Identification of a novel PKC-phosphorylation site in the metabotropic glutamate receptor 5 that regulates calmodulin binding, receptor trafficking, and downstream signaling

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Identification of a novel PKC-phosphorylation site in the metabotropic glutamate receptor 5 that regulates calmodulin binding, receptor trafficking, and downstream signaling

Directed by Professor Young Soo Ahn

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끝으로 어떤 선택을 하든지 저를 믿고 지지해주는 평생 동반자이자 좋은 친구인 나의 지혜로운 아내에게 진심으로 사랑하고 고맙다는 말을 전합니다.

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ABSTRACT

Identification of a novel PKC-phosphorylation site in the metabotropic glutamate receptor 5 that regulates calmodulin binding, receptor trafficking, and downstream signaling

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(Directed by Professor Young Soo Ahn)

Metabotropic glutamate receptor 5 (mGluR5) is a G-protein couple receptor (GPCR) and plays an important role in synaptic plasticity, neuronal development, and neurodegeneration. mGluR5 is coupled to Gq protein, phospholipase C and protein kinase C (PKC). It has been known that the phosphorylation of GPCRs and consequential changes in receptor trafficking such as endocytosis is a typical mechanism of GPCR desensitization. Although PKC phosphorylation is also implicated in mGluR5 desensitization, the precise mechanism is not clear. In this study, a novel PKC phosphorylation site, serine 901 (S901), in mGluR5 C-terminus was identified using phosphopeptide map analysis. In HeLa cells expressing mGluR5 and in primary cultured neurons, PKC activator PMA and mGluR5 agonist DHPG induced the phosphorylation of S901 in mGluR5, as detected using a S901 phosphorylation state-specific

antibody. In addition, agonist-induced S901 phosphorylation was inhibited by PKC inhibitor bisindolylmaleidmide I. S901 is located within the calmodulin (CaM) binding site in the mGluR5 C-terminus. Therefore, the effect of the phosphorylation of S901 on CaM binding was evaluated and the result showed that the phosphorylation of S901 by PKC inhibited the CaM binding to mGluR5. In Total Internal Reflection Fluorescence Microscope (TIRFM) and ELISA measuring cell surface mGluR5, it was found that the phosphorylation of the residue decreased the level of mGluR5 surface expression. Interestingly, CaM overexpression diminished the agonist-induced decrease of wild-type mGluR5 surface expression, whereas CaM knock-down dramatically enhanced that of S901A mGluR5 which had not been changed in response to the agonist. Furthermore, preventing S901 phosphorylation prolonged Ca^{2+} oscillation triggered by mGluR5 activation. Therefore, our data suggest that mGluR5 activation induces S901 phosphorylation by PKC, thereby indicating that agonist-induced S901 phosphorylation links CaM binding, receptor trafficking and downstream signaling.

Key Word: Metabotropic glutamate receptor 5, Calmodulin, Receptor trafficking, PKC, Phosphorylation

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I. INTRODUCTION

Glutamate is a major excitatory neurotransmitter and plays an important role in various functions of the central nervous system (CNS)[1]. The action of glutamate is mediated by the activation of glutamate receptors. There are two kinds of glutamate receptors. One is the ionotropic glutamate receptors (iGluRs) that are ion channels such as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kinate receptor. The other is the metabotropic glutamate receptors (mGluRs). mGluRs, which consists of subtypes including mGluR1 to mGluR8, are G-protein coupled receptors (GPCRs) that are coupled to second messenger signaling via G-protein[2]. They are expressed on the presynaptic (e.g. mGluR7) or postsynaptic (e.g. mGluR5) membrane and regulate the synaptic transmission in

the CNS[2, 3]. Like other GPCRs, mGluRs have extracellular N-terminus where agonist can bind, seven transmembrane domain and intracellular C-terminus where many proteins can bind and posttranslational modification such as phosphorylation can occur[2]. mGluRs are classified into three group (e.g. Group I, II, and III) according to the preferred G protein and signaling pathway. Among them, mGluR5 belongs to Group I mGluRs that are coupled to Gq protein and phospholipase C (PLC) signaling pathway[2]. Therefore, when mGluR5 receptor is activated by agonist, consequentially activated PLC hydrolyzes phosphatidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is linked to IP_3 receptor and the increase of intracellular calcium and DAG induces the activation of protein kinase C (PKC)[2]. Then, activated PKC can phosphorylate its substrates. Functionally, mGluR5 is involved in the synaptic plasticity such as the formation of long-term depression (LTD)[4] and plays an important role in several neurological diseases such as pain[5], addiction[6], and fragile X syndrome[7].

In terms of the relationship between PKC and mGluR5 function, there have been some electrophysiological data showing that the PKC activity regulates mGluR5 desensitization[8]. Considering that the receptor phosphorylation and consequent endocytosis is a typical mechanism of GPCR desensitization[9], we can infer that mGluR5 phosphorylation by PKC might

affect the receptor trafficking like the internalization in a similar way as mGluR5 desensitization. However, there still have been lack of biochemical evidences of PKC phosphorylation and the desensitization mechanism by PKC remains unclear.

Metabotropic glutamate receptor 5 has long intracellular C-terminal domain and interacts with many proteins like calmodulin (CaM), the E3 ligase Siah1A, Homer, and Tamalin within this region[10]. Among them, CaM, highly ubiquitous Ca^{2+} sensing protein[11], is particularly interesting because it plays a key role in synaptic plasticity[12] and CaM binding to mGluR5 and receptor phosphorylation show an antagonistic relationship[13]. Moreover, it has been known that PKC phosphorylation can induce the dissociation of CaM binding to other GPCRs such as mGluR7[14], 5-HT_{1A} receptor[15]. In addition, CaM can regulate the trafficking of those receptors[14, 16]. Therefore, it is likely that PKC activity might affect mGluR5 functions via the regulation of CaM binding and receptor trafficking.

Taken together, it can be speculated that the agonist induced phosphorylation of mGluR5 by PCK might affect receptor internalization as a desensitizing mechanism by mediating the potential interacting protein, CaM. The present study shows that the PKC activity directly mediates agonist-induced serine 901 (S901) phosphorylation on mGluR5. S901 phosphorylation

by PKC decreases CaM binding and mGluR5 surface expression. In addition, CaM binding to mGluR5 stabilizes the receptor surface expression. Furthermore, the mutation of serine 901 to alanine (S901A) that prevents S901 phosphorylation dramatically prolongs mGluR5-induced Ca^{2+} oscillation. Therefore, our study suggests that S901 phosphorylation by PKC affects mGluR5 receptor trafficking via the regulation of CaM binding and this could be the underlying mechanism of PKC-mediated mGluR5 desensitization.

II. MATERIALS AND METHODS

1. DNA constructs and antibodies

Myc-tagged mGluR5 construct was made by inserting a Myc epitope into the N-terminus of mGluR5 (between a.a. 22 and 23). A GST-fusion protein containing the C-terminus of mGluR5 was made by subcloning first one-third of the mGluR5 C-terminus (a.a. 828-944) into pGEX vectors (Amersham Biosciences, Buckinghamshire, UK). Mutations of S901 including Myc-mGluR5 or GST-mGluR5-Cprox were made by using site-directed mutagenesis. The anti-Myc monoclonal antibody (9E10) was purchased from Sigma (St. Louis, MO, USA). Anti-His monoclonal antibody and Alexa 488- and 633-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit and chicken anti-mGluR5 antibodies were generated against a peptide corresponding to the mGluR5 C-terminus[17]. The S901 phosphoantibody was generated against the phospho-peptide N-RL[pSer]VHINKKENPNQC-C, and was purified using the phosphopeptide conjugated to a SulfoLink gel (Pierce, Rockford, IL, USA).

