Phosphorylation and Calmodulin Binding of the Metabotropic Glutamate Receptor Subtype 5 (mGluR5) Are Antagonistic in Vitro*

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Metabotropic glutamate receptors, which are members of a G protein-coupled receptor family, mediate the glutamate responses by coupling to the intracellular signal transduction pathway. We herein report that calmodulin (CaM) interacts with the metabotropic glutamate receptor subtype 5 (mGluR5) in a Ca²⁺-dependent manner in vitro. CaM is capable of binding on two distinct sites in the COOH-terminal intracellular region of the receptor with different affinities. The CaM binding domains are separated by an alternatively spliced exon cassette present in one of the splicing isoforms of mGluR5. By using fusion proteins and synthetic peptides we showed that protein kinase C phosphorylates both CaM binding regions. This phosphorylation is inhibited by the binding of CaM to the receptor, and conversely the binding is inhibited by the phosphorylation. These antagonisms of the CaM binding and phosphorylation thus suggest the possibility that they regulate the receptor responses in vivo.

Glutamate is the main excitatory neurotransmitter in the mammalian brain and is important in memory acquisition and learning. Glutamate receptors are categorized into two groups: ionotropic receptors and metabotropic receptors. The ionotropic glutamate receptors function as a glutamate-gated cation channel, whereas the metabotropic glutamate receptors (mGluRs) are coupled to G proteins (1) and evoke a variety of functions by mediating intracellular signal transduction (2). Eight members of the mGluRs have been identified so far, and metabotropic glutamate receptor subtype 1 (mGluR1) and subtype 5 (mGluR5) were shown to activate phospholipase C (3–5).

Three splice variants called mGluR1a to -1c have been described (6, 7). The mGluR1a possesses a long COOH-terminal intracellular domain which is homologous to mGluR5 in the amino acid sequence. The COOH-terminal domain of mGluR1b is much smaller than that of mGluR1a since an additional exon containing an in-frame stop codon is inserted into the domain. In mGluR1c, a distinct insertion results in a similar truncation of the COOH terminus. In contrast to the large fast transient responses induced by mGluR1a, the shorter proteins mGluR1b and mGluR1c elicited a small and more slowly generated oscillatory current in Xenopus oocytes (7). Moreover, the subcellular distribution of mGluR1b expressed in cells was different from that of mGluR1a (8). We previously reported the presence of a variant of mGluR5 and named it mGluR5b (9). An extra 96-base pair exon was inserted into mGluR5a, which had originally been reported as mGluR5. Because of this insertion, the mGluR5b cDNA is able to encode a protein longer than mGluR5a by 32 amino acids. The mRNA levels of the two isoforms were regulated in a development-specific manner (10). No difference of these two isoforms could be seen regarding their pharmacological properties (11, 12). The two mGluR5 variants revealed common functional characteristics with mGluR1a: the generation of the rapid and transient responses when expressed into Xenopus oocytes and the transduction mechanism and subcellular localization in transfected cells (12). Although it was suggested that the long COOH-terminal domain of these three receptors may play a similar role, their structural determinants for the properties still remain unknown. The identification of proteins which interact with the COOH-terminal domain of mGluR5 may give a clue for the role of the domain and may suggest the biological function of the spliced cassette of mGluR5.

In the present study, we created several fusion proteins of glutathione S-transferase (GST) which possess various portions of the COOH-terminal intracellular region of mGluR5 and used them to detect proteins which could interact with them. A 17-kDa protein associating with them was identified as calmodulin (CaM). To our knowledge, none of the G protein-coupled receptors have been reported to interact with CaM directly although several ion channels are regulated by direct CaM binding (13, 14). We characterized the two distinct CaM binding sites which apparently have different affinities for CaM. The two binding sites are separated by the alternatively spliced exon cassette of mGluR5b. We further showed that protein kinase C (PKC) phosphorylates the fusion proteins and the peptides which have one of the two CaM binding regions. PKC phosphorylation is inhibited by CaM binding, and conversely the formation of the complex with CaM is blocked by this phosphorylation. These results suggest that the direct binding of CaM on the COOH-terminal region of the mGluR5 may thus affect the PKC-mediated modulation of the receptor while also indicating that the phosphorylation state may regulate the effect of CaM binding on the receptor.

