A Relationship between Protein Kinase C Phosphorylation and Calmodulin Binding to the Metabotropic Glutamate Receptor Subtype 7*

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Metabotropic glutamate receptor subtype 7 (mGluR7) is coupled to the inhibitory cyclic AMP cascade and is selectively activated by a glutamate analogue, 1,2-amino-4-phosphonobutyrate. Among 1,2-amino-4-phosphonobutyrate-sensitive mGluR subtypes, mGluR7 is highly concentrated at the presynaptic terminals and is thought to play an important role in modulation of glutamatergic synaptic transmission by postsynaptic inhibition of glutamate release. To gain further insight into the intracellular signaling mechanisms of mGluR7, with the aid of glutathione S-transferase fusion affinity chromatography, we attempted to identify proteins that interact with the intracellular carboxyl terminus of mGluR7. Here, we report that calmodulin (CaM) directly binds to the carboxyl terminus of mGluR7 in a Ca2+ dependent manner. The CaM-binding domain is located immediately following the 7th transmembrane segment. We also show that the CaM-binding domain of mGluR7 is phosphorylated by protein kinase C (PKC). This phosphorylation is inhibited by the binding of Ca2+/CaM to the receptor. Conversely, the Ca2+/CaM binding is prevented by PKC phosphorylation. Collectively, these results suggest that mGluR7 serves to cross-link the cyclic AMP, Ca2+, and PKC phosphorylation signal transduction cascades.

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‡ The abbreviations used are: mGluR, metabotropic glutamate receptor; IP3, inositol trisphosphate; L-AP4, L-2-amino-4-phosphonobutyrate; CaM, calmodulin; PKC, protein kinase C; anti-CaM mAb, mouse monoclonal antibody against CaM; GST, glutathione S-transferase; ct-mGluR7, carboxyl terminus of mGluR7 (ct-mGluR7). We show that a 17-kDa protein, which was identified as calmodulin (CaM), binds directly to ct-mGluR7 in a Ca2+ dependent manner. In addition, we show that the Ca2+/CaM binding is inhibited by protein kinase C (PKC)-evoked phosphorylation. Furthermore, PKC phosphorylation of ct-mGluR7 is inhibited by Ca2+/CaM binding.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following sources: bovine CaM from Sigma; mouse monoclonal antibody against CaM (anti-CaM mAb) from Upstate Biotechnology, Inc. (Lake Placid, NY); rat brain PKC from Calbiochem; catalytic subunit of bovine heart protein kinase A (PKA) from Boehringer Mannheim Co., Ltd. (Nagoya, Japan); cAMP-dependent protein kinase from Sigma; [γ-32P]ATP from Amersham Pharmacia Biotec. Recombinants—Proteins—ct-mGluR2 (Glu929-Leu1077), ct-mGluR3 (Glu829-Leu977), ct-mGluR4 (His846-Ile912), ct-mGluR6 (His840-Leu872), and ct-mGluR7 (His846-Ile915) were amplified, using the corresponding mGluR cDNAs (10, 21, 22) as templates by polymerase chain reaction (PCR). ct-mGluR5 (His846-Ile908) was amplified with rat brain total RNA by reverse transcriptase-mediated PCR (23). Primers for mGluR5 are coupled to the stimulation of the inositol triphosphate (IP3)/Ca2+ signaling pathway. Group 2 (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR6, mGluR7, and mGluR8) receptors are coupled to the inhibitory cyclic AMP cascade in heterologously expressing cells but differ in their agonist selectivity. Among the mGluR family, mGluR7 is the most highly conserved across different mammalian species (5) and is widely distributed throughout the central nervous system (6–9). This receptor is selectively activated by 1,2-amino-4-phosphonobutyrate (L-AP4) (10, 11). L-AP4 has been shown to exert a potent presynaptic inhibition of glutamate release (12, 13). At synapses, mGluR7 is located in close proximity to synaptic vesicle release sites (9, 14, 15). Recent gene targeting analysis has indicated that mGluR7 deficiency causes a reduction in high frequency synaptic transmission, post-tetanic potentiation, and short term potentiation in the CA1 synapses of hippocampal slices (16). In behavioral analyses, these knockout mice showed a deficit in fear response and conditioned taste aversion (17). Therefore, mGluR7 has been postulated to play an important role in synaptic modulation and plasticity. However, it remains elusive whether a variety of cellular and physiological functions of mGluR7 all result from coupling to the inhibitory cyclic AMP cascade mediated by this receptor subtype.