2. Fusion protein phosphorylation and pull-down assay

GST fusion proteins were obtained according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). *In vitro* phosphorylation was performed as previously described[18, 19]. Briefly, GST fusion proteins were reacted with 25 ng of purified PKC in a reaction buffer (pH 7.4) containing 20 mM HEPES, 1.67 mM $CaCl_2$, 1 mM dithiothreitol, 10 mM

$MgCl_2$, 10 μM cold ATP, 1 pmol of [γ-^{32}P]ATP (3000 Ci/mmol) at 30 ºC for 30 min. The reaction was stopped by adding the SDS-sample buffer and boiling for 5 min. Fusion proteins were loaded on SDS-PAGE and transferred to nitrocellulose membranes for autoradiography. After the phosphorylated fusion proteins were visualized by autoradiography, the bands were excised for two-dimensional phosphopeptide mapping. For pull-down assays, GST fusion proteins bound to Sepharose beads were phosphorylated as described above, but without adding [γ-^{32}P]ATP. Phosphorylated fusion proteins were reacted with recombinant CaM (1 μg, Upstate, Lake Placid, NY, USA) in a total volume of 500 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 0.5% Nonidet P-40, protease inhibitors, and phosphatase inhibitors) for 2 h at 4°C. The beads were washed 4 times with binding buffer, and the bound proteins were eluted by adding SDS-PAGE sample buffer and boiling for 5 min. Samples resolved by SDS-PAGE were transferred to a polyvinyl difluoride membrane (PVDF, Millipore, Bedford, MA, USA) and visualized by Western blotting. The binding of CaM to phosphorylated mGluR5 was analyzed by immunoblotting with CaM antibody (Upstate, Lake Placid, NY, USA), and phosphorylation of S901 was confirmed by immunoblotting with the S901 phosphoantibody.

3. Two-dimensional phosphopeptide mapping

Peptide mapping was performed as previously described[18, 19]. Briefly, the excised bands of phosphorylated GST-fusion proteins bound to nitrocellulose were soaked for 1 h in tubes containing 1 mL of 1%

polyvinylpyrrolidone-40 in 100 mM acetic acid. After washing with 0.4% NH_4HCO_3, the membranes were digested with 0.5 mg/mL trypsin overnight at 37 °C. The supernatant containing digested peptides of fusion proteins were dried in a SpeedVac. After several washing and drying cycles, the peptides were dissolved in 5 μL of H_2O. One or two μL of peptides were spotted onto a cellulose thin layer chromatography plate (Merck, Darmstadt, Germany). The phosphopeptides were resolved in the first dimension by electrophoresis in buffer containing 2.5% formic acid and 7.8% acetic acid. Separation by ascending chromatography in the second dimension was performed using buffer containing 62.5% isobutyric acid, 4.8% pyridine, 1.9% butanol, and 2.9% acetic acid. The thin layer chromatography plate was air-dried, and the peptide map was visualized using a Storm PhosphorImager (GE Healthcare, Piscataway, NJ, USA).

4. Immunoblotting and immunoprecipitation

Transiently transfected HeLa cells or primary cultured neurons were prepared as previously described[20] and were lysed with 1% Triton X-100 in PBS. Cell lysates were immunoprecipitated with mouse anti-Myc or rabbit anti-mGluR5 or rabbit S901 phosphorylation state-specific antibody overnight. This mixture was incubated with protein G or A agarose beads for 2 h. After 5 times washing of protein G or A agarose beads with 1% Triton X-100 in PBS, the beads were incubated with SDS sample buffer at 37 ºC for 10 min. The eluted proteins were loaded on SDS-PAGE gel and transferred to PVDF membrane. Immunoblotting was performed using mouse anti-His antibody (Clontech,

Mountain View, CA, USA), chicken anti-mGluR5 antibody, or rabbit anti-mGluR5 antibody.

5. Cell surface biotinylation assay

HeLa cells expressing mGluR5 (wild-type, S901D or S901A) were rinsed three times with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS++) and then incubated with 1 mg/mL EZ-link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA) in PBS++ for 20 min at 4 °C. Cells were rinsed three times with cold 50 mM glycine in PBS++ to quench unreacted biotin. Cells were lysed in PBS containing 1% Triton X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) for 10 min at room temperature. Cell lysates were centrifuged at 13,000x g for 10 min. The supernatant was incubated with neutravidin agarose beads (Pierce, Rockford, IL, USA) for 2 h at 4 °C and washed four times with PBS containing 1% Triton X-100. Precipitates were eluted and analyzed by immunoblotting with the rabbit anti-mGluR5 antibody.

6. shRNA CaM knock-down

Three distinct human CaM genes encode proteins with an identical amino acid sequence[21]. Because relative transcriptional activity of each calmodulin gene is tissue-specific, it was necessary to select shRNA-CaMs that effectively suppress the expression of CaM in HeLa cells. Three pLKO.1-puro shRNA plasmid target sets directed against human Calm1 (GenBank accession number NM_006888), Calm2 (GenBank accession number NM_001743),

Calm3 (GenBank accession number NM_005184), and Non-Target shRNA plasmid were obtained from MISSION shRNA Libraries (Sigma, St. Louis, MO, USA). Each shRNA plasmid target set consists of five individual shRNA clones containing different target sequences. HeLa cells were grown to ~40% confluency on 12 well culture plates. We transfected 0.8 μg of each of the fifteen individual shRNA plasmids into HeLa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours later, whole cell lysates were prepared. The amount of endogenous CaM in cell lysates was evaluated by immunoblotting with anti-CaM antibody (Abcam, Cambridge, UK). Among these clones, we selected the most effective clones for each CaM gene silencing. Optimal shRNA clones targeting CaM 2 and 3 were found. The target sequences are 5'-GCAGAGTTACA GGACATGATT-3' (Calm2) and 5'-GCCAGGTCAATTATGAAGAGT-3' (Calm3). A mixture of equal amounts of these two clones was used to suppress CaM expression. We used the Mission non-target shRNA as a negative control. For immunoblotting, we transfected 0.8 μg of shCaM plasmid into HeLa cells grown on 12 well tissue culture dishes. For ELISA experiments, we transfected 0.2 μg of Myc-mGluR5 wild-type or Myc-mGluR5 S901A plasmids and mixture of 0.2 μg of each shRNA against Calm2 and Calm3 plasmid into HeLa cells grown on 24 well tissue culture dishes.

7. Total internal reflection fluorescence microscopy (TIRFM)

HeLa cells transiently expressing mGluR5-mRFP wild-type or mGluR5-mRFP S901A were grown to ~30% confluency on 40-mm glass

bottom culture dishes (MatTek, Ashland, MA, USA), washed in modified HBSS (140 mM NaCl, 10 mM HEPES, 11 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$), and allowed to equilibrate for at least 30 min at 37ºC prior to imaging. TIRFM was carried out using Nikon TIRF-2 w/ TE2000-PFS and Chamber System (Nikon, Tokyo, Japan) and its temperature-controlled chamber was set at 37°C for live cell imaging. mGluR5-mRFP was excited with the 532 nm laser and illumination intensity was attenuated as needed by a neutral density (ND) filter. Incident illumination passing through the Apo TIRF 100×, 1.49NA oil-immersion objective was angled to obtain TIRF images. The emitted fluorescence was captured by a Hamamatsu C9100-12 Camera System (Hamamatsu photonics, Hamamatsu, Japan). Prior to adding 500 μM (final concentration) of L-glutamate dissolved in modified HBSS, the Perfect Focus System (PFS) unit (Nikon, Tokyo, Japan) was turned on to correct the focus drift caused by the addition of any reagents. MetaMorph 6.0 software (Universal Imaging Corp., Downingtown, PA, USA) was used for image analysis. Data are presented as the percentage of integrated optical density of spots at each time point divided by the integrated optical density at Time 0. Images were acquired every 3 sec for 10 min with a 100 msec exposure time.