EXPERIMENTAL PROCEDURES

Construction and Isolation of GST/fusion Proteins—cDNA fragments were obtained from cloned cDNA encoding human mGluR3a or mGluR5b (11) by restriction enzyme digestion, and a BamHI linker was ligated with the 5'-end of the fragment followed by blunting the end if necessary. Each fragment was cloned in-frame into the GST gene fusion vector pGEX-2T. The fusion protein was expressed in Escherichia coli and was purified by glutathione-Sepharose beads (Pharmacia) according to the manufacturer’s protocol. The experiments of Figs. 1 and 3A...
were done with the immobilized fusion proteins on the beads whereas the experiments of Figs. 3B and 5 were done with the fusion proteins eluted from the matrix.

**Metabolic Labeling of P19 Cells and NG108-15 Cells**—P19 cells and NG108-15 cells were cultured as described above for each experiment. Dibutylated P19 cells were used for the experiments on day 3 after treatment with retinoic acid. For metabolic labeling with [35S]methionine and [35S]cysteine, the cells were preincubated with cystine-, methionine-, and cysteine-free Dulbecco’s modified Eagle’s medium (ICN) supplemented with 1% fetal bovine serum. After a 30-min incubation in the medium, the cells were treated with 200 µM Nω-nitro-arginine (L-NAME) and incubated for additional 4 h at 37 °C. The cells were then scraped and washed with phosphate-buffered saline. The pelleted cells were resuspended in 1 mL of buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 1.5 µM pepstatin, 5 µg/mL aprotinin, and 1 mM sodium orthovanadate and lyzed by incubating for 30 min at 4 °C. The lysates were clarified by centrifugation at 14,000 × g at 4 °C for 30 min.

**Preparation of Mouse Brain Homogenate**—Whole brain from an 11-week-old mouse (C57BL) was homogenized in 10 volumes of buffer A containing protease inhibitor mixture and then lysed and centrifuged as described above.

**Protein-binding Assays**—The immobilized fusion protein (3 µg) was incubated with or without S-labeled cell extracts (400 µL), the mouse brain homogenate (400 µL), or bovine CaM (5 µg) in a total volume of 400 µL for 1–2 h at 4 °C. The incubation buffer was as described above for the 35S-labeled cell extracts and buffer B (buffer A with 2 mM CaCl2) for buffer C (buffer A with 5 mM EGTA) for the mouse brain homogenate and bovine CaM (Seikagaku Corp.). The beads were then washed with the incubation buffer. The retained proteins were removed from the beads with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and then were resolved by SDS-PAGE. In Fig. 1B, the gels were fixed with 10% glacial acetic acid and 30% methanol for 20 min and dried. The radioactivities were then image-analyzed (FUJIX BAS 1000). In Fig. 1, C and D, the samples were subjected to SDS-PAGE in which the gel and the electrophoresis buffer contained 5 mM EGTA and were then subjected to Western blotting. The fusion protein (25 pmol) or the synthetic peptide (400 pmol) was incubated with or without CaM in a total volume of 5 µL (20 mM Tris-HCl, pH 7.5, and 2 mM CaCl2) for 40 min at room temperature. The mixture was then washed with the incubation buffer. The retained proteins were transferred to a polyvinylidene difluoride membrane and visualized by an immunoblot analysis with a Vistra ECF Western blotting system (Amersham). Immunoblots were performed as described (15) using an anti-CaM antibody (Seikagaku Corp.).

