjugated secondary antibodies and enhanced chemiluminescence were used to visualize proteins.

Phosphorylation reactions in vitro. GST, GST-fusion proteins, or synthetic peptides were incubated with CaMKIIα or protein kinase A (PKA) (Millipore) for 30 min or indicated durations at 30°C in a volume of 25 μl of the reaction buffer containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM DTT, 0.1 mg/ml BSA, 50 μM ATP, and 2.5 μCi/tube [γ-^{32}P]ATP ($\sim$3000 Ci/mmol; PerkinElmer Life and Analytical Sciences) with or without 0.5 mM $CaCl_2$ and 1 μM CaM. The phosphorylation reactions were stopped by adding LDS sample buffer and boiling for 3 min. Phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

Autophosphorylation of CaMKIIα. Autophosphorylation reactions were performed in 25 μl of reaction buffer containing 500 ng of CaMKIIα, 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 mM $CaCl_2$, 50 μM ATP, and 1 μM CaM for 10 min at 30°C. The reaction was stopped by adding EGTA (5 mM final concentration) on ice. Aliquots (5 μl) of the autophosphorylated kinases were immediately used in *in vitro* binding assays.

Dephosphorylation with calf-intestinal alkaline phosphatase. For dephosphorylation of phosphorylated GST-fusion proteins, proteins were incubated with active CaMKIIα (100 ng) in 25 μl reaction buffer containing 10 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM DTT, 50 μM ATP, and 2.5 μCi/tube [γ-^{32}P]ATP ($\sim$3000 Ci/mmol, PerkinElmer). After 30 min at 30°C, GST-fusion proteins were precipitated and the supernatant containing CaMKIIα was removed. Precipitates were washed twice. They were then suspended in a solution containing calf-intestinal alkaline phosphatase (CIP) (100 U/ml; Roche) and incubated for 1 h at 37°C. Samples were then subjected to standard gel electrophoresis and autoradiography.
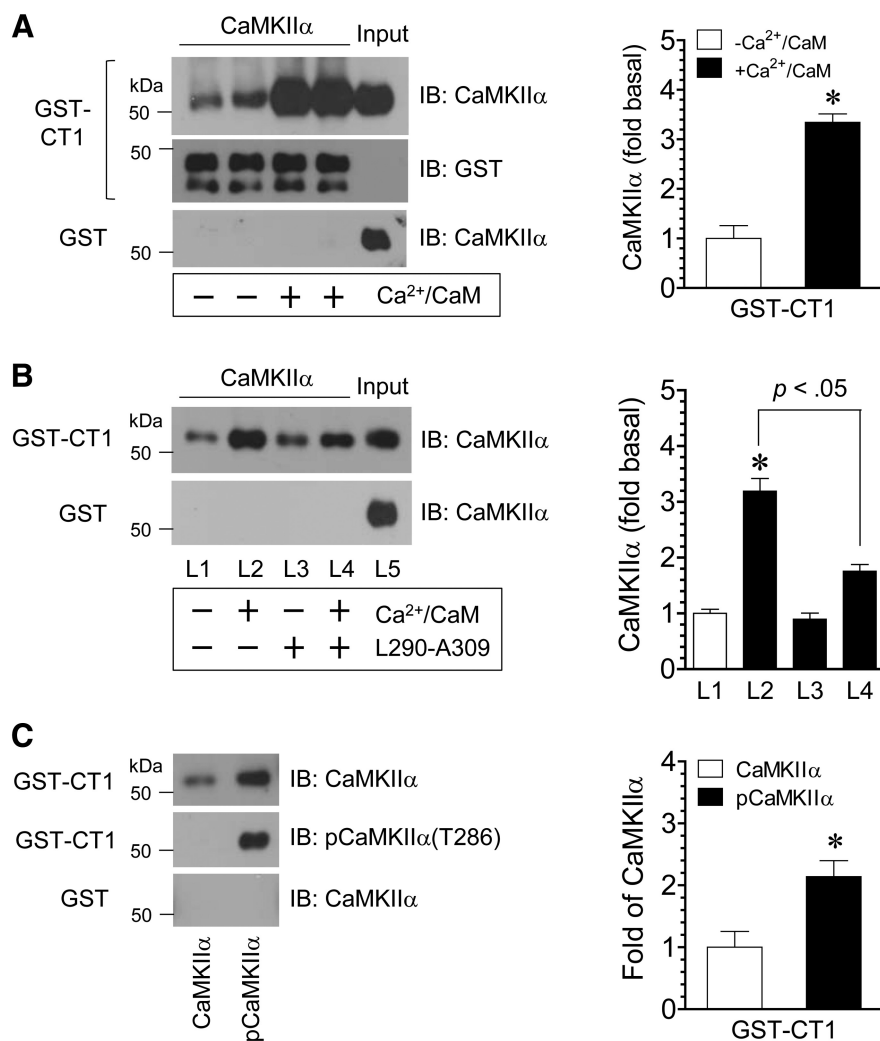

Figure 2. Ca^{2+}/CaM and T286-autophosphorylation potentiate CaMKIIα binding to mGluR1a. ***A***, Ca^{2+}/CaM increased the binding of CaMKIIα to mGluR1a–CT1. ***B***, The inhibitory peptide (L290–A309) reduced the Ca^{2+}/CaM-induced potentiation of CaMKIIα–mGluR1a binding. ***C***, T286-autophosphorylated CaMKIIα showed a higher level of binding to mGluR1a–CT1 compared with equally loaded, unphosphorylated CaMKIIα. Binding assays were performed between CaMKIIα or pCaMKIIα and immobilized GST or GST–mGluR1a–CT1 in the presence or absence of $CaCl_2$ (0.5 mM), CaM (1 μM), or L290–A309 (5 μM) as indicated. EGTA (1 mM) was added in the assays lacking $CaCl_2$. Bound CaMKIIα or pCaMKIIα proteins were visualized by immunoblots (IB). The input was 5% of total CaMKIIα proteins used in the assays (***A***, ***B***). Representative immunoblots are shown to the left of the quantified data. Data are presented as means ± SEM ($n = 3$–5 per group). *$p < 0.05$ versus Ca^{2+}/CaM-free samples (***A***, ***B***) or CaMKIIα (***C***).

IP_3 assays. The intracellular level of IP_3 was measured using a Hit-Hunter IP_3 Fluorescence Polarization Assay Kit from DiscoveRx according to the protocol of the manufacturer. The assay is a competitive binding analysis and is a rapid and direct measurement of cellular IP_3. In the assay, cellular IP_3 displaces a fluorescent derivative of IP_3 (tracer) from a specific binding protein, which reduces the amount of bound IP_3 tracers and lowers fluorescence polarization signals. Rat striatal slices were lysed by perchloric acid after drug treatment. Cell lysates or IP_3 standards were pipetted into a 96-well plate. The IP_3 tracer (20 μl) was added into each well. After shaking (5 min), the IP_3 binding protein (40 μl) was added. The fluorescence polarization of the IP_3 tracer (fluorescein) was detected in a BioTek Synergy 2 multi-detection microplate reader with a fluorescence polarization filter, using the 485 nm excitation wavelength and 530 nm emission wavelength. The IP_3 concentration of each sample was calculated from the plotted standard curve.

