

Activated CaMKII α Binds to the mGlu₅ Metabotropic Glutamate Receptor and Modulates Calcium Mobilization[§]

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ABSTRACT

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and metabotropic glutamate receptor 5 (mGlu₅) are critical signaling molecules in synaptic plasticity and learning/memory. Here, we demonstrate that mGlu₅ is present in CaMKII α complexes isolated from mouse forebrain. Further in vitro characterization showed that the membrane-proximal region of the C-terminal domain (CTD) of mGlu_{5a} directly interacts with purified Thr286-autophosphorylated (activated) CaMKII α . However, the binding of CaMKII α to this CTD fragment is reduced by the addition of excess Ca²⁺/calmodulin or by additional CaMKII α autophosphorylation at non-Thr286 sites. Furthermore, in vitro binding of CaMKII α is dependent on a tribasic residue motif Lys-Arg-Arg (KRR) at residues 866–868 of

the mGlu_{5a}-CTD, and mutation of this motif decreases the coimmunoprecipitation of CaMKII α with full-length mGlu_{5a} expressed in heterologous cells by about 50%. The KRR motif is required for two novel functional effects of coexpressing constitutively active CaMKII α with mGlu_{5a} in heterologous cells. First, cell-surface biotinylation studies showed that CaMKII α increases the surface expression of mGlu_{5a}. Second, using Ca²⁺ fluorimetry and single-cell Ca²⁺ imaging, we found that CaMKII α reduces the initial peak of mGlu_{5a}-mediated Ca²⁺ mobilization by about 25% while doubling the relative duration of the Ca²⁺ signal. These findings provide new insights into the physical and functional coupling of these key regulators of postsynaptic signaling.

Introduction

The ability of excitatory glutamatergic synapses to undergo dynamic changes in strength, termed synaptic plasticity, is critical for many behaviors. It is well established that glutamate activation of diverse ionotropic and metabotropic receptors is critical for short-term and long-term control of many neuronal responses (Niswender and Conn, 2010), and that these responses require a complex and incompletely understood

network of signaling proteins. Among the seven members of the metabotropic glutamate (mGlu) receptor family, mGlu₁ and mGlu₅ specifically couple through G $\alpha_{q/11}$ to stimulate multiple signaling pathways, including phosphoinositide hydrolysis and mobilization of intracellular Ca²⁺ stores. These receptors have long been implicated in multiple forms of long-term depression that require new protein synthesis (Oliet et al., 1997; Palmer et al., 1997; Huber et al., 2001) or increased endocannabinoid signaling (Lüscher and Huber, 2010). Despite many similarities, mGlu₁ and mGlu₅ can be differentially regulated by various mechanisms and have been shown to have different neuronal roles. For instance, in hippocampus, mGlu₁ increases the frequency of spontaneous inhibitory postsynaptic currents, whereas mGlu₅ potentiates *N*-methyl-D-aspartate receptor currents (Mannaioni et al., 2001). In particular, mGlu₅ has been specifically implicated in a number of neuropsychiatric disorders including addiction, schizophrenia, fragile X syndrome, obsessive compulsive disorder, and Alzheimer's disease (Grueter et al., 2008; Michalon et al., 2012; Ronesi et al., 2012; Hu et al., 2014; Ade et al., 2016; Foster and Conn, 2017).

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ABBREVIATIONS: AM, acetoxymethyl ester; ANOVA, analysis of variance; bp, base pair; CA, constitutively active; CA-CaMKII, constitutively active Ca²⁺/calmodulin-dependent protein kinase II; CaM, calmodulin; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; CaMKAP, Ca²⁺/calmodulin-dependent protein kinase II-associated protein; CTD, C-terminal domain; D₂R, D₂ dopamine receptor; D₃R, D₃ dopamine receptor; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; F/F₀, fluorescence intensity ratio; GST, glutathione S-transferase; HA, hemagglutinin; HEK293A, human embryonic kidney 293A; HRP, horseradish peroxidase; IR, infrared; KO, knockout; mApp, mApple control vector; mGlu, metabotropic glutamate receptor; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMSF, phenylmethane sulfonyl fluoride; sulfo-NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; TTBS, Tween Tris-buffered saline; WT, wild type.

Like mGlu₅, Ca²⁺/calmodulin (CaM)-dependent protein kinase II α (CaMKII α) is a key signaling protein in dendritic spines. CaMKII α is activated by Ca²⁺/CaM binding and undergoes autophosphorylation at Thr286 (Miller et al., 1988; Mukherji et al., 1994; Rich and Schulman, 1998; Yang and Schulman, 1999; Baucum et al., 2015). Thr286 autophosphorylation increases the affinity for Ca²⁺/CaM and stabilizes the kinase in a constitutively active conformation. This constitutive activity is essential for normal synaptic plasticity in many brain regions (Silva et al., 1992a,b; Giese et al., 1998; Zhou et al., 2007; Mockett et al., 2011; Coultrap et al., 2014; Shonesy et al., 2014; Jin et al., 2015) including mGlu_{1/5}-dependent long-term depression in the hippocampus (Huber et al., 2001; Mockett et al., 2011). Interestingly, both CaMKII α - and mGlu₅ knock-out (KO) mice display deficits in learning and memory and hippocampal synaptic plasticity (Jia et al., 1998; Huber et al., 2001; Simonyi et al., 2005). Although both mGlu₅ and CaMKII are critical to many forms of plasticity, a functional link between the two has not been widely investigated.

The intracellular C-terminal domains (CTDs) of mGlu₁ and mGlu₅ have emerged as important loci for regulation by protein binding and phosphorylation (Enz, 2012; Mao and Wang, 2016). Although there are two splice variants of mGlu₅ (mGlu_{5a} and mGlu_{5b}), most studies have focused on mGlu_{5a}, and a few pharmacological differences between splice variants have been identified (Joly et al., 1995; Minakami et al., 1995; Romano et al., 1996). The mGlu₅-CTD has been shown to bind to a number of different proteins including Ca²⁺/CaM and Homer to regulate cell-surface expression of the receptor (Roche et al., 1999; Saito et al., 2002; Lee et al., 2008; Choi et al., 2011). Protein kinase A (PKA) and protein kinase C (PKC) also regulate mGlu₅ surface expression through phosphorylation of the CTD (Mao et al., 2008; Uematsu et al., 2015). It was recently reported that CaMKII can bind to the CTD and intracellular loop 2 of both mGlu₁ and mGlu₅ (Jin et al., 2013a,b; Raka et al., 2015) and that CaMKII modulates mGlu₅ agonist-induced internalization and ERK1/2 activation (Raka et al., 2015). Here, we identify three basic residues (Lys⁸⁶⁶-Arg⁸⁷⁷-Arg⁸⁸⁸) in the membrane proximal region of the mGlu_{5a}-CTD that are essential for a direct interaction with activated CaMKII α , and provide novel insights into multiple factors that modulate the interaction. We also show that CaMKII binding to the CTD is important for the regulation of mGlu₅ surface expression and Ca²⁺ mobilization. These data provide novel insights into the molecular basis and function of the mGlu₅-CaMKII interaction that may be involved in synaptic plasticity.

Materials and Methods

DNA Constructs

The glutathione *S*-transferase (GST)-mGlu_{5a}-CTD expression construct was created by polymerase chain reaction amplification of the region encoding residues 827–964 of mGlu_{5a} (NP_058708.1) using forward primer 5'-CTGGAAGTTCTGTTCCAGGGGCCCCGATCCA-AACCGGAGAGAAAT-3' and reverse primer 5'-GCCGCAAGCTTGT-CGACGGAGCTCGAATTCTTAGGTCCCAAAGCGCTT-3' and inserting the product into BamHI/EcoRI sites of pGEX6P using a sequence- and ligation-independent cloning protocol (Li and Elledge, 2012).

The pCGN plasmid to express wild-type (WT) mGlu_{5a} with an N-terminal hemagglutinin (HA) tag was made by amplifying the entire rat mGlu_{5a} coding sequence (forward primer: 5'-TGACGTGCCTGACT-ATGCCTCTAGAATGGTCTTCTGTTGATCCT-3'; reverse primer:

5'-ACTCACCTGAAGTTCTCAGGATCCTCACAACGATGAAGAACT-CT-3') and inserting the fragment into XbaI and BamHI restriction sites of the empty pCGN plasmid [a gift from Dr. Winship Herr, Université de Lausanne, Switzerland; Addgene (Cambridge, MA) plasmid ID 53308].

The K^{866RR} mutation to AAA in mGlu_{5a} was generated by site-directed mutagenesis of the pGEX6P or pCGN constructs (see above) using a Quick Change protocol (Agilent Technologies, Santa Clara, CA) with the following primers: forward, 5'-GGGTTTCCCCAGAGGAG-CGGGCGGCGGCCACAGGTTGACTAGGCTGCT-3'; and reverse, 5'-AGCAGCCTAGTCAACCTGTGGGCCGCCGCGGCTCCTCTG-GGGAAACCC-3'.

We used pcDNA3.1 constructs to express untagged and mApple-tagged WT-CaMKII α and a constitutively active (CA) T286D/T305A/T306A triple mutant of CaMKII α (CA-CaMKII α), as previously described (Jiao et al., 2008; Jalan-Sakrikar et al., 2012; Stephenson et al., 2017). In the CA-CaMKII α , the phospho-mimetic T286D mutation results in constitutive CaMKII α activity and the phospho-null T305A/T306A mutations prevent CaMKII α phosphorylation at these sites, which interferes with binding of Ca²⁺/CaM and α -actinin (Jalan-Sakrikar et al., 2012).

