Mechanisms of Signal Transduction:

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Protein Kinase C Phosphorylation of the Metabotropic Glutamate Receptor mGluR5 on Serine 839 Regulates Ca^{2+} Oscillations*

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The activation of Group 1 metabotropic glutamate receptors, mGluR5 and mGluR1α, triggers intracellular calcium release; however, mGluR5 activation is unique in that it elicits Ca^{2+} oscillations. A short region of the mGluR5 C terminus is the critical determinant and differs from the analogous region of mGluR1α by a single amino acid residue, Thr-840, which is an aspartic acid (Asp-854) in mGluR1α. Previous studies show that mGluR5-elicited Ca^{2+} oscillations require protein kinase C (PKC)-dependent phosphorylation and identify Thr-840 as the phosphorylation site. However, direct phosphorylation of mGluR5 has not been studied in detail. We have used biochemical analyses to directly investigate the phosphorylation of the mGluR5 C terminus. We showed that Ser-839 on mGluR5 is directly phosphorylated by PKC, whereas Thr-840 plays a permissive role. Although Ser-839 is conserved in mGluR1α (Ser-853), it is not phosphorylated, as the adjacent residue (Asp-854) is not permissive; however, mutagenesis of Asp-854 to a permissive alanine residue allows phosphorylation of Ser-853 on mGluR1α. We investigated the physiological consequences of mGluR5 Ser-839 phosphorylation using Ca^{2+} imaging. Mutations that eliminate Ser-839 phosphorylation prevent the characteristic mGluR5-dependent Ca^{2+} oscillations. However, mutation of Thr-840 to alanine, which prevents potential Thr-840 phosphorylation but is still permissive for Ser-839 phosphorylation, has no effect on Ca^{2+} oscillations. Thus, we showed that it is phosphorylation of Ser-839, not Thr-840, that is absolutely required for the unique Ca^{2+} oscillations produced by mGluR5 activation. The Thr-840 residue is important only in that it is permissive for the PKC-dependent phosphorylation of Ser-839.

Metabotropic glutamate receptors (mGluRs)[1] play important roles throughout the nervous system, including the activation of ion channels and the regulation of synaptic plasticity (1). In addition, they have been implicated in a variety of neurological diseases (2–5). There are eight different mGluRs, and these are subdivided into three groups based on sequence identity and pharmacological properties. Group 1 mGluRs (mGluR1 and mGluR5) are linked to phospholipase C, whereas Group 2 mGluRs (mGluR2 and mGluR3) and Group 3 mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8) are negatively linked to adenylate cyclase. Activation of Group 1 mGluRs triggers phospholipase C, resulting in increases in IP_3 and diacylglycerol production and the concomitant release of intracellular Ca^{2+} and activation of protein kinase C (PKC) (6). Group 1 mGluR-elicited Ca^{2+} release stimulates PKC translocation to the plasma membrane and oscillations of both PKC activation and IP_3 production (7, 8).

Although both mGluR1 and mGluR5 stimulate intracellular Ca^{2+} release, they differ in that mGluR5 activation results in Ca^{2+} oscillations, whereas mGluR1 activation results in a single Ca^{2+} transient with or without subsequent low frequency oscillations (9, 10). A previous study (10) demonstrated that a short stretch of amino acids in the C terminus of mGluR5 was the critical determinant regulating Ca^{2+} oscillations, specifically the identity of a single amino acid residue, Thr-840. In addition, it was reported that the direct phosphorylation of Thr-840 by PKC was the mechanism regulating the Ca^{2+} oscillations (10). In the current study, we have investigated the direct phosphorylation of mGluR5 by PKC using phosphopeptide analysis. Surprisingly, we demonstrated that Thr-840 is not a PKC substrate but only plays a permissive role for PKC phosphorylation of the adjacent amino acid Ser-839. Consequently, it is the PKC phosphorylation of Ser-839 that regulates mGluR5-elicited Ca^{2+} oscillations. Furthermore, we showed that the analogous serine in mGluR1, Ser-853, is not phosphorylated due to an adjacent aspartic acid residue but can be phosphorylated when the adjacent amino acid is mutated to a permissive residue. Therefore, we have found that Thr-840 of mGluR5 and Asp-854 of mGluR1 are the critical determinants of the distinct Ca^{2+} transients elicited by mGluR5 and mGluR1, respectively. However, our data clearly demonstrate that this is because of the differential effect these residues have on the PKC phosphorylation of a conserved adjacent serine, not because of the direct PKC phosphorylation of Thr-840 on mGluR5.

EXPERIMENTAL PROCEDURES

DNA Constructs and Site-directed Mutagenesis—The mGluR5 cDNA was a generous gift from Dr. S. Nakanishi (Kyoto University). The full-length C terminus (amino acids 828–1171) and first one-third of the C terminus (amino acids 828–944) of mGluR5 were amplified from rat mGluR5 cDNA using PCR. The first one-third of the C terminus (amino acids 842–958) of rat mGluR1α was also amplified by PCR. The PCR products were then digested with EcoRI and subcloned in-frame into pGEX-4T-1 GST fusion vector (Amersham Biosciences). mGluR5 (S839A, T840A, T840D, S834A, T837A, and T838A) and mGluR1α (S853A, D854A, D854T, and S853A/D854A) mutations on GST fusion constructs or pRK5-mGluR5 were generated using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) following the manufacturer's instructions. Mutations were confirmed by sequence analysis.

