Calmodulin dynamically regulates the trafficking of the metabotropic glutamate receptor mGluR5

Jeong Ho Lee*, Jinu Lee†, Kyu Yeong Choi‡, Regine Hepp§, Jae-Youn Lee*, Mi Kyung Lim*, Mayumi Chatani-Hinze‡, Paul A. Roche§, Dong Goo Kim*, Young Soo Ahn*, Chul Hoon Kim*¶, and Katherine W. Roche‡

*Department of Pharmacology, Brain Research Institute, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea; †Department of Pharmacology, College of Medicine, Pochon CHA University, Sungnam 463-836, Korea; ‡National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892; and §National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Edited by Roger A. Nicoll, University of California, San Francisco, CA, and approved June 16, 2008 (received for review December 20, 2007)

Metabotropic glutamate receptors (mGluRs) 1–8 are G protein-coupled receptors (GPCRs) that modulate excitatory neurotransmission, neurotransmitter release, and synaptic plasticity. PKC regulates many aspects of mGluR function, including protein–protein interactions, Ca^{2+} signaling, and receptor desensitization. However, the mechanisms by which PKC regulates mGluR function are poorly understood. We have now identified calmodulin (CaM) as a dynamic regulator of mGluR5 trafficking. We show that the major PKC phosphorylation site on the intracellular C terminus of mGluR5 is serine 901 (S901), and phosphorylation of this residue is up-regulated in response to both receptor and PKC activation. In addition, S901 phosphorylation inhibits mGluR5 binding to CaM, decreasing mGluR5 surface expression. Furthermore, blocking PKC phosphorylation of mGluR5 on S901 dramatically affects mGluR5 signaling by prolonging Ca^{2+} oscillations. Thus, our data demonstrate that mGluR5 activation triggers phosphorylation of S901, thereby directly linking PKC phosphorylation, CaM binding, receptor trafficking, and downstream signaling.

phosphorylation | protein kinase C | receptor trafficking

The group I metabotropic glutamate receptor mGluR5 is highly expressed in the forebrain, where it regulates synaptic plasticity (1, 2). In addition, mGluR5 plays a role in pain (3) and addiction (4) and in neurological disorders such as fragile X syndrome (5, 6). Group I mGluRs are G protein-coupled receptors (GPCRs), which are coupled to phospholipase C, and receptor activation triggers phosphoinositide turnover, release of intracellular Ca^{2+}, and activation of Protein Kinase C (PKC) (7). Although PKC activity regulates mGluR5-mediated Ca^{2+} signaling and receptor function (8–11), there are no studies linking PKC phosphorylation of mGluR5 to receptor surface expression, endocytosis, or intracellular trafficking.

Like other GPCRs, mGluR5 interacts with many proteins in addition to the guanine nucleotide-binding proteins (G proteins). Most of the binding sites for these protein–protein interactions reside within the long intracellular C-terminal domain of mGluR5, suggesting that this region is critical in the functional regulation of mGluR5. For example, the Homer proteins bind to the PPxxFR motif within the distal C terminus, the Tamalin protein associates with the distal C terminus, and calmodulin (CaM) and the E3 ligase Siah-1A bind to the first one-third of the mGluR5 C terminus (12–15). However, the dynamic regulation of these protein–protein interactions has not been described. CaM is a particularly intriguing candidate as an mGluR5 regulator because of its Ca^{2+} dependence and its key role in synaptic plasticity (16, 17). Furthermore, CaM binding to other GPCRs, including dopamine, opioid, and serotonin receptors, has been documented, consistent with a conserved regulatory role for CaM in regulating GPCRs (18–20). We now show that PKC phosphorylation of serine 901 (S901) on mGluR5 inhibits CaM binding and decreases mGluR5 surface expression. Furthermore, preventing S901 phosphorylation led to a dramatic increase in mGluR5-mediated Ca^{2+} oscillations, revealing a direct mechanistic link among mGluR5 activation, phosphorylation, trafficking, and downstream signaling.

Results

We characterized the direct phosphorylation of mGluR5 by PKC by using several biochemical techniques. GST fusion proteins containing the proximal one-third of the mGluR5 C terminus (GST–mGluR5–Cprox) were incubated with purified PKC *in vitro*, and receptor phosphorylation was evaluated by using phosphopeptide map analysis. There were multiple PKC phosphorylation sites, and mutation of S901 to alanine eliminated the most prominent phosphopeptides, indicating that S901 is directly phosphorylated by PKC (Fig. 1*A*). We also evaluated the phosphorylation of GST–mGluR5–Cprox by PKC by using mass spectrometry, which unambiguously identified S901 as the predominant phosphorylation site (data not shown). HeLa cells expressing Myc-tagged wild-type mGluR5 or mGluR5 S901A were treated with the PKC activator phorbol 12-myristate 13-acetate (PMA), and we observed phosphorylation of wild-type mGluR5, but not mGluR5 S901A, by using a S901 phosphorylation state-specific antibody. Treating the cells with PMA to stimulate PKC rapidly increased the phosphorylation of mGluR5 on S901 (Fig. 1 *B* and *C*). Furthermore, application of the mGluR5 agonist dihydroxyphenylglycol (DHPG) dramatically increased S901 phosphorylation on mGluR5 in heterologous cells (Fig. 1 *D* and *E*). To examine S901 phosphorylation of endogenous mGluR5, phosphorylated receptor was immunoprecipitated from cell lysates prepared from cultured hippocampal neurons by using the S901 phosphorylation state-specific antibody in both nondenaturing and denaturing conditions. We found that PKC activation increased S901 phosphorylation of mGluR5 in hippocampal neurons (Fig. 1 *F* and *G*). In addition, DHPG increased S901 phosphorylation, which was blocked by the PKC inhibitor bisindolylmaleimide I, demonstrating that agonist-induced S901 phosphorylation is mediated by PKC (Fig. 1 *H–J*).