Recombinant Protein Purification

Expression and purification of recombinant mouse CaMKII α has been described before (McNeill and Colbran, 1995). To express GST-tagged proteins, pGEX6P-1 plasmids were transformed into BL21(DE3) bacteria cells. Cells were grown in Lysogeny broth media at 37°C to reach an optical density of ~0.6. Cells were cooled to room temperature, and isopropyl β -D-1-thiogalactopyranoside (0.2 mM) was then added to induce protein expression for 12–16 hours. Inducing protein expression at room temperature substantially reduced the protein degradation seen when proteins were expressed at 37°C. Expressed proteins were purified using Pierce Glutathione Agarose Beads (cat. no. 16101; Thermo Fisher Scientific, Waltham, MA) following manufacturer instructions. Eluted proteins were then dialyzed in 10 mM HEPES pH 7.5, 25 μ M phenylmethane sulfonyl fluoride (PMSF), 62.5 μ M benzamidine, 62.5 μ M EDTA, and 0.1% Triton X-100 overnight with one buffer change.

CaMKII α Autophosphorylation

Purified mouse CaMKII α was autophosphorylated under two different conditions. Typically, CaMKII α was incubated with 50 mM HEPES, pH 7.5, 10 mM Mg(CH₃-COO)₂, 0.5 mM CaCl₂, 2 μ M CaM, and 40 μ M ATP on ice for 90 seconds before the addition of EDTA and EGTA (20 mM final) to terminate phosphorylation by chelation of Mg²⁺ and Ca²⁺. Similar conditions were previously shown to result in the selective autophosphorylation of Thr286 (McNeill and Colbran, 1995). Where indicated, identical autophosphorylation reactions were incubated for 10 minutes at 30°C to perform a more extensive phosphorylation at several additional sites (Baucum et al., 2015).

GST Pull-Down and CaM Binding Competition

Purified GST-mGlu₅-CTD (1 μ M) and CaMKII α (62.5 nM; preautophosphorylated as indicated in the figure legends) were incubated at 4°C in GST pull-down buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% (v/v) Triton X-100] with either 2 mM EGTA or 2.5 mM CaCl₂ plus 10 μ M CaM, as indicated. An aliquot (5%) of each incubation was saved as an input sample. After 1 hour, prewashed Pierce Glutathione Agarose Beads (cat. no. 16101; Thermo Fisher Scientific) (15 μ l of a 50:50 slurry) were added, and incubation was continued at 4°C for an additional 1 hour. The beads were then separated by centrifugation (2000g, 30 seconds) and washed three times with GST pull-down buffer containing either 2 mM EGTA or 2.5 mM CaCl₂, respectively. Beads were then incubated at 4°C with GST pull-down buffer containing 20 mM glutathione, adjusted to pH 8.0, for 10 minutes. After centrifugation, eluted proteins were transferred to a new tube, mixed with 4 \times SDS-PAGE buffer and heated for 10 minutes at 90°C prior to SDS-PAGE and Western blot analysis.

Cell Culture, Transfection and Immunoprecipitation

HEK293A cells (cat. no. R70507; Thermo Fisher Scientific) were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate, at 37°C in a humidified incubator containing 5% CO₂ and 95% O₂. Vectors encoding mApple-CaMKII α (WT or CA) and mGlu_{5a} (3 μ g DNA each) or empty vector controls (3 μ g) were cotransfected into one 10-cm dish of 60%–70% confluent HEK293A cells using 3 μ l/ μ g DNA of Fugene 6 (cat. no. E2691; Promega, Madison, WI). About 48 hours later, cells were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5% NP40 (v/v), 0.5% deoxycholate (v/v), 0.2 mM PMSF, 1 mM benzamide, 10 μ g/ml leupeptin, 10 μ M pepstatin, and 1 μ M microcystin. Cell lysates were cleared by centrifugation (10 minutes at 12,000g), and a 30 μ l sample of the input was saved for SDS-PAGE. The remaining supernatant was incubated at 4°C for 1 hour with rabbit anti-HA antibodies and 20 μ l prewashed Dynabeads Protein A (50% v/v; cat. no. 10001D; Thermo Fisher Scientific). Beads were isolated magnetically, washed three times using lysis buffer, and eluted using 2 \times Laemmli sample buffer for 10 minutes at room temperature prior to SDS-PAGE and Western blotting.

Biotinylation and Cell Surface Expression

Transfected HEK293A cells (see above) were placed on ice, the media were gently removed, and the cells were immediately washed two times using ice-cold phosphate-buffered saline (PBS). Cells were then scraped into ice-cold PBS, transferred to a 1.5-ml tube, centrifuged at 4°C (500g; 3 minutes), and gently resuspended in 1 ml of cold PBS containing 2 mg of EZ-Link sulfo-NHS-SS-biotin (Thermo-Fisher). After gently rocking for 1 hour, excess reagent was quenched by the addition of 50 mM Tris HCl, pH 8.0, and cells were centrifuged and washed again in 1 ml of 50 mM Tris HCl. Cells were then suspended in 1 ml of ice-cold lysis buffer (25 mM Tris HCl, pH 7.4; 150 mM NaCl; 1% NP40; 0.5% sodium deoxycholate containing 0.2 mM PMSF; 1 mM benzamide; 10 μ g/ml leupeptin; and 10 μ M pepstatin) and incubated on ice for 30 minutes. Insoluble material was removed by centrifugation (16,000g; 10 minutes, at 4°C), and a 30- μ l aliquot of the supernatant was saved for an input sample for SDS-PAGE (Cho et al., 2014). The remaining supernatants were mixed for 1 hour at 4°C with magnetic NeutrAvidin beads (30 μ l; 50% slurry; Thermo Fisher Scientific). The beads were separated magnetically and washed three times with lysis buffer. Biotinylated proteins were dissociated from the beads in SDS sample buffer containing 150 mM DTT for 10 minutes at room temperature. The biotinylated and total protein samples were analyzed by Western blotting for mGlu₅.

Immunoblotting and Semiquantitative Analysis

Since heating samples results in aggregation of full-length mGlu₅ protein, all samples that were blotted for the full-length receptor were incubated for 10 minutes at room temperature before SDS-PAGE. SDS-polyacrylamide gels were transferred to nylon-backed nitrocellulose membranes in 10 mM 3-(cyclohexylamino)propanesulfonic acid buffer. After blocking in Tween Tris-buffered saline [TTBS; 50 mM Tris-HCl, pH 7.5, 0.1% (v/v) Tween 20, 150 mM NaCl] containing 5% nonfat milk, membranes were incubated for either 2 hours at room temperature for purified protein studies or overnight at 4°C in HEK293A cell and brain lysate samples with primary antibodies diluted in TTBS with 5% milk. Membranes were washed five times in TTBS and incubated for 1 hour at room temperature with secondary antibodies conjugated to horseradish peroxidase (HRP) (Promega; or Santa Cruz Biotechnology, Dallas, TX), or infrared (IR) dyes (LI-COR Biosciences, Lincoln, NE) diluted in TTBS with 5% milk. Antibody signals were visualized via enzyme-linked chemiluminescence using the Western Lightning Plus-ECL enhanced chemiluminescent substrate (PerkinElmer, Waltham, MA) and visualized using Premium

X-ray Film (Phenix Research Products, Candler, NC). Secondary antibodies conjugated to IR dyes (LI-COR Biosciences) were used for development with an Odyssey System (LI-COR Biosciences). Images were quantified using ImageJ software.

Antibodies

The following antibodies were used for immunoblotting at the indicated dilutions: total CaMKII α (1:5000; cat. no. MA1-048; Thermo Fisher Scientific) and p-Thr286 CaMKII α (1:3000; cat. no. sc-12886-R; Santa Cruz Biotechnology); mGlu₅-specific antibody (1:3000; cat. no. AB5675; MilliporeSigma, Burlington, MA); rabbit anti-HA (5 μ l for immunoprecipitation; cat. no. sc805; Santa Cruz Biotechnology); and goat-GST antibody (1:10,000; cat. no. ab181652; Abcam, Cambridge, UK).

Secondary Antibodies. Secondary antibodies were as follows: HRP-conjugated anti-rabbit (1:3000; cat. no. W4011; Promega), HRP-conjugated anti-mouse (1:3000; cat. no. W4021; Promega), and HRP-conjugated anti-goat (1:3000; cat. no. sc-2056; Santa Cruz Biotechnology); IR dye-conjugated donkey anti-rabbit 800CW (1:10,000; cat. no. 926–32213; LI-COR Biosciences), and IR dye-conjugated donkey anti-mouse 680LT (1:10,000; cat. no. 926–68022; LI-COR Biosciences).