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[1] The abbreviations used are: mGluR, metabotropic glutamate receptor; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; GST, glutathione *S*-transferase; PMA, phorbol 12-myristate 13-acetate.

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Fusion Protein Production and in Vitro Phosphorylation—Fusion proteins for wild-type and mutant C termini of mGluR5 or mGluR1α were purified for the most part in accordance with the protocol provided by the manufacturer (Amersham Biosciences). In brief, DNA constructs were transformed into BL21 bacterial cells. 50-ml cultures were grown until the cultures reached the mid-log phase of growth (A_{550} = 0.5–1.0) after adding 1 ml of overnight cultures. Isopropyl 1-thio-β-D-galactopyranoside (1 mM) was added to cultures to induce fusion protein expression. After 4 h of induction, the cells were lysed with 5 ml of B-PER bacterial protein extraction reagent (Pierce, Rockford, IL). The bacterial lysates were incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 30 min, and then the beads were washed with 10× bed volume of phosphate-buffered saline. The fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione. The fusion proteins were phosphorylated in 20 mM HEPES, pH 7.4, 1.67 mM $CaCl_2$, 1 mM dithiothreitol, 10 mM $MgCl_2$, 200 mM cold ATP, 1 pmol of [γ-^{32}P]ATP (3000 Ci/mmol) with 25 ng of purified PKC (Promega, Madison, WI) at 30 °C for 30 min. The reactions were stopped by adding SDS-PAGE sample buffer, and the samples were boiled for 5 min. The phosphorylated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The bands were visualized by autoradiography and were excised for *in vitro* phosphopeptide mapping.

Two-dimensional Phosphopeptide Mapping—Peptide mapping was performed as previously described (11, 12). Briefly, phosphorylated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The relevant bands were excised and 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 proteins on the membrane were digested with trypsin overnight at 37 °C. Supernatants containing the tryptic digestion products were dried in a SpeedVac, washed twice with 900 μl of H_2O, and resuspended in 5 μl of H_2O. One or two μl of the dissolved phosphopeptides were spotted onto a cellulose thin layer chromatography plate (Merck). 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 by PhosphorImager analysis.

Measurement of Ca^{2+} Oscillations—HeLa cells (American Type Culture Collection CCL-2) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum on coverglasses. The cells were transfected with wild-type or mutant constructs of mGluR5 using CalPhos mammalian calcium phosphate transfection kit (BD Biosciences). The cells were incubated with transfectants 12 h before changing to fresh medium and analyzed 36–48 h following transfection. The cells were loaded with 5 μM Oregon Green 488 BAPTA-1 AM (Molecular Probes, Eugene, OR) for 30 min at 37 °C and then washed three times with HEPES-buffered solution containing 135 mM NaCl, 5.4 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM HEPES. After 1 h of incubation in the same buffer, confocal microscopy was performed on a Zeiss LSM-510 laser-scanning microscope after the treatment of a mGluR5 agonist (500 μM glutamate). A time scan was performed for 300 s for each sample. Cells exhibiting a Ca^{2+} response to agonist application were classified as oscillating or non-oscillating, with oscillating cells defined as those having oscillation frequencies >1/min with constant amplitude and frequency and non-oscillating cells defined as those having any calcium transients other than oscillations defined above, including a single calcium peak, a single sustained calcium plateau, a single calcium peak or a plateau with slow oscillations in a later phase, or a single peak with a shoulder (8–10).

Generation of Phosphorylation State-specific Antibodies and Immunoblotting—Rabbit phosphospecific antibodies against phosphorylated Ser-839 of mGluR5 were generated by BIOSOURCE International (Camarillo, CA). Rabbits were immunized with a synthetic peptide N-C(EE)FTT[pS]TVVR-C. The sera were collected and affinity-purified using the antigen peptide. Amino acids in parentheses represent additional glutamic acid residues that were added to the N terminus of peptides to promote solubility. Fusion proteins (wild-type GST-mGluR5-Cprox, GST-mGluR5-Cprox (S839A), and GST alone) were phosphorylated by purified PKC *in vitro* as described under "Fusion Protein Production and *in Vitro* Phosphorylation." Proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Phosphorylation of Ser-839 on mGluR5 C-terminal fusion proteins was analyzed by immunoblotting with the pSer839 phosphospecific antibody.