8. ELISA for quantification of receptor surface expression

HeLa cells were grown to ~60% confluency on 24-well tissue culture dishes and transfected with Myc-mGluR5 wild-type or Myc-mGluR5 S901A. After 36~48 h, cells were washed in modified HBSS (see above) 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, washed again, and nonspecific binding was blocked with 1% BSA/PBS for 45 min. Cells were incubated with Myc antibody (9E10) diluted 1:1000 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, Buckinghamshire, UK) diluted 1:5000 in 1% BSA. Cells were washed and incubated with the colorimetric horseradish peroxidase substrate, O-phenylenediamine dihydrate (1 mg/mL; Sigma, St Louis, MO, USA) 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 using a microplate reader at 492 nm. The value obtained for untransfected cells was subtracted as background optical density.

9. Receptor trafficking in neurons

A fluorescence-based antibody uptake internalization assay was performed as previously described[20] 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 using calcium phosphate co-precipitation. After 48 hours, Myc antibody was added to media to label surface-expressed mGluR5 for 40 min. Neurons were washed with PBS twice and then treated with 100uM of group I mGluR agonist DHPG for 30 min at 37°C or with conditioned media alone. 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, Carlsbad, CA, USA) for 30 min at room temperature for staining the surface receptors, and were permeabilized with 0.25% Triton X-100 for 5 min. After blocking with 10% normal goat serum, the neurons were incubated with Alexa 488-conjugated (green) anti-mouse secondary antibody (Molecular Probes, Carlsbad, CA, USA) for 30 min to label intracellular pools of receptors. After washing with PBS, ProLong Antifade reagent (Molecular Probes, Carlsbad, CA, USA) was applied to neurons prior to mounting. Images were analyzed with a 40x objective on a Zeiss LSM 510 confocal microscope. 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, Carlsbad, CA, USA) was excited with a 543 nm HeNe laser and presented as white. No cross talk between channels was detected under these settings. Series of optical sections were collected at intervals of 0.4 μm. Figures show maximum projections. For quantitative analysis, images from three or four dendrites per neuron (at least fifteen 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., Downingtown, PA, USA).

10. Statistics

Data were analyzed with Student's unpaired t-test or one-way ANOVA

and subsequent Tukey-Kramer test. Statistical significance was accepted at $*p < 0.05$ or $**p < 0.01$. P-values were presented with F value and degree of freedom as needed. Results were reported as mean ± SEM or ± SD as indicated.

III. RESULTS

1. Serine 901 (S901) phosphorylation by PKC in heterologous cells

In order to find out new PKC phosphorylation site on mGluR5-C terminus, GST-fusion protein containing the proximal portion (a.a. 828-944) of mGluR5 C-terminus (GST-mGluR5-Cprox) were generated. Then, they were incubated with PKC and [γ-^{32}P] ATP *in vitro* and the phosphorylation status was measured using two dimensional phosphopeptide map analysis. It was found that there were several PKC phosphorylation sites (Fig. 1A). However, when serine 901 (S901) was mutated to alanine (S901A), it was observed that the most prominent phosphorylated peptide was eliminated (Fig. 1A). Next, S901 phosphorylation state-specific antibody was generated and confirmed by immunoblotting with PKC-phosphorylated fusion protein (GST-mGluR5 Cprox wild-type or S901A) (Fig. 1B). Then, it was examined whether S901 could be phosphorylated by PKC in cells. After Myc-tagged mGluR5 wild-type (WT) or S901A were transfected into HeLa cell, these cells were treated with PKC activator, PMA, and mGluR5 agonist, DHPG. The immunoprecipitation was performed from cells lysates using anti-myc antibody and it was observed that PMA and DHPG increased the S901 phosphorylation in mGluR5 WT using S901 phosphorylation state specific antibody, not in mGluR5 S901A (Fig. 1C-F)

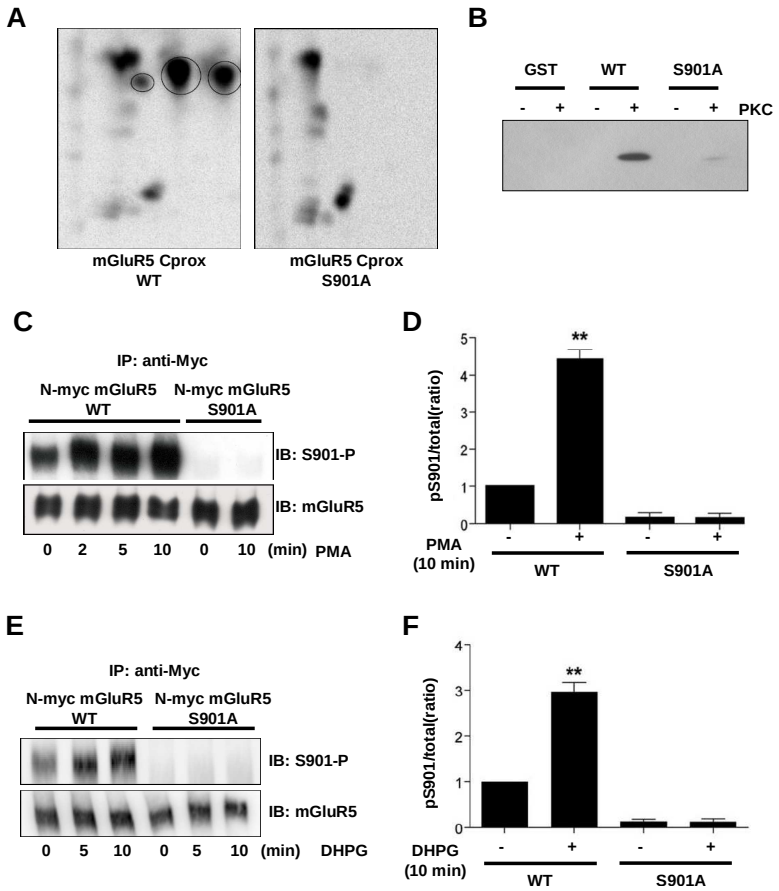

FIGURE 1. Serine 901 (S901) on mGluR5 is phosphorylated by PKC in HeLa cells. (*A*) GST-mGluR5-Cprox wild-type and GST-mGluR5-Cprox S901A were incubated with PKC and [γ-^{32}P] ATP *in vitro*. Phosphorylation status was measured using two-dimensional phosphopeptide mapping analysis. (*B*) GST-mGluR5-Cprox wild type and S901A were phosphorylated by PKC *in vitro* and S901 phosphorylation was detected using immunoblotting with S901 phosphorylation state-specific antibody generated in our laboratory. (C-*F*) Myc-mGluR5 wild-type or Myc-mGluR5 S901A was transfected into HeLa cells. They were treated with PMA (1 μM) or DHPG (100 μM) as indicated. Cell

lysates were immunoprecipitated with Myc antibody and S901 phosphorylation was measured by immunoblotting with S901 phosphoantibody. Quantification of immunoblotting (*D* and *F*) was shown as a histogram (n=3). Statistical significance is indicated as $**p < 0.01$

2. S901 phosphorylation by PKC in neurons

Next, it was examined whether PMA and DHPG can also induce S901 phosphorylation of endogenous mGluR5. Primary cultured neurons were treated with PMA and DHPG and then phosphorylated receptors were immunoprecipitated using S901 phosphorylation state specific antibody both in denaturing and non-denaturing condition. It was found that PMA and DHPG dramatically induced S901 phosphorylation (Fig. 2A-D). Moreover, it was also found that PCK specific inhibitor, bisindolymaleimide I (Bis I), prevented the DHPG induced S901 phosphorylation of endogenous mGluR5 (Fig. 2E). Therefore, these data indicate that the agonist induced S901 phosphorylation of mGluR5 is mediated by PKC.