S901 is located within a region of the mGluR5 C terminus that contains a CaM-binding site (Fig. 2*A*), and previous studies have shown that the CaM–mGluR5 interaction is regulated by PKC phosphorylation *in vitro* (21). We, therefore, evaluated CaM binding to the mGluR5 C terminus by using a GST pull-down assay. As anticipated, wild-type mGluR5 bound to CaM robustly, and the

Author contributions: J.H.L., J.L., and K.Y.C. contributed equally to this work; C.H.K. and K.W.R. designed research; J.H.L., J.L., K.Y.C., R.H., M.C.-H., and C.H.K. performed research; J.H.L., J.-Y.L., and M.K.L. contributed new reagents/analytic tools; J.H.L., J.L., K.Y.C., R.H., J.-Y.L., M.C.-H., P.A.R., D.G.K., Y.S.A., and C.H.K. analyzed data; and C.H.K. and K.W.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¶To whom correspondence should be addressed. E-mail: kimhoon@yuhs.ac.

This article contains supporting information online at www.pnas.org/cgi/content/full/0712033105/DCSupplemental.

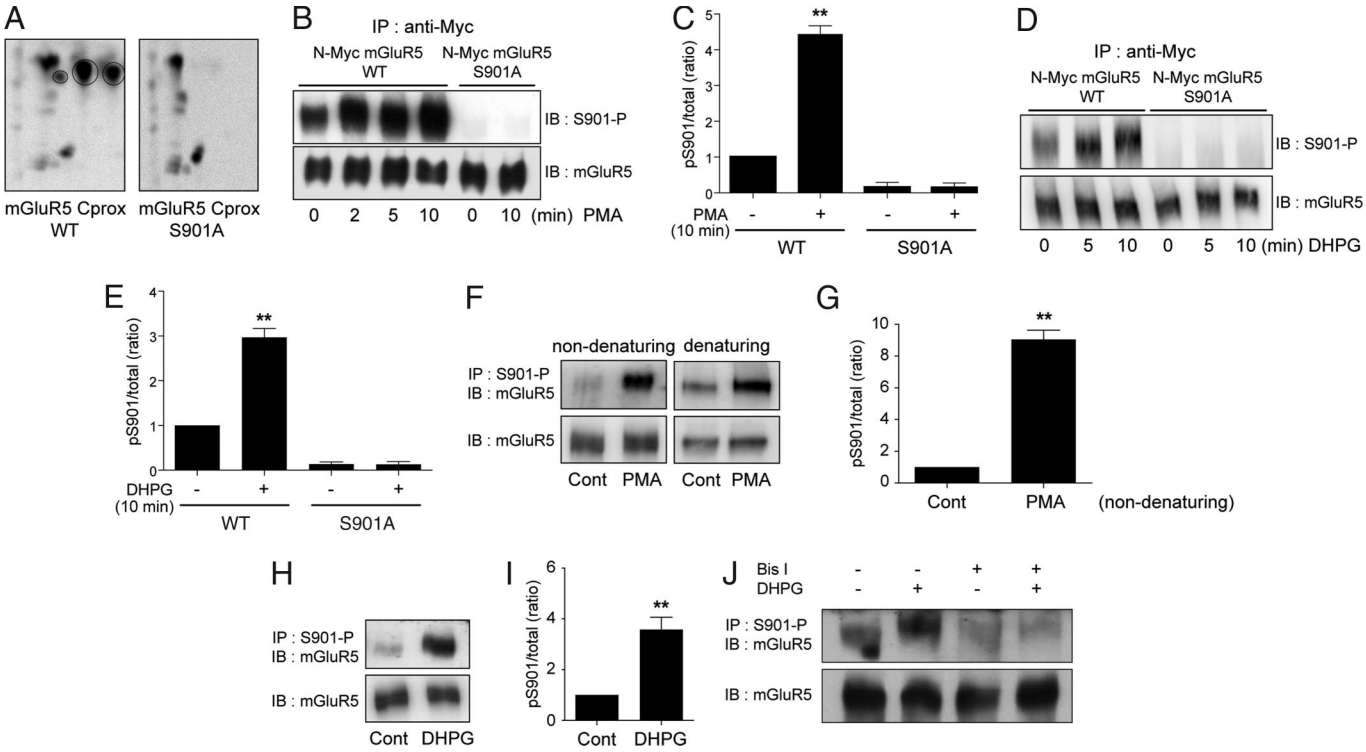

Fig. 1. mGluR5 is phosphorylated by PKC on S901. (*A*) Wild-type GST–mGluR5–Cprox and GST–mGluR5–Cprox (S901A) were phosphorylated by PKC *in vitro* by using [γ-^{32}P]ATP and analyzed by 2D phosphopeptide mapping. (*B–E*) HeLa cells expressing wild-type Myc–mGluR5 or Myc–mGluR5 S901A were treated with PMA (1 μM) or DHPG (100 μM) as indicated. mGluR5 was immunoprecipitated with Myc antibody, and S901-phosphorylated mGluR5 was detected by using rabbit S901 phosphoantibody. (*F* and *G*) Cultured hippocampal neurons were treated with PMA (1 μM for 10 min), S901-phosphorylated mGluR5 was immunoprecipitated under nondenaturing and denaturing conditions, and proteins were immunoblotted with mGluR5 antibody. (*H–J*) Cultured hippocampal neurons were treated with DHPG (100 μM for 10 min) with or without preincubation with bisindolylmaleimide I (Bis I) (5 μM for 30 min). S901-phosphorylated mGluR5 was immunoprecipitated, and proteins were immunoblotted with mGluR5 antibody. Quantification of immunoblotting (*C*, *E*, *G*, and *I*) is depicted as a histogram including results from three independent experiments. Statistical significance is indicated as ** ($P < 0.01$).

interaction was disrupted by PKC phosphorylation (Fig. 2*A*). However, PKC-dependent inhibition of CaM binding to mGluR5 was eliminated when S901 on mGluR5 was mutated to alanine (Fig. 2*A*), highlighting a central role for S901 in regulating CaM binding to mGluR5. The CaM–mGluR5 interaction is abolished by the addition of EGTA to the binding reaction mixture, indicating that it is Ca^{2+}-dependent (Fig. 2*A*). We also performed coimmunoprecipitation experiments by using full-length mGluR5 and CaM expressed in HeLa cells, and we found that mutation of S901 to aspartic acid, a phosphomimetic mutation of S901, profoundly