Recent biochemical and molecular studies have indicated that protein-protein interactions play a pivotal role in regulation and signal transduction of group 1 mGluRs (18–20). The identification of molecules that interact with receptors would thus provide an important clue for understanding the receptor function. In this study, we attempted to identify proteins that interact with mGluR7 by glutathione S-transferase (GST) affinity chromatography using the intracellular carboxyl terminus of mGluR7 (ct-mGluR7). We show that a 17-kDa protein, which was identified as calmodulin (CaM), binds directly to ct-mGluR7 in a Ca2+ dependent manner. In addition, we show that the Ca2+/CaM binding is inhibited by protein kinase C (PKC)-evoked phosphorylation. Furthermore, PKC phosphorylation of ct-mGluR7 is inhibited by Ca2+/CaM binding.
PCR or reverse transcriptase-mediated PCR were designed as follows: the nucleotide sequence immediately following the 7th transmembrane segment and covering 19–21 base pairs of the downstream sequence of each individual ct-mGluR was preceded by an appropriate restriction cleavage site and used as a 5′ primer. The sequence containing a stop codon and the upstream sequence of each respective ct-mGluR was followed by a restriction cleavage site and used as a 3′ primer. GST fusion proteins containing different ct-mGluRs (GST-ct-mGluRs) were generated by inserting the PCR products in-frame into a multiple cloning site downstream of the GST-coding region of the pGEX4T series of vectors (Amersham Pharmacia Biotech). Truncated forms of ct-mGluR7 fused to the GST protein were similarly constructed by starting with an appropriate amino-terminal sequence in ct-mGluR7 and ending with the TAA stop codon. All ct or truncated forms of mGluR7 were designed to contain the common linker amino acid sequence between GST and the inserts. A proper in-frame insertion and the absence of any sequence errors of the PCR products were confirmed by DNA sequencing in both strands of all constructs. The GST fusion proteins were expressed in an Escherichia coli strain BL21 and purified by glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The GST-ct-mGluR7 protein was further purified by cation exchange chromatography on a MonoS column (Amersham Pharmacia Biotech) using a gradient elution from 150 to 405 mM NaCl in a solution containing 50 mM Hepes, pH 7.0, 2 mM EDTA, and 1 mM dithiothreitol. Fractions containing the GST-ct-mGluR7 protein were combined, dialyzed against 25 mM Hepes, pH 7.4, containing 150 mM NaCl, and then concentrated with Ultrafree (Millipore, Bedford, MA).

Affinity Chromatography of Brain Extracts—Brains from adult Sprague-Dawley rats were homogenized on ice using a glass-Teflon homogenizer and concentrated with Ultrafree (Millipore, Bedford, MA). Brain cytosolic fractions were prepared and loaded to glutathione-Sepharose 4B beads and tested for their ability to immobilize on beads: lane 1, GST-ct-mGluR2; lane 2, GST-ct-mGluR7; lane 3, GST alone; lane 4, without any immobilized GST proteins. Lane M, molecular mass markers (kDa).

For phosphorylation of PKC or PKA, the incubation mixture (10 μl) contained 8.75 mM Hepes, pH 7.4, 52.5 mM NaCl, and 1 mM CaCl₂. Phosphorylation was started by addition of the following solution (10 μl): for PKC phosphorylation, 40 mM Tris-Cl, pH 7.5, 20 mM MgCl₂, 200 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was preclarified by passing Sepharose 4B beads. GST fusion proteins (100 μl) were immobilized on glutathione-Sepharose 4B beads (50 μl) and incubated with the preclarified brain supernatant for 4 h at 4°C. The beads were washed with Buffer A five times, and bound proteins were eluted by adding the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The phosphorylated proteins were separated by SDS-PAGE (4–20%) and stained with Coomassie Brilliant Blue R-250. The concentration of proteins was measured using the DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

Amino Acid Sequencing—Amino acid sequences were determined essentially according to the method of Matsudaira (24). Proteins were subjected to SDS-PAGE and electrophoresed to a polyvinylidene difluoride membrane (Schleicher & Schuell). The blotted membrane was briefly stained with Coomassie Brilliant Blue R-250. After extensive washing with water, membrane pieces containing the proteins of interest were excised and then examined with a Procise 492 gas-phase sequencer (Applied Biosystems Division, Perkin Elmer).

