

**Ca²⁺-dependent binding of calcium-binding protein 1 to presynaptic group III
metabotropic glutamate receptors and blockage by phosphorylation of the
receptors**

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Abstract

Presynaptic group III metabotropic glutamate receptors (mGluRs) and Ca^{2+} channels are the main neuronal activity-dependent regulators of synaptic vesicle release, and they use common molecules in their signaling cascades. Among these, calmodulin (CaM) and the related EF-hand Ca^{2+} -binding proteins are of particular importance as sensors of presynaptic Ca^{2+} , and a multiple of them are indeed utilized in the signaling of Ca^{2+} channels. However, despite its conserved structure, CaM is the only known EF-hand Ca^{2+} -binding protein for signaling by presynaptic group III mGluRs. Because the mGluRs and Ca^{2+} channels reciprocally regulate each other and functionally converge on the regulation of synaptic vesicle release, the mGluRs would be expected to utilize more EF-hand Ca^{2+} -binding proteins in their signaling. Here I show that calcium-binding protein 1 (CaBP1) bound to presynaptic group III mGluRs competitively with CaM in a Ca^{2+} -dependent manner and that this binding was blocked by protein kinase C (PKC)-mediated phosphorylation of these receptors. As previously shown for CaM, these results indicate the importance of CaBP1 in signal cross talk at presynaptic group III mGluRs, which includes many molecules such as cAMP, Ca^{2+} , PKC, G protein, and Munc18-1. However, because the functional diversity of EF-hand calcium-binding proteins is extraordinary, as exemplified by the regulation of Ca^{2+} channels, CaBP1 would provide a distinct way by which presynaptic group III mGluRs fine-tune synaptic transmission.

Keywords: mGluR; calcium-binding protein 1; calmodulin; presynaptic; calcium channel; glutamate receptor

Abbreviations: mGluR, metabotropic glutamate receptor; CaM, calmodulin; CaBP1, calcium-binding protein 1, VILIP-2, visinin-like protein-2; NCS-1, neuronal calcium sensor-1; PKC, protein kinase C; ct, intracellular C-terminal tail; NA-HRP, horseradish peroxidase-conjugated NeutrAvidin

1. Introduction

Presynaptic group III metabotropic glutamate receptors (mGluR4, 7 and 8) are G protein-coupled receptors expressed in the presynaptic active zone, and they reduce synaptic vesicle release upon agonist stimulation [1-3]. However, this classical ligand-dependent signaling mechanism is not enough to account for all the roles of these receptors in the regulation of synaptic transmission [4-6]. We recently described a ligand-independent mechanism based on an intracellular dynamic protein complex, in which presynaptic group III mGluRs bind and release Munc18-1, an essential protein in synaptic transmission [7], in a Ca^{2+} and Ca^{2+} / calmodulin (CaM) -dependent fashion [8]. This mechanism was shown to be responsible for the expression of short-term synaptic facilitation, crucial for information processing in the nervous system [8, 9], and highlights the importance of a Ca^{2+} -binding protein, CaM, in mGluR-mediated presynaptic signaling.

The importance of Ca^{2+} -binding proteins is also indicated in the case of presynaptic voltage-dependent Ca^{2+} channels, in which CaM and related EF-hand Ca^{2+} -binding proteins, namely, calcium-binding protein 1 (CaBP1), visinin-like protein-2 (VILIP-2), and neuronal calcium sensor-1 (NCS-1), modulate these channels [10-16]. Among these Ca^{2+} -binding proteins, CaM, CaBP1 or VILIP-2 binds directly to

a C-terminal domain of a Ca^{2+} channel, differentially mediating short-term synaptic plasticities [10, 12, 13, 15, 16].

These findings indicate the crucial roles of EF-hand Ca^{2+} -binding proteins for both mGluR- and Ca^{2+} channel-mediated regulation of synaptic transmission at the presynaptic active zone. Although multiple EF-hand Ca^{2+} -binding proteins differentially regulate Ca^{2+} channels, CaM is currently the only known EF-hand Ca^{2+} -binding protein to be utilized by presynaptic group III mGluRs [8, 17, 18]. Considering the conserved structure of EF-hand proteins, together with the localization of the receptors and the channels at the active zone and their functional convergence on synaptic transmission, I assumed some of the EF-hand Ca^{2+} -binding proteins for Ca^{2+} channels can also bind to group III mGluRs to activity-dependently regulate synaptic transmission. Here, I show that CaBP1 binds to presynaptic group III mGluRs in a Ca^{2+} and protein kinase C (PKC) phosphorylation-dependent manner, reminiscent of the way by which CaM bind to the mGluRs.