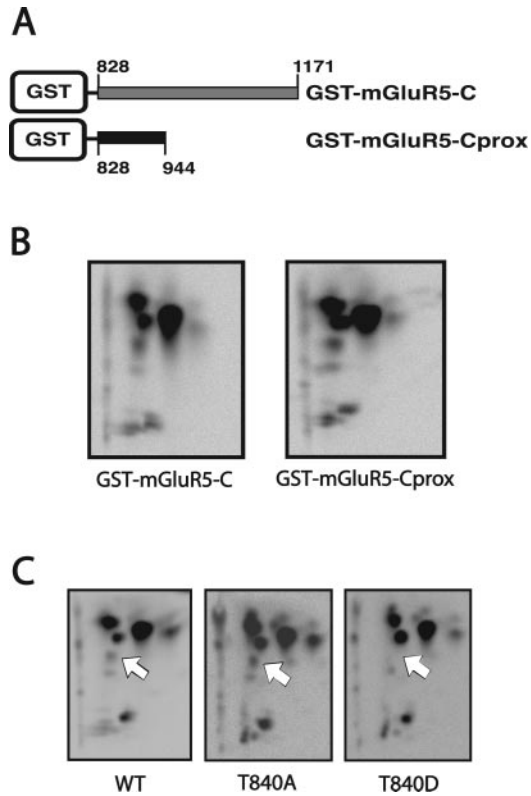

FIG. 1. **The proximal one-third of the mGluR5 C terminus is phosphorylated by PKC on sites other than T840.** *A*, diagram of C-terminal GST fusion proteins of the mGluR5 C terminus. *B*, the first one-third of the mGluR5 C terminus contains the majority of the sites phosphorylated by PKC. GST-mGluR5-C (amino acids 828–1171) and GST-mGluR5-Cprox (amino acids 828–944) were phosphorylated by purified PKC *in vitro* using [γ-^{32}P]ATP and then subjected to two-dimensional phosphopeptide mapping as described under "Experimental Procedures." *C*, threonine 840 is not directly phosphorylated by PKC. Wild-type GST-mGluR5-Cprox, GST-mGluR5-Cprox (T840A), and mGluR5-Cprox (T840D) were phosphorylated by PKC *in vitro* using [γ-^{32}P]ATP and then subjected to two-dimensional phosphopeptide mapping as described under "Experimental Procedures." The phosphopeptide that includes Thr-840 is indicated with a *white arrow*. The figures include representative phosphopeptide maps selected from 3–4 independent experiments.

RESULTS

PKC is an important modulator of mGluR function (10, 13–17); however, the biochemical analyses characterizing the direct phosphorylation of mGluRs by PKC have been lacking. Therefore, we began to characterize the mGluR5 C terminus as the region most likely to contain PKC phosphorylation sites. To evaluate precisely the direct phosphorylation of the mGluR5 C terminus by PKC, we generated a GST fusion protein containing the entire mGluR5 C terminus (GST-mGluR5-C; Fig. 1*A*). Previous studies have reported the phosphorylation of specific residues, including Thr-840, on mGluR5 within the proximal C terminus near the seventh transmembrane domain (10, 18); therefore, we also generated a GST fusion protein containing the first one-third of the mGluR5 C terminus, GST-mGluR5-Cprox (Fig. 1*A*). We performed an *in vitro* phosphorylation assay to evaluate the direct phosphorylation of mGluR5 by PKC. GST-mGluR5 fusion proteins were incubated with [γ-^{32}P]ATP and PKC for 30 min at 30 °C as described under "Experimental Procedures." The fusion proteins were then resolved by SDS-PAGE, the phosphorylated proteins were proteolyzed with trypsin, and the peptide fragments were resolved in two dimensions, resulting in a distinct phosphopeptide map. The peptide map of the mGluR5 C terminus revealed multiple phosphopeptides that varied in their intensity (Fig. 1*B*). Inter-

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estingly, the peptide maps of GST-mGluR5-C and GST-mGluR5-Cprox were indistinguishable (Fig. 1*B*). This was compelling evidence that the major PKC phosphorylation sites on the mGluR5 C terminus were contained within the proximal one-third of this region. Therefore we used the GST-mGluR5-Cprox fusion protein for further analysis.

We next wanted to evaluate the phosphorylation of Thr-840 within mGluR5, which had been reported to be the PKC substrate responsible for regulating the Ca^{2+} oscillations (10). We began by evaluating PKC phosphorylation of the wild-type mGluR5 C terminus and comparing it to the mGluR5 C terminus containing an alanine mutation of the critical threonine (GST-mGluR5-Cprox T840A). We performed *in vitro* phosphorylation of the wild-type GST-mGluR5-Cprox along with the GST-mGluR5-Cprox (T840A). However, we found no change in the peptide maps (Fig. 1*C*), which was surprising, as Thr-840 had been reported to be a PKC substrate (10). We also investigated the threonine to aspartic acid mutation (GST-mGluR5-Cprox T840D) described in that study (10). In contrast to T840A, we found that T840D did result in the disappearance of a distinct phosphopeptide on the map compared with wild-type mGluR5 (Fig. 1*C*). These data are therefore inconsistent with the direct phosphorylation of Thr-840 by PKC, as both the alanine and aspartic acid mutation would abolish direct phosphorylation of Thr-840. Instead, these experiments suggest that the T840D mutant specifically disrupted the phosphorylation of some other site on mGluR5.

We therefore tested the alternative hypothesis that the identity of the 840 amino acid affects the substrate specificity of PKC for a nearby residue. The first one-third of the mGluR5 C terminus contains many potential PKC phosphorylation sites, with several serines and threonines included within the predicted tryptic peptide fragment containing Thr-840 (^{834}SAFTTSTVVR^{843}). One candidate residue for PKC phosphorylation was the adjacent Ser-839 (Fig. 2*A*). To test this hypothesis, we mutated Ser-839 to alanine (GST-mGluR5-Cprox S839A) and evaluated it using the *in vitro* phosphorylation assay. We found that PKC phosphorylation of GST-mGluR5-Cprox (S839A) resulted in a phosphopeptide map with a phosphopeptide missing (Fig. 2*B*), yielding a map with the same pattern as the peptide map of the T840D mutant (Fig. 1*C*). This was consistent with the T840D mutation disrupting the PKC phosphorylation of the adjacent Ser-839. Furthermore, point mutations of the other serine and threonines within the predicted peptide fragment (S834A, T837A, T838A) did not affect phosphorylation and resulted in peptide maps that did not differ from wild type (Fig. 2*C*). In addition to the peptide map analysis, we also generated a phosphorylation state-specific antibody recognizing mGluR5 phosphorylated on Ser-839 to probe whether or not Ser-839 is directly phosphorylated. We performed an *in vitro* PKC phosphorylation assay of GST, wild-type GST-mGluR5-Cprox, and GST-mGluR5-Cprox (S839A), resolved the proteins by SDS-PAGE, and immunoblotted with our Ser-839 phosphoantibody. We found that the antibody specifically recognized PKC-phosphorylated wild-type GST-mGluR5-Cprox and did not recognize PKC-phosphorylated GST-mGluR5-Cprox (S839A), consistent with the direct phosphorylation of Ser-839 *in vitro* (Fig. 2*D*).