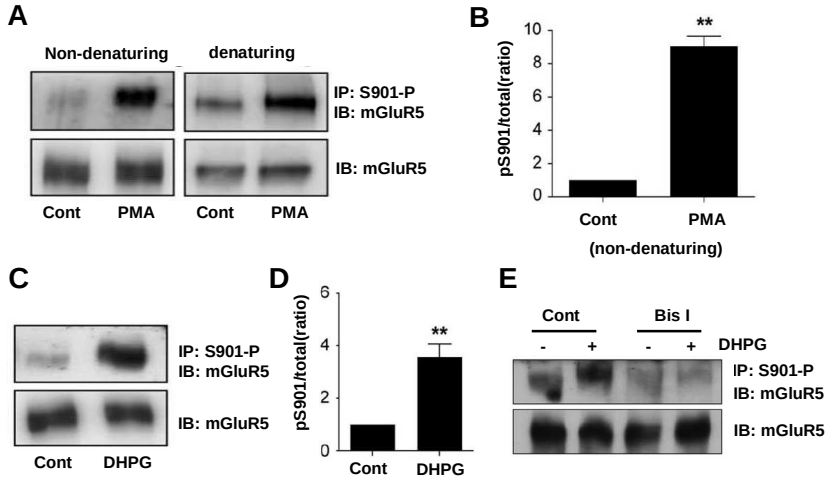

FIGURE 2. S901 on mGluR5 is phosphorylated by PKC in neurons. (*A-B*) Cultured cortical neurons were treated with PMA (1 μM) for 10min. Neuronal lysates were immunoprecipitated with S901 phosphospecific antibody both in non-denaturing and denaturing condition. Then, S901 phosphorylated mGluR5 was measured using immunoblotting with mGluR5 antibody. (C-*D*) Cultured neurons were treated with DHPG (100 μM) for 10min. Phosphorylated receptors were immunoprecipitated with S901 phosphospecific antibody and measured using immunoblotting with mGluR5 antibody. (*E*) Cultured neuron preincubated with bisindolylmaleimide I (Bis I, 5 μM for 30 min) were treated with DHPG (100 μM for 10min). S901 phosphorylated mGluR5 was immunoprecipitated and then measured with mGluR5 antibody. Quantification of immunoblotting (*B* and *D*) was shown as a histogram (n=3). Statistical significance is indicated as $^{**}p < 0.01$

3. S901 phosphorylation induced dissociation of CaM-mGluR5 interaction

Calmodulin (CaM) is highly ubiquitous Ca^{2+} sensing proteins in the cytoplasm. In the CNS, CaM is involved in synaptic plasticity[12]. The reason that CaM was studied is because it has been known that the phosphorylation of mGluR5 by PKC and CaM binding shows the antagonistic relationship[13]. In that, mGluR5 phosphorylation by PKC prevents CaM binding to mGluR5. More interestingly, S901 is located within CaM binding site on mGluR5 (Fig. 3A). In order to examine whether S901 phosphorylation by PKC can affect CaM binding to mGluR5, GST-full down assay was performed and it was found that S901 phosphorylation by PKC dramatically inhibited CaM-mGluR5 WT interaction (Fig. 3B-C). However, CaM-mGluR5 S901A interaction was not affected by PKC treatment. It was also found that the addition of EGTA completely prevented CaM binding to mGluR5, suggesting that CaM-mGluR5 interaction is Ca^{2+} dependent (Fig. 3B). Furthermore, co-immunoprecipitation was performed from full-length mGluR5 and CaM expressing HeLa cells. It was found that the mutation S901 to aspartic acid (S901D), a phosphomimetic mutation, significantly inhibited CaM binding to full-length mGluR5 (Fig. 3D-E). These data suggest that S901 phosphorylation plays a key role in regulating CaM binding to mGluR5.

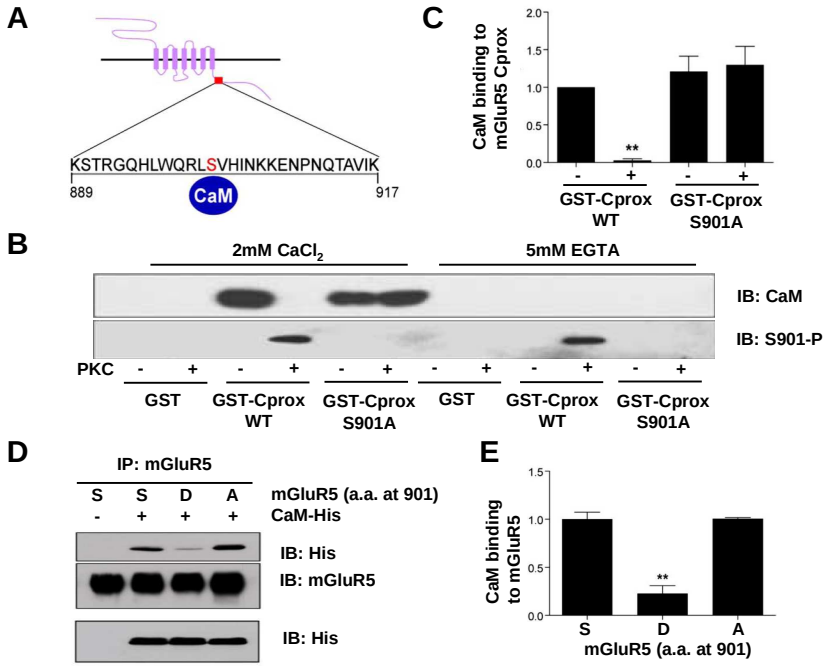

FIGURE 3. S901 phosphorylation inhibits CaM binding to mGluR5. (*A*) A cartoon shows that S901 is located within CaM-binding site on the mGluR5 C-terminus (*B-C*) GST-mGluR5 Cporx wild-type or S901A was treated with PKC and incubated with recombinant CaM in the addition of 2mM $CaCl_2$ or 5mM EGTA. The binding of CaM to phosphorylated mGluR5 was measured by immunoblotting with CaM antibody. S901 phosphorylation by PKC was analyzed using S901 phsophospecific antibody. Quantification of immunoblotting was shown as a histogram (n=4). (*D-E*) His tagged CaM and various mGluR5 constructs (wild-type, S901D, or S901A) were co-transfected into HeLa cells. Cell lysates were immunoprecipitated with mGluR5 antibody and His-CaM binding to mGluR5 was measured by immunoblotting with His antibody. Quantification of immunoblotting was shown as a histogram (n=3). Statistical significance is indicated as $^{**}p < 0.01$ [$F(2, 6)=47.95$]