Striatal slice preparation. Striatal slices were prepared as described previously (Liu et al., 2009). Briefly, rats were decapitated after anesthesia, and brains were removed and placed in ice-cold artificial CSF (ACSF) containing (in mM) 10 glucose, 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 26
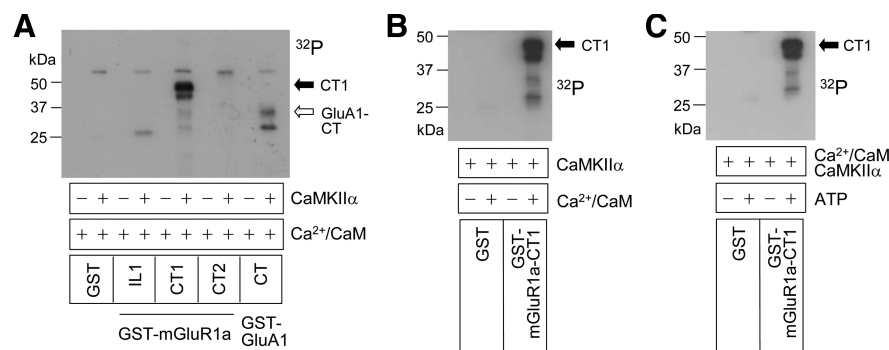
Figure 3. Phosphorylation of mGluR1a by CaMKIIα. **A**, An autoradiograph illustrating phosphorylation of GST–mGluR1a–CT1, but not GST, GST–mGluR1a–IL1, and GST–mGluR1a–CT2, in the presence of Ca²⁺/CaM. **B**, An autoradiograph illustrating phosphorylation of GST–mGluR1a–CT1 in the presence but not absence of Ca²⁺/CaM. **C**, An autoradiograph illustrating phosphorylation of GST–mGluR1a–CT1 in the presence but not absence of ATP. Phosphorylation reactions were performed at 30°C for 30 min with or without [γ-³²P]ATP. The reactions were then subjected to gel electrophoresis, followed by autoradiography. Filled and open arrows indicate phosphorylated GST–mGluR1a–CT1 and GST–GluA1–CT, respectively.

$NaHCO_3$, 2 $MgSO_4$, and 2 $CaCl_2$, bubbled with 95% O_2–5% CO_2, pH 7.4. Coronal slices were prepared using a vibratome (VT1200S; Leica). The striatum was dissected from the slices in ice-cold ACSF. Slices were pre-incubated in ACSF in an incubation tube at 30°C under constant oxygenation with 95% O_2–5% CO_2 for 60 min. The solution was replaced with fresh ACSF for an additional preincubation (10–20 min). Drugs as specified in each experiment were added and incubated at 30°C. After drug treatment, slices were frozen and stored at −80°C until assayed.

Peptide synthesis. Peptides, including Tat-fusion peptides, were synthesized and purified by Peptide 2.0 (Chantilly, VA). Tat-fusion peptides gain cell permeability by containing an arginine-enriched cell-membrane transduction domain of the human immunodeficiency virus type 1 Tat protein (YGRKKRRQRRR) (Schwarze et al., 1999). The CaMKII inhibitory peptide, CaMKIINtide (KRPPKLGQIGRSKRVVIEDDR) derived from the CaMKII inhibitory protein CaMKIIN (Chang et al., 1998), was synthesized to contain a Tat domain (Vest et al., 2007). Kemptide (LRRASLG; Kemp et al., 1977) was purchased from ANAspec.

Antibodies and pharmacological agents. Antibodies used in this study include a rabbit antibody against mGluR1a (1:1000; Millipore), CaMKIIα (1:1000; Santa Cruz Biotechnology), pCaMKIIα(T286) (1:1000; Cell Signaling Technology), or GST (1:1000; Sigma), or a mouse antibody against mGluR1a (1:1000; BD Biosciences), or CaMKIIα (1:1000; Santa Cruz Biotechnology), or a goat antibody against CaMKIV (1:1000; Santa Cruz Biotechnology). Pharmacological agents, including (RS)-3,5-dihydroxyphenylglycine (DHPG), 3-methyl-aminothiophene dicarboxylic acid (3-MATIDA), and 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP) were purchased from Tocris Cookson. Ionomycin, KN93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl) amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), and KN92 (2-[N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate) were purchased from Sigma. All drugs were freshly prepared at the day of experiments.

Statistics. The results are presented as means ± SEM and were evaluated using a one- or two-way ANOVA, as appropriate, followed by a Bonferroni's (Dunn's) comparison of groups using least-squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

Results

CaMKIIα binds to the C-terminal region of mGluR1a

We first explored possible protein–protein interactions between CaMKIIα and mGluR1a. We focused on the CT domain of mGluR1a because it is much larger than other intracellular domains. GST-fusion proteins containing FL or fragments of mGluR1a–CT were synthesized (Fig. 1A). Using these immobilized baits in pull-down assays, we found that GST–mGluR1a–CT–FL(K841–L1199) pulled down CaMKIIα from soluble rat

striatal lysates (Fig. 1B). The N-terminal fragment of CT, GST–mGluR1a–CT1(K841–T1000), also pulled down CaMKIIα, whereas GST alone and the C-terminal fragment of CT, GST–mGluR1a–CT2(P1001–L1199), did not. No GST-fusion proteins pulled down CaMKIV (Fig. 1B). These data indicate that CaMKIIα interacts with mGluR1a–CT1. To determine whether CaMKIIα directly binds to mGluR1a, we performed binding assays with purified proteins. We found that purified CaMKIIα bound to immobilized GST–mGluR1a–CT–FL and GST–mGluR1a–CT1 but not GST–mGluR1a–CT2 or GST alone (Fig. 1C). Thus, CaMKIIα can directly interact with the CT1 region of mGluR1a–CT. To further identify the CaMKIIα-binding region in the CT1 segment, we generated GST-fusion proteins containing different parts of CT1 (CT1a–CT1c; Fig. 1D). CT1a(K841–N885) harbored the binding of CaMKIIα (Fig. 1E). CT1b(A886–K931) and CT1c(N925–T1000) did not (Fig. 1E). Thus, the CaMKIIα-binding site seems to locate in the CT1a region that contains the first 45 aa of mGluR1a–CT. All blots were probed in parallel with a GST antibody to ensure equivalent protein loading (data not shown).

We also tried to identify the CaMKIIα region specific for the interaction with mGluR1a. To this end, we prepared GST-fusion proteins containing distinct subdomains of the kinase. We used these GST-fusion proteins in binding assays to precipitate mGluR1a–CT–FL. Bound mGluR1a–CT proteins were visualized in the following Western blots with an antibody against mGluR1a–CT. The N-terminal catalytic domain of CaMKIIα, GST–CaMKIIα–CD(L91–S272), like GST–CaMKIIα–FL(M1–H478), precipitated mGluR1a–CT (Fig. 1F). The regulatory domain, GST–CaMKIIα–RD(H273–S314), and the C-terminal association domain, GST–CaMKIIα–AD(G315–H478), produced no precipitation. Moreover, GST–CaMKIV-FL did not precipitate mGluR1a–CT. Thus, CaMKIIα–CD seems to be the subdomain responsible for the mGluR1a interaction.