Based on our mutagenesis studies of mGluR5, it followed that mGluR1α might be phosphorylated by PKC on serine 853, which is analogous to Ser-839 of mGluR5 (see alignment in Fig. 2*A*), when we replace the adjacent aspartic acid (Asp-854 in wild-type mGluR1α) with a permissive amino acid. To characterize the direct phosphorylation of mGluR1α, we made a GST fusion protein of the proximal one-third of the mGluR1α C terminus (GST-mGluR1α-Cprox). We performed an *in vitro* phosphorylation assay comparing the PKC phosphorylation of wild-type GST-mGluR1α-Cprox and GST-mGluR1α-Cprox mutants. Interestingly, the phosphopeptide map for mGluR1α-

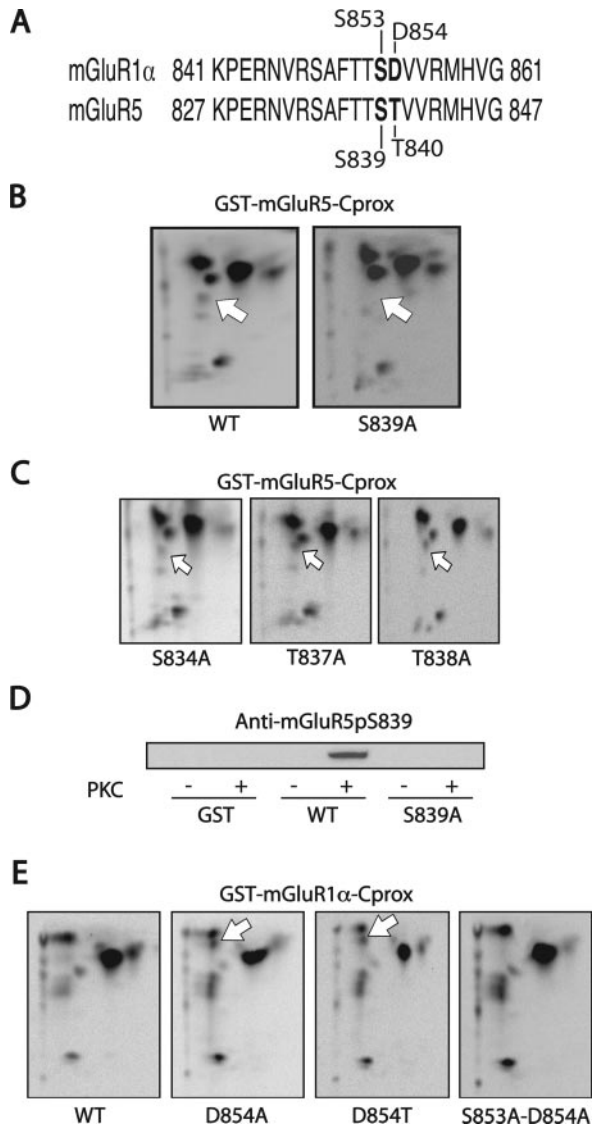

FIG. 2. **Identification of Ser-839 as a PKC phosphorylation site on mGluR5.** *A*, alignment of mGluR1α (amino acids 841–861) and mGluR5 (amino acids 827–847). *B*, serine 839 on mGluR5 was directly phosphorylated by PKC. Wild-type (*WT*) GST-mGluR5-Cprox and GST-mGluR5-Cprox (S839A) were phosphorylated by purified PKC *in vitro* using [γ-^{32}P]ATP and then subjected to two-dimensional phosphopeptide mapping, as described under "Experimental Procedures." The phosphopeptide that contains Ser-839 and Thr-840 is indicated with a *white arrow*. *C*, other serine and threonine residues in the same region of the mGluR5 C terminus were not phosphorylated by PKC. GST-mGluR5-Cprox (S834A), GST-mGluR5-Cprox (T837A), and mGluR5-Cprox (T838A) were phosphorylated by purified PKC *in vitro* using [γ-^{32}P]ATP and then subjected to two-dimensional phosphopeptide mapping, as described under "Experimental Procedures." *D*, serine 839 was phosphorylated *in vitro* as detected by a phosphorylation state-specific antibody. Wild-type GST-mGluR5-Cprox, GST-mGluR5-Cprox (S839A), and GST alone were phosphorylated by purified PKC *in vitro*. The proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antibodies specifically recognizing phosphorylated Ser-839. *E*, serine 853 on mGluR1α is directly phosphorylated by PKC upon mutation of Asp-854 to a permissive amino acid. Wild-type (*WT*) GST-mGluR1α-Cprox, GST-mGluR1α-Cprox (D854A), GST-mGluR1α-Cprox (D854T), and mGluR1α-Cprox (S853A/D854A) were phosphorylated by purified PKC *in vitro* using [γ-^{32}P]ATP and then subjected to two-dimensional phosphopeptide mapping, as described under "Experimental Procedures." The figures include representative phosphopeptide maps selected from 2–3 independent experiments for each mutant.