4. Role of S901 phosphorylation in the trafficking of mGluR5 in heterologous cells

As mentioned earlier, receptor phosphorylation and consequent endocytosis is a typical mechanism of GPCR desensitization. Therefore, it is likely that S901 phosphorylation can affect the internalization of mGluR5. It was examined whether S901 phosphorylation could regulate the trafficking of mGluR5. The trafficking of mGluR5 on the plasma membrane of HeLa cells expressing mRFP-tagged mGluR5 was monitored in real-time using TIRFM. It was observed that fluorescence spots of mGluR5 WT were disappeared in response to the glutamate (Fig. 4A), consistent with the agonist induced endocytosis of mGluR5. However, when these fluorescence spots were analyzed using MetarMorph software and compared mGluR5 WT to S901A, it was found that fluorescence spots of mGluR5 S901A did not change over the time in response to the agonist (Fig. 4B). In addition, using ELISA assay for surface receptor, it was observed that the agonist treatment induced the decrease of mGluR5 WT surface expression, not mGluR5 S901A (Fig. 4C). Using surface biotinylation assay, it was also found that the surface expression level of mGluR5 was significantly decreased in HeLa cells expressing mGluR5 S901D mutant, mimicking the phosphorylation of entire pool of mGluR5, compared to mGluR5 WT (Fig. 4D-E). These data demonstrate that the agonist induced S901 phosphorylation regulates the internalization and surface expression level of mGluR5.

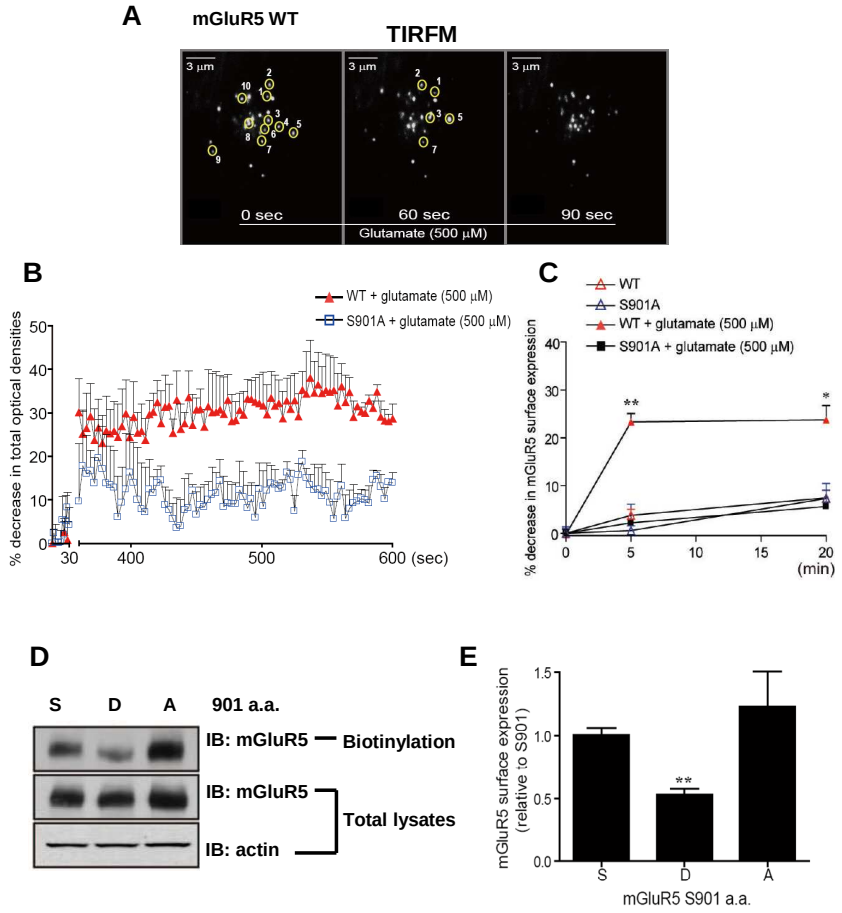

FIGURE 4. S901 phosphorylation regulates the trafficking of mGluR5 in HeLa cells. (*A*) In response to glutamate (500 μM), the trafficking of mGluR5 on the membrane of HeLa expressing wild-type mGluR5-mRFP was monitored over time using total internal reflection fluorescence microscopy (TIRFM). (*B*) After glutamate treatment, changes of mGluR5 wild-type and S901A fluorescence were monitored over time by TIRFM and analyzed by MetaMorph software. Total optical densities of mGluR5 wild-type and S901A fluorescence are presented in a line graph. (*C*) Changes of Myc tagged mGluR5 wild-type or

S901A surface expression following -/+ glutamate addition were measured using ELISA for surface receptor. The expression levels are presented in a line graph. *$p < 0.05$ [$F(3, 20)=4.68$], **$p < 0.01$ [$F(3, 23)=12.63$] compared with S901A + glutamate (*D-E*) The expression of surface mGluR5 (wild-type, S901D, or S901A) in HeLa was analyzed by biotinylation assay. Biotin-labeled membrane proteins were isolated from cell lysates using neutravidin agarose and then analyzed by immunoblotting with mGluR5 or actin antibodies. Quantification of immunoblotting was shown as a histogram. **$p < 0.01$ [$F(2, 12)=4.55$] compared with mGluR5 wild-type

5. Role of CaM binding in the trafficking of mGluR5 in heterologous cells

When it is considered that S901 phosphorylation regulates CaM binding to mGluR5, it needs to be examined whether changes in CaM expression can alter the trafficking of mGluR5. First, it was evaluated whether overexpression and knock-down of CaM in HeLa cells can change the basal surface expression level of mGluR5. It was found that changes in CaM expression did not affect the basal surface expression of mGluR5 (Fig. 5A). However, CaM overexpression inhibited the agonist induced decrease of mGluR5 WT surface expression (Fig. 5B). Moreover, knock-down of CaM dramatically decreased the agonist-induced surface level of mGluR5 S901A (Fig. 5C). Taken together with the previous data, these data demonstrate that the mGluR5 trafficking and the agonist induced decrease of mGluR5 surface expression is due to the agonist induced S901 phosphorylation and consequent CaM dissociation.