Ca²⁺ and autophosphorylation enhance CaMKIIα binding to mGluR1a

Ca²⁺-activated CaM binds to the regulatory domain of CaMKII to activate the kinase. This activation usually regulates the affinity of CaMKII for its targets. To determine whether Ca²⁺ regulates the CaMKII binding to mGluR1a, we tested the binding in the presence of Ca²⁺/CaM. In the absence of Ca²⁺/CaM (with a Ca²⁺ chelator EGTA, 1 mM), CaMKIIα constitutively bound to mGluR1a–CT1 (Fig. 2A), similar to the results observed above. In the presence of Ca²⁺ (0.5 mM) and CaM (1 μM), the CaMKIIα binding was remarkably increased. This increase was attenuated by pretreatment with the L290–A309 fragment, a peptide that corresponds to the CaM binding domain of CaMKII and thereby competitively antagonizes the CaM binding to CaMKII (Colbran and Soderling, 1990) (Fig. 2B). These results demonstrate that, although there exists a detectable level of constitutive binding between CaMKIIα and mGluR1a, Ca²⁺/CaM further enhance the affinity of CaMKIIα for mGluR1a.

Activated CaMKIIα undergoes autophosphorylation at T286, which keeps the kinase active even after initial Ca²⁺ signals subside (Hudmon and Schulman, 2002). To determine the binding

A　mGluR1a-CT1(K841-T1000)
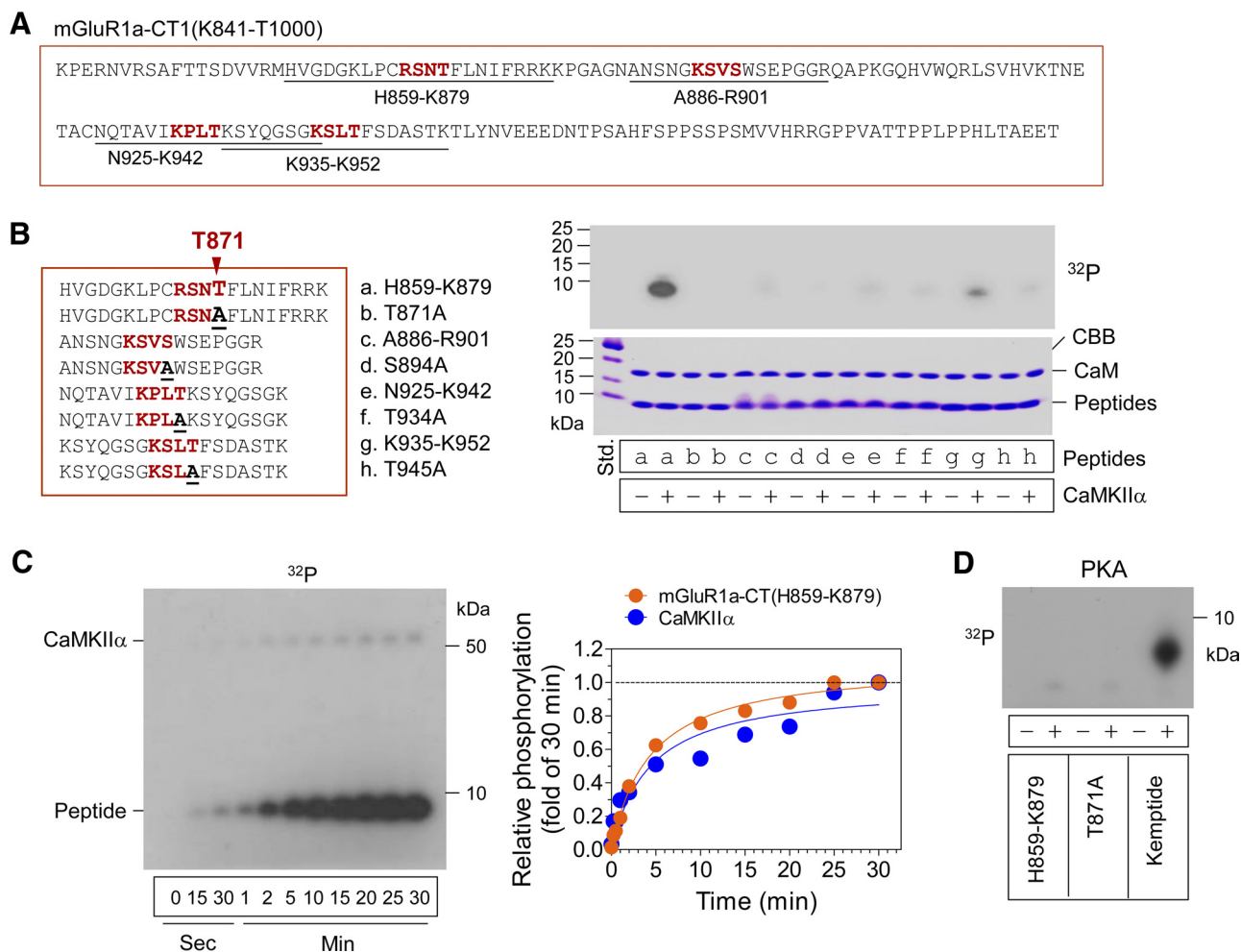

B

C

D

Figure 4. Phosphorylation of mGluR1a T871 by CaMKIIα. **A**, Analysis of amino acid sequence of entire mGluR1a–CT1(K841–T1000). Four fragments flanking the consensus CaMKII phosphory-lation motif are underlined. **B**, Phosphorylation of synthetic peptides (a, c, e, and g) and their corresponding mutants (b, d, f, and h). A representative autoradiograph is shown above the Coomassie Brilliant Blue (CBB) protein staining. Note that the H859–K879 peptide was markedly phosphorylated by CaMKIIα. **C**, Time courses of mGluR1a–CT(H859–K879) phosphorylation and CaMKIIα autophosphorylation. An autoradiograph is shown to the left of the quantified data. **D**, PKA-mediated phosphorylation of mGluR1a–CT(H859–K879) and kemptide. Note that PKA phosphorylated its classical substrate kemptide but not mGluR1a–CT(H859–K879). Phosphorylation reactions were performed at 30°C for 30 min with [γ-^{32}P]ATP in the presence of active PKA. The reactions were then subjected to gel electrophoresis, followed by autoradiography. Data are presented as means ± SEM ($n = 3$ per group).

of autophosphorylated CaMKIIα to mGluR1a, we subjected CaMKIIα to Ca^{2+}/CaM activation and autophosphorylation in a reaction solution containing ATP (a preferred phosphate donor). We then used autophosphorylated CaMKIIα in binding assays in the presence of EGTA (1 mM). We found that autophosphory-lated CaMKIIα exhibited a higher level of binding to mGluR1a–CT1 compared with unphosphorylated CaMKIIα (Fig. 2C). Thus, T286 autophosphorylation sustains the kinase at a high level of binding to mGluR1a.