**Phosphorylation of Fusion Proteins or Synthetic Peptides by PKC**—The fusion protein (25 pmol) or the synthetic peptide (400 pmol) was incubated with or without CaM in a total volume of 5 µL (20 mM Tris-HCl, pH 7.5, and 2 mM CaCl2) for 40 min at room temperature. The components for phosphorylation were then added in a total buffer volume of 20 µL. The phosphorylation buffer contained 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.5 mM CaCl2, 0.25% bovine serum albumin, 0.5 mM dithioerythritol, 100 µg/mL phosphatidylserine, 100 µM 4-(2-PATP, and 0.1 mM calmodulin). Reactions were incubated at 30 °C. For quantification of 32P-labeled fusion proteins, the reaction was stopped by the addition of the SDS-PAGE sample buffer, and 12.5% SDS-PAGE was performed. The gels were fixed with 10% glacial acetic acid and 30% methanol for 20 min and dried. The radioactivities were then image-analyzed (FUJIX BAS 1000). For quantification of the phosphorylated peptide, aliquots were spotted from the reaction mixture onto PS1 phosphocellulose paper (Whatman) and then were subjected to washing and Cerenkov counting (16). In some experiments, we performed SDS-PAGE with Tricine buffer (17) and quantified 32P-labeled peptides to confirm the propriety of the procedure with PS1 papers (data not shown). To estimate the maximal levels of 32P incorporation into the peptides, the incubation was done with 0.005 milliunit of PKC for 20–25 h for the peptide R1 and 2–4 h for the peptide R2. No further increase in the phosphorylation of the peptides was seen after incubation for the additional 1 h.

For the experiments examining the interaction of phosphorylated fusion protein with immobilized CaM, fusion protein (10 pmol) was incubated with PKC (0.005 milliunit) either in the absence or presence of 100 µM P20 at 30 °C. The solution (20 µL) was then mixed with a 37.5-µL bed volume of CaM-Sepharose (Sigma) in a total volume of 200 µL of buffer B. After a 1-h incubation at room temperature, the resin was washed with 7.7 volumes of the same buffer. The fraction was denoted by Unbound in Fig. 5. The resin was then washed twice with 12 volumes of buffer B (CaCl2 concentration in buffer B was reduced to 0.01 mM) and eluted with 12 volumes of buffer C. Each fraction was concentrated by Ultrafree-MC, UFC5LGC00 (Millipore) and then 25% of the unbound, wash, and eluted fractions as well as 2.5% of the input were resolved by 12.5% SDS-PAGE. An immunoblot analysis was done with an anti-GST antibody (Pharmacia Biotech Inc.). Image analyzing was performed for the phosphorylated fusion protein.

**Nondenaturing Polyacrylamide Gel-shift Assays of CaM-Peptide Complexes**—The synthetic peptides (R1, 15 µmol; R2, 3 nmol) were incubated in the phosphorylation reaction buffer with or without PKC (0.04 milliunit) for 13 h at 30 °C. The concentration of the components was the same as the standard condition except that the ATP concentration was changed to 2 mM and bovine serum albumin was omitted. The peptide solution was then diluted to varying concentrations with the reaction buffer. The diluted peptide solution (10 µL) was added to 300 pmol of CaM (2.5 µL) diluted peptide solution (50 µL) was added to 300 pmol of CaM and was then further incubated for 40 min at room temperature. The aliquots were then resolved by 12.5% nondenaturing polyacrylamide gel containing 50 mM Tris-HCl, pH 8.8, and 2 mM CaCl2 and visualized by Coomassie Blue staining.

**RESULTS**

**Detection of a Cellular Protein That Interacts with the mGluR5 Fusion Protein**—We considered the possibility that a COOH-terminal intracellular domain of mGluR5 specifically interacts with other cellular proteins and thus regulates the receptor responses. To test the possibility, we screened for cellular proteins that interact with the mGluR5 COOH terminus fused to GST using undifferentiated P19 cells and the P19 cells differentiated into neurons and astroglia (18). The protein-binding assay in Fig. 1B revealed that a 17-kDa protein interacts with the immobilized cytoplasmic domain of both mGluR5a (GST-MGR5a-g46-1073, Fig. 1A) and mGluR5b (GST-MGR5b-g46-1073, Fig. 1A). The undifferentiated and differentiated P19 cells had the 17-kDa protein (Fig. 1B), and the neureoblastoma × glioma hybrid cells NG108-15 also had one (data not shown). To identify the domains which bind the 17-kDa protein, we examined the ability of the GST-fusion proteins containing various lengths of the COOH terminus of mGluR5a (Fig. 1A) to bind the 17-kDa protein using NG108-15 cells. Two distinct regions, the 31-amino acid sequence (at the residue numbers 846–876 corresponding to human mGluR5b; denoted by site I) and the 54-amino acid sequence (at residue numbers 917–970 in human mGluR5b; denoted by site II) have been shown to be responsible for the binding (Fig. 1, A and B). The amounts of the 17-kDa protein associated with the fusion protein containing site II were larger than those with the site I fusion protein (Fig. 1B). The mGluR5b-specific exon is located between site I and site II in the mGluR5b (Fig. 1A).