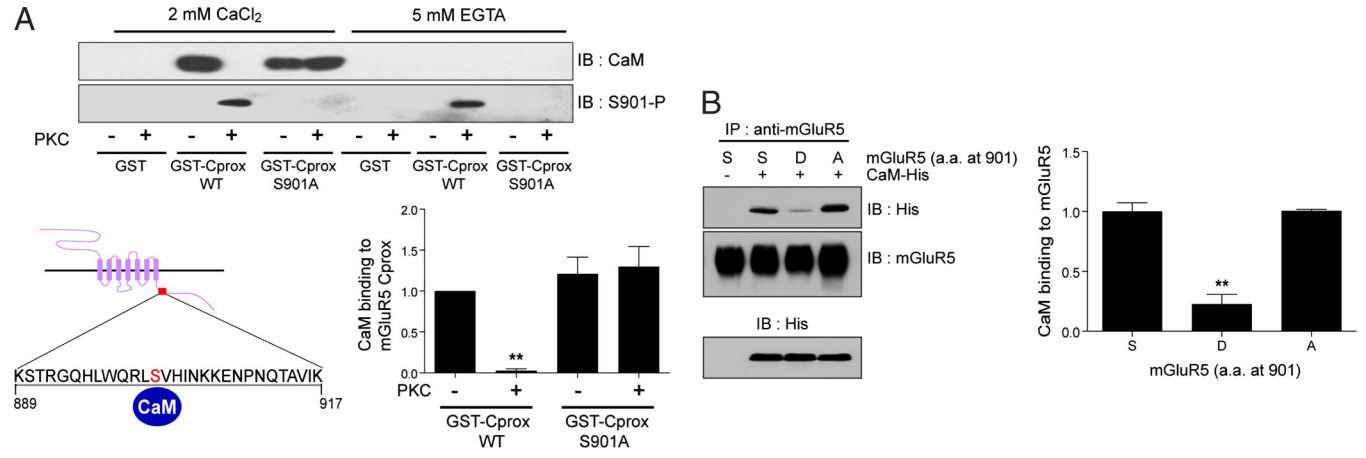

Fig. 2. PKC phosphorylation of mGluR5 on S901 inhibits CaM binding. (*A*) S901 regulates mGluR5 binding to CaM. Wild-type and mutant GST-mGluR5 fusion proteins were phosphorylated with or without PKC and incubated with recombinant CaM in the presence of $CaCl_2$ or EGTA. Proteins were resolved by SDS/PAGE and immunoblotted with CaM antibody or S901 phosphoantibody. A schematic figure shows the CaM-binding motif on the mGluR5 C terminus, and quantification is depicted as a histogram ($n = 4$). (*B*) HeLa cells were cotransfected with mGluR5 (wild-type, S901A, or S901D) and CaM–His. mGluR5 was immunoprecipitated from cell lysates with mGluR5 antibody, resolved by SDS/PAGE, and immunoblotted with His antibody. Quantitative analysis of the blots is depicted as a histogram ($n = 3$). Statistical significance is indicated as ** ($P < 0.01$).