CaMKIIα phosphorylates mGluR1a
To determine whether CaMKIIα phosphorylates mGluR1a, we monitored the CaMKIIα-catalyzed incorporation of ^{32}P into mGluR1a in sensitive autoradiography. Active CaMKIIα in the presence of Ca^{2+}/CaM strongly phosphorylated GST–mGluR1a–CT1 but not GST alone and GST–mGluR1a–CT2 (Fig. 3A). GST–mGluR1a–IL1 only showed much weaker phos-phorylation. In assays with another glutamate receptor, i.e., the AMPA receptor, a known substrate of CaMKII (Barria et al., 1997; Mammen et al., 1997), we observed phosphorylation of the CT fragment of AMPA receptor GluA1 subunits (Fig. 3A), which served as a positive control. Inactive CaMKIIα in the absence of

Ca^{2+}/CaM did not phosphorylate mGluR1a–CT1 (Fig. 3B), nei-ther did active CaMKIIα in the absence of ATP (Fig. 3C). De-phosphorylation treatment of a duplicate reaction with CIP significantly reduced mGluR1a–CT1 phosphorylation (data not shown). These data identify mGluR1a as a preferred substrate of CaMKIIα and primary phosphorylation site(s) are re-stricted to CT1.

CT1 contains a total of four sites that are consistent with the consensus CaMKII phosphorylation sequence, R/K*XX*S/T (White et al., 1998) (Fig. 4A). We thus synthesized four peptides flanking these potential phosphorylation sites. Using these pep-tides together with their site-directed mutants, we performed a series of phosphorylation assays to identify accurate phospho-accepting site(s) within CT1 (Fig. 4B). Interestingly, CaMKIIα intensely phosphorylated only one peptide (H859–K879) (Fig. 4B). In contrast, CaMKIIα produced little or no phosphorylation signals in other three peptides (A886–R901, N925–K942, and K935–K952). The positive H859–K879 peptide contains an 868–RSNT–871 motif that aligns well with the consensus sequence. When mutating threonine 871 (T871) to alanine (T871A), no phosphorylation was detected in the peptide. This complete loss of phosphorylation signals supports T871 as the primary site of
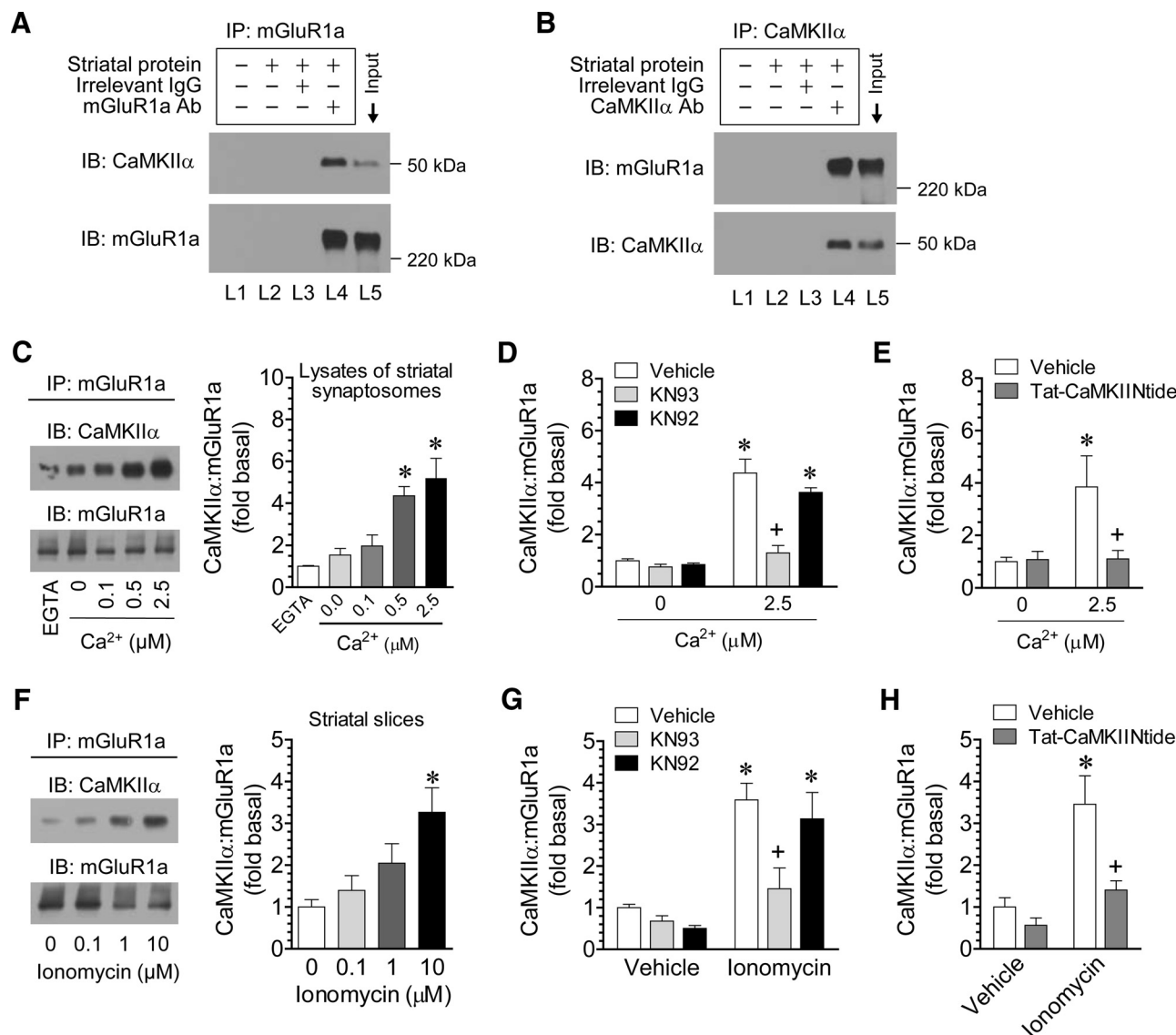
Figure 5. Interactions of CaMKIIα with mGluR1a in striatal neurons. ***A***, ***B***, Coimmunoprecipitation (IP) of CaMKIIα and mGluR1a in rat striatal synaptosomes. Lanes 2 and 3 showed no specific bands because of the lack of a precipitating antibody (L2) and the use of an irrelevant IgG (L3). The input was 1 and 6% of total solubilized proteins used in the CaMKIIα and mGluR1a coimmunoprecipitation, respectively. ***C***, Effects of Ca^{2+} (10 min) on the association of CaMKIIα with mGluR1a. ***D***, Effects of KN93 and KN92 on the Ca^{2+}-stimulated CaMKIIα–mGluR1a association. Ca^{2+} (2.5 μM) was cotreated with KN93 or KN92 (20 μM) for 10 min. ***E***, Effects of Tat–CaMKIINtide on the Ca^{2+}-stimulated CaMKIIα–mGluR1a association. Tat–CaMKIINtide (2 μM) was applied 45 min before and during Ca^{2+} (2.5 μM, 10 min). ***F***, Effects of ionomycin (10 min) on the association of CaMKIIα with mGluR1a. ***G***, Effects of KN93 and KN92 on the ionomycin-stimulated CaMKIIα–mGluR1a association. Ionomycin (10 μM) was cotreated with KN93 or KN92 (20 μM) for 10 min. ***H***, Effects of Tat–CaMKIINtide on the ionomycin-stimulated CaMKIIα–mGluR1a association. Tat–CaMKIINtide (2 μM) was applied 45 min before and during ionomycin (10 μM, 10 min). Lysates of striatal synaptosomes (***C–E***) or striatal slices (***F–H***) were used. Precipitated proteins were visualized by immunoblots (IB). Data are presented as means $\pm$ SEM ($n = 3$–4 per group). *$p < 0.05$ versus EGTA, vehicle, or vehicle + vehicle. $^{+}p < 0.05$ versus Ca^{2+} or vehicle + ionomycin.