**Identification of CaM as an mGluR5-binding Protein**—We next tested whether the protein specifically associated with the two regions of mGluR5 is calmodulin (CaM), a Ca2+-binding protein and an intracellular Ca2+ transducer that regulates the activity of a variety of structurally distinct proteins (19). The fusion protein-immobilized agarose beads were incubated with mouse brain homogenate. The bound proteins were then analyzed by an immunoblot analysis using an anti-CaM antibody, which revealed the 17-kDa protein to be CaM (Fig. 1C). In Fig. 1D, the agarose resin was incubated with CaM from the bovine brain, and the CaM binding on the affinity resin was detected. This strongly suggests that the association between CaM and the immobilized fusion protein is direct. The CaM binding was abolished by the removal of Ca2+ and the addition of 5 mM EGTA (Fig. 1, C and D). Thus, the CaM binding to the fusion protein is Ca2+-dependent. We confirmed that the signal intensity of CaM control did not decrease by incubation with EGTA prior to SDS-PAGE (data not shown), which excludes the possibility that the signals may thus be missing in the +EGTA/−Ca2+ lanes of Fig. 1, C and D, because the CaM antibody binding to CaM is Ca2+-dependent. The slower migrating bands seen in Fig. 1D may represent aggregated CaM (20). CaM tends to wash off the blotted matrix (15) probably because
the protein is small and highly acidic. The aggregated CaM may be retained on the filter matrix and thus be detected by the immunoblotting more efficiently than the CaM monomer. We also observed the extra bands by Coomassie Blue staining, which showed a similar mobility to the upper bands in Fig. 1D but were much fainter than the band corresponding to the CaM monomer (data not shown).

The mobility of CaM shown in Fig. 1, C and D, was slightly different from that in Fig. 1B. The polyacrylamide gel and the electrophoresis buffer for the experiments shown in Fig. 1, C and D, contained EGTA whereas the gel and the buffer of the experiment of Fig. 1B did not. It is known that CaM shows Ca\(^{2+}\)-dependent shifts in electrophoretic mobility and also exhibits apparent heterogeneity at the subsaturation levels of Ca\(^{2+}\) (21) as shown in Fig. 1B.

CaM bound less efficiently to site I\(I_1\) than site II in the experiments with either the brain homogenate (Fig. 1C) or CaM (Fig. 1D), and these findings coincided with the results by the \(^{35}\)S-labeled cell lysate in Fig. 1B. Since site I\(I_1\) begins at the amino acid residue 846 and thus lacks the NH\(_2\)-terminal region of the cytoplasmic domain, we constructed and expressed the GST-MGR5\(_{\text{site I}}\) containing the amino acid sequence at positions 827–876 (Fig. 1A). The amounts of the associated CaM to the GST-MGR5\(_{\text{site I}}\) and those to the GST-MGR5\(_{\text{site II}}\) were similar (Fig. 1C). This indicates that the lower affinity between the GST-MGR5\(_{\text{site I}}\) and CaM is not due to the lack of the NH\(_2\)-
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A minor band was observed in the peptide-free lane which may be the aggregated CaM. This fraction moved upward as the peptide:CaM molar ratio increased. These bands may represent the complexes of the aggregated CaM and increasing amounts of peptides.