Mice

CaMKII-KO mice were generated in the Vanderbilt Transgenic Mouse Core as a by-product of published CRISPR/Cas9-mediated experiments directed at creating a knockin E183V mutation of CaMKII α (Stephenson et al., 2017). We selected a founder containing a deletion of 11 base pairs (bp) (TGCTGAGGAAG) from exon 8, leading to a frame shift and early translational termination. Primers used to genotype the CaMKII α KO mice are as follows: forward, 5'-GATAC-CTCTCCCCAGAGGAC-3', reverse, 5'-TGCAGTGGTAAGGAGTG-GTG-3' for WT; and forward, 5'-GGACAGTACAACCCAGCTT-3', and reverse, 5'-CCCGTACGGGTCTTCTCTCA-3' for KO, generating a 206-bp band for WT, a 351-bp band for KO, and a 557-bp band for all mice. The CaMKII α KO was confirmed by immunoblotting brain lysates. All mice were on a mixed B6D2 [C57BL/6J (B6) \times DBA/2J (D2)] background and were housed (2–5 per cage) on a 12-hour light/dark cycle with food and water ad libitum. WT and KO experimental mice (littermates) were generated using an HETXHE breeding strategy. All animal procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Mouse Brain Tissue Preparation and Immunoprecipitation

Both male and female mice (30–60 days old) were anesthetized with isoflurane and decapitated, and forebrains were quickly dissected. Half of a forebrain (cut along the mid-line) was homogenized using at least 20 strokes with a dounce homogenizer in 1.5 ml of an isotonic buffer containing 150 mM KCl, 50 mM Tris-HCl, 1 mM DTT, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.2 mM PMSF, 1 mM benzamide, 10 μ g/ml leupeptin, 10 μ M pepstatin, and 1 μ M microcystin. The homogenate was rotated end over end at 4°C for 30 minutes and then centrifuged at 10,000g for 30 minutes to remove insoluble material. A 30- μ l input sample was saved before CaMKII α (MA1-048) antibody, and 20 μ l of magnetic Protein G beads (cat. no. 10003D; Invitrogen) were added to 1 ml of homogenate and rotated end over end for 3–4 hours. Beads were separated magnetically and washed three times with homogenization buffer. Immunoprecipitated complexes were eluted using 2 \times Laemmli Sample Buffer containing 150 mM DTT for 10 minutes at room temperature and analyzed by immunoblotting.

Ca²⁺ Imaging in 96-Well Plates

A FlexStation II liquid handler/plate reader (Molecular Devices, Sunnyvale, CA) was used for intracellular Ca²⁺ measurements in HEK293A cells stably expressing low amounts of the rat mGlu_{5a}

receptor (293A-5a^{LOW} cells), as previously described (Hammond et al., 2010; Gregory et al., 2012; Noetzel et al., 2012). The cells were maintained at 37°C in complete DMEM supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES pH 7.5, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic/antimycotic solution (Thermo Fisher Scientific), and 500 µg/ml G418 in a humidified incubator containing 5% CO₂/95% O₂. For experiments, 10-cm dishes were transfected with 3 µg of mApple control vector (mApp) or 3 µg of mApp-CaMKIIα (WT or CA; see above). On the following day, cells were transferred to clear-bottomed, black-walled, poly-D-lysine-coated 96-well plates (BD BioCoat; BD Biosciences, San Jose, CA) (3 × 10⁴ cells/well) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate, and incubated overnight at 37°C in 5% CO₂. Approximately 24 hours later, medium was manually removed and replaced with Hanks' balanced salt solution containing 20 mM HEPES, 2.5 mM probenecid, and 2 µM Fluo-4/acetoxymethyl ester (AM) dye (pH 7.4), and plates were incubated for 30 minutes (37°C, 5% CO₂). This medium was manually removed and replaced with 40 µl of calcium assay buffer (Hanks' balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid, pH 7.4). Glutamate additions were performed after a 30-second baseline to construct concentration-response curves, and plates were monitored for a total of 120 seconds using an excitation wavelength of 488 nm, an emission wavelength of 525 nm, and a cutoff wavelength of 515 nm. Data were collected with SoftMax Pro (Molecular Devices) then transformed, and agonist concentration-response curves were fitted to a four-parameter logistic equation with GraphPad Prism (GraphPad Software, San Diego, CA). For area under the curve measurements, time parameters were set to measure the area under the curve between the time points of 10–60 seconds to capture the initial Ca²⁺ peak. Raw values were generated by SoftMax Pro and normalized to the maximum response of control cells.

Single Cell Ca²⁺ Imaging

HEK293A cells were transiently transfected to express full-length mGlu_{5a} (WT or mutated as indicated: 0.3 µg DNA) and either mApple or mApple-CA-CaMKIIα (3 µg DNA). On the following day, transfected cells were plated in clear glass-bottomed, poly-D-lysine-coated 29 mm dishes (D29-10-1.5-N; Cellvis, Mountain View, CA) (5 × 10⁴ cells) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (Thermo Fisher Scientific) and incubated overnight at 37°C in 5% CO₂. On the day of the experiment, cells were incubated in media supplemented with 2 µM Fura-2/AM (Thermo Fisher Scientific) for 20 minutes at 37°C in 5% CO₂, and then transferred to a Ca²⁺ imaging solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES; at pH 7.5 and ~313 mOsm). After incubation for 20 minutes at 37°C in 5% CO₂, fluorescence imaging was performed using a Nikon (Tokyo, Japan) Eclipse TE2000-U Microscope equipped with an epifluorescence illuminator (Sutter Instrument, Novato, CA), a charge-coupled device camera (model HQ2; Photometrics, Tucson, AZ), and Nikon Elements software. Cells were perfused at 37°C at a flow rate of 2 ml/min with Ca²⁺ imaging solution. First, the field of view was imaged using 568-nm excitation to detect cells expressing mApple. Then, ratios of emitted fluorescence (at 510 nm) from mApple-positive cells were measured after excitation at 340 and 380 nm (F340/F380); ratios were measured every 3 seconds for a 1-minute baseline period. Then the cells were treated with 100 µM glutamate (added to the Ca²⁺ imaging solution) for 10 minutes, during which time the Fura-2 F340/F380 ratios were collected every 3 seconds. Relative changes in Ca²⁺ levels of the mApple-expressing cells were analyzed using Nikon Elements software. The F340/F380 ratios of each cell were normalized to the first F340/F380 ratio acquired for that cell during the baseline period [fluorescence intensity ratio (F/F0) = (340/380 value)/(baseline 340/380 value)] and then analyzed using Clampfit software (Molecular Devices). Peak Ca²⁺ responses for all cells were aligned (at 45 seconds), a ROUT test was first used to identify outliers in the maximal ΔF/F0 values for all cells within each

experimental group, and then ΔF/F0 values for each time point were averaged together for each dish of cells. The maximal Ca²⁺ response was defined as the average of all the peak ΔF/F0 values on an experimental day. An average trace for each day of experiments was generated to calculate the half-life of the Ca²⁺ signal; the decline of the average ΔF/F0 values in each dish were normalized to the average maximal Ca²⁺ response in that dish and then fitted to a nonlinear one-phase exponential decay fit constrained to y0 = 1 using GraphPad Prism version 6.0. We determined ΔF/F0 and half-lives in five independent experiments (transfections) on separate days (19–124 cells/condition per day) and tested for differences using a Student's *t* test. All values are presented as the mean ± S.E.M.

Results

Mouse Forebrain Lysates Contain CaMKIIα-mGlu₅ Complexes. To confirm that mGlu₅ specifically associates with CaMKII in the brain, we incubated forebrain lysates from WT or CaMKIIα-KO mice with a CaMKIIα-specific monoclonal antibody or a control IgG. The resulting immune complexes were isolated and then immunoblotted for mGlu₅ and CaMKIIα. Note that the mGlu₅ antibody used for these studies recognizes an epitope that is shared by the two known mGlu₅ splice variants, mGlu_{5a} and mGlu_{5b}, which are differentially expressed during development (Minakami et al., 1995; Romano et al., 1996). Input samples from WT and CaMKIIα KO tissue prepared in parallel contain similar levels of the monomeric and dimeric forms of mGlu₅ (Fig. 1), although the ratio of monomeric and dimeric species varied between independent experiments (data not shown). CaMKIIα complexes isolated from WT mouse forebrain contained both monomeric and dimeric forms of mGlu₅, with the ratio of these forms reflecting variability in the ratio detected in the inputs. However, very little mGlu₅ could be detected in IgG control complexes isolated from WT tissue, or in CaMKIIα complexes isolated from CaMKIIα KO tissue (Fig. 1). Thus, mGlu₅ is a bona fide component of the CaMKIIα complexes present in mouse brain lysates.