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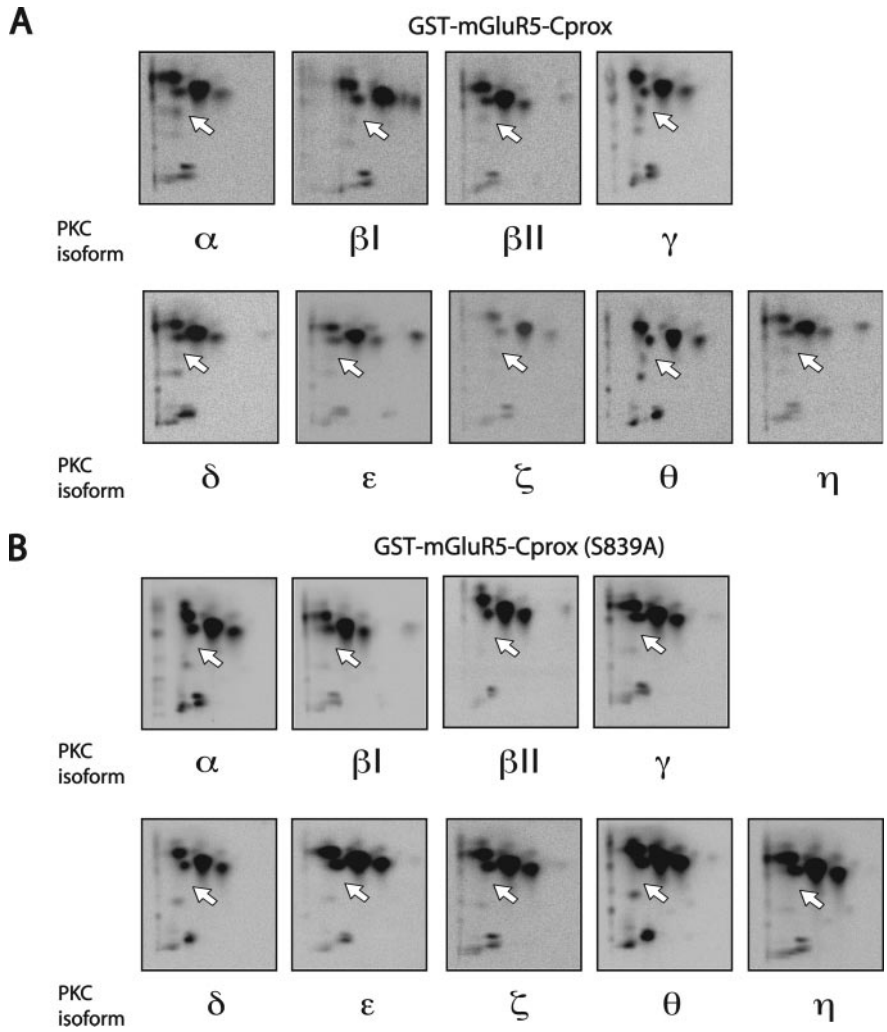

FIG. 3. **Several different PKC isoforms phosphorylate Ser-839 but not Thr-840.** GST-mGluR5-Cprox and GST-mGluR5-Cprox (S839A) were phosphorylated *in vitro* by the recombinant PKC isoforms α, βI, βII, γ, δ, ϵ, ζ, θ, and η (25 ng each) using the same reaction conditions of 30 °C for 30 min. The phosphopeptide containing Ser-839 and Thr-840 is indicated with a *white arrow*. Phosphopeptide mapping was performed as described under "Experimental Procedures."

Cprox D854A and mGluR1α-Cprox D854T revealed the appearance of a new phosphopeptide (Fig. 2*E*). To confirm that the new phosphopeptide was the result of the phosphorylation of Ser-853, we made a double mutation, including both S853A and D854A (GST-mGluR1α-Cprox S853A/D854A). PKC phosphorylation of this fusion protein resulted in a peptide map lacking the critical phosphopeptide and resembling the peptide map of wild-type mGluR1α (Fig. 2*E*). These results strongly suggest that the endogenous aspartic acid (Asp-854 of mGluR1α) affects the phosphorylation of the adjacent residue on mGluR1α (Ser-853), just as the adjacent threonine or aspartic acid affects the phosphorylation of Ser-839 on mGluR5.

Our data, thus far, was entirely consistent with the direct phosphorylation of Ser-839 on mGluR5 by PKC and an absence of PKC phosphorylation of Thr-840. However, we wanted to explore the possibility that certain isoforms of PKC might preferentially phosphorylate Thr-840 or other nearby residues in addition to Ser-839. All of our phosphorylation assays had used a PKC mixture that consisted predominantly of α, β, and γ PKC. We therefore extended our analyses using a battery of PKC isoforms to phosphorylate wild-type GST-mGluR5-Cprox or GST-mGluR5-Cprox (S839A) (Fig. 3). We found that the phosphopeptide maps derived from the phosphorylation assays of different PKC isoforms were quite similar, indicating that most of the PKC phosphorylation sites could be phosphorylated by several different isoforms of PKC. Interestingly, many of the PKC isoforms phosphorylated the peptide containing Ser-839 and Thr-840 (Fig. 3*A*), with PKCα, PKCγ, and PKCθ being most efficient. In contrast, PKCδ, -ϵ, and -ζ did not appear to phosphorylate Ser-839 at all. It should be noted that our experiments only addressed the differential specificity of the PKC isoforms *in vitro* and therefore do not necessarily reflect isoform specificity *in vivo*, where other factors, such as compartmentalization, may also play a critical role. However, importantly, the same peptide fragment was never phosphorylated by any PKC isoform when Ser-839 was mutated to alanine (Fig. 3*B*). These data demonstrated that Ser-839 was the only residue within the peptide that was phosphorylated by the variety of PKC isoforms tested. Under no conditions was Thr-840, or any other nearby residue, contained within that particular tryptic peptide, phosphorylated by these same PKC isoforms. These data further confirm the specificity of the PKC phosphorylation of serine 839.