PKC Phosphorylation of the mGluR5 and Effect of CaM on It—Many receptors including ion channels and G protein-coupled receptors are known to be modulated by phosphorylation. No strict consensus sequences are detected among the phosphorylation sites by PKC except for the fact that basic amino acid residues near the phosphorylation site may be required (23, 24). The two CaM binding regions described above possess several threonine and serine residues which have basic amino acids around them. We thus examined whether the recombinant fusion protein serves as a substrate of PKC in vitro. As shown in Fig. 3A, the GST-MGR5 site I and the GST-MGR5 site II were phosphorylated by PKC. In contrast, PKC could not phosphorylate GST-MGR5 site II, which has the mGluR5b-specific region but does not have either site I or site II. Recently, PKC-dependent phosphorylation of threonine residue at position 840 of rat mGluR5 was reported (22). Since GST-MGR5 site I lacks the threonine residue at position 841 of human mGluR5 that corresponds to position 840 of rat mGluR5, we tested whether GST-MGR5 site I could be phosphorylated. Its phosphorylation rate was similar to that of GST-MGR5 site I (Fig. 3A), which indicated that the PKC phosphorylation of GST-MGR5 site I could occur at other serine/threonine residue(s). The phosphorylation rate of site II was much faster than site I or site I (Fig. 3, A and B).

We next determined whether PKC phosphorylation was affected by the binding of CaM on its target sequence. Fig. 3A shows that the phosphorylation of site I, site II, and site II is almost fully inhibited at the CaM:fusion protein ratio of 10:1. We did not observe any inhibition of phosphorylation by CaM in a control experiment using histone H1 as a substrate of PKC (data not shown). As shown in Fig. 3B, site II phosphorylation was affected by CaM more readily than site I phosphorylation. The different sensitivity to CaM between site I and site II could thus be interpreted by their different affinity for CaM.

The peptides R1 and R2 could also be phosphorylated by PKC, and the 32P incorporation into the R1 peptide was much slower than the incorporation into the R2 peptide (Fig. 4A). We quantified the maximal levels of 32P incorporation into the peptides. The calculation of stoichiometry indicated that PKC catalyzed the incorporation of approximately 0.61 mol of phosphate/mol of peptide R1 and 0.79 mol of phosphate/mol of peptide R2 (mean of duplicate determinations, from two separate experiments). These results thus indicated that the enzyme phosphorylates at least one residue in peptides R1 and R2. The phosphorylation of the two peptides was inhibited by preincubation with CaM (Fig. 4, B and C), and the phosphoryl-
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Fig. 3. Autoradiographs showing the effect of CaM on PKC phosphorylation of the mGluR5 fusion proteins. A, the GST-fusion protein or GST backbone on glutathione-Sepharose beads was preincubated with either CaM (CaM:fusion protein ratio is 10; denoted by +) or the appropriate volume of buffer (denoted by −) as described under “Experimental Procedures.” The components necessary for phosphorylation were then added including [γ-32P]ATP and 0.005 milliunit of PKC, and the mixture was incubated at 30 °C for the times indicated. The reactions were stopped by adding SDS-PAGE sample buffer. The samples were processed by 12.5% SDS-PAGE. The positions of the protein standards and their sizes (in kDa) are shown on the right. The prolonged incubation up to 1 h did not reveal the phosphorylation of the GST-MGR5bb-specific, nor the GST backbone (data not shown). B, effect of CaM on phosphorylation of the site I and site II. The GST fusion proteins were preincubated with varying molar amounts of CaM. The GST:fusion protein ratios are indicated in the figure. The components necessary for phosphorylation including [γ-32P]ATP and 0.005 milliunit of PKC were then added. Phosphorylated bands of the GST fusion proteins were then added including [γ-32P]ATP and 0.005 milliunit of PKC, and the mixture was incubated at 30 °C for the times indicated. The reactions were stopped by adding SDS-PAGE sample buffer. The samples were processed by 12.5% SDS-PAGE. The positions of the protein standards and their sizes (in kDa) are shown on the right. The prolonged incubation up to 1 h did not reveal the phosphorylation of the GST-MGR5bb-specific, nor the GST backbone (data not shown). B, effect of CaM on phosphorylation of the site I and site II. The GST fusion proteins were preincubated with varying molar amounts of CaM. The GST:fusion protein ratios are indicated in the figure. The components necessary for phosphorylation including [γ-32P]ATP and 0.005 milliunit of PKC were then added. Phosphorylated bands of the GST fusion proteins which were resolved on 12.5% SDS-PAGE are indicated by arrows.