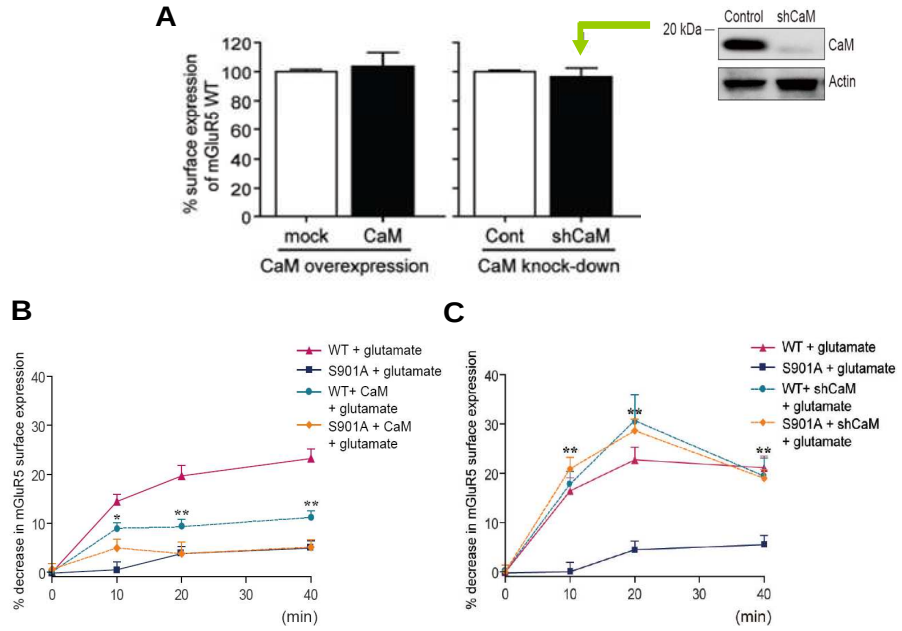

FIGURE 5. Changes of CaM expressions alter the trafficking of mGluR5 in HeLa cells. (*A*) Myc tagged mGluR5 construct and CaM or shRNA targeting CaM (shCaM) were co-transfected into HeLa cells. Surface expression of mGluR5 was measured by ELISA for surface receptor with Myc antibodies. The knockdown of endogenous CaM by shCaM was confirmed using immunoblotting with CaM and actin antibodies (Inset). (*B*-*C*) HeLa cells expressing Myc-mGluR5 (wild-type or S901A) with -/+ CaM (*B*) or -/+ shCaM (*C*) were treated with glutamate. Surface expression of mGluR5 was evaluated by ELISA over time. Changes of surface expression were presented as a line graph (*B*) $*p < 0.05$ [$F(3, 83)=23.83$ for 10 min], $**p < 0.01$ [$F(3, 88)=17.19$ for 20min, $F(3, 84)=49.55$ for 40min] compared with WT + glutamate. (*C*) $**p < 0.01$ [$F(3, 76)=18.75$ for 10 min, $F(3, 76)=19.58$ for 20min, $F(3, 76)=12.84$ for 40 min] compared with S901A + glutamate.

6. Role of S901 phosphorylation and CaM binding in the trafficking of mGluR5 in neurons

Next, it needs to be examined whether S901 phosphorylation and CaM binding can affect the trafficking of mGluR5 in hippocampal neurons. In order to measure the receptor trafficking in neurons, fluorescence-based antibody uptake assay was performed. In this assay, green spots mean the internalized mGluR5, whereas red spots mean the surface expressed mGluR5 (Fig. 6A). It was found that the DHPG treatment significantly induced the internalization of mGlu5 in hippocampal neurons (Fig. 6A). Using this technique, the agonist induced internalization of mGluR5 S901A was compared to that of mGluR5 WT. It was found that the agonist induced internalization of mGluR5 S901A was significantly decreased (Fig. 6B-C). Moreover, It was found that co-transfection of CaM decreased the agonist induced internalization of mGluR5 WT (Fig. 6B-C). Therefore, these findings demonstrate that S901 phosphorylation and CaM binding is a key mechanism regulating the agonist induced internalization of mGluR5 both in heterologous cells and neurons.

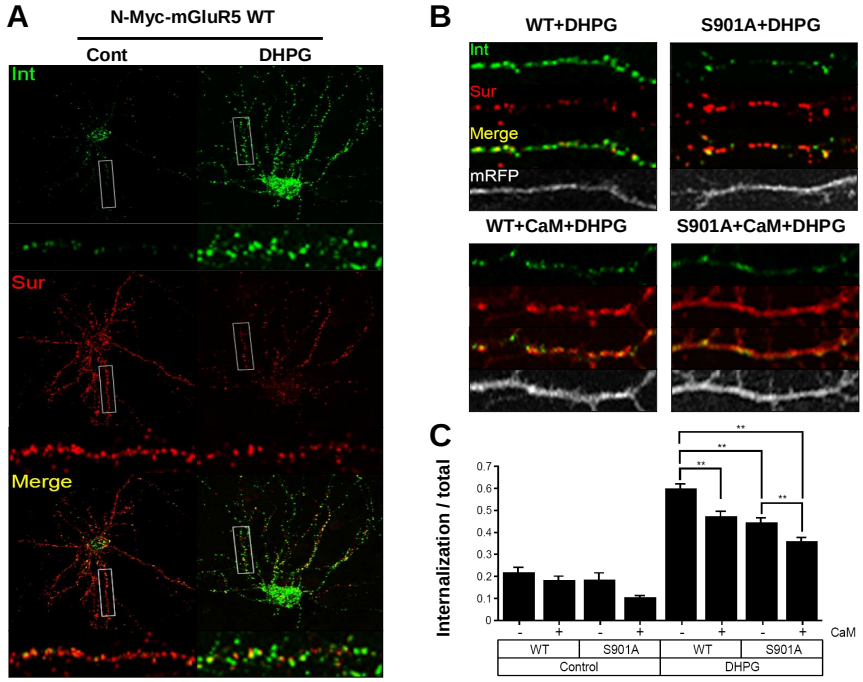

FIGURE 6. Agonist induced internalization of mGluR5 is regulated by S901 phosphorylation and CaM binding in neurons. (*A*) Culture hippocampal neurons expressing Myc-mGluR5 were preincubated with Myc antibody for 45 min. After the treatment of DHPG (100 μM) for 30 min, the surface receptors were labeled with red fluorescence, whereas the internalized receptors with green fluorescence (details in Materials and Methods). Fluorescence-labeled receptors were visualized by confocal micrscopy. Merged images are presented on the bottom. (*B*) Myc-mGluR5 wild-type or S901A with CaM-mRFP or mRFP only were co-transfected into hippocampal neurons. After treatment of DHPG, the surface and internalized receptors were visualized as indicated. mRFP signals were presented as white for clarification. (*C*) Quantification of confocal imaging was shown as a histogram. Y-axis indicates the ratio of the internalized receptors among the total receptors. $^{**}p < 0.01$. (n=180 for DHPG group, n=40 for Control group)

7. Role of S901 phosphorylation in the downstream signaling of activated mGluR5

Finally, it was examined whether S901 phosphorylation can affect the mGluR5 signaling. It has been well known that the activated mGluR5 by the agonist triggers Ca^{2+} oscillation[22]. Moreover, it has been also known that the frequency of Ca^{2+} spike is correlated with the mGluR5 receptor density on the plasma membrane[23]. Using fura-2-AM and ratiometric spectrofluoremeter, it was found that the agonist treatment induced Ca^{2+} oscillation in mGluR5 WT expressing HeLa cells (Fig. 7A). The frequency of Ca^{2+} spike (14.27 ± 3.49 mHz) and the half time of Ca^{2+} to return to the prestimulated level (151.54 ± 112.83 s) in mGluR5 WT expressing HeLa cells (Fig. 7C and 7E) were also measured. However, in mGluR5 S901A expressing HeLa cells, the frequency of Ca^{2+} spike was significantly increased as 26.75 ± 7.70 mHz and the half time of Ca^{2+} to return to the prestimulated level was dramatically prolonged as 886.85 ± 236.97 s (Fig. 7B, 7D, and 7F). Therefore, these data indicate that the agonist induced signaling of mGluR5 S901A is severely prolonged, compared to that of mGluR5 WT. This is consistent with that the prevention of S901 phosphorylation inhibits the desensitization of mGluR5 signaling.