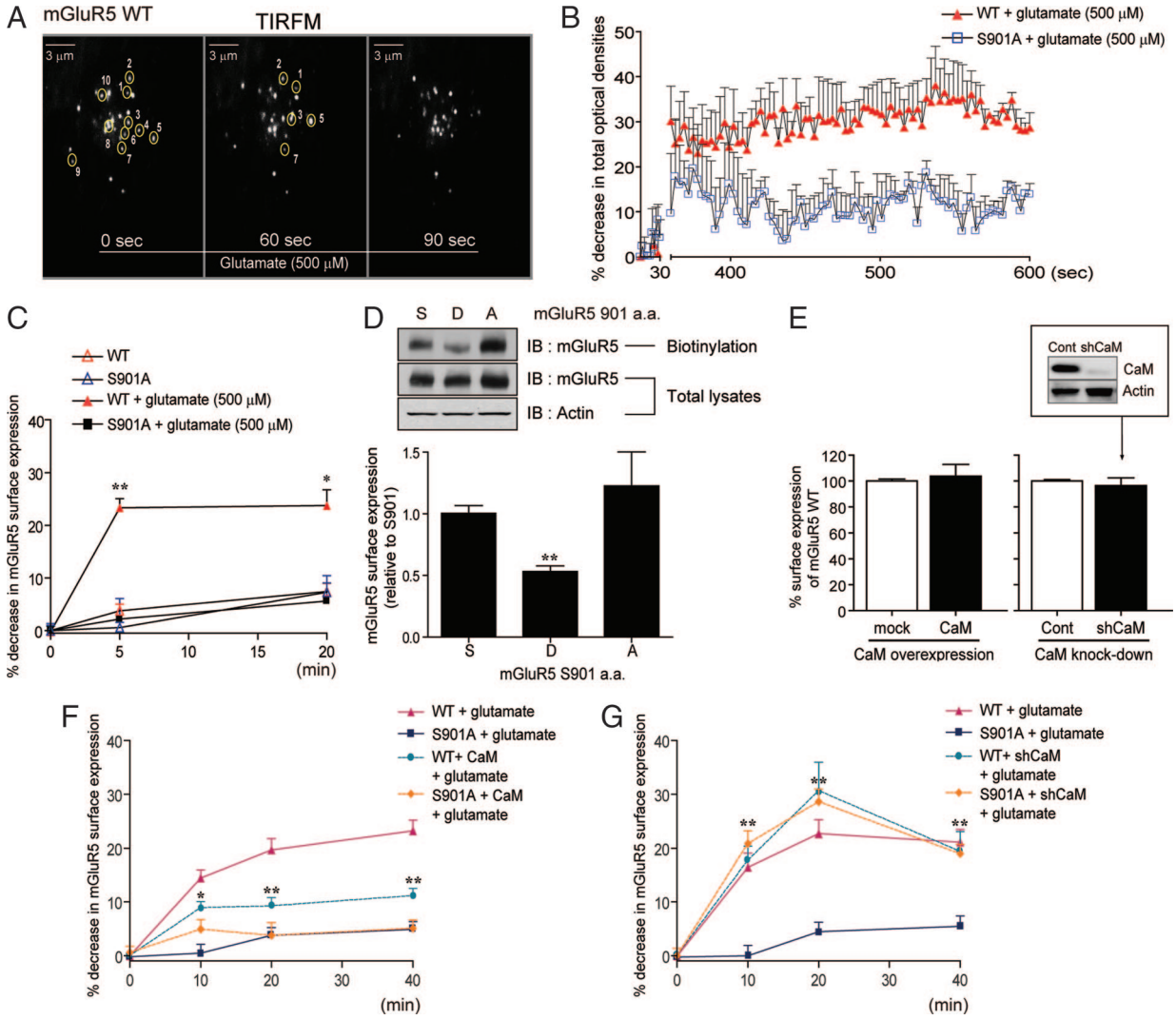

Fig. 3. Phosphorylation of S901 and CaM binding regulate mGluR5 trafficking and surface expression in heterologous cells. (*A*) TIRFM imaging of wild-type mGluR5–mRFP in HeLa cells. Glutamate was applied to HeLa cells expressing mGluR5–mRFP, and the disappearance of mGluR5 fluorescence on or near the cell surface was monitored over time. (*B*) Changes in the intensity of mGluR5–mRFP (wild-type or S901A) fluorescence from TIRFM images after glutamate application are depicted in a line graph. (*C*) Surface expression of mGluR5 was analyzed in HeLa cells expressing wild-type Myc–mGluR5 or Myc–mGluR5 S901A, treated with or without glutamate by using a quantitative colorimetric assay. *, $P < 0.05$; **, $P < 0.01$ compared with S901A plus glutamate. (*D*) Surface expression of mGluR5 (wild-type, S901A, or S901D) was analyzed in HeLa cells by biotinylation. Surface-expressed proteins were isolated from cell lysates by using neutravidin agarose, and total lysates and biotinylated fractions were immunoblotted with mGluR5 or actin antibodies. Surface-expressed mGluR5 was normalized by mGluR5 expression from total lysates. Quantification is depicted as a histogram, including results from five independent experiments. **, $P < 0.01$ compared with wild-type (S901S). (*E*) HeLa cells were cotransfected with Myc–mGluR5 and CaM or Myc–mGluR5, and shRNA targeting CaM (shCAM) and mGluR5 surface expression was measured with a Myc antibody (9E10)-based ELISA. Expression of endogenous CaM in HeLa cells was inhibited by expressing shRNAs targeting human Calm2 and Calm3 but not with the expression of nontargeting control shRNA (Cont). Cell lysates were subjected to SDS/PAGE and immunoblotted with CaM or actin antibodies (*Inset*). (*F*) HeLa cells expressing Myc–mGluR5 (wild-type or S901A) with or without CaM were incubated with glutamate. The surface expression of mGluR5 was quantitated by using a Myc antibody-based ELISA. *, $P < 0.05$; **, $P < 0.01$ compared with wild-type plus glutamate. (*G*) HeLa cells cotransfected with Myc–mGluR5 (wild-type or S901A) and shRNAs targeting human Calm2 and Calm3 were treated with glutamate. The surface expression of mGluR5 was measured with a Myc antibody-based ELISA. **, $P < 0.01$ compared with S901A plus glutamate.

inhibited mGluR5 binding to CaM (Fig. 2*B*). Thus, these findings identify phosphorylation of S901 as the critical mechanism regulating mGluR5 binding to CaM.

We next directly examined the role of PKC phosphorylation and CaM binding on the trafficking of mGluR5. We monitored the trafficking of mGluR5 at or near the plasma membrane in real time by using total internal reflection fluorescence microscopy (TIRFM) (Fig. 3*A*). Fluorescent spots of mGluR5–monomeric red fluorescent protein (mRFP) moved away from the plasma membrane after agonist treatment, consistent with agonist-induced endocytosis. In contrast to wild-type mGluR5, the distribution of mGluR5 S901A did not change significantly over time (Fig. 3*B*). By using an ELISA-based assay, we found that the surface expression of wild-type mGluR5 decreased over time with agonist exposure, whereas the surface expression of mGluR5 S901A did not (Fig. 3*C*). Therefore, our findings support a role for S901 phosphorylation in regulating mGluR5 trafficking and surface expression. To mimic S901 phosphorylation of the entire pool of mGluR5, we mutated S901 to aspartic acid, a phosphomimetic mutation, and measured surface expression of wild-type mGluR5 and mGluR5 S901D by using a biotinylation assay. Compared to wild-type mGluR5, the surface level of mGluR5 S901D was dramatically decreased (Fig. 3*D*).

Because S901 regulates binding of CaM, we explored whether changes in CaM expression altered mGluR5 surface expression.