In Vitro Binding Assay—GST or GST fusion proteins (2 μg each) were incubated with glutathione-Sepharose 4B beads (20 μl). CaM (1 μg) was incubated with GST or GST fusion protein-immobilized beads in 500 μl of Buffer B (25 mM Hepes, pH 7.4, 150 mM NaCl, and 1% Triton X-100) in the presence of either 2 mM CaCl₂ or 5 mM EGTA without addition of CaCl₂ for 2 h at 4°C. The beads were washed with the incubation buffer, and bound proteins were eluted by the SDS-PAGE loading buffer and incubated for 30 min at 60°C. Proteins were separated by SDS-PAGE (15% gel), and bound proteins were eluted by the SDS-PAGE loading buffer, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. The concentration of proteins was measured using the DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

Identification of an mGluR7-interacting Protein—We attempted to identify proteins that interact with mGluR7 by affinity chromatography using the ct region of mGluR7 fused to GST. GST alone or GST-ct-mGluR2 was used as control. Rat brain cytosolic fractions were prepared and loaded to glutathione-Sepharose 4B beads coated with each GST protein. After extensive washing, bound proteins were eluted by addition of the SDS-PAGE loading buffer, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. A prominent band with a mobility of approximately 17 kDa was detected in the eluate from GST-ct-mGluR7 affinity beads (Fig. 1, lane 2). No such 17-kDa protein was retained with affinity beads attached with (lane 3) or without (lane 4) GST. The specificity of interaction toward ct-mGluR7 was confirmed by using GST-ct-mGluR2 which showed no detectable binding of the 17-kDa protein (lane 1). Peptide sequencing of this 17-kDa protein and subsequent data base analysis indicated that the partial sequence determined (Thr-Ile-Asp-Phe-Pro-Glu-Phe-Leu-Thr-Met-Met-Ala-Arg-Lys-Met-Lys) precisely corresponded to the sequence of rat CaM.

Characterization of CaM Binding to ct-mGluR7—Next, we examined the specificity of interaction between CaM and the ct regions of group 2 and group 3 mGluRs using GST fusion protein affinity chromatography. The GST proteins fused to the ct regions of group 2 and group 3 mGluRs were immobilized on glutathione-Sepharose 4B beads and tested for their ability to retain bovine CaM (Fig. 2, upper). CaM interacted exclusively with ct-mGluR7. In contrast, no detectable interaction was observed with group 2 ct-mGluRs and other members of group 3.
3 ct-mGluRs as well as GST alone. Furthermore, the interaction between CaM and ct-mGluR7 was completely abolished by replacing 2 mM CaCl$_2$ with 5 mM EGTA in the binding solution. Staining of the SDS-polyacrylamide gel with Coomassie Brilliant Blue R-250 confirmed that comparable amounts of GST or GST fusion proteins were present in these experiments (Fig. 2, lower). The results indicate that among group 2 and group 3 mGluR subtypes, CaM interacts specifically with ct-mGluR7 in a Ca$^{2+}$-dependent manner.

To define a CaM-interacting domain in ct-mGluR7, we constructed a series of GST fusion proteins possessing different truncation forms of ct-mGluR7 (Fig. 3A). The truncated forms containing at least residues Val$^{856}$-Leu$^{892}$ (Tr1-mGluR7 and Tr4-mGluR7) showed a strong Ca$^{2+}$-dependent interaction with CaM comparable to that of ct-mGluR7 (Fig. 3B, upper). The presence and absence of Cys$^{893}$, which is implicated as a possible palmitoylation site (25), had no effect on Ca$^{2+}$/CaM binding. In contrast, removal of a cluster of basic residues at the amino-terminal region of ct-mGluR7 drastically reduced Ca$^{2+}$/CaM binding (Tr2-mGluR7 and Tr3-mGluR7). In these experiments, comparable amounts of GST fusion proteins were confirmed by Coomassie Brilliant Blue R-250 staining (Fig. 3B, lower).

The affinity of Ca$^{2+}$-dependent CaM binding to ct-mGluR7 and Tr4-mGluR7 was determined by incubating a fixed amount of the GST fusion proteins immobilized onto glutathione-Sepharose 4B beads with increasing concentrations of CaM in the presence of 2 mM CaCl$_2$ (Fig. 4, A and B). Analysis of saturation curves of bound CaM showed virtually identical values of dissociation constant ($K_d$) of CaM binding for ct-mGluR7 and Tr4-mGluR7, 38.8 $\pm$ 8.3 nM for ct-mGluR7 and 45.5 $\pm$ 13.6 nM for Tr4-mGluR7 (mean $\pm$ S.D., n = 2). The results indicate that the segment consisting of Val$^{856}$-Leu$^{892}$ is sufficient for interaction between CaM and ct-mGluR7.