phosphorylation within mGluR1a–CT1. Of note, T871 lies within the center of the CaMKIIα binding motif in mGluR1a–CT1. Additional studies that aimed to characterize T871 phosphorylation discovered that phosphorylation of T871-containing peptides was rapid and comparable with autophosphorylation of CaMKIIα (Fig. 4*C*). No phosphorylation was seen in the H859–K879 peptide in response to active PKA, whereas active PKA readily phosphorylated its classical substrate peptide, kemptide (Fig. 4*D*).

Interactions of CaMKIIα with mGluR1a *in vivo*

We next wanted to examine the interaction between native CaMKIIα and mGluR1a in neurons *in vivo*. We thus performed coimmunoprecipitation with the solubilized synapto-

somal fraction (P2) from the rat striatum. In the first set of coimmunoprecipitation with an anti-mGluR1a antibody, a CaMKIIα-immunoreactive band was consistently seen in the mGluR1a precipitates (Fig. 5*A*). In reverse coimmunoprecipitation with a CaMKIIα antibody, the mGluR1a immunoreactivity was displayed in the CaMKIIα precipitates (Fig. 5*B*). The irrelevant IgG did not precipitate either protein. Thus, there exists the interaction between CaMKIIα and mGluR1a in striatal neurons *in vivo*.

Ca^{2+} enhances CaMKIIα–mGluR1a interactions in striatal neurons

Ca^{2+} enhanced CaMKIIα–mGluR1a–CT1 binding *in vitro*. To determine whether this reflects the case in neurons, we evaluated
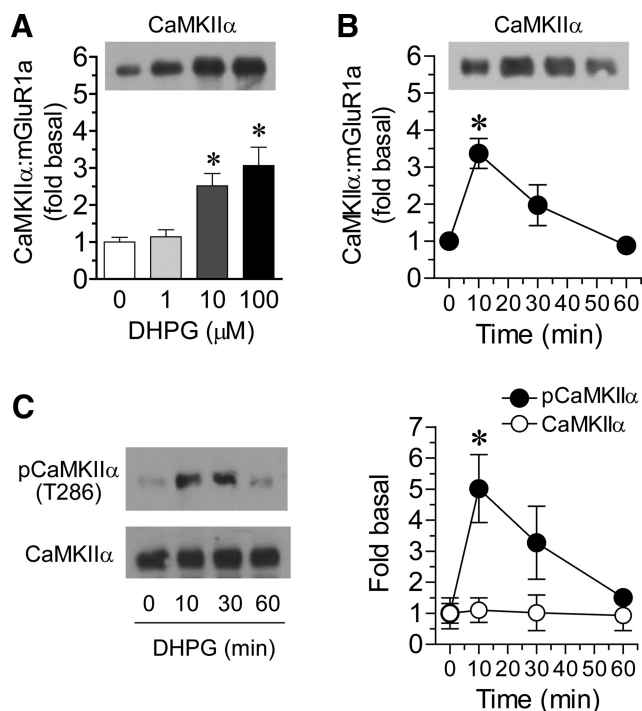
Figure 6. Effects of DHPG on CaMKIIα–mGluR1a interactions in rat striatal neurons. **A**, Concentration-dependent effects of DHPG (10 min) on CaMKIIα–mGluR1a interactions in striatal slices. **B**, Time-dependent effects of DHPG (100 μM) on CaMKIIα–mGluR1a interactions in striatal slices. **C**, Time-dependent effects of DHPG (100 μM) on cellular levels of pCaMKIIα(T286) and CaMKIIα in striatal slices. Proteins were visualized by immunoblots. Data are presented as means ± SEM ($n = 3–5$ per group). *$p < 0.05$ versus basal levels.

the Ca^{2+}-regulated interaction between the two endogenous proteins in rat striatal neurons using coimmunoprecipitation. Direct addition of Ca^{2+} to lysates of striatal synaptosomes (0.1-2.5 μM, 10 min) concentration-dependently elevated the amount of CaMKIIα bound to mGluR1a (Fig. 5C). This elevation seems to result from Ca^{2+} activation of CaMKIIα because it was blocked by KN93 (20 μM), an inhibitor that inhibits activation of CaMKII by preventing the CaM binding, but not by KN92, an inactive analogy of KN93 (Fig. 5D). Another cell-permeable and highly selective CaMKII inhibitory peptide, Tat–CaMKIINtide (Chang et al., 1998; Vest et al., 2007), incubated at 2 μM (45 min before Ca^{2+} addition) also blocked the Ca^{2+}-mediated elevation (Fig. 5E). These results demonstrate a Ca^{2+}-sensitive nature of interactions between native CaMKIIα and mGluR1a. To assay the interaction in living neurons, we subjected rat striatal slices to a Ca^{2+} ionophore, ionomycin. Like Ca^{2+} addition to lysates, applying ionomycin to slices (0.1–10 μM, 10 min) produced a concentration-dependent increase in CaMKIIα–mGluR1a interactions (Fig. 5F). This increase was blocked by KN93 (Fig. 5G) and Tat–CaMKIINtide (Fig. 5H). Neither KN93 nor Tat–CaMKIINtide altered basal CaMKIIα–mGluR1a interactions. These results establish that Ca^{2+} augments CaMKIIα–mGluR1a interactions in striatal neurons.

Agonist-induced interactions between CaMKII and mGluR1a
Activation of mGluR1 leads to intracellular Ca^{2+} release. To define whether activation of mGluR1 affects CaMKIIα–mGluR1a interactions, we investigated pharmacological effects of the group I mGluR agonist DHPG on coimmunoprecipitation of CaMKIIα and mGluR1a. In rat striatal slices, applying DHPG (10 min) substantially increased the amount of CaMKIIα coimmunoprecipitated with mGluR1a (Fig. 6A). The increase was evidently

concentration dependent (Fig. 6A) and dynamic (Fig. 6B). The transient response reached peak at 10 min and gradually returned to the normal level by 60 min (Fig. 6B). Similar to this, corresponding increases in autophosphorylated CaMKIIα levels were induced, whereas total cellular levels of CaMKIIα remained stable (Fig. 6C). These data reveal a feedback interaction between CaMKIIα and mGluR1a. In response to the agonist, CaMKIIα was recruited to the activated receptor.