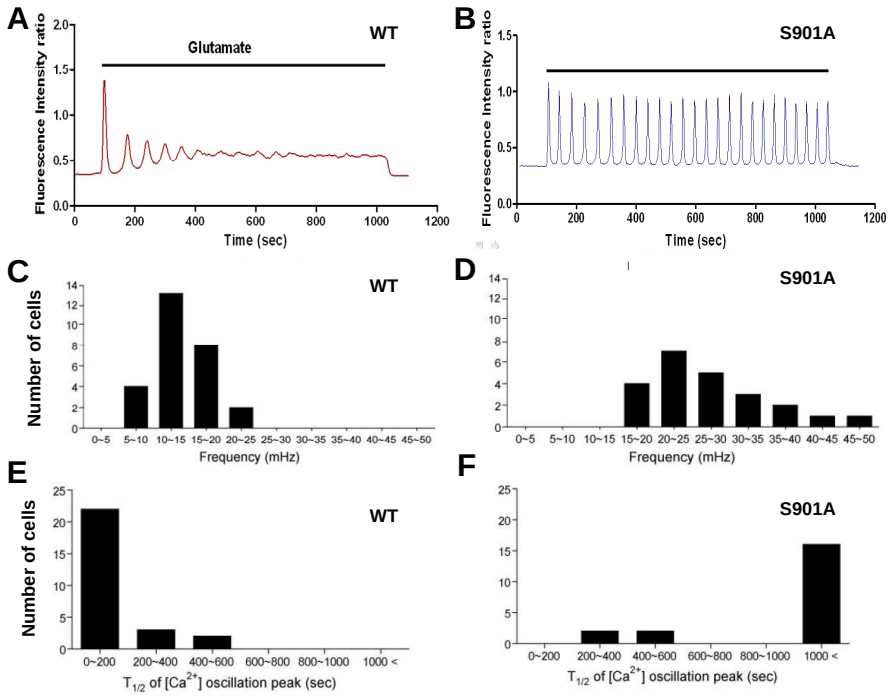

FIGURE 7. S901 phosphorylation regulates the frequency and half time of Ca^{2+} oscillation in mGluR5 expressing HeLa cells. (*A-B*) Typical examples of agonist-induced Ca^{2+} oscillation pattern in mGluR5 wild-type (*A*) or S901A (*B*) expressing HeLa cells were shown. Ca^{2+} oscillations were analyzed using fura-2AM and ratiometric spectrofluorophotometer. (*C-D*) The frequency of Ca^{2+} spikes was measured in mGluR5 wild-type or S901A expressing HeLa cells. Data are represented as a histogram (n=27 for wild-type; n=23 for S901A). (*E-F*) The half-time of Ca^{2+} spikes returning to pre-stimulated level was measured and presented as a histogram (n=27 for wild-type; n=20 for S901A).

IV. DISCUSSION

In the present study, the novel PKC phosphorylation site, S901 on mGluR5 C-terminus was identified. Agonist-induced mGluR5 activation and PKC activity regulated S901 phosphorylation, which profoundly inhibited CaM binding to mGluR5. Importantly, agonist-induced S901 phosphorylation decreased mGluR5 surface expression. It was also shown that S901 phosphorylation and consequential changes in receptor trafficking were mediated by CaM, whose binding to mGluR5 stabilized the surface expression of mGluR5. Furthermore, it was found that agonist-stimulated mGluR5 signaling in mGluR5 S901A was severely prolonged, indicating that preventing S901 phosphorylation inhibits the desensitization of mGluR5 induced by agonist. Therefore, it can be speculated as a hypothetical model that CaM binding stabilizes mGluR5 surface expression, but agonist induced S901 phosphorylation by PKC and consequent CaM dissociation decrease mGluR5 surface expression (Fig. 8).

There have been evidences that the direct phosphorylation of S839 on mGluR5 C-terminus plays an important role in regulating Ca^{2+} oscillations that are PKC dependent[19, 22]. The present study suggests that S901 is another key phosphorylation site that regulates the frequency and duration of Ca^{2+} oscillations. In addition, our data strongly indicate that the decrease of mGluR5 surface expression by S901 phosphorylation is the underlying mechanism of PKC mediated mGluR5 desensitization.

CaM is a universal Ca^{2+} sensor protein ubiquitously expressed in most

eukaryotic cells, which relay Ca^{2+} mediated intracellular signaling[11]. Especially, it is highly abundant in brain[24] and enriched in postsynaptic density[25] and synaptic vesicles[26] of neurons, thereby regulating ion channel function[27, 28], GPCR signaling[29-31], and synaptic plasticity[27, 28, 31]. Intriguingly, there have been accumulating evidences suggesting that CaM directly interacts with various GPCRs expressed in neurons such as dopamine[29], opioid[32], serotonin[15, 30], and metabotropic glutamate receptors (mGluRs)[13, 33]. In some cases, CaM is involved in the GPCR trafficking. For examples, CaM binding to mGluR7 disrupts its interaction with PDZ protein PICK1, increasing the internalization of the receptor[14] and the agonist-induced endocytosis of 5-HT_{1A} receptor is prevented by CaM inhibitor[16]. In addition, there are some evidences that CaM regulates the function of G protein-coupled receptor kinases, which are implicated in endocytosis of GPCRs, indicating a possible role of CaM in regulating GPCR trafficking[34, 35]. Therefore, when it comes to the dynamic regulation of GPCR trafficking by CaM binding, this study shows the direct evidences that CaM stabilizes mGluR5 surface expression.

As mentioned earlier, CaM is highly ubiquitous and abundant proteins. Although GPCRs such as mGluR5 and mGluR7 can be expressed together in one neuron, the trafficking of receptors are regulated by CaM in an opposite way. Then, how can different functions of CaM in each GPCR be explained? Various stimuli including the activation of Gq-coupled metabotropic and tyrosine kinase receptors can induce the activation of protein kinase Cs (PKCs) that is crucial for synaptic plasticity[36]. The activation of PKC is especially implicated in the phosphorylation of various CaM binding GPCRs including 5-

HT_{1A} and 5-HT_{2A} receptor[15, 30], mGluR5[13], and mGluR7[33]. The phosphorylation of CaM binding sites by activated PKC can induce the dissociation of CaM binding from its target GPCRs. For example, the phosphorylation on GPCRs such as 5-HT_{1A} receptor[15], mGluR5[13] and mGluR7[33] by PKC and CaM binding to receptors are antagonistic. Moreover, CaM binding can affect the interaction of GPCRs with other binding proteins. For example, CaM binding site on mGluR5 overlays Siah-1A binding sites[10]. CaM and Siah-1A competitively bind to mGluR5 C-terminus[37]. In addition, CaM binding to mGluR7 competitively decreases the PICK1 binding[14]. In that, the phosphorylation of GPCRs by PKC and consequential dissociation of CaM from receptors can allow the binding of other interacting proteins such as Siah-1A and PICK1.