CaMKIIα Directly Binds to the mGlu₅ C-Terminal Domain. Like most prior studies, we chose to use the mGlu_{5a} splice variant for our molecular studies. It was previously

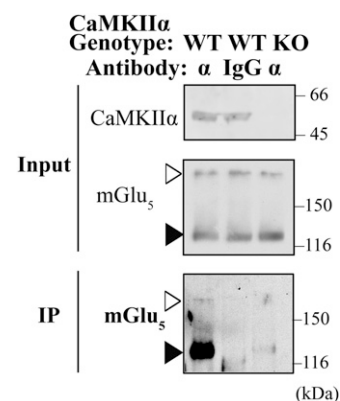


Fig. 1. Coimmunoprecipitation of mGlu₅ with mouse forebrain CaMKIIα. Solubilized fractions from WT or CaMKIIα KO mouse forebrain were immunoprecipitated (IP) using CaMKIIα-specific (α) or control (IgG) antibodies, as indicated. Inputs and immune complexes were analyzed by immunoblotting: mGlu₅ was detected only in immune complexes isolated from WT tissue using the CaMKIIα antibody. Open and closed arrowheads indicate dimeric and monomeric species of mGlu₅. The figure is representative of three similar experiments.

reported that residues 827–964 of the mGlu_{5a}-CTD bind to inactive CaMKII α , but that CaMKII autophosphorylation disrupted the interaction (Jin et al., 2013b). To confirm this finding, we generated a GST-tagged mGlu_{5a}-CTD construct containing residues 827–964 (GST-mGlu_{5a}-CTD) for use in glutathione agarose cosedimentation experiments. Initial studies detected weak binding of inactive CaMKII α to GST-mGlu_{5a}-CTD that was not consistently above background binding to a GST negative control (data not shown). Therefore, we systematically tested interactions of GST-mGlu_{5a}-CTD with CaMKII α in various activation states.

Purified CaMKII α was autophosphorylated in the presence of Ca²⁺/CaM in vitro at either 4°C or 30°C. Similar total levels of Thr286 autophosphorylation were detected by immunoblotting after incubation at either 4°C or 30°C (Fig. 2A), but the 30°C autophosphorylation reduced the electrophoretic mobility of CaMKII α . These observations are consistent with those of prior studies showing that incubation at 4°C results in selective Thr286 autophosphorylation (McNeill and Colbran, 1995), whereas incubation at 30°C allows for extensive autophosphorylation at several other sites (Baucum et al., 2015).

We then performed glutathione-agarose cosedimentation experiments to test the interaction of GST-mGlu_{5a}-CTD with CaMKII α in these different activation states (after terminating the autophosphorylation reactions by chelating metal ions with excess EGTA and EDTA). The selective Thr286-autophosphorylation (4°C) protocol resulted in a robust enhancement of CaMKII α binding to GST-mGlu_{5a}-CTD relative to the nonphosphorylated kinase, but this interaction was substantially reduced after more extensive in vitro phosphorylation at 30°C (Fig. 2B). The short exposure times used for the development of these immunoblots failed to detect weak binding of inactive CaMKII α to GST-mGlu_{5a}-CTD. In combination, these data show that although activation and Thr286 autophosphorylation of CaMKII α strongly enhance binding to the mGlu_{5a}-CTD, the interaction can be reduced by autophosphorylation at additional non-Thr286 sites.

Binding of Activated CaMKII α to the GST-mGlu_{5a}-CTD Is Disrupted by Ca²⁺/CaM. Ca²⁺/CaM binds to residues 889–917 within the CTD of mGlu_{5a} with important functional consequences (Minakami et al., 1997; Lee et al., 2008; Choi et al., 2011). Moreover, it was previously reported that excess Ca²⁺/CaM disrupts the binding of inactive

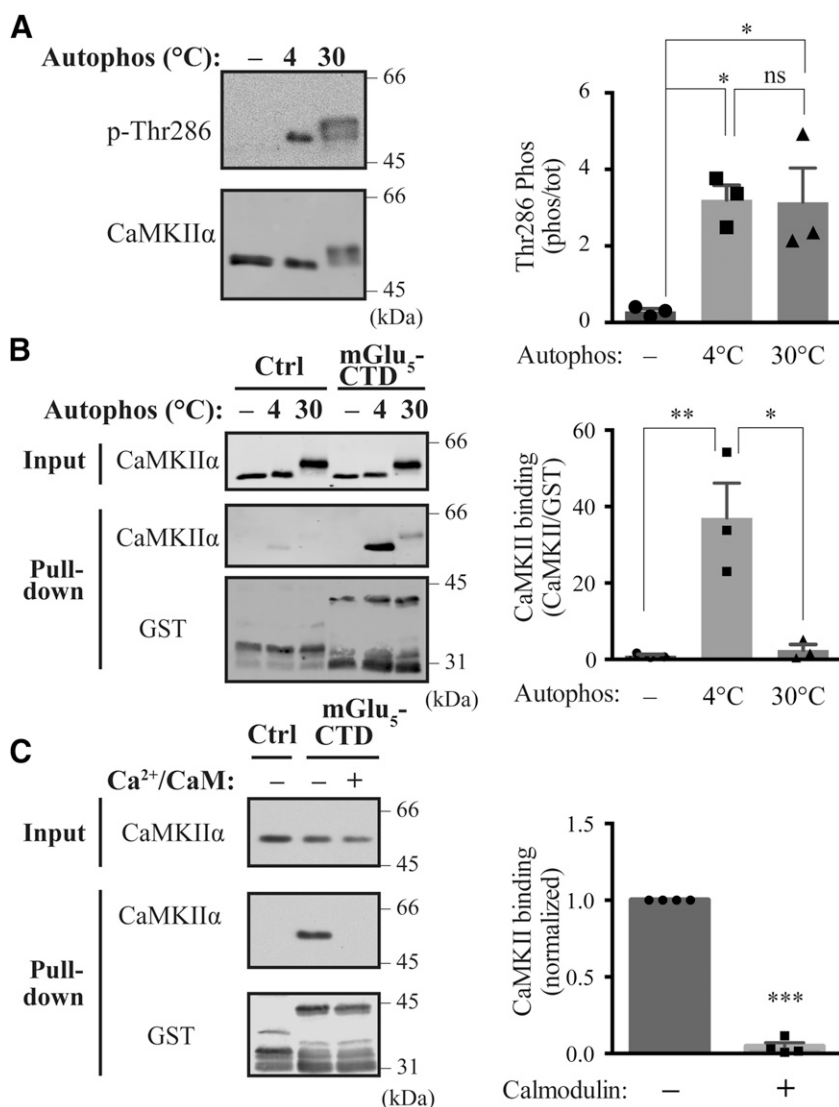


Fig. 2. CaMKII autophosphorylation at Thr286 enhances binding to the mGlu₅ CTD. (A) Autophosphorylation (Autophos) of purified CaMKII α . Purified CaMKII α was incubated with Mg(C₂H₃O₂)₂, CaCl₂, CaM, and ATP for either 90 seconds at 4°C or 10 minutes at 30°C, and samples were immunoblotted for total or phospho-Thr286 (p-Thr286) CaMKII. Although quantitative analysis (right) indicated that there was a similarly robust Thr286 autophosphorylation using these two conditions, the 10-minute/30°C incubation resulted in a substantial reduction in electrophoretic mobility due to phosphorylation at additional unidentified sites. Data are plotted as the mean \pm S.E.M. ($n = 3$) and analyzed using a one-way ANOVA ($P = 0.0167$, $F = 8.746$, $R^2 = 0.7446$) with Sidak's post hoc test for multiplicity adjusted P values: control (Ctrl) vs. 4°C, $P = 0.031$; control vs. 30°C, $P = 0.035$; 4°C vs. 30°C, $P = 1.00$. (B) The GST-mGlu_{5a}-CTD binds CaMKII α after selective autophosphorylation at Thr286. GST-mGlu_{5a}-CTD was incubated with purified CaMKII α that had been preincubated as in (A), and complexes were isolated using glutathione agarose. Immunoblot analyses revealed that CaMKII α binding to the CTD was strongly enhanced by selective Thr286 autophosphorylation at 4°C, but that the autophosphorylation of additional sites on CaMKII at 30°C substantially reduced binding. Data are plotted as the mean \pm S.E.M. ($n = 3$) and were analyzed using a one-way ANOVA ($P = 0.005$, $F = 2.477$, $R^2 = 0.829$) with Sidak's post hoc test for multiplicity-adjusted P values: control vs. 4°C, $P = 0.009$; 4°C vs. 30°C, $P = 0.011$; control vs. 30°C, $P = 1.00$. (C) Binding of activated CaMKII α to GST-mGlu_{5a}-CTD is disrupted by Ca²⁺/CaM. Purified CaMKII α was autophosphorylated for 90 seconds at 4°C [see (A)] and then incubated with GST-mGlu_{5a}-CTD in the absence or presence of excess Ca²⁺/CaM (see Materials and Methods). Complexes were isolated using glutathione-agarose and then immunoblotted as indicated. Data are plotted as the mean \pm S.E.M.; excess Ca²⁺/CaM significantly reduced CaMKII α binding ($P < 0.0001$ relative to a theoretical value of 1.00, one-sample t test; $n = 4$). In all panels, the symbols *, ** and *** indicate $P < 0.05$, 0.01 and 0.001, respectively, with n.s. indicating non-significant ($P > 0.05$).

CaMKII α to the mGlu₅-CTD (Jin et al., 2013b). Therefore, we tested whether excess Ca²⁺/CaM also disrupts the binding of activated CaMKII α to GST-mGlu₅-CTD. Thr286-autophosphorylated CaMKII α (4°C protocol) robustly binds to GST-mGlu₅-CTD, as noted above, but this interaction was essentially eliminated by the inclusion of excess Ca²⁺/CaM in the binding assay (Fig. 2C). Thus, binding of activated CaMKII α to the mGlu₅ CTD is also blocked by Ca²⁺/CaM, suggesting that multiple Ca²⁺-sensitive proteins are involved in the regulation of mGlu₅ signaling.