As aforementioned, CaMKIIα binds to the membrane-proximal CT region of mGluR1a. To identify a sufficient binding motif from this region, we synthesized a 14 aa interfering peptide (KLPCRSNTFLNIFR, mGluR1a-i) flanking the T871 phosphorylation site. We then tested whether this peptide competes with mGluR1a-CT1 for binding to CaMKIIα. In binding assays *in vitro*, the peptide substantially blocked the binding of GST–mGluR1a–CT1 to CaMKIIα (Fig. 7A). However, a sequence-scrambled control peptide (SIFNRKNRCLPLFT, mGluR1a-c) did not (Fig. 7A). Thus, a CT1 region corresponding to these 14 residues, including the T871 phosphorylation site, constitutes a core motif for CaMKIIα binding. To determine the effect of these peptides in striatal neurons, we synthesized the interfering and control peptide together with Tat (YGRKKRRQRRR), an arginine-enriched domain known to render fusion peptides cell permeability (Aarts et al., 2002). Adding Tat–mGluR1a-i (10 μM, 45 min before DHPG) to rat striatal slices significantly reduced the DHPG-induced interaction of CaMKIIα with mGluR1a (Fig. 7B). In contrast, Tat–mGluR1a-c had no effect (Fig. 7B). Thus, the mGluR1a-i-sensitive site on mGluR1–CT is important for harboring CaMKIIα binding.

CaMKII desensitizes mGluR1a
The interaction of CaMKIIα with mGluR1a may have functional consequences. To clarify this, we investigated the functional role of CaMKIIα in the regulation of mGluR1a-associated signaling. Activation of Ga_q-coupled mGluR1a is known to stimulate PLCβ1, which in turn hydrolyzes PI molecules to DAG and IP_3 (Niswender and Conn, 2010; Traynelis et al., 2010). We thus measured the IP_3 yield as the functional output of mGluR1a signaling. To this end, we used a fluorescence polarization tool to directly assay rapid rises in cytosolic IP_3 in response to mGluR1a agonist stimulation. In rat striatal slices, applying DHPG at 100 μM for different durations (5, 10, 20, 30, 60, 120, or 180 s) produced a rapid and transient elevation of IP_3 (Fig. 8A). A peak increase was observed at ~20 s incubation of DHPG. Pretreatment of striatal slices with the mGluR1a antagonist 3-MATIDA (10 μM, 30 min before DHPG) partially blocked the IP_3 formation induced by DHPG (100 μM, 20 s) (Fig. 8B). Similarly, the mGluR5 antagonist MTEP (10 μM, 30 min before DHPG) induced a partial decrease in IP_3 responses (Fig. 8B). Co-pretreatment with both antagonists almost completely blocked the effect of DHPG (Fig. 8B). These data validate our IP_3 assays as a sensitive method for measuring IP_3 production in striatal neurons and establish a sufficient mGluR1a component in mediating IP_3 responses to DHPG. The effect of DHPG on IP_3 formation was then analyzed in the presence of MTEP in the following experiments to selectively study the mGluR1a signaling.

Most GPCRs undergo feedback desensitization (i.e., agonist-dependent) after prolonged or repeated agonist stimulation (Aronica et al., 1993; Gereau and Heinemann, 1998; Ferguson, 2001; Kelly et al., 2008). This process involves phosphorylation of the receptor by either second-messenger-dependent protein kinases (e.g., PKA, PKC, or CaMKII) or GPCR kinases. A number of previous studies have demonstrated desensitization of
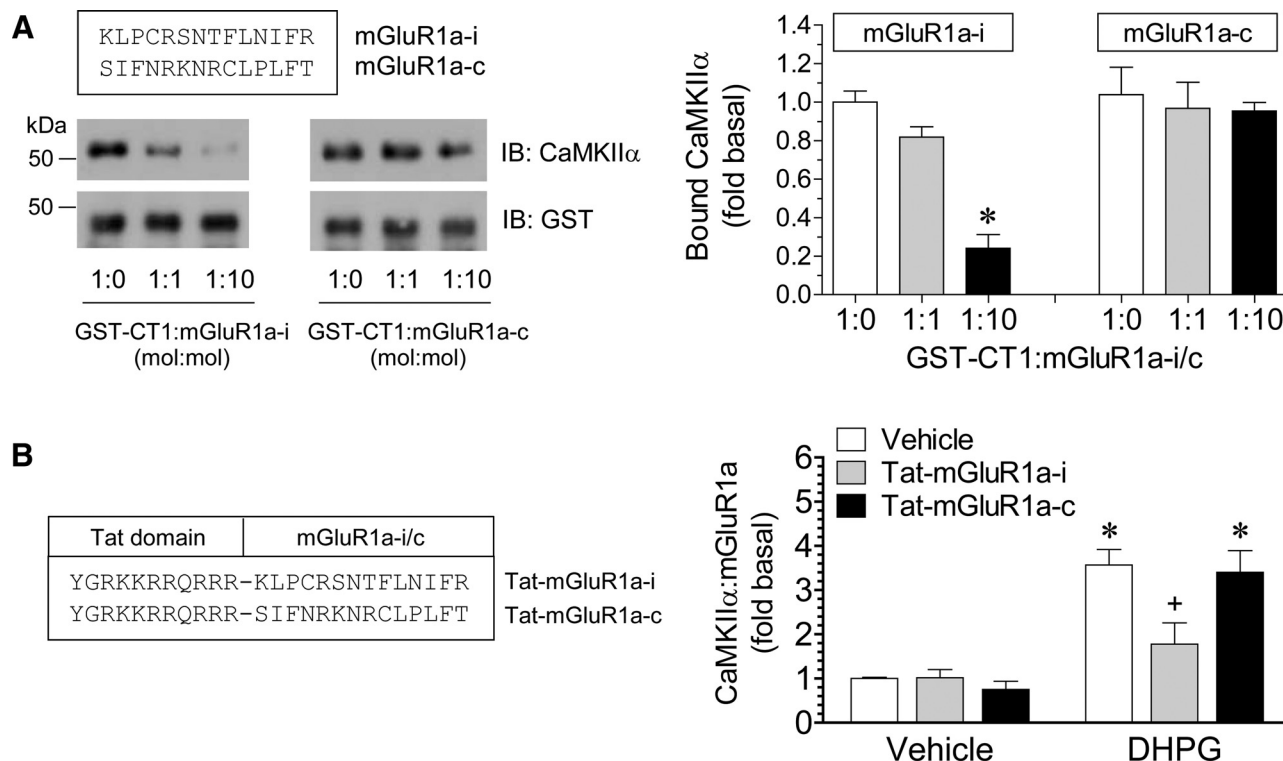
Figure 7. Effects of Tat peptides on CaMKIIα–mGluR1a interactions. **A**, Effects of mGluR1a-i and mGluR1a-c on the in vitro binding of CaMKIIα to GST–mGluR1a–CT1. Representative immunoblots (IB) are shown to the left of the quantified data. **B**, Effects of Tat–mGluR1a-i and Tat–mGluR1a-c on the DHPG-stimulated CaMKIIα–mGluR1a association in striatal slices. Note that Tat–mGluR1a-i but not Tat–mGluR1a-c attenuated the DHPG-stimulated CaMKIIα–mGluR1a interaction. Tat peptides (10 μM) were applied 45 min before and during DHPG (100 μM, 10 min). Proteins were visualized by immunoblots. Data are presented as means ± SEM (n = 3–5 per group). *$p < 0.05$ versus basal levels. +$p < 0.05$ versus vehicle + DHPG.