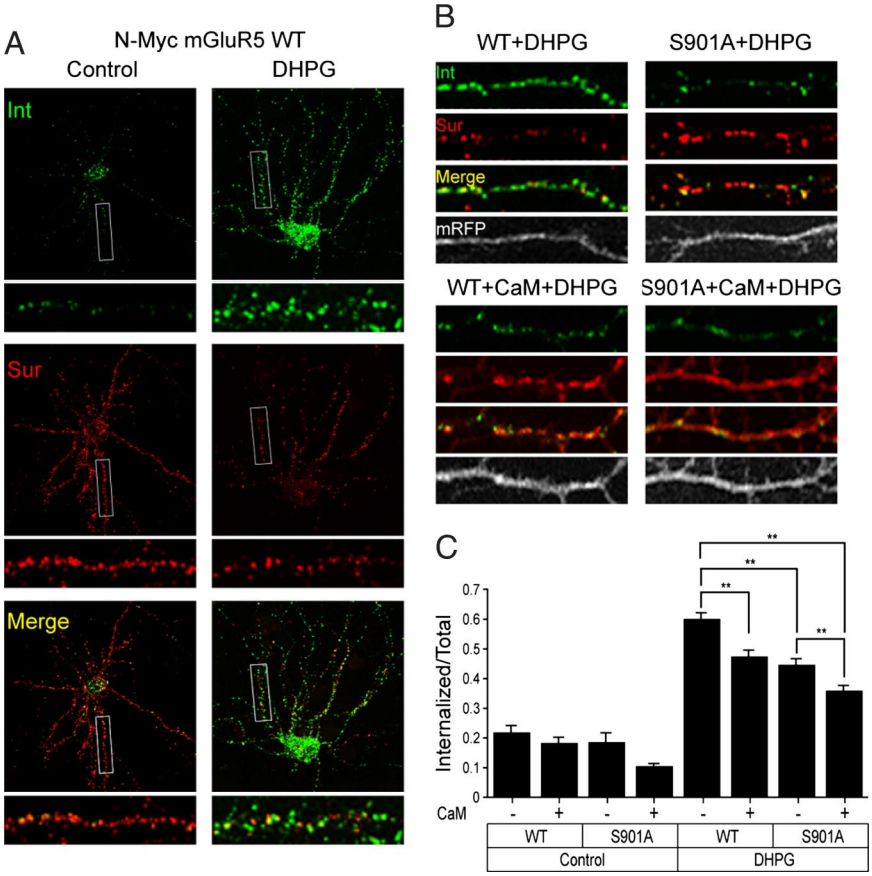

Fig. 4. Agonist-induced internalization of mGluR5 is regulated by S901 and CaM binding in neurons. (*A*) Cultured hippocampal neurons expressing Myc–mGluR5 were incubated with Myc antibody for 45 min at room temperature. The neurons were incubated with DHPG at 37°C for 30 min as indicated. The surface (Sur) pool (red) and internalized (Int) receptor (green) were visualized as described in the *Methods* and analyzed by laser scanning confocal microscopy. The merge of the two signals is shown. The region in the white box is shown at higher magnification below. (*B*) Myc-tagged mGluR5 (wild-type or S901A) was cotransfected with mRFP-tagged CaM or mRFP only. The internalized pool (green) and surface pool (red) are indicated. mRFP signal is shown as white for clarification. (*C*) Quantification of the fluorescence microscopy is presented as a histogram. The values indicate internalization fraction as compared with the total. The data represent means ± SEM; $n \geq 180$ neuronal processes analyzed for DHPG and $n \geq 40$ for control. **, $P < 0.01$.

Although overexpression or knockdown of CaM did not affect the steady-state surface expression level of mGluR5 (Fig. 3*E*), overexpression of CaM inhibited agonist-induced changes in mGluR5 surface expression (Fig. 3*F*). Furthermore, knockdown of CaM by using shRNA CaM decreased the surface expression of mGluR5 S901A after agonist exposure (Fig. 3*G*), a finding that was consistent with the mGluR5 S901A effects on trafficking being a direct result of increased CaM binding to the receptor.

We also examined the role of S901 phosphorylation and CaM binding on the trafficking of mGluR5 expressed in hippocampal neurons. By using a fluorescence-based antibody uptake assay, we observed robust endocytosis (internalized receptor, green) and a concomitant reduction in surface-expressed wild-type mGluR5 (surface, red) with agonist treatment (Fig. 4*A*). We found a significant decrease in the amount of internalized mGluR5 S901A compared to wild-type mGluR5 (Fig. 4 *B* and *C*). In addition, cotransfection of mGluR5 with CaM resulted in a significant decrease in mGluR5 endocytosis, for both wild-type mGluR5 and mGluR5 S901A (Fig. 4 *B* and *C*). These findings demonstrate that mGluR5 trafficking is regulated by S901 phosphorylation and CaM binding in both heterologous cells and in neurons.

mGluR5 activation triggers Ca^{2+} oscillations after agonist treatment, and the frequency of the Ca^{2+} spikes is correlated with mGluR5 receptor density on the plasma membrane (8, 22). Therefore, regulation of mGluR5 surface expression by S901 phosphorylation is likely to affect mGluR5-initiated signaling. To test this hypothesis, HeLa cells expressing mGluR5 (wild-type or S901A) were loaded with fura-2-AM, and agonist-simulated Ca^{2+} oscillation patterns were analyzed by using a ratiometric spectrofluorophotometer. Compared to wild-type mGluR5, mGluR5 S901A showed an increase in Ca^{2+} oscillation frequency (14.27 ± 3.49 mHz for wild-type vs. 26.75 ± 7.70 mHz for S901A; $P < 0.01$) (Fig. 5*A* and *B*). The half-time for Ca^{2+} to return to prestimulated levels was also measured, revealing that mGluR5 S901A activation resulted in more prolonged Ca^{2+} oscillations compared to wild-type mGluR5 (151.54 ± 112.83 s for wild-type vs. 886.85 ± 236.97 s for S901A; $P < 0.01$) (Fig. 5*C*). These data, therefore, demonstrate that mGluR5 S901A-induced signaling is prolonged relative to that observed in wild-type mGluR5-expressing cells.

Discussion

In this study, we have identified S901 as the major PKC phosphorylation site on the intracellular C terminus of mGluR5. Phosphorylation of S901 was dynamically regulated by PKC activity and receptor activation. Importantly, we found that phosphorylation of S901 profoundly inhibited CaM binding to mGluR5. In addition, we found that PKC phosphorylation of S901 decreased mGluR5 surface expression, providing the first evidence that PKC activation directly regulates mGluR5 trafficking. Furthermore, we show that overexpression of CaM increases mGluR5 surface expression, whereas knockdown of CaM decreases mGluR5 surface expression, demonstrating that CaM specifically mediates the PKC-dependent regulation of mGluR5 trafficking. Thus, we show that CaM stabilizes the surface expression of a GPCR. Our findings are consistent with a model in which mGluR5 surface expression is stabilized by CaM binding, but after receptor stimulation, PKC activity increased S901 phosphorylation, disrupted CaM binding, and reduced mGluR5 surface expression (Fig. 6).