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ivation of R2 was more severely blocked by CaM than that of R1 (Fig. 4D). These results thus suggested that the direct CaM association to the peptide prevents the access of PKC.

Phosphorylation of mGluR5 Interferes with the Interaction between mGluR5 and CaM—Since the binding of CaM on mGluR5 inhibited the phosphorylation of mGluR5, it seemed likely that the phosphorylation would conversely inhibit the CaM binding. We thus examined whether the mGluR5 phosphorylation interferes with the ability of mGluR5 to associate with CaM. The fusion protein GST-MGR5site I and GST-MGR5site II was incubated with PKC in the absence and presence of ATP; the former condition served as a control reaction. Both unphosphorylated and phosphorylated proteins were further incubated with the CaM-agarose beads, and the elution pattern of each was analyzed (Fig. 5). The uppermost band in Fig. 5, A or B, corresponds to the band shown in Fig. 5C. This could thus represent the fusion protein in full-length whereas the extra bands in Fig. 5, A or B, could indicate the incompletely translated proteins or the degraded proteins which include the GST backbone degraded from the GST-fusion. The unphosphorylated fusion protein of site II bound the CaM-agarose resin quite tightly; it did not flow through the resin nor was it washed off (Fig. 5A, right). Significant amounts of GST-MGR5site I passed through the resin, and some of the retained protein was released by washing, thus indicating that its binding on the immobilized CaM is weaker than the site II binding (Fig. 5A, left). Unphosphorylated fusion proteins of site I and site II which bound the CaM-agarose beads were eluted with the buffer containing 5 mM EGTA (Fig. 5A). In contrast, the majority of the phosphorylated proteins GST-MGR5site I and GST-MGR5site II either flowed through or washed off the CaM-agarose resin (Fig. 5, B and C).

To verify the effect of phosphorylation on the CaM binding, we performed a gel-shift assay with the phosphorylated R1 or R2 peptide (Fig. 2C). The lower arrowheads indicate free CaM; much higher molecular weight proteins may be aggregated CaM as mentioned before. The CaM-peptide complexes indicated by the upper arrowheads were missing when the phosphorylated peptides were added for both the R1 and R2 peptides. With peptide R2, the mobility shift of the monomer or aggregated CaM was almost completely blocked by the phosphorylation. With peptide R1, only a small change in the mobility of both the CaM monomer and the aggregated CaM occurred by incubation with the peptide which had been subjected to PKC phosphorylation. The extent of the mobility shifts at the phosphorylated R1 peptide:CaM ratio of 20:1 (Fig. 2C) was similar to or less than that at the unphosphorylated peptide:CaM ratio of 1:1 (Fig. 2B). There are two possible reasons for the fact that the small shift still occurred with peptide R1 in Fig. 2C. 1) One possibility is that the unphosphorylated R1 peptide is responsible for the mobility shift of CaM. We changed the phosphorylation condition to achieve the maximal phosphorylation of the peptide. The ATP concentration and the amount of PKC was increased, and the incubation period was elongated. Nevertheless, it may be possible that a small fraction of the R1 peptide still remained unphosphorylated. Even if this happens, the unphosphorylated peptide could be less than one-twentieth of the peptide based on the results of Fig. 2, B and C. 2) The other possibility is that the phosphorylated peptide has a weak affinity for CaM. As shown in Fig. 5C, the majority of the 32P-incorporated fusion protein flowed through the CaM-agarose resin. A small amount of the phosphorylated protein, however, was released by washing and EGTA treatment. This indicates that some of the fusion protein was retained on the CaM-agarose resin and suggests that the phosphorylated protein had a weak interaction with CaM. It is thus conceivable that the phosphorylated peptides may also have a weak affinity for CaM. In any event, the primary effect of phosphorylation is thus to prevent CaM binding. Therefore, the findings on both the fusion proteins and the peptides provide convincing evidence that the phosphorylation of the CaM binding site either prevents or hampers its interaction with CaM.