If PKC phosphorylation of Ser-839 is critical for mGluR5-elicited Ca^{2+} oscillations, then the S839A mutation should alter Ca^{2+} transients elicited by mGluR5 activation. To investigate this, we expressed full-length mGluR5 wild type or mutants in HeLa cells, and changes in intracellular Ca^{2+} concentration in response to agonist application (500 μM glutamate, 250 s) were analyzed using a confocal microscope. The intracellular Ca^{2+} transients were classified as oscillating (representative response, Fig. 4*A*), or non-oscillating (see "Experimental Procedures") (8–10). Consistent with a critical role for Ser-839 phosphorylation in the regulation of Ca^{2+} signaling, the S839A mutation significantly prevented the oscillating Ca^{2+} response compared with mGluR5 wild type

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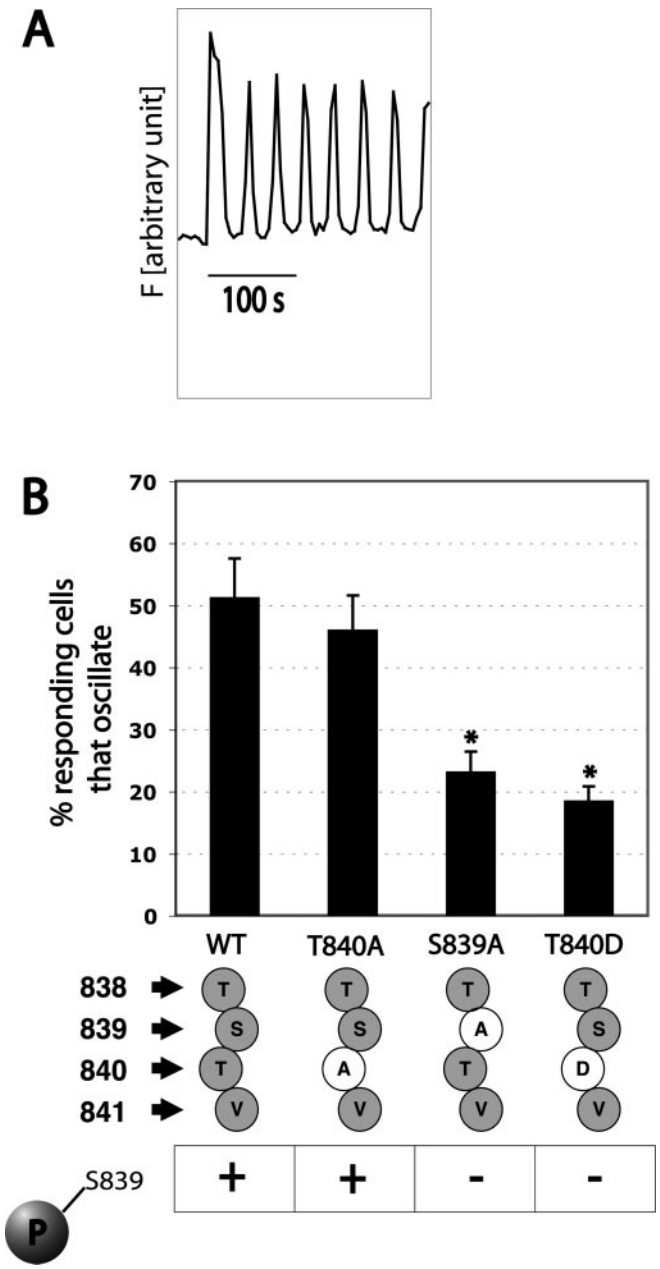

FIG. 4. **PKC phosphorylation of Ser-839 on mGluR5 regulates Ca^{2+} responses evoked by activation of mGluR5.** HeLa cells were transfected with mGluR5 wild type (*WT*), mGluR5 S839A, mGluR5 T840A, or mGluR5 T840D full-length expression constructs. Calcium responses were monitored under confocal microscope for 250 s in time scan mode after treatment of glutamate (500 μM). Calcium responses were classified as oscillating or non-oscillating, as described under "Experimental Procedures." *A*, representative trace of oscillating Ca^{2+} response to agonist application in cell expressing wild-type mGluR5. *B*, the number of cells that displayed the calcium oscillations was counted and depicted as a percentage of the total number of cells that showed any calcium response. The percentages of responding cells that oscillated were 51.1 ± 6.4, 45.9 ± 6.4, 22.9 ± 3.4, and 18.3 ± 2.5% for mGluR5 wild-type, mGluR5 T840A, mGluR5 S839A, and mGluR5 T840D, respectively. *, $p < 0.01$ *versus* wild-type control. All results are expressed as means ± S.E. for four independent experiments. Total numbers of cells counted over four independent experiments were 234, 197, 219, and 221 for mGluR5 S839A, mGluR5 T840D, mGluR5 T840A, and mGluR5 wild-type, respectively. Analysis of variance was used to assess the difference between multiple groups.