CaM interacts with a variety of proteins and, via these interactions, regulates neuronal processes such as ion channel function (23, 24), GPCR signaling (18–20, 25), and synaptic plasticity (23–25). CaM has been shown to bind to several GPCRs (18–21, 26–28), and in some cases, CaM is implicated in the regulation of GPCR trafficking. For example, agonist-induced endocytosis of the 5-HT_{1A} receptor is prevented by pharmacological inhibitors of CaM (29). In addition, CaM regulates the activity of G protein-

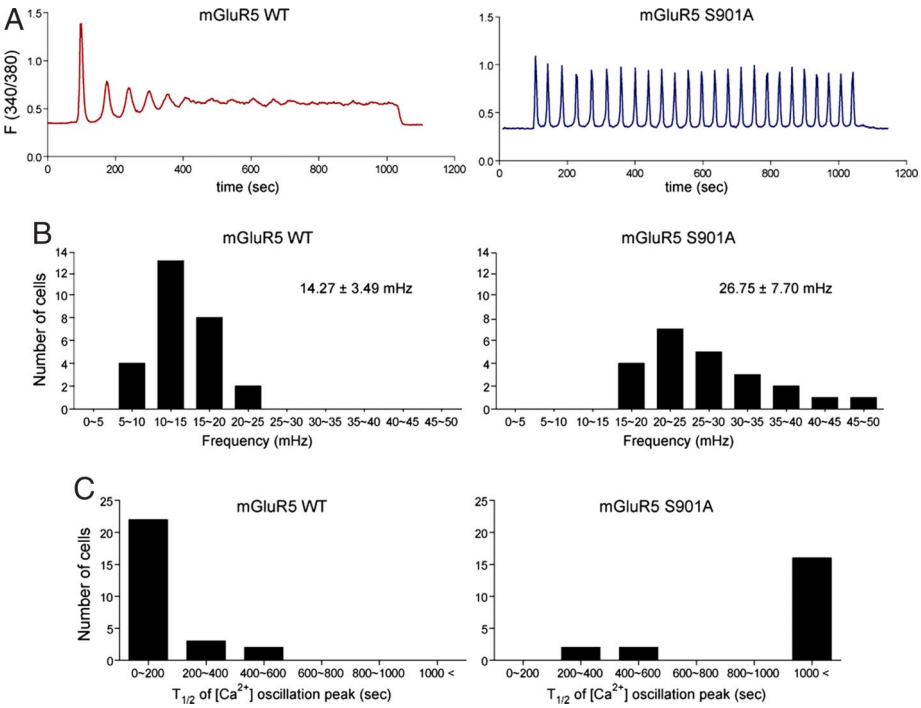

Fig. 5. S901 phosphorylation regulates Ca^{2+} oscillation frequency and decay time in mGluR5-expressing HeLa cells. (*A*) Representative Ca^{2+} oscillation patterns induced by mGluR5 wild-type and mGluR5 S901A. HeLa cells expressing mGluR5 wild-type or S901A were loaded with fura-2-AM and incubated with glutamate (100 μM). Ca^{2+} oscillations were analyzed by using a ratiometric spectrofluorophotometer. (*B*) Histograms of Ca^{2+} oscillation frequency in HeLa cells expressing mGluR5 wild-type or mGluR5 S901A (n = 27 for wild-type; n = 23 for S901A). The data are represented as means ± SD. (*C*) Histograms of Ca^{2+} oscillation decay half-life in HeLa cells expressing mGluR5 wild-type or mGluR5 S901A (n = 27 for wild-type; n = 20 for S901A).

coupled receptor kinases, which play important roles in endocytosis of GPCRs, suggesting an indirect role of CaM in regulating GPCR trafficking (30, 31). Recent findings also show that CaM binding to mGluR7 disrupts its interaction with the PDZ protein PICK1, increasing mGluR7 endocytosis (32). We now describe the direct stabilization of mGluR5 surface expression via CaM binding, and these data are, to our knowledge, the first to describe the dynamic regulation of GPCR trafficking by CaM binding in live cells.

PKC regulates many aspects of mGluR5 function. Early studies have demonstrated that mGluR5 activity elicits high-frequency Ca^{2+} oscillations that are PKC-dependent. These oscillations are regulated by the direct phosphorylation of the mGluR5 C terminus, with phosphorylation of S839 being required (8, 10). We now show that phosphorylation of a second site, S901, is a key mechanism in the regulation of mGluR5-mediated high-frequency Ca^{2+} oscillations. These data strongly suggest that the effect of PKC phosphorylation of S901 on Ca^{2+} oscillations is mediated by phosphorylation-dependent decreases in mGluR5 surface expression.

CaM interacts with ionotropic glutamate receptors in addition to mGluRs. CaM binding to NMDA receptors regulates both receptor localization and channel function, thereby contributing to the modulation of synaptic plasticity (17, 23, 24, 33, 34). CaM binding