DISCUSSION

We have shown in this study that CaM directly binds the COOH terminus of the mGluR5 in vitro. We identified the two binding sites which are separated by the mGluR5b-specific alternative exon. There is growing evidence that the ion chan-
NMR channels can be regulated by CaM through direct binding. In some cases, the binding reduces the channel activity; in others, it activates the channels. The binding of CaM on the olfactory cyclic nucleotide-gated cation channel reduces the channel activity (25), and in contrast, the Paramecium Na^1 channels are activated by CaM (26). Direct CaM binding might thus be a general mechanism for the Ca^2^1-dependent regulation of ion channel properties. There has however been no previous report of direct CaM binding on G protein-coupled receptors as far as we know. In a recent study, a subunit of the N-methyl-D-aspartate receptor, which belongs to the glutamate-gated ion channels, was also reported to be a target of CaM binding (14).

Two CaM binding sites of the subunit NR1 were found which have different affinities for CaM. It was demonstrated by patch-clamp recording techniques that the interaction of CaM with the NR1 subunit causes an inactivation of the channels. We showed in this paper that CaM also interacts with the subtype 5 of the metabotropic glutamate receptors at two distinct sites. It is interesting that direct CaM binding has been demonstrated in members of both categories of glutamate receptors: namely, ionotropic receptors and metabotropic receptors. The degree of cooperativity between the two CaM binding

**Fig. 4. Effect of CaM on phosphorylation of peptides R1 and R2.**

A. Time course of phosphorylation. R1 (open circles) or R2 (filled circles) was incubated with [γ- ^32P]ATP and PKC (0.0005 milliunit, n = 1; 0.0009 milliunit, n = 3) at 30 °C. The incubations were terminated at the times indicated, and the radioactivity incorporated into the peptide was measured as described under "Experimental Procedures." The radioactivity for the phosphorylated R2 peptide after incubation with PKC for 40 min was arbitrarily assigned a value of 100, and the radioactivities of the other samples are expressed as a percentage of this value. The results represent the mean ± S.E. (n = 4). B and C, R1 (B) or R2 (C) was preincubated with varying amounts of CaM as described under "Experimental Procedures." The CaM:peptide ratios are as follows: 0, open circles; 0.2, filled circles; 0.5, open triangles; 1, filled triangles; 2, open squares. The components necessary for the phosphorylation were then added including PKC (0.005 milliunit for the R1 and 0.0005 milliunit for the R2), and the mixture was incubated at 30 °C for the times indicated. The values were normalized against the quantity of the phosphorylated peptide after incubation with PKC in the absence of CaM for 40 min. The results represent the mean ± S.E. (R1, n = 7; R2, n = 6). D, the quantity of the phosphorylated peptide R1 (open circles) or R2 (filled circles) after incubation with PKC for 20 min in the experiments shown in B or C is plotted against the molar ratio of CaM/peptide. The radioactivity in the absence of CaM was taken as 100. The results represent the mean ± S.E. (R1, n = 7; R2, n = 6). *, p < 0.01, significance by t test comparing the R1 phosphorylation to the R2 phosphorylation under the same peptide:CaM ratio.
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sites is unknown in both the case of NR1 and the case of mGlur5. Although both of the fusion proteins derived from mGlur5a and mGlur5b associated with CaM in vitro, the affinities for CaM might be different in the two splice variants of the native form. It is also conceivable that a conformational change of the receptor by agonist stimulation might also affect the accessibility of CaM to the receptor. The functional consequences of the direct binding of CaM on mGlur5 thus remain to be elucidated.