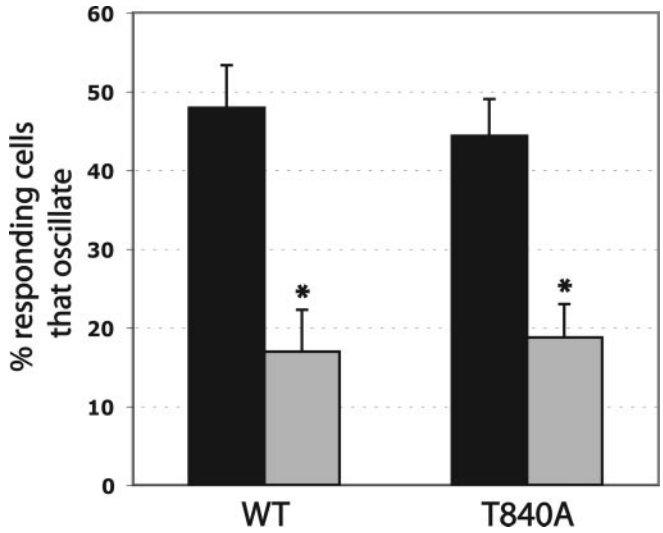

FIG. 5. **Mutation of threonine 840 does not eliminate the PKC regulation of mGluR5-elicited Ca^{2+} oscillations.** HeLa cells transfected with mGluR5 wild-type (*WT*) or mGluR5 T840A full-length expression constructs were treated with phorbol 12-myristate 13-acetate (*PMA*) for 12 h to down-regulate PKC. Calcium responses to glutamate were assessed as described in the legend to Fig. 4. The percentages of responding cells that oscillated were 47.9 ± 5.4% for −PMA wild-type *versus* 16.9 ± 5.3% for +PMA wild-type, and 44.4 ± 4.7% for −PMA T840A *versus* 18.7 ± 4.2% for +PMA T840A. *, $p < 0.01$ *versus* −PMA control. All results are expressed as means ± S.E. for three independent experiments. Total numbers of cells counted over three independent experiments were 119, 108, 102, and 98 for mGluR5 T840A (+PMA), mGluR5 T840A (−PMA), mGluR5 wild-type (+PMA), and mGluR5 wild-type (−PMA), respectively. Student's *t* test was used to assess the difference between −PMA (*black bar*) and +PMA (*shaded bar*).

(Fig. 4*B*). Similar to previous studies (9, 10) and in agreement with our phosphorylation data, activation of mGluR5 T840D also resulted in a pronounced reduction of oscillating Ca^{2+} responses (Fig. 4*B*). However, consistent with our finding that it is Ser-839 and not Thr-840 phosphorylation that is important for regulating Ca^{2+} signaling, T840A (which is permissive for Ser-839 phosphorylation) exhibited Ca^{2+} oscillations that were indistinguishable from mGluR5 wild-type Ca^{2+} responses (Fig. 4*B*). Thus phosphorylation of Ser-839 is the critical molecular determinant for the Ca^{2+} oscillations induced by mGluR5 activation.

Previous studies (8, 10) have characterized the mGluR5-elicited Ca^{2+} oscillations pharmacologically. For example, using several PKC inhibitors, Kawabata *et al.* (9, 10) determined that the oscillations were dependent on PKC but not on other kinases, such as cAMP-dependent protein kinase, protein kinase G, or calcium/calmodulin-dependent protein kinase II. Therefore, we also investigated the PKC dependence of mGluR5-elicited Ca^{2+} oscillations, specifically comparing wild-type mGluR5 to mGluR5 T840A. Cells expressing mGluR5 T840A were treated with PMA for 12 h to down-regulate PKC activity (8). Under these conditions, both mGluR5 wild-type and mGluR5 T840A no longer consistently produced Ca^{2+} oscillations (Fig. 5), further confirming that PKC phosphorylation of Thr-840 is not the essential PKC event regulating Ca^{2+} oscillations.

DISCUSSION

Many studies have implicated PKC as an important regulator of mGluR5 function (10, 14–16). One of the most convincing examples of PKC regulating mGluR signaling is that of mGluR5-mediated Ca^{2+} oscillations. The activation of the Group 1 metabotropic glutamate receptors, mGluR1α and mGluR5, results in intracellular Ca^{2+} release, but mGluR5 activation is unique in that it triggers Ca^{2+} oscillations. It is known that the proximal region of the mGluR5 C terminus is the critical determinant regulating the Ca^{2+} oscillations, and until now, it has been widely accepted that PKC phosphorylation of Thr-840 within that region of mGluR5 was the essential

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mechanism (8–10, 19–22). This is based on mutagenesis data in which altering Thr-840 to aspartic acid perturbs mGluR5 Ca^{2+} oscillations. We have used biochemical phosphorylation assays to specifically characterize the PKC phosphorylation of the portion of the mGluR5 C terminus surrounding Thr-840. Strikingly, we found that Thr-840 is not a PKC substrate, and its phosphorylation does not play any role in the PKC regulation of mGluR5-elicited Ca^{2+} oscillations. Instead, an adjacent residue, Ser-839, is directly phosphorylated by PKC, and phosphorylation of this residue regulates mGluR5-dependent Ca^{2+} oscillations.