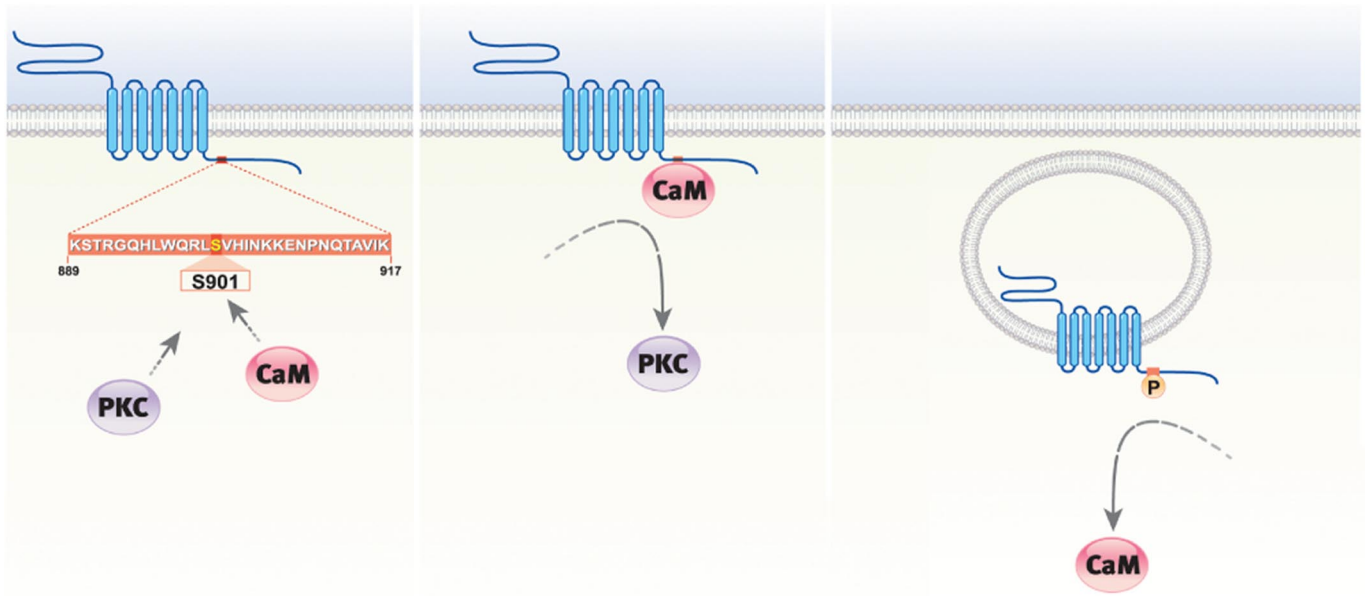

Fig. 6. Model of PKC–CaM regulation of mGluR5 surface expression. Our data support a model in which competition between PKC phosphorylation of S901 and CaM binding to S901 on mGluR5 determines trafficking of mGluR5 (*Left*). CaM binding acts to stabilize receptor surface expression (*Middle*), whereas PKC phosphorylation of S901 inhibits CaM binding and decreases receptor surface expression (*Right*).

to receptors often competes with other protein–protein interactions, and CaM competition with spectrin, and α-actinin binding to NMDA receptors is one such example (17, 24, 33, 34). Similarly, it has also been shown that CaM binding to mGluR5 inhibits the binding of the E3 ligase Siah-1A *in vitro* (13). Recently Siah-1A has been shown to promote monoubiquitination of α-synuclein, leading to its aggregation (35). It is possible that the effects of CaM on mGluR5 trafficking observed in our study are a consequence of changes in Siah-1A-dependent ubiquitination of mGluR5; however, direct evidence for this hypothesis awaits further experimentation.

Our findings suggest that the ability of CaM to regulate the binding activities of glutamate receptor-interacting proteins at excitatory synapses may allow it to act as a signal integrator or modulator of glutamate receptor-induced synaptic plasticity. As we now show for mGluR5, the rapid Ca^{2+}-dependent regulation of CaM binding mediates receptor expression at synapses and will likely regulate mGluR5-dependent processes *in vivo* as well.

Methods

Detailed information on TIRFM, DNA constructs, antibodies, and additional methods is available in supporting information (SI) *Methods*.

ELISA for Quantification of Receptor Surface Expression. HeLa cells were grown to ≈60% confluence on 24-well tissue culture dishes and transfected with wild-type Myc–mGluR5 or Myc–mGluR5 S901A. After 36–48 h, cells were washed in modified HBSS and allowed to equilibrate for at least 30 min at 37°C. Cells were treated with 500 μM L-glutamate for the indicated time intervals. The cells were washed, fixed with 4% paraformaldehyde in PBS for 5 min at room temperature, and washed again, and nonspecific binding was blocked with 1% BSA/PBS for 45 min. Cells were incubated with Myc antibody (9E10) diluted 1:1,000 in 1% BSA/PBS for 60 min, washed, and blocked again with 1% BSA/PBS for 15 min and incubated for 60 min with anti-mouse IgG–HRP (Amersham Biosciences) diluted 1:5,000 in 1% BSA. Cells were washed and incubated with the colorimetric horseradish peroxidase substrate *O*-phenylenediamine dihydrate (1 mg/ml; Sigma) in OPD buffer (50 mM citric acid, 65 mM sodium phosphate, and 0.03% hydrogen peroxide, pH 5.0) with gentle shaking for 12 min. An equal volume of 0.1N H_2SO_4 was added to stop the reaction. The optical density of each well was determined by using a microplate reader at 492 nm. The value obtained for untransfected cells was subtracted as background optical density.

Receptor Trafficking in Neurons. A fluorescence-based antibody uptake internalization assay was performed as described previously (36), with some modifications. Cultured hippocampal neurons prepared from E18 Sprague–Dawley rats were transfected with Myc–mGluR5 (wild-type or S901A) and mRFP–CaM or mRFP alone by using Ca^{2+} phosphate coprecipitation. After 2 days, Myc antibody was added to the media to label surface-expressed mGluR5 for 45 min. Neurons were washed with PBS twice and then treated with the group I mGluR agonist DHPG (100 μM) for 30 min at 37°C or with conditioned media alone at room temperature. After washing with PBS, the cells were fixed with 4% paraformaldehyde/4% sucrose in PBS for 15 min. Neurons were incubated with Alexa 633-conjugated (red) anti-mouse secondary antibody (Molecular Probes) for 30 min at room temperature to label the surface receptors and were washed, followed by permeabilization with 0.25% Triton X-100 for 5 min. After blocking with 10% normal goat serum, neurons were incubated with Alexa 488-conjugated (green) anti-mouse secondary antibody (Molecular Probes) for 30 min to label intracellular pools of receptors. After washing with PBS, ProLong Antifade reagent (Molecular Probes) was applied to neurons before mounting. Images were analyzed with a ×40 objective on an LSM 510 confocal microscope (Zeiss). Alexa 488 dye was excited with a 488-nm Ar laser and appeared green. The Alexa 633 dye (excited with a 633 nm HeNe laser) was presented as red for clarification. mRFP (Invitrogen) was excited with a 543-nm HeNe laser and presented as white. No crosstalk between channels was detected under these settings. Series of optical sections were collected at intervals of 0.4 μm. The figures show maximum projections. For quantitative analysis, images from three or four dendrites per neuron (at least 15 neurons per experiment) were collected, and the amount of internalization was measured based on the data collected in three independent experiments with MetaMorph 6.0 software (Universal Imaging Corp.). The values represent means±SEM. Significance was determined by using a Student's unpaired *t* test.

ACKNOWLEDGMENTS. This work was supported by Korea Research Foundation Grant KRF-2006-331-E00063 (to C.H.K.), funded by the Korean Government (MOEHRD Basic Research Promotion Fund); Korea Science and Engineering Foundation Grant R11-2007-040-01006-0 (to C.H.K.), funded by the Korean Government (MEST); and the National Institute of Neurological Disorders and Stroke (K.Y.C., M.C.H., and K.W.R.) and National Cancer Institute (R.H. and P.A.R.) Intramural Research Programs, National Institutes of Health.