The eight mGlur subtypes can be classified into three groups (27). The Group I mGlurs, which comprise mGlur1 and mGlur5, are coupled to phospholipase C, which catalyzes phosphoinositide hydrolysis. It results in the production of both inositol trisphosphate, which induces the release of Ca²⁺ from intracellular stores, and diacylglycerol, which stimulates PKC. PKC is a serine/threonine kinase that is thought to play some role in diverse cellular processes. There are many serine and threonine residues at the COOH terminus of mGlur1 and mGlur5, which suggests that the residues could be targets of kinases that could thus regulate receptor activity. We herein demonstrated that the two CaM binding sites in mGlur5 are substrates for PKC. The changes in the cellular response by direct stimulation of PKC using phorbol esters have been previously investigated in many studies. The mGlur5-mediated phosphoinositide hydrolysis was inhibited by the PKC stimulation in cerebellar granule cells (28) and in rat hippocampal slices (29). The diacylglycerol production by the agonist stimulation of mGlurs was also inhibited by the direct stimulation of PKC in cerebocortical nerve terminals (30). The receptor-mediated response was inhibited by the preactivation of PKC in baby hamster kidney (BHK) cells transfected with mGlur1a (31) whereas receptor phosphorylation was also observed in response to agonist activation of the receptor in the same BHK cells (32). These findings have led to the suggestion that the PKC phosphorylation of Group I receptor may result in receptor desensitization. The phosphorylation site responsible for the desensitization has yet to be identified, and this study thus suggests some likely candidates. Preliminary results suggest that a higher rate of phosphate incorporation appears in the fusion protein derived from mGlur5b than in the fusion protein from mGlur5a under the same reaction conditions. Although the fusion protein which has only the mGlur5b-specific region is not phosphorylated, the region might be phosphorylated when linked to site I and/or site II. It is also conceivable that the mGlur5b-specific exon between site I and site II affects the accessibility of the PKC enzyme to the two sites. The alternative exon might serve as a modulatory domain which affects the phosphorylation state of the receptor.

The CaM-dependent inhibition of the phosphorylation was demonstrated at both sites in mGlur5. The phosphorylation of the site II protein, which showed a higher affinity for CaM, was more sensitively suppressed by CaM. We observed similar results with peptides R1 and R2, where the latter was more sensitive in phosphorylation suppression by CaM. Thus the most likely reason for the reduced phosphorylation is that CaM blocks access of PKC to the phosphorylation site. We further showed that the phosphorylation of the two sites prevents their interaction with CaM. These results thus indicated that the CaM binding and the PKC phosphorylation are mutually antagonistic.

Myristoylated alanine-rich protein kinase C substrate (MARKCS) is a major cellular substrate of PKC, and it binds to both CaM and actin. PKC phosphorylation inhibits CaM and actin binding, and CaM binding prevents PKC phosphorylation and actin binding (33, 34). The CaM binding domains of mGlur5 might thus be involved in cytoskeletal association in a manner analogous to the MARKCS protein. The relative timing of surges of internal Ca²⁺ transient (and the Ca²⁺-CaM complexes it produces) and PKC activity thus determines the extent of MARKCS protein phosphorylation (35). It is thus conceivable that the different times of arrival of Ca²⁺ and PKC signal to the membrane-associated mGlur5 might thus affect the extent of the phosphorylation of mGlur5.

Whereas the two mGlur5 variants and mGlur1a possess a long cytoplasmic domain, the splice variants of mGlur1 named mGlur1b and mGlur1c have a smaller COOH terminus than the three receptors because of an in-frame stop codon in the alternative exon (6, 7). The shorter proteins elicited a small and slowly generated oscillatory current in Xenopus oocytes (7) whereas the subcellular distribution of mGlur1b was different from mGlur1a and the two mGlur5 isomers when expressed in cells (8). The COOH-terminal domain of mGlur1a contains regions homologous to site I and site II of mGlur5, whereas the smaller cytoplasmic regions in mGlur1b and mGlur1c also have a region corresponding to site I but do not have a region corresponding to site II because of the truncation by the in-frame stop codon. We could thus speculate that a loss of a target sequence of CaM or PKC or possibly a binding domain of actin in mGlur1b and mGlur1c might thus confer the characteristics shown in the shorter receptor. Our findings in this study may provide new leads for approaching the Ca²⁺-mediated modulation of the mGlurs as well as the biological function of the spliced cassette of the receptors.

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