Identification of a CaMKII α -Binding Determinant in the mGlu₅ CTD. As an initial approach to identify key CaMKII α -binding determinants in the mGlu₅ CTD, we compared residues 827–964 of mGlu₅ with CaMKII α -binding domains that have been previously identified in other proteins. Our laboratory recently showed that activated CaMKII α binds to the N-terminal domains of Cav1.2 and Cav1.3 L-type voltage-gated Ca²⁺ channels, and that this interaction is disrupted by the mutation of three basic residues (Arg⁸³-Lys-Arg⁸⁵) to alanine (Wang et al., 2017). Similar tribasic residue motifs are also present within CaMKII α -binding domains that have been previously identified in the intracellular loops of the D₂ dopamine receptor (D₂R) and D₃ dopamine receptor (D₃R) (Liu et al., 2009; Zhang et al., 2014) and the mGlu₁-CTD (Jin et al., 2013a). Notably, the CaMKII α -binding fragment of the mGlu₅-CTD also contains a tribasic residue motif (residues Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸) (Fig. 3A). We found that substituting alanines for Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸ in the mGlu₅-CTD essentially abolished the binding of activated CaMKII α to GST-mGlu₅-CTD in vitro (Fig. 3B). These data identify a key determinant for CaMKII binding to the CTD of mGlu₅.

CaMKII α Activation Increases CaMKII α -mGlu₅ Association in Heterologous Cells. To better understand the interaction of CaMKII α with full-length mGlu₅ we conducted coimmunoprecipitation experiments from lysates of transfected HEK293A cells. We first tested the hypothesis that CaMKII α activation would increase the association with full-length mGlu₅, as with in vitro binding of CaMKII α to GST-mGlu₅-CTD. We expressed mApple-tagged WT CaMKII α in the absence or presence of mGlu₅ with an N-terminal HA-epitope tag in HEK293A cells. Prior to HA immunoprecipitation, the cell lysates were split into two aliquots and preincubated with either excess EGTA and EDTA or with Ca²⁺/CaM, Mg²⁺, ATP, and phosphatase inhibitors

to stimulate CaMKII α autophosphorylation. CaMKII activation in the lysates resulted in a robust increase in autophosphorylation at Thr286, without the large shift in electrophoretic mobility that was observed after autophosphorylation of purified CaMKII α at 30°C (Fig. 4A). HA-immunoprecipitation from the two preincubated lysates yielded similar amounts of the monomeric and dimeric species of HA-mGlu₅, but CaMKII α activation resulted in a statistically significant ~3-fold increase in the amount of coimmunoprecipitated CaMKII α (Fig. 4A). These data show that full-length mGlu₅ preferentially interacts with activated WT CaMKII α .

Association of Activated CaMKII α with Full-Length mGlu₅ Requires Arg⁸³-Lys-Arg⁸⁵. We next investigated whether the association of activated CaMKII α with full-length mGlu₅ involves the CTD. To avoid complications that might arise from preincubating cell lysates to activate WT-CaMKII α , we used an mApple-tagged CA-CaMKII α (mApple-CA-CaMKII α); the phosphomimetic T286D mutation results in constitutive CaMKII α activity, and the phospho-null T305A/T306A mutations prevent CaMKII α phosphorylation at these sites, which interferes with the binding of Ca²⁺/CaM and α -actinin (Jalan-Sakrikar et al., 2012). The mApple-CA-CaMKII α was expressed alone, or coexpressed with either HA-mGlu₅ or HA-mGlu₅-AAA (with Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸ mutated to alanines). HA-immunoprecipitation from cell lysates confirmed a robust association of mApple-CA-CaMKII α with WT mGlu₅ that was partially (~50%) reduced by the triple alanine mutation in the CTD (Fig. 4B). These data demonstrate that the Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸ residues in the mGlu₅-CTD play an important role in the association of activated CaMKII α with the full-length mGlu₅ receptor.

CaMKII α Increases Basal mGlu₅ Surface Expression. Since the CTD is known to modulate mGlu₅ cell-surface expression and consequently mGlu₅ signaling, we investigated the effect of CaMKII α on the cell-surface expression of full-length mGlu₅. Intact HEK293A cells expressing mGlu₅ with or without mApple-CA-CaMKII α were incubated with sulfo-NHS-SS-biotin to biotinylate all surface-expressed proteins. Streptavidin-conjugated magnetic beads were then used to isolate cell-surface proteins from cell lysates. Immunoblotting of total cell lysates and isolated cell-surface proteins revealed that the coexpression of mApple-CA-CaMKII α increased the proportion of mGlu₅ expressed on the cell surface by 3.0 \pm 0.7-fold (S.E.M.) under basal conditions (P = 0.036; one-

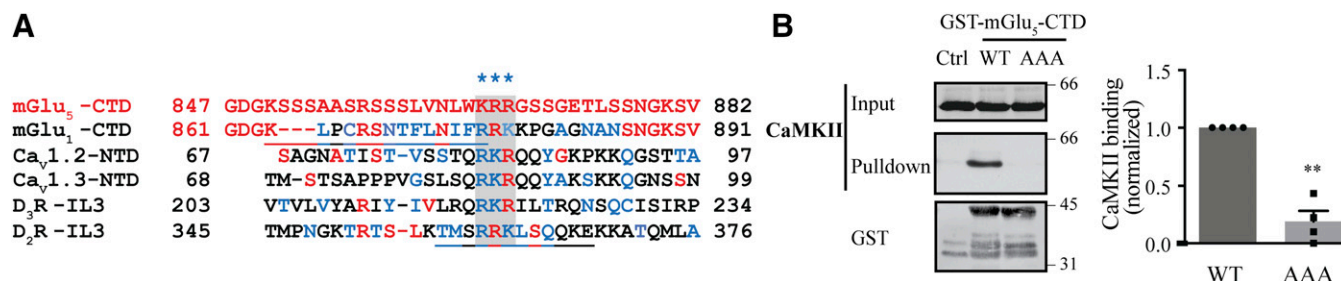


Fig. 3. Identification of CaMKII-binding determinants in the mGlu₅-CTD. (A) Alignment of part of the mGlu₅-CTD with amino acid sequences surrounding known CaMKII-binding domains. Tribasic residue motifs (highlighted with blue asterisks above) were identified within CaMKII-binding domains from other proteins, as well as within the CaMKII binding fragment in the CTD of mGlu₅. Mutation of R⁸³K⁸⁴R⁸⁵ to AAA in the Cav1.3 N-terminal domain disrupts the binding of CaMKII (Wang et al., 2017). The red and blue fonts indicate residues in each domain that are identical and homologous, respectively, with residues in the mGlu₅ sequence. Underlined residues in the mGlu₁-CTD and the D₂R and D₃R (IL3, third intracellular loop) demark the sequences of synthetic peptides that were shown to compete for CaMKII binding (Jin et al., 2013a; Zhang et al., 2014). (B) Mutation of the tribasic residue motif in the mGlu₅-CTD disrupts CaMKII binding. Thr286 autophosphorylated CaMKII α (90 seconds/4°C protocol) was incubated with GST-mGlu₅-CTD (WT or with a K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸ to AAA mutation), and complexes were analyzed as in Fig. 1. The K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA mutation essentially abolishes CaMKII binding. Data are plotted as the mean \pm S.E.M. (P = 0.003, one-sample t test; n = 4). In panel B, the symbol ** indicates P < 0.01.

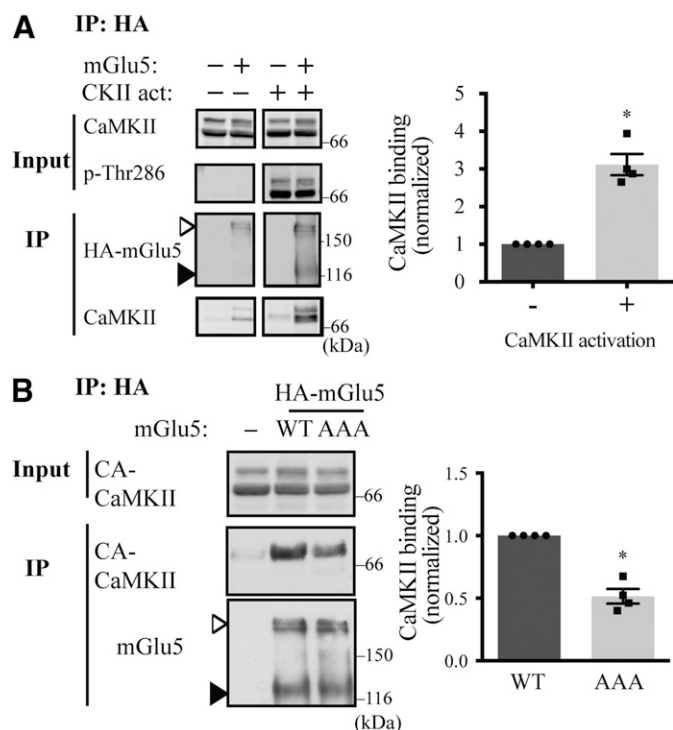


Fig. 4. Role of the CTD in full-length mGlu_{5α} binding to activated CaMKII α . (A) CaMKII activation enhances interaction with full length mGlu₅. Solubilized fractions of HEK293A cells expressing HA-tagged mGlu_{5α} and/or mApple-tagged WT CaMKII α (as indicated above lanes) were pre-incubated with Ca²⁺/CaM, MgAc₂, and ATP in the presence or absence of excess EDTA (\pm activation, respectively) and then immunoprecipitated (IP) using antibodies to the HA epitope. Lysates and immune complexes were analyzed by immunoblotting, as indicated. CaMKII α activation results in robust Thr-286 autophosphorylation, which increases CaMKII α association with HA-mGlu_{5α}. Data are plotted as the mean \pm S.E.M. ($P = 0.043$, one-sample t test; $n = 4$). Open and closed arrowheads indicate dimeric and monomeric species of mGlu_{5α}. (B) CaMKII association with full-length mGlu_{5α} is disrupted by mutation of the CTD tribasic residue motif. Solubilized fractions of HEK293A cells expressing HA-mGlu₅ (WT or with the K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA mutation) and mApple-tagged CA-CaMKII α were immunoprecipitated using antibodies to the HA epitope. Lysates and the immune complexes were analyzed by immunoblotting, as indicated. The K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA mutation reduced the association of CA-CaMKII α with HA-mGlu₅. Data are plotted as the mean \pm S.E.M. ($P = 0.028$, one sample t test; $n = 4$). p-Thr286, phospho-Thr286. In the bar graphs for both panels, * indicates $P < 0.05$.