1. Bortolotto ZA, Bashir ZI, Davies CH, Collingridge GL (1994) A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. *Nature* 368:740–743.
2. Huber KM, Kayser MS, Bear MF (2000) Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* 288:1254–1257.
3. Spooren WP, Gasparini F, Salt TE, Kuhn R (2001) Novel allosteric antagonists shed light on $mglu_5$ receptors and CNS disorders. *Trends Pharmacol Sci* 22:331–337.
4. Kenny PJ, Markou A (2004) The ups and downs of addiction: Role of metabotropic glutamate receptors. *Trends Pharmacol Sci* 25:265–272.
5. Bear MF, Huber KM, Warren ST (2004) The mGluR theory of fragile X mental retardation. *Trends Neurosci* 27:370–377.
6. Dölen G, *et al.* (2007) Correction of fragile X syndrome in mice. *Neuron* 56:955–962.
7. Conn PJ, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* 37:205–237.
8. Kawabata S, *et al.* (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature* 383:89–92.
9. Gereau RW IV, Heinemann SF (1998) Role of protein kinase C phosphorylation in rapid desensitization of metabotropic glutamate receptor 5. *Neuron* 20:143–151.
10. Kim CH, Braud S, Isaac JT, Roche KW (2005) Protein kinase C phosphorylation of the metabotropic glutamate receptor mGluR5 on Serine 839 regulates Ca^{2+} oscillations. *J Biol Chem* 280:25409–25415.
11. Kim CH, Lee J, Lee JY, Roche KW (2008) Metabotropic glutamate receptors: Phosphorylation and receptor signaling. *J Neurosci Res* 86:1–10.
12. Tu JC, *et al.* (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron* 21:717–726.
13. Ishikawa K, *et al.* (1999) Competitive interaction of seven in absentia homolog-1A and Ca2+/calmodulin with the cytoplasmic tail of group 1 metabotropic glutamate receptors. *Genes Cells* 4:381–390.
14. Kitano J, *et al.* (2002) Tamalin, a PDZ domain-containing protein, links a protein complex formation of group 1 metabotropic glutamate receptors and the guanine nucleotide exchange factor cytohesins. *J Neurosci* 22:1280–1289.
15. Enz R (2007) The trick of the tail: Protein-protein interactions of metabotropic glutamate receptors. *Bioessays* 29:60–73.
16. Xia Z, Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci* 6:267–276.
17. Wechsler A, Teichberg VI (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *EMBO J* 17:3931–3939.
18. Bofill-Cardona E, *et al.* (2000) Binding of calmodulin to the D_2-dopamine receptor reduces receptor signaling by arresting the G protein activation switch. *J Biol Chem* 275:32672–32680.
19. Turner JH, Raymond JR (2005) Interaction of calmodulin with the serotonin $5\text{-hydroxytryptamine}_{2A}$ receptor. A putative regulator of G protein coupling and receptor phosphorylation by protein kinase C. *J Biol Chem* 280:30741–30750.
20. Wang D, Sadee W, Quillan JM (1999) Calmodulin binding to G protein-coupling domain of opioid receptors. *J Biol Chem* 274:22081–22088.
21. Minakami R, Jinnai N, Sugiyama H (1997) Phosphorylation and calmodulin binding of the metabotropic glutamate receptor subtype 5 (mGluR5) are antagonistic in vitro. *J Biol Chem* 272:20291–20298.
22. Nash MS, *et al.* (2002) Determinants of metabotropic glutamate receptor-5-mediated Ca2+ and inositol 1,4,5-trisphosphate oscillation frequency. Receptor density versus agonist concentration. *J Biol Chem* 277:35947–35960.
23. Ehlers MD, Zhang S, Bernhadt JP, Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* 84:745–755.
24. Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. *J Neurosci* 19:1165–1178.
25. O'Connor V, *et al.* (1999) Calmodulin dependence of presynaptic metabotropic glutamate receptor signaling. *Science* 286:1180–1184.
26. Nakajima Y, Yamamoto T, Nakayama T, Nakanishi S (1999) A relationship between protein kinase C phosphorylation and calmodulin binding to the metabotropic glutamate receptor subtype 7. *J Biol Chem* 274:27573–27577.
27. Nickols HH, Shah VN, Chazin WJ, Limbird LE (2004) Calmodulin interacts with the V2 vasopressin receptor: Elimination of binding to the C terminus also eliminates arginine vasopressin-stimulated elevation of intracellular calcium. *J Biol Chem* 279:46969–46980.
28. Thomas WG, Pipolo L, Qian H (1999) Identification of a Ca^{2+}/calmodulin-binding domain within the carboxyl-terminus of the angiotensin II (AT_{1A}) receptor. *FEBS Lett* 455:367–371.
29. Della Rocca GJ, *et al.* (1999) Serotonin 5-HT1A receptor-mediated Erk activation requires calcium/calmodulin-dependent receptor endocytosis. *J Biol Chem* 274:4749–4753.
30. Pronin AN, Satpaev DK, Slepak VZ, Benovic JL (1997) Regulation of G protein-coupled receptor kinases by calmodulin and localization of the calmodulin binding domain. *J Biol Chem* 272:18273–18280.
31. Chuang TT, Paolucci L, De Blasi A (1996) Inhibition of G protein-coupled receptor kinase subtypes by Ca2+/calmodulin. *J Biol Chem* 271:28691–28696.
32. Suh YH, *et al.* (2008) Corequirement of PICK1 binding and PKC phosphorylation for stable surface expression of the metabotropic glutamate receptor mGluR7. *Neuron* 58:736–748.
33. Wyszynski M, *et al.* (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* 385:439–442.
34. Zhang S, Ehlers MD, Bernhardt JP, Su CT, Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* 21:443–453.
35. Lee JT, Wheeler TC, Li L, Chin LS (2008) Ubiquitination of alpha-synuclein by Siah-1 promotes alpha-synuclein aggregation and apoptotic cell death. *Hum Mol Genet* 17:906–917.
36. Lavezzari G, McCallum J, Dewey CM, Roche KW (2004) Subunit-specific regulation of NMDA receptor endocytosis. *J Neurosci* 24:6383–6391.