Competition between PKC Phosphorylation and CaM Binding of mGluR7 in Vitro—The CaM-binding domain in ct-mGluR7 contains consensus sequences for both PKC phosphorylation, (Ser/Thr)-X-(Arg/Lys), and PKA phosphorylation, (Arg/Lys)-X-(Ser/Thr) (26). We examined whether ct-mGluR7 serves as a phosphorylation substrate for PKC or PKA and, if so, whether phosphorylation of these kinases and CaM binding are mutually affected by each other. We first examined the effects of CaM binding on PKC phosphorylation by incubating a fixed amount of either GST-ct-mGluR7 or GST-Tr4-mGluR7 with PKC and [$\gamma$-$^{32}$P]ATP in the absence and presence of increasing concentrations of CaM. Time courses and extents of phosphorylation of ct-mGluR7 or Tr4-mGluR7 were determined by autoradiography of a $^{32}$P-labeled product run on an SDS-polyacrylamide gel (Fig. 5). In the absence of CaM, both ct-mGluR7 and Tr4-mGluR7 were rapidly phosphorylated in a time-dependent manner with about one-third of the proteins estimated to be phosphorylated at 1 h in both cases. Neither GST nor CaM was phosphorylated by PKC. Furthermore, there was no obvious difference in the time course and extent of PKC phosphorylation between ct-mGluR7 (Fig. 5A) and Tr4-mGluR7 (Fig. 5B). We also examined possible PKA phospho-
rylation of GST-ct-mGluR7 by incubating with PKA and \([\gamma^{32P}]ATP\). Regardless of the presence and absence of Ca\(^{2+}\)/CaM, GST-ct-mGluR7 was not appreciably phosphorylated by PKA (data not shown). These results show that the CaM-binding domain is a major site of PKC phosphorylation within the ct-mGluR7 sequence. In contrast, this site does not serve as a target for PKA phosphorylation.

Importantly, phosphorylation of both ct-mGluR7 and Tr4-mGluR7 was progressively inhibited by adding increasing amounts of CaM to the reaction mixture. At the molar ratio of 1:1 between the PKC substrates and CaM, phosphorylation of both ct-mGluR7 and Tr4-mGluR7 was almost completely inhibited by Ca\(^{2+}\)/CaM. Furthermore, no obvious difference in the sensitivity of inhibition of PKC phosphorylation by Ca\(^{2+}\)/CaM binding was observed between the two PKC substrates. This finding is consistent with the results above, which show a similar affinity of these mGluR7 segments for Ca\(^{2+}\)/CaM binding. The results indicate that the interaction of Ca\(^{2+}\)/CaM with the ct domain of mGluR7 prevents phosphorylation by PKC.

Finally, we examined the effect of PKC phosphorylation on Ca\(^{2+}\)/CaM binding to ct-mGluR7 (Fig. 6). GST-ct-mGluR7 or GST-Tr4-mGluR7 was incubated with PKC in the presence and absence of ATP. The resultant GST fusion proteins were coupled to glutathione-Sepharose 4B beads and tested for their ability to bind to CaM in a Ca\(^{2+}\)-dependent manner. Non-phosphorylated GST fusion proteins could bind to Ca\(^{2+}\)/CaM, but once phosphorylated, they lost their ability to bind to Ca\(^{2+}\)/CaM. The results indicate that Ca\(^{2+}\)/CaM binding is inhibited by PKC phosphorylation.

**DISCUSSION**

In this study, *in vitro* analysis indicates that CaM directly binds to ct-mGluR7 in a Ca\(^{2+}\)-dependent fashion. Among the mGluR family, mGluR5 has been shown to interact with Ca\(^{2+}\)/CaM at two distinct sites of ct-mGluR5 with different affinities (27). Consistent with the sequence homology between mGluR1 and mGluR5, we have found that Ca\(^{2+}\)/CaM also binds to ct-mGluR1. Interestingly, the group 3 mGluR subtypes (mGluR4, mGluR7, and mGluR8) have highly homologous ct tails, but no significant interaction was observed between CaM and mGluR4 or mGluR8. Therefore, the binding of Ca\(^{2+}\)/CaM is specific to group 1 mGluRs and mGluR7 within the mGluR family and thus is not relevant to intracellular second messengers involving these receptor subtypes. The CaM-binding domain of ct-mGluR7 diverges from those of group 1 mGluRs but possesses several structural characteristics of CaM binding (28) as follows: 1) one face possessing basic and polar residues in an \(\alpha\)-helical wheel representation, whereas the other face contains a stretch of hydrophobic amino acids; 2) a cluster of basic amino acids at the amino-terminal portion, followed by a sequence that contains relatively few basic amino acids but often possesses a consensus sequence for phosphorylation (Fig. 3). Interestingly, some of these features are shared by mGluR4 and mGluR8. However, these two subtypes show no apprecia-