sample t test vs. hypothetical value of 1) (Fig. 5). To determine whether CaMKII α interaction with the mGlu_{5α}-CTD is important for this effect, we examined the cell-surface expression of mGlu_{5α}-AAA. In the absence of coexpressed CaMKII, the surface expression of mGlu_{5α}-AAA was not significantly different from those of WT mGlu_{5α} [1.6 ± 0.6 -fold (S.E.M.); $n = 5$; $P = 0.35$; one-sample t test vs. hypothetical value of 1]. Moreover, the coexpression of mApple-CA-CaMKII α had no effect on cell-surface expression of mGlu_{5α}-AAA. These data demonstrate that interaction with the mGlu_{5α}-CTD is necessary for CaMKII α -mediated increases in mGlu_{5α} cell-surface expression.

CaMKII α Reduces mGlu_{5α}-Stimulated Peak Ca²⁺ Mobilization. To investigate the effect of CaMKII α on mGlu_{5α} signaling, we measured glutamate-induced Ca²⁺ mobilization in populations of 293A-5a^{LOW} cells that stably express mGlu_{5α} and were transiently transfected to coexpress mApple or mApple-tagged CaMKII α (either WT or CA). A similar fraction of the total cells expressed detectable levels of

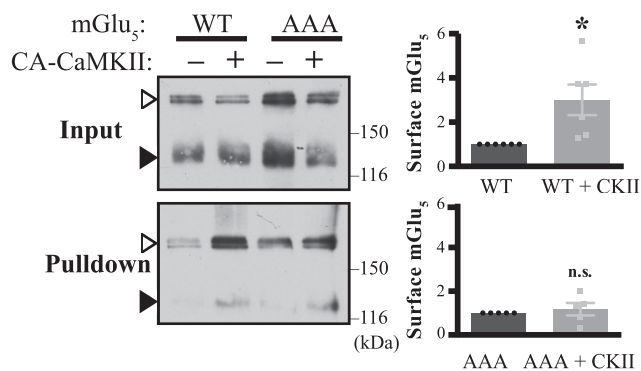


Fig. 5. CaMKII enhances the cell-surface expression of mGlu_{5α} via interaction with the CTD. Cell-surface biotinylation analyses of HEK293A cells expressing mGlu_{5α} (WT or with K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA mutation in the CTD) with either mApple or mApple-tagged CA-CaMKII α . The coexpression of CA-CaMKII α increased steady-state surface expression levels of WT mGlu_{5α} ($P = 0.036$, one-sample t test; $n = 6$), but not of the K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA mutant. Data are plotted as the mean \pm S.E.M. ($P = 0.569$, one-sample t test; $n = 5$). * $P < 0.05$; n.s., non-significant.

mApple-tagged WT- or CA-CaMKII α in each transfection (typically $\sim 60\%$). After loading glutamate-starved cells with Fluo-4-AM, a fluorescent Ca²⁺ indicator, we measured fluorescence responses of total cell populations to increasing glutamate concentrations (0.01–100 μ M) (Fig. 6A). An overlay of raw traces from cells expressing mApple, mApple-WT-CaMKII α , or mApple-CA-CaMKII α in a representative experiment is shown in Fig. 6B. Peak Ca²⁺ responses (increased fluorescence) at each glutamate concentration were expressed as a ratio to the maximum response to a saturating concentration of glutamate (100 μ M) in mApple-expressing control cells for each individual experiment, and then data were averaged across five independent experiments. Glutamate increased the peak fluorescence in a concentration-dependent manner, with an apparent EC₅₀ value of 0.38 ± 0.03 μ M in control cells, similar to previous analyses (Schoepp et al., 1999; Hammond et al., 2010). The glutamate response was unaffected by the coexpression of mApple-WT-CaMKII α , but the coexpression of mApple-CA-CaMKII α reduced peak Ca²⁺ responses at the highest concentrations of glutamate by approximately 20%, without affecting the apparent EC₅₀ value (Fig. 6C). As an alternative measure of Ca²⁺ responses, we determined the area under the curve of the initial Ca²⁺ peak at the highest glutamate concentration. There was no difference in the area under the curve between cells expressing mApple or mApple-CaMKII α -WT, but the coexpression of mApple-CA-CaMKII α significantly reduced the area under the curve (control, 109.2 ± 2.7 ; WT, 101.3 ± 7.4 ; CA, 82.6 ± 4.1 ; one-way analysis of variance (ANOVA), $P = 0.011$, $F = 6.280$; Sidak's post hoc test for multiplicity-adjusted P values: WT vs. control, $P = 0.51$; CA vs. control, $P = 0.0073$) (data not shown). Since mApple-CA-CaMKII α is expressed in only a fraction of the cell population in each well, the measured reductions in maximal Ca²⁺ responses presumably underestimate the actual impact of expressing CA-CaMKII α in each cell. However, these data cannot differentiate whether this effect reflects decreased Ca²⁺ mobilization within each cell or a decrease in the fraction of responsive cells. Nevertheless, the data indicate that the coexpression of CA-CaMKII α , but not WT-CaMKII α , can reduce mGlu_{5α}-stimulated peak Ca²⁺ mobilization.