mGluR1 in response to agonist stimulation, although underlying mechanisms remain to be fully elucidated (Schoepp and Johnson, 1988; Thomsen et al., 1993; Alaluf et al., 1995; Herrero et al., 1998; Sato et al., 2004; Dhami and Ferguson, 2006). To determine whether CaMKII contributes to such feedback desensitization, we performed a set of pharmacological experiments in rat striatal slices. We first tested the occurrence of mGluR1 desensitization in terms of IP_3 production. For this purpose, we subjected slices to a relatively prolonged preexposure (first treatment) to DHPG (100 μM, 5 min) (Fig. 9A). We then washed off DHPG and inserted a 5 or 30 min interval before a challenge treatment (second treatment) with DHPG (100 μM). IP_3 production was assayed at the peak, i.e., 20 s after the second addition of DHPG. At a 5 min internal, the second DHPG treatment produced typical IP_3 responses in slices pretreated with vehicle (Fig. 9B). However, in slices preexposed to DHPG, the subsequent DHPG treatment failed to trigger a significant IP_3 response, indicating the development of desensitization. When DHPG was given at a longer interval (30 min), substantial IP_3 responses resumed (Fig. 9B), indicating a full recovery of desensitization. Remarkably, KN93 (20 μM, 30 min before and during the first DHPG treatment) significantly reversed the desensitized IP_3 response to the second DHPG treatment (5 min after the first DHPG stimulation), whereas KN92 did not (Fig. 9C). This suggests that CaMKII inhibits mGluR1 activity and contributes to desensitization of the receptor. Like KN93, Tat–mGluR1a-i but not Tat–mGluR1a-c (10 μM, 45 min before and during the first DHPG stimulation) partially restored IP_3 responses to the DHPG challenge (Fig. 9D). KN93, KN92, or Tat peptides alone had no effect on basal IP_3 production (data not shown). These data support a model that CaMKII, through interacting with mGluR1, participates in form-
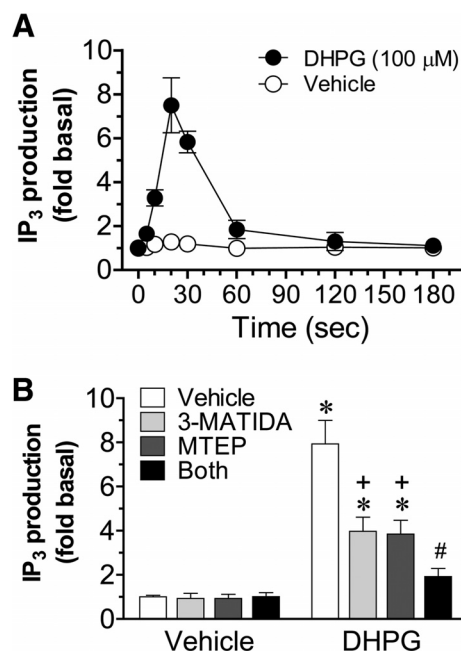

Figure 8. IP_3 production after stimulation of mGluR1/5 in striatal neurons. **A**, Effects of DHPG (100 μM for 5, 10, 20, 30, 60, 120, or 180 s) on cytosolic IP_3 levels. Note that the agonist triggered a rapid and transient production of IP_3. **B**, Effects of the mGluR1 or 5 antagonist on the DHPG-stimulated IP_3 formation. Note that either antagonist alone partially blocked IP_3 responses to DHPG, whereas coincubation of both antagonists additively blocked the IP_3 production. Experiments were conducted on rat striatal slices. The antagonists (3-MATIDA and MTEP) were applied alone or together at 10 μM 30 min before and during 20 s incubation of DHPG (100 μM). Data are presented as means ± SEM (n = 3–5 per group). *$p < 0.05$ versus vehicle + vehicle. +$p < 0.05$ versus vehicle + DHPG. #$p < 0.05$ versus 3-MATIDA + DHPG or MTEP + DHPG.

ing a negative feedback loop controlling agonist-induced desensitization of the receptor.

Discussion

In this study, we investigated the protein–protein interaction between CaMKII and mGluR1a and the regulation of mGluR1a by CaMKII via a phosphorylation mechanism in neurons. We found that CaMKIIα directly bound to the mGluR1a–CT. The affinity of CaMKIIα for mGluR1a was enhanced by Ca^{2+} *in vitro*. In striatal neurons, the mGluR1 agonist DHPG activated the IP_3–Ca^{2+} pathway, which in turn activated CaMKIIα and led to the recruitment of active CaMKIIα to mGluR1a. The direct association with CaMKIIα implies that mGluR1a may be a biochemical substrate of the enzyme. Indeed, CaMKIIα phosphorylated mGluR1a at a threonine site (T871) in the CT. Physiologically, the activity- and Ca^{2+}-dependent interaction of CaMKIIα with mGluR1a forms a negative feedback loop critical for processing the agonist-induced desensitization of mGluR1a.

Compared with other GPCR subfamilies, mGluR1a has relatively small intracellular loops (IL1, IL2, and IL3) and a surprisingly large CT. This confers the CT as a primary site for protein–protein interactions. In fact, most mGluR1a interacting partners discovered so far interact with the CT (Enz, 2012; Fagni, 2012). Likewise, CaMKIIα in this study bound to the mGluR1a–CT. The binding of CaMKIIα is isoform selective because another isoform, CaMKIV, did not show any binding to the receptor. Noticeably, the binding site in the mGluR1a–CT contains R/K*XXS*/T residues, a consensus substrate recognition motif for CaMKII (White et al., 1998). This R/K*XXS*/T-containing binding region is conserved among long (1a) and short (1b, 1c, and 1d) variants of mGluR1 given the fact that all these common variants share the identical first 46 aa in the proximal CT. Another important characteristic of the CaMKIIα–mGluR1a binding is its Ca^{2+} sensitivity. Adding Ca^{2+} substantially enhanced the binding *in vitro*. Ca^{2+} also enhanced the association of endogenous CaMKIIα with mGluR1a in striatal neurons. Thus, Ca^{2+} positively regulates the interaction between the two proteins. The similar regulation has been seen in interactions of CaMKIIα with other binding partners, including NMDA glutamate receptor NR2B subunits (Gardoni et al., 1998; Leonard et al., 1999), dopamine D_3 receptors (Liu et al., 2009), muscarinic M_4 receptors (Guo et al., 2010), and others (Colbran, 2004). Of note, Ca^{2+}-sensitive CaM directly bound to the mGluR5–CT and competitively inhibited the binding of other proteins to mGluR5 (Minakami et al., 1997; Ishikawa et al., 1999; Lee et al., 2008; Wang et al., 2009). However, CaM did not bind to mGluR1 (Choi et al., 2011) and can therefore affect the CaMKII–mGluR1 interaction solely by its binding to CaMKII.