**FIG. 4.** Determination of affinities of CaM binding to ct-mGluR7 and Tr4-mGluR7. GST-ct-mGluR7 (A) and GST-Tr4-mGluR7 (B) (60 pmol each) were immobilized on glutathione-Sepharose 4B beads and incubated with increasing concentrations of CaM in the presence of 2 mM CaCl\(_2\). Amounts of bound CaM were determined from densitometric analysis of immunoblotting with anti-CaM mAb after SDS-PAGE. *Data points and bars show mean ± S.D., respectively, in representative experiments done in duplicate.*

**FIG. 5.** Inhibitory effect of Ca\(^{2+}\)/CaM binding on PKC phosphorylation of GST-ct-mGluR7 and GST-Tr4-mGluR7. A fixed amount of GST-ct-mGluR7 (A) and GST-Tr4-mGluR7 (B) (60 pmol each) was incubated with PKC and \([\gamma^{32P}]ATP\) in the absence and presence of increasing amounts of CaM (18, 60, and 180 pmol) as indicated with molar ratios between the GST fusion proteins and CaM. The reaction was terminated at the indicated times (min), and the reaction product was subjected to SDS-PAGE, followed by autoradiography of a \(^{32P}\)-labeled product. GST alone was also subjected to the phosphorylation reaction in the absence of CaM and run on SDS-PAGE (lane GST). Molecular sizes (kDa) of marker proteins are shown on the left.

\(^{2}\) K. Ishikawa, Y. Nakajima, and S. Nakanishi, unpublished observations.
**Calmodulin Binding and PKC Phosphorylation of mGluR7**

Calmodulin binding and PKC phosphorylation of mGluR7 is more reminiscent to the regulation of myristoylated alanine-rich protein kinase C substrate (MARCKS) by the Ca$^{2+}$/CaM binding and protein phosphorylation. MARCKS is a major cellular substrate of PKC, with PKC phosphorylation inhibiting CaM binding and CaM binding preventing PKC phosphorylation. The brain MARCKS is highly concentrated at the presynaptic junction and is thought to be phosphorylated by PKC through an increase in [Ca$^{2+}$], triggered by neuronal cell stimulation (34). It has recently been reported that activation of PKC suppresses the activity of group 3 mGlus to inhibit transmission at glutamatergic synapses (13). Furthermore, mGluR7 is largely located at the presynaptic terminals. Therefore, it is conceivable that mGluR7 is under dual regulation by PKC and CaM at the presynaptic terminals, when neuronal cells are stimulated and increase a Ca$^{2+}$ influx through activated Ca$^{2+}$ channels. Thus, our observations suggest that mGluR7 serves to cross-link the cyclic AMP, Ca$^{2+}$, and PKC phosphorylation signal transduction cascades. In closing, this process may play an important role in modulating synaptic transmission in concert with the function of CaM which recognizes changes in [Ca$^{2+}$].

**REFERENCES**


**FIG. 6. Inhibitory effect of PKC phosphorylation on Ca$^{2+}$/CaM binding to ct-mGluR7.** GST-ct-mGluR7, GST-Tr4-mGluR7, and GST alone (5 μg each) were subjected to PKC phosphorylation reaction for 3 h in the presence and absence of ATP. The resultant GST fusion proteins or GST alone were immobilized on glutathione-Sepharose 4B beads and tested for their ability to bind to CaM in the presence of either 2 mM CaCl$_2$ or 5 mM EGTA. The lower panel shows a Coomassie Brilliant Blue R-250 staining, indicating no difference in retention of the phosphorylated and non-phosphorylated proteins on the glutathione beads.

![Western blot analysis of CaM binding in the presence of ATP](image)

**ATP**

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**TABLE 1. Summary of CaM binding and PKC phosphorylation of mGluR7.**

- **GST:** Glutathione-S-transferase
- **ct:** c-terminal
- **Tr4:** fourth transmembrane domain

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