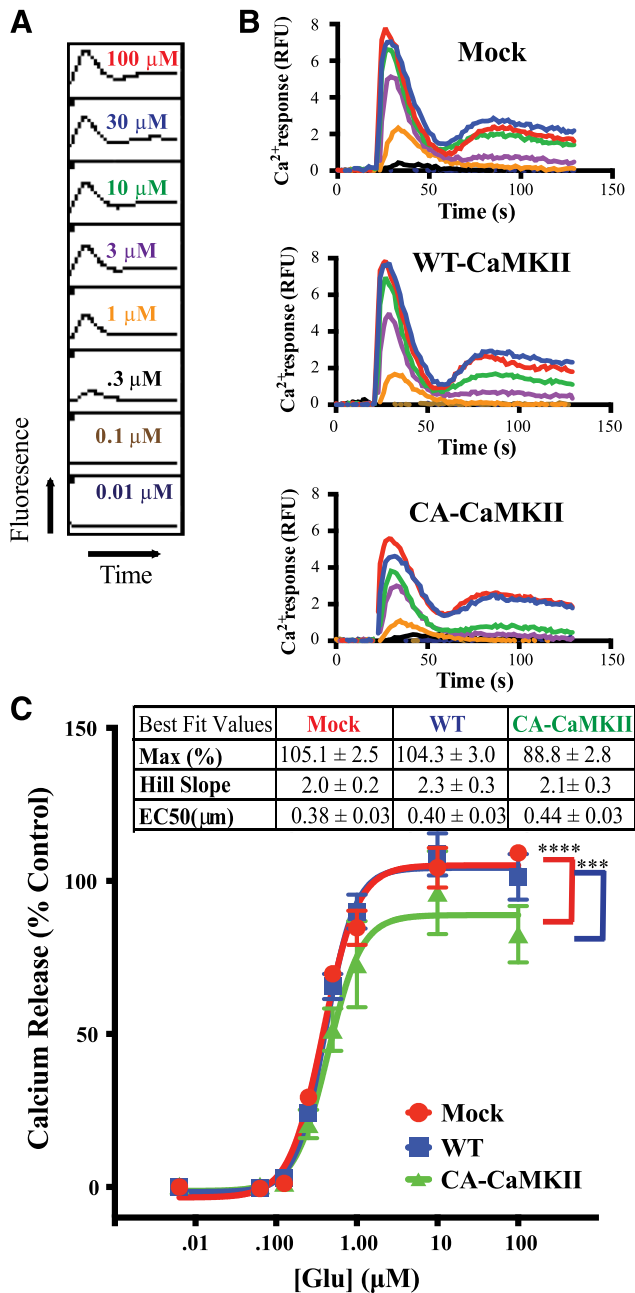


Fig. 6. CaMKII α regulates mGlu₅ α -stimulated Ca²⁺ mobilization in 293A-5a^{LOW} cells. Time courses of intracellular Ca²⁺ responses to glutamate were measured by changes in Fluo-4 fluorescence in stable 293A-5a^{LOW} cells in 96-well plates. (A) Time courses of Ca²⁺ responses. Example of calcium responses to increasing glutamate concentrations was collected in a row of eight wells. (B) Overlay of individual Ca²⁺ responses to increasing concentrations of glutamate [labeled by colors in (A)] from 293A-5a^{LOW} cells transiently transfected to express mApple control, mApple-CaMKII α -WT, or mApple-CA-CaMKII α from a representative experiment. (C) Concentration-response curves. Initial peak Ca²⁺ responses ($\Delta F/F_0$) at each concentration were normalized to the maximal glutamate-stimulated response in control (mApple-transfected) cells within each experiment. Normalized Ca²⁺ responses are plotted as the mean \pm S.E.M. ($n = 5$ experiments) as a function of glutamate concentration. The expression of CaMKII α -WT had no impact on the Ca²⁺ response curve, but the expression of CA-CaMKII α reduced peak Ca²⁺ responses (multiple-comparisons two-way ANOVA: sources of variation: CaMKII, $P < 0.0001$; interaction, $P = 0.029$. Tukey's post hoc test for multiplicity-adjusted P values: mApple vs. WT, $P = 0.926$; mApple vs. CA-CaMKII α , $P < 0.0001$; WT vs. CA-CaMKII α , $P = 0.0002$). The inset table shows the maximum response (Max), EC₅₀ (μM), and Hill coefficient (\pm S.E.M.) obtained by fitting the data in GraphPad Prism. In panel C, *** and **** indicate $P < 0.001$ and 0.0001 , respectively.

CaMKII α Prolongs mGlu₅ α -Mediated Ca²⁺ Signaling.

To address caveats associated with studies investigating the effects of mApple-CA-CaMKII α on Ca²⁺ mobilization in 293A-5a^{LOW} cells, we also examined Ca²⁺ mobilization in single HEK293A cells transfected to express full-length mGlu₅ α with either mApple alone (control) or mApple-CA-CaMKII α . After loading all cells with Fura-2/AM, a ratiometric Ca²⁺ indicator, single cells were selected for analysis based on the presence of mApple as a marker of transfection. Application of 100 μM glutamate to cells coexpressing soluble mApple or mApple-CA-CaMKII α with WT mGlu₅ α produced an initial peak of Fura-2 fluorescence followed by highly variable changes of fluorescence over the next 10 minutes (Fig. 7A). In a majority of cells in each group (53%–68%) Ca²⁺ signals waned over time, sometimes with a secondary shoulder, but subpopulations of the cells displayed clear Ca²⁺ oscillations that either returned to baseline between oscillations (10%–21%) or were superimposed on a more sustained Ca²⁺ elevation (18%–25%) (Supplemental Fig. 1, A–C). However, the percentage of WT mGlu₅ α cells exhibiting Ca²⁺ oscillations was unaffected by the coexpression of mApple-CA-CaMKII α (Supplemental Fig. 1D). Since it is unclear whether oscillating and nonoscillating cell responses cause different physiologic effects, we developed an approach to analyze the responses of all cells (both oscillating and nonoscillating) across five independent experiments, revealing that the initial peak fluorescence was significantly reduced ($P = 0.009$) in cells expressing mApple-CA-CaMKII α versus cells expressing mApple alone (Fig. 7B), consistent with data from stably transfected cell populations (Fig. 6). Moreover, the Ca²⁺ signal was relatively prolonged in cells expressing mApple-CA-CaMKII α versus cells expressing mApple alone, as reflected by a statistically significant increase in the half-life of the fluorescence signal (Fig. 7A, inset; Fig. 7C). We also analyzed responses in subsets of the cells within each population that exhibited at least three baseline Ca²⁺ oscillations (Supplemental Fig. 2A). There was no statistically significant difference in the total number of mGlu₅ α -mediated Ca²⁺ oscillations between transfection conditions (Supplemental Fig. 2B). However, coexpression of mApple-CA-CaMKII α reduced the relative rate of decay of peak Ca²⁺ signals in successive oscillations (Supplemental Fig. 2C). Coexpression of mApple-CA-CaMKII α also increased the frequency of Ca²⁺ oscillations, as reflected by a reduction of the interevent intervals (Supplemental Fig. 2D). In combination, these data confirm that CaMKII α can reduce the amplitude of initial mGlu₅ α -dependent Ca²⁺ mobilization and indicate that the relative duration of Ca²⁺ signals is extended, with increases of the frequency of oscillations when they are present.

To test the hypothesis that CaMKII α binding to the mGlu₅ α -CTD is necessary for the modulation of Ca²⁺ mobilization, we examined the effect of coexpressing mApple-CA-CaMKII α with mGlu₅ α -AAA (Fig. 7D). The replacement of Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸ in the CTD with alanines had little effect on glutamate-stimulated Ca²⁺ mobilization in cells expressing mApple. Moreover, the coexpression of mApple-CA-CaMKII α with mGlu₅ α -AAA had no statistically significant effect on either the initial peak (Fig. 7E) or the duration (Fig. 7F) of the glutamate-stimulated Ca²⁺ signal relative to control cells expressing mApple alone. Furthermore, the CTD mutation had no statistically significant effect on the responses of cells displaying baseline Ca²⁺ oscillations (Supplementary

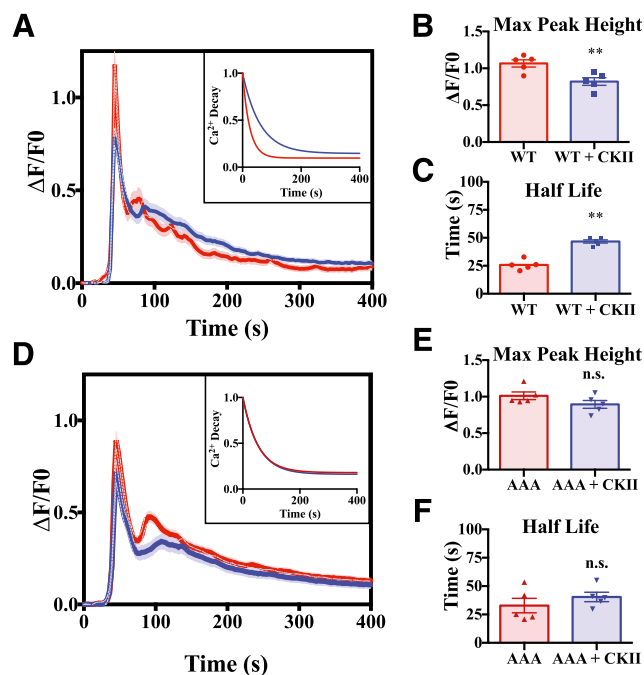


Fig. 7. CaMKII α binding to the CTD is required for the modulation of mGlu_{5α}-stimulated Ca²⁺ mobilization. HEK293A cells were transiently transfected to express mGlu_{5α} (WT or K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA) with either mApple or mApple-CA-CaMKII α for single-cell Fura-2 Ca²⁺ imaging (see *Materials and Methods*). Representative data from a single experiment. Averaged normalized changes in fluorescence from 58 to 114 cells ($\Delta F/F_0$, mean \pm S.E.M.) expressing mGlu_{5α}-WT (A) or mGlu_{5α}-K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA (D) in the presence (blue lines) or absence (red lines) of mApple-CA-CaMKII α . The inset graphs show line fits for time courses of the decline of Ca²⁺ signals from the peak $\Delta F/F_0$ under each condition. (B, C, E, and F) Summary data. The bar graphs depict mean \pm S.E.M. values for peak Ca²⁺ signals ($\Delta F/F_0$) (B and E) and half-lives for the decline in Ca²⁺ signals (C and F) with superimposed data points from each experiment ($n = 5$). The expression of constitutively active mApple-CA-CaMKII decreases the peak Ca²⁺ signal but increases the half-life of the Ca²⁺ signal with mGlu_{5α}-WT [(B) $P = 0.009$; (C) $P = 0.001$], but has no significant effect on the mGlu_{5α}-K⁸⁶⁶R⁸⁶⁷R⁸⁶⁸/AAA mutant that disrupts CaMKII binding to the CTD [(E), $P = 0.155$; (F), $P = 0.415$]. Paired Student's t tests were used for statistical comparisons in each panel. Max, maximal. In all panels, ** indicates $P < 0.01$ and n.s. indicates non-significant.

Fig. 1D) but abrogated the CaMKII-dependent modulation, as reflected by a lack of effect on the peak height decay of successive Ca²⁺ oscillations (Supplemental Fig. 2C) and the Ca²⁺ oscillation frequency (Supplemental Fig. 2D). These data indicate that binding to the mGlu_{5α}-CTD is important for both the increase of initial peak Ca²⁺ signals and for the prolonged Ca²⁺ signaling induced by coexpression of CA-CaMKII α .

Discussion

In a previous report (Jin et al., 2013b), the membrane proximal region of the mGlu_{5α}-CTD was shown to bind inactive CaMKII. Here we extend these findings by further characterizing the physical and functional relationship between these key regulators of synaptic transmission. We confirmed that CaMKII α and mGlu₅ specifically interact in mouse brain. However, our data show that mGlu_{5α}-CTD residues 827–964 bind more strongly to CaMKII α in an active, Thr286-autophosphorylated conformation, but that this interaction is disrupted by excess Ca²⁺/CaM or by robust

CaMKII autophosphorylation at additional undefined sites. Furthermore, our data indicate that CaMKII binding to the CTD exerts complex effects on mGlu_{5α} surface expression and downstream Ca²⁺ mobilization.