Siah-1A can be a highly possible candidate as a major regulator, enhancing the internalization of mGluR5. Siah-1A, E3 ubiquitin ligase, induces the ubiquitination and degradation of its substrates including mGluR1 and mGluR5[38]. Recently, it has been known that several E3 ubiquitin ligases such as Mdm2 and Cbl can be implicated both in the mono- or poylubiquitination[39]. The monoubiquitination of membrane receptors such as receptor tyrosine kinases (RTKs) and α-factor receptor plays a role in the receptor trafficking by enhancing the internalizing and lysosomal degradation of receptors[40, 41]. Siah-1A also can promote the monoubiquitination of α-synuclein[42]. Therefore, it is likely that agonist-induced phosphorylation of mGluR5 dissociates CaM binding and may enhance Siah-1A binding to the receptor and Siah-1A then may increase the internalization and degradation of mGluR5 as a major regulator. On the other hand, PICK1 is a PDZ and BAR

domain containing proteins that bind to AMPA receptor subunits such as GluR2 and GluR3[43]. It has been known that PICK1 is a key regulator of AMPA receptor trafficking[43]. In mGluR7, the PKC phosphorylation and dissociation of CaM enhance PICK1 binding to the receptor, thereby increasing the surface expression of mGluR7[14]. Therefore, these evidences suggest that CaM may play a role as an integrator of the GPCRs trafficking, coordinating the binding of regulators to each GPCRs in response to the receptor phosphorylation by PKC.

Increasingly, it is becoming evident that CaM interacts with numerous GPCRs such as vasopressin and angiotensin receptor, as well as GPCRs specific in neurons[44-46]. This study is the first evidence to describe the dynamic regulation of GPCR trafficking by CaM binding in living cells. In spite of the rapid recent progress in dissecting the functional role of CaM in GPCRs, the role of CaM as an integrator of GPCRs trafficking still remains unanswered regarding the major regulators that control each GPCRs trafficking and signaling combined with the dissociation of CaM. Therefore, the identification of GPCRs interacting proteins that are antagonistic to CaM binding may provide a mechanistic linking between the phosphorylation and trafficking and signaling of GPCRs. I believe that the current scientific interests in these topics will be answered in the near future.

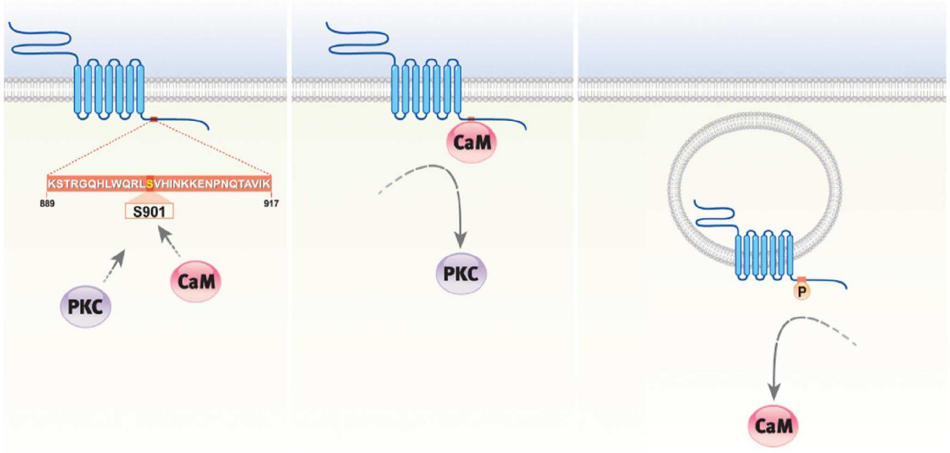

FIGURE 8. Hypothetical model of the PKC-CaM that regulates mGluR5 trafficking. The model that competition between PKC phosphorylation of S901 and CaM binding to mGluR5 regulates receptor trafficking can be speculated (*Left*). CaM binding to mGluR5 stabilizes receptor surface expression (*Middle*). However, S901 phosphorylation by PKC prevents CaM binding and enhances the internalization of receptor (*Right*).

V. CONCLUSION

The present study shows the direct mechanistic link between PKC phosphorylation of mGluR5, CaM binding, receptor trafficking, and downstream signaling. We concluded that:

1. The agonist-induced S901 phosphorylation on mGluR5 is mediated by PKC.

2. S901 phosphorylation is a critical mechanism regulating CaM binding to mGluR5.

3. S901 phosphorylation and CaM binding regulate mGluR5 receptor trafficking.

4. S901 phosphorylation regulates Ca^{2+} oscillation frequency and decay time.

This study demonstrates that CaM plays a role in regulating the binding activities of glutamate receptor-interacting proteins and the receptor functions. These are consistent with the possible role of CaM as an integrator or modulator of glutamate receptor-induced synaptic plasticity. As shown here, CaM will dynamically regulate receptor expression and mGluR5-mediated synaptic plasticity *in vivo* as well.

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ABSTRACT (IN KOREA)

Metabotropic glutamate receptor 5 에서 calmodulin 결합, 수용체 trafficking 그리고 downstream signaling 을 조절하는 새로운 PKC 인산화 위치 규명

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Metabotropic glutamate receptor 5 (mGluR5)는 G 단백 연결 수용체로서 신경가소성, 신경발달 및 퇴행성 신경질환에 중요한 역할을 하며, 신호전달과 관련하여 Gq 단백과 연관되어 phospholipase C 와 protein kinase C (PKC)를 활성화 시키는 것으로 알려져 있다. G 단백 연결 수용체의 중요한 민감소실(desensitization) 기전에는 수용체 인산화와 그 결과로 나타나는 세포 내 이입(endocytosis)과 같은 수용체 trafficking 이 있다. PKC 의 활성화가 mGluR5 의 민감소실과 관련이 있는 것으로 알려져 있으나 현재 그 정확한 기전을 알려져 있지 않다. 본 연구에서는 phosphopeptide mapping 분석을 통하여 mGluR5 세포 내 C-말단 부위에 있는 serine 901(S901)이 PKC 의하여 인산화 됨을 확인하였다. mGluR5 를 발현하는 HeLa 세포와 일차 배양한 신경세포 모두에서 PKC 활성제인 PMA 와 mGluR5 효현제인 DHPG 를 처리하였을 때 S901 인산화가 유도됨을 S901 인산화 항체를

이용하여 확인하였다. 또한 PKC 억제제인 bisindolylmaleimide I 을 세포에 처리한 경우 mGluR5 효현제에 의해 유도되는 S901 인산화가 억제됨을 확인하였다. 흥미롭게도 S901 은 mGluR5 C-말단에 위치한 calmodulin(CaM) 결합 부위에 위치하고 있다. 따라서 CaM 의 결합이 S901 인산화에 의해 조절되는지를 살펴보았다. PKC 에 의한 S901 인산화에 의해 CaM 과 mGluR5 와 결합이 억제되는 것이 관찰되었다. Total Internal Reflection Fluorescence Microscope (TIRFM)와 ELISA 를 이용한 실험에서 S901 인산화가 mGluR5 의 세포막 발현을 감소시키는 결과를 얻었다. 또한 CaM 을 과발현(overexpression) 시킨 경우 효현제에 의한 mGluR5 세포막 발현 감소가 억제 되나, CaM 을 knock-down 시킨 경우 효현제에 의해 별 변화가 없던 S901A mutant mGluR5 세포막 발현을 크게 감소시킨 것을 확인 하였다. 더 나아가 S901 인산화를 봉쇄한 경우 Ca^{2+} oscillation 을 지속시킴으로써 mGluR5 신호전달에 영향을 주는 것을 확인하였다. 따라서 이상의 결과를 통해서 볼 때, 활성화된 mGluR5 는 PKC 를 통하여 S901 인산화를 유도하고 이것은 PKC 를 통한 인산화, CaM 결합, 수용체 trafficking 과 신호전달을 직접적으로 연결하는 중요한 역할을 함을 알 수 있었다.

핵심되는 말: Metabotropic glutamate receptor 5, Calmodulin, Receptor trafficking, PKC, Phosphorylation

PUBLICATION LIST

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