In addition to Ca^{2+}, the autophosphorylation facilitates the binding of CaMKIIα to mGluR1a. CaMKIIα once activated phosphorylates its own phosphorylation site (T286) in addition to exogenous substrates. This autophosphorylation endows the kinase with
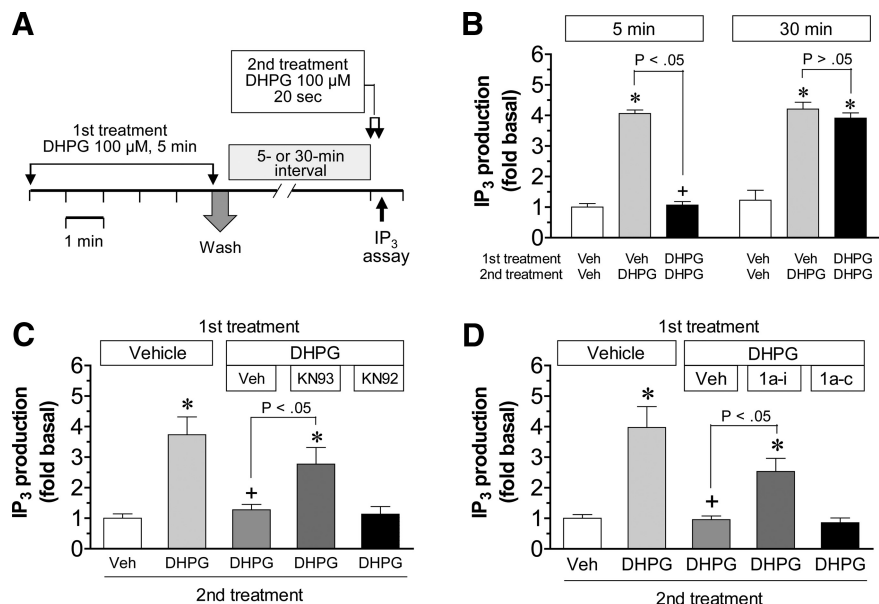
Figure 9. CaMKII desensitizes the mGluR1-mediated IP_3 production in striatal neurons. **A**, An agonist treatment paradigm leading to desensitization of mGluR1. Rat striatal slices were preexposed to 100 μM DHPG for 5 min (first treatment). DHPG was then washed off, followed by a 5 or 30 min recovery period. IP_3 production was assayed after a 20 s challenge of 100 μM DHPG (second treatment). In all experiments, MTEP (10 μM) was added to block mGluR5. **B**, Desensitization of mGluR1-mediated IP_3 responses. Note that the mGluR1-mediated IP_3 production was desensitized and recovered 5 and 30 min after the first DHPG treatment, respectively. **C**, Effects of KN93 and KN92 on desensitization of mGluR1. KN93 or KN92 (20 μM) was added 30 min before and during the first DHPG treatment. **D**, Effects of Tat peptides on desensitization of mGluR1. Tat–mGluR1a-i (1a-i) or Tat–mGluR1a-c (1a-c) at 10 μM was added 45 min before and during the first DHPG treatment. IP_3 production in response to the second DHPG treatment (100 μM, 20 s) was assayed 5 min after the preexposure to DHPG (**C, D**). Data are presented as means ± SEM ($n = 3$–6 per group). *$p < 0.05$ versus vehicle + vehicle. +$p < 0.05$ versus vehicle + DHPG. Veh, Vehicle.

a Ca^{2+}-independent (autonomous) activity (Hudmon and Schulman, 2002; Colbran and Brown, 2004). In this study, we found a high affinity of autophosphorylated CaMKIIα for mGluR1a. This is thought to be important for sustaining the interaction of the kinase with mGluR1a and thus prolonging the regulation of receptor function after a transient Ca^{2+} response to agonist stimulation.

Phosphorylation serves as an important mechanism for modulating protein function. Phosphorylation of mGluR1 is therefore thought to have an impact on receptor physiology (Dhami and Ferguson, 2006; Kim et al., 2008; Mao et al., 2008; this study). Our functional assays show that the CaMKII inhibitor KN93 that blocked the binding of CaMKIIα to mGluR1a and presumably reduced the CaMKII-mediated phosphorylation of the receptor resensitized the receptor for its response to subsequent DHPG administration. Moreover, disrupting the CaMKIIα–mGluR1a association with an interaction-dead peptide (Tat–mGluR1a-i) achieved the same result. Thus, CaMKII acts as a critical regulator in a feedback loop aimed to facilitate mGluR1 desensitization. This CaMKII-sensitive feedback model is in accordance with the early observations that (1) DHPG readily activated CaMKII in striatal and hippocampal neurons (Choe and Wang, 2001; Mockett et al., 2011), and (2) the internalization of mGluR1a triggered homologously by glutamate required CaMKII activation (Mundell et al., 2004). The fact that the CaMKII binding and phosphorylation site (T871) in the mGluR1a CT is immediately adjacent to the G-protein-coupling domain of mGluR1a is noteworthy (Dhami and Ferguson, 2006).

The Ca^{2+}- and state-dependent nature of the CaMKII–mGluR1 interaction may have an important functional consequence. Under basal conditions, CaMKII has a minimal influence over mGluR1 signaling, corresponding to a low level of interac-

tive activity between CaMKII and the receptor. After ligand occupation, the mGluR1a-associated $G\alpha_q$–PLC–IP_3 pathway is activated. This triggers a Ca^{2+} transient and subsequent activation of CaMKII. The activated kinase then interacts with and phosphorylates mGluR1a to facilitate the transition of receptors to a desensitized state. In this process, interference with the CaMKII–mGluR1a coupling by Tat–mGluR1a-i could remove the CaMKII-mediated desensitization and augment receptor responsiveness to agonist. Of note, mGluR1 activity is linked to synaptic plasticity. One classical form of synaptic plasticity, i.e., long-term depression (LTD), required selective activation of mGluR1 but not mGluR5 for its expression in corticostriatal glutamatergic synapses (Gubellini et al., 2001). Thus, it is intriguing to explore the role of CaMKII–mGluR1a interactions in striatal LTD in future studies.

The mGluR1a has been demonstrated previously to be regulated by a phosphorylation mechanism involving PKC (Manzoni et al., 1990; Catania et al., 1991; Thomsen et al., 1993; Alaluf et al., 1995). Active PKC phosphorylated a consensus PKC phosphorylation site (T695) within the mGluR1a–IL2 *in vitro* (Medler and Bruch, 1999). This phosphorylation appears to mediate feedback desensitization of mGluR1α in HEK293 cells (Francesconi and Duvoisin, 2000). Given a similar role of CaMKIIα demonstrated in this study, PKC and CaMKIIα may function in concert to orchestrate desensitization of the receptor.

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