Although Thr-840 is not directly phosphorylated, both our study and that of Kawabata *et al.* (10) show that a threonine residue at the 840 position, not an aspartic acid, is essential for mGluR5-elicited Ca^{2+} oscillations. We then demonstrated that this could be attributed to a permissive or structural role for Thr-840 in allowing the phosphorylation of Ser-839 on mGluR5. Although the original study by Kawabata *et al.* used peptide map analysis to analyze the phosphorylation of the critical region of mGluR5, they never evaluated the T840A mutation. This experiment would have revealed that Thr-840 was not the residue directly phosphorylated by PKC.

We also showed that the analogous residue Asp-854 in mGluR1α inhibits the PKC phosphorylation of the conserved serine in mGluR1α, Ser-853. When mGluR1α Asp-854 is mutated to a permissive residue, D854A, we observed PKC phosphorylation of Ser-853. These findings are entirely consistent with the original findings in which activation of mGluR5 containing the T840D mutation resulted in mGluR1α-like intracellular calcium release, and activation of mGluR1α containing the D854T mutation resulted in mGluR5-like Ca^{2+} oscillations.

Although our experiments are the first to demonstrate that Thr-840 is not directly phosphorylated by PKC, other studies suggest that Thr-840 may not be a substrate for PKC. For example, the T840A and T840D mutations in mGluR5 have been reported to differentially regulate PKC oscillation patterns upon mGluR5 activation (7, 21). These results cannot be explained by the direct phosphorylation of Thr-840 on mGluR5 by PKC (or lack of phosphorylation of serine 854 of mGluR1α), because both of these mutations would abolish phosphorylation of this particular residue and would be predicted to have similar phenotypes. However, in neither study was an alternative mechanism for PKC regulation of mGluR5 signaling identified. Our findings that PKC directly phosphorylates Ser-839 and that Thr-840 is permissive for Ser-839 phosphorylation are consistent with these reports and reconcile much of the data found in the literature. Taken together, these data are consistent with a role for PKC phosphorylation of Ser-839 specifically regulating mGluR5-elicited Ca^{2+} (and perhaps PKC and IP_3) oscillations.

We used an *in vitro* phosphorylation assay followed by phosphopeptide map analyses to determine that Ser-839 is phosphorylated by PKC but that Thr-840 is not. Initially we used a PKC mixture that consisted predominantly of α, β, and γ isoforms in all of our phosphorylation assays. However, we wanted to explore the possibility that particular PKC isoforms might preferentially phosphorylate Ser-839 or Thr-840. A recent study reported that PKCδ specifically phosphorylates Thr-840, although no biochemical assays of phosphorylation were conducted (21). To address the possibility that specific isoforms might preferentially phosphorylate Thr-840, we used a battery of PKC isoforms to phosphorylate wild-type GST-mGluR5-Cprox or GST-mGluR5-Cprox (S839A) (Fig. 3). We found that Thr-840 was not phosphorylated by any of the PKC isoforms used in our assay, including PKCδ. Once again these data supported a purely permissive or structural role for Thr-840 in allowing Ser-839 to be a substrate for PKC phosphorylation. Finally, to explore whether the mGluR5 T840A Ca^{2+} oscillations were PKC-dependent, we treated cells expressing mGluR5 T840A with PMA for a prolonged period of time to down-regulate PKC. We found that PKC down-regulation inhibited the oscillations. Thus, mGluR5 T840A-elicited Ca^{2+} oscillations were indeed PKC-dependent, consistent with PKC directly phosphorylating Ser-839 and not Thr-840.

Our results clearly demonstrated a central role for the PKC phosphorylation of Ser-839 in regulating mGluR5 Ca^{2+} oscillations in heterologous cells. Furthermore, our studies revealed a unique substrate-specific phosphorylation event that differentiates the regulation of the Group 1 mGluR-mediated intracellular Ca^{2+} release. Such a precisely regulated difference in intracellular signaling between mGluR5 and mGluR1α is extremely provocative, and our studies defining the precise PKC substrate within mGluR5 that is phosphorylated will facilitate more rigorous studies. However, it is also clear that the regulation of mGluR5-elicited Ca^{2+} oscillations must be more complicated than the PKC phosphorylation of a single residue. The fact that all responding cells do not oscillate (Fig. 4) lends support to the notion that other factors are at play. This is also apparent by the many complexities in the Group 1 mGluR Ca^{2+} signaling literature (8, 23–25).

The role of Ca^{2+} oscillations in neurons is not understood, although it is clear that the frequency, duration, and spatial location of calcium signals are critical for the precise regulation of signal transduction and gene expression in other cellular systems (26–28). A recent study reported that mutating Asp-854 on mGluR1α to threonine (to mimic mGluR5) does not yield oscillations in Purkinje neurons (22), making it unclear how Ca^{2+} oscillations in glia and heterologous cells relate to Ca^{2+} transients in neurons. These recent findings suggest that, at least in Purkinje neurons, the regulation of Ca^{2+} transients may be more complex than previously believed and illustrate the importance of evaluating mGluR signaling in neurons. By precisely characterizing the molecular determinants underlying PKC phosphorylation of mGluR5, our study will facilitate more vigorous and accurate analyses of mGluR-regulated Ca^{2+}, PKC, and IP_3 oscillations *in vivo*.

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