There is a growing appreciation that specific physiologic actions of CaMKII are modulated in part through dynamically regulated interactions with CaMKII-associated proteins (CaMKAPs). Several CaMKAPs preferentially interact with activated conformations of CaMKII; these CaMKAPs can be subclassified based on differences between the amino acid sequences of their CaMKII-binding domains. CaMKII-binding domains in the *N*-methyl-D-aspartate receptor GluN2B subunits and calcium channel $\beta 1$ and $\beta 2$ subunits resemble the CaMKII regulatory domain (Strack et al., 2000; Grueter et al., 2008). In contrast, the amino acid sequence of a CaMKII-binding domain in densin has similarity with a naturally occurring CaMKII inhibitor protein (Jiao et al., 2011). Here, we show here that the binding domain for activated CaMKII in the mGlu_{5α}-CTD does not resemble these CaMKAPs. Rather, this novel interaction requires three basic residues (Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸), similar to the recently identified interaction of activated CaMKII with the N-terminal domains of L-type voltage-gated Ca²⁺ channels (Wang et al., 2017). Interestingly, triple basic residue motifs can also be identified in CaMKII-binding domains of other G protein-coupled receptors, including intracellular loops of the G_{α_i} -coupled D₂R and D₃R (Liu et al., 2009; Zhang et al., 2014) and the CTD of the mGlu₁ receptor (Jin et al., 2013a,b), which also couples to $G_{\alpha_{q11}}$ (Fig. 3). Thus, it will be interesting to investigate the role of these triple basic residue motifs in CaMKII binding to additional G protein-coupled receptors.

One unusual aspect of CaMKII binding to the mGlu_{5α}-CTD is that, whereas the *in vitro* interaction requires CaMKII α activation and Thr286 autophosphorylation, additional autophosphorylation at non-Thr286 sites after incubation at 30°C reduces the binding. Our recent proteomics analyses of purified CaMKII α autophosphorylated *in vitro* using a similar 30°C protocol detected 17 autophosphorylation sites, in addition to Thr-286 (Baucum et al., 2015). Presumably, the autophosphorylation at one or more of these non-Thr286 sites interferes with *in vitro* CaMKII α binding to mGlu_{5α}. Although this is a potentially interesting finding, parallel proteomics analyses of CaMKII isolated from mouse brain failed to detect phosphorylation at many of these *in vitro* sites (Baucum et al., 2015). However, it is possible that this observation explains why Jin et al. (2013b) found that autophosphorylated CaMKII did not bind to mGlu_{5α} *in vitro* because their autophosphorylation reactions were incubated at 30°C.

Our data show that CaMKII α activation enhances the association with full-length mGlu_{5α}, and that this interaction involves the Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸ motif in the CTD (Fig. 3). However, triple alanine substitution of CTD residues 866–868 reduced the interaction by only ~50%, suggesting that CaMKII may interact with additional regions in mGlu_{5α} or bind to the receptor through an indirect interaction. Indeed, a CaMKII interaction with the second intracellular loop of mGlu₅ has been reported previously (Raka et al., 2015), although we have been unable to detect direct binding of purified CaMKII to a GST fusion protein containing the mGlu₅ second intracellular loop (data not shown). Although our data cannot preclude a role for a secondary or indirect interaction, our analyses in heterologous cells indicate that CaMKII α interaction with the CTD is critical for several novel functional effects of CaMKII on mGlu_{5α}.

signaling. First, we show here that CaMKII α can increase cell-surface expression of mGlu_{5a}. Second, we found that CaMKII α has complex effects on mGlu_{5a}-dependent Ca²⁺ mobilization. As noted previously, mGlu₅ activation can induce temporally diverse intracellular Ca²⁺ responses in heterologous cells and in neurons (Flint et al., 1999; Mao and Wang, 2003; Kim et al., 2005; Uematsu et al., 2015; Jong and O'Malley, 2017). The coexpression of CaMKII had little effect on the proportion of cells exhibiting different oscillatory or nonoscillatory response patterns (Supplementary Fig. 1). However, we found that the coexpression of CA-CaMKII α reduces the amplitude of the initial peak Ca²⁺ signals (Fig. 6C; Fig. 7B) but prolongs the duration of the Ca²⁺ signals (Fig. 7C; Supplementary Fig. 2C) in either the total responding cell population or only in cells that exhibit baseline Ca²⁺ oscillations. The coexpression of CA-CaMKII α also increases the frequency of baseline Ca²⁺ oscillations (Supplementary Fig. 2D). All of these effects are prevented by the triple alanine substitution for Lys⁸⁶⁶-Arg⁸⁶⁷-Arg⁸⁶⁸ in the CTD (Fig. 7, D–F; Supplementary Fig. 2, C and D). Presumably, the effect of CaMKII α to increase basal cell-surface expression contributes to the prolongation of Ca²⁺ signaling, but the mechanisms underlying the reduced initial peak Ca²⁺ signal, observed in both stable 293A-5a^{LOW} cell populations and in single transiently transfected cells, remains unclear. Taken together, our data show that binding of CaMKII α can play an important role in modulating cellular responses to mGlu_{5a} activation. Further examination into the contribution of these mechanisms in synaptic plasticity and neuronal Ca²⁺ signaling are warranted in future studies.

Interestingly, cell-surface expression of mGlu_{5a} is also modulated by direct binding of Ca²⁺/CaM to the CTD, similar to the effects of CaMKII α binding to the CTD reported herein, and Ca²⁺/CaM also prolongs mGlu₅-mediated Ca²⁺ signaling (Lee et al., 2008). The Ca²⁺/CaM binding domain involved in mediating these effects is located 30–40 residues C-terminal to the tribasic residue motif that is critical for CaMKII binding. Nevertheless, we found that Ca²⁺/CaM competes for the binding of activated CaMKII to the mGlu_{5a}-CTD in vitro (Fig. 2C). Taken together, our data suggest an intriguing model in which the binding of CaM might confer a relatively transient Ca²⁺-dependent modulation of mGlu_{5a} surface expression and signaling, but that increased CaMKII α autophosphorylation at Thr286 would result in sustained binding to the CTD and longer-term modulation of mGlu_{5a} surface expression and Ca²⁺ mobilization. Since Thr286 autophosphorylation of CaMKII is sensitive to changes in the source, duration, or frequency of Ca²⁺ signals originating from multiple channels (Pasek et al., 2015), such as those occurring during synaptic plasticity, as well as to the regulated activities of protein phosphatases, this may provide a mechanism for cross talk with other signaling pathways.

As noted above, CaMKII has also been shown to interact with a membrane-proximal region in the CTDs of mGlu₁ (Jin et al., 2013a), and the CaMKII-binding domain in mGlu₁ contains a tri-basic residue motif, similar to the motif we have identified here as being critical for CaMKII binding to the mGlu_{5a}-CTD. However, CaMKII was shown to desensitize mGlu₁ signaling, whereas we found that CaMKII prolongs mGlu₅ signaling. This apparently differential modulation of mGlu₁ and mGlu₅ by CaMKII may contribute to their distinct neuronal roles (Mannaioni et al., 2001; Valenti et al., 2002; Volk et al., 2006). Interestingly, the effects of CaMKII on mGlu₁ signaling are mediated in part by phosphorylation at

Thr871, which lies within the CaMKII-binding domain. Therefore, it will be interesting to investigate whether phosphorylation is required for the effects of CaMKII on mGlu₅ signaling, as well as the physical interaction demonstrated here.

The effects of CaMKII α on mGlu_{5a} must also interface with the known modulation of mGlu₅ signaling by other mechanisms. Prior studies have shown that several protein kinases modulate mGlu₅ via the CTD. For example, PKC phosphorylates Ser901 in the mGlu_{5a}-CTD to inhibit Ca²⁺/CaM binding and antagonize the aforementioned modulation by Ca²⁺/CaM (Lee et al., 2008). In addition, PKA phosphorylates Ser870 in mGlu_{5a}, prolonging Ca²⁺ mobilization, similar to the effects of CaMKII reported here, and enhancing ERK activation (Uematsu et al., 2015). However, it was previously reported that CaMKII reduces mGlu₅-stimulated ERK1/2 activation and increases agonist-induced mGlu₅ internalization (Raka et al., 2015). It is possible that the enhanced agonist-induced internalization in part results from the increased basal surface expression reported herein (Fig. 5). Although the mechanistic relationships between these different modes of mGlu₅ regulation remain to be more clearly established, the convergence of Ca²⁺/CaM, CaMKII, PKA, and PKC actions within an ~60–amino acid region in the long CTD (345 amino acids) suggests that the actions of mGlu₅ are tightly controlled across different time frames, presumably fine-tuning neuronal responses such as different forms of synaptic plasticity.

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Authorship Contributions

Participated in research design: Marks, Shonesy, Wang, Niswender, Colbran.

Conducted experiments: Marks.

Contributed new reagents or analytic tools: Marks, Wang, Stephenson, Niswender.

Performed data analysis: Marks, Shonesy.

Wrote or contributed to the writing of the manuscript: Marks, Shonesy, Colbran.

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