2. Materials and methods

2.1. Plasmids

Constructs expressing the intracellular C-terminal tail of mGluRs (ct-mGluRs) were made in pGEX4T-1 (GE Healthcare) as described previously [8, 17]. CaM (GenBank ID: NM_031969), CaBP1 (GenBank ID: NM_133529), VILIP-2 (GenBank ID: NM_017357), and NCS-1 (GenBank ID: NM_024366) cDNAs were PCR-amplified from rat brain cDNA (Marathon Ready, Clontech). Constructs for bacterial expression of the biotinylated Ca^{2+} -binding proteins were made as follows: pET43.1a(+) (Novagen) was cut with Nde I and BamH I to remove the Nus-Tag sequence, and a short nucleotide

sequence coding for a biotinylation peptide, AviTag [19], was ligated in place. I adopted Ser following the initiation Met in AviTag to confer proteolytic stability [19]. The coding sequence of each Ca²⁺-binding protein was ligated to this vector downstream of the nucleotide sequence of AviTag. The regulatory element of the bacterial expression vector pPROLar.A122 (Clontech, Xho I to EcoRI) was replaced with the promoter/operator element from pQE30 (Qiagen, Xho I to EcoR I) to make pPROT5A122, which has a p15A origin of replication and can be induced by IPTG alone. The coding region of biotin protein ligase (BirA) was amplified by PCR from genomic DNA of the bacterial strain XL1-Blue (Stratagene), and ligated to this vector. The integrity of PCR products was confirmed by DNA sequencing of both strands of all constructs. The amino acid residues of ct-mGluRs and data base accession numbers are as follow: ct-mGluR4, His 848 - Ile 912 (GenBank ID: NP_073157.1); ct-mGluR4-1, His 848 - Leu 889; ct-mGluR4-2, Cys 890 - Ile 912; ct-mGluR7, His 851 - Ile 915 (GenBank ID: NP_112302.1); ct-mGluR7-1, His 851 - Leu 892; ct-mGluR7-2, Cys 893 - Ile 915. The coding nucleotide sequences of the rat Ca²⁺-binding proteins were the same as those of the proteins with the corresponding accession numbers, and that sequence of BirA was the same as the one in GenBank (GenBank ID: NC_010473.1). All the constructs used for functional comparisons were designed to have common linker amino acid sequences between tag sequences and inserts.

2.2. Protein purification

The GST-ct-mGluRs were expressed in an Escherichia coli strain BL21 and purified by glutathione-Sepharose 4B (GE Healthcare) as described previously [8, 17]. Each Ca²⁺-binding protein and BirA were simultaneously expressed in a bacterial strain

BL21(DE3), and the biotinylated Ca²⁺-binding proteins were purified on Monomeric Avidin Resin (Pierce) according to the manufacturer's protocol. Protein concentrations were determined by use of a DC protein assay kit (Bio-Rad) with BSA as a standard.

2.3. *In vitro* binding assay

In vitro binding assays were conducted as described previously [8, 17]. Briefly, GST or GST fusion proteins (0.6 μM) were immobilized on glutathione Sepharose 4B (20-μl bed). Biotinylated Ca²⁺-binding protein (1 μM) was incubated with GST or GST-fusion protein-immobilized beads in 500 μl of a binding buffer [8, 17] for 2 hours at 4 °C. The beads were then washed with the binding buffer, and bound proteins were eluted with SDS-PAGE loading buffer and incubated for 30 min at 60 °C. After separation by SDS-PAGE and transfer to nitrocellulose membrane, bound proteins were visualized by using horseradish peroxidase-conjugated NeutrAvidin, a deglycosylated form of avidin (Pierce).

2.4. PKC phosphorylation

PKC phosphorylation was conducted for 3 hours at 30 °C as described [17]. In this study, PKC was used at 35 micro units per pmole of GST or GST-fusion proteins.

3. Results

3.1. Identification of CaBP1 binding to group III ct-mGluRs

To elucidate mechanisms by which presynaptic group III mGluRs neuronal activity-dependently regulate synaptic transmission, I asked whether known EF-hand Ca²⁺-binding proteins that modulate Ca²⁺ channels [10, 12-15] could bind to presynaptic

group III mGluRs. In this and subsequent experiments, *in vivo* biotinylated Ca²⁺-binding proteins purified from *E. coli* were used as described in Materials and methods. The GST-tagged ct region of group III mGluRs was immobilized on glutathione-Sepharose 4B beads, and the bound proteins were tested for their ability to bind CaBP1 (Fig. 1, upper). CaBP1 interacted specifically with ct-mGluR4 and 7, with no detectable interaction observed with GST alone. Furthermore, the interaction between CaBP1 and ct-mGluR4 or 7 was abolished by replacing the 2 mM CaCl₂ in the binding buffer with 2 mM EGTA. 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. 1, lower). These results indicate that CaBP1 interacted specifically with ct-mGluR4 and 7 in a Ca²⁺-dependent manner. We also conducted the same binding assay for VILIP-2 and NCS-1, and found they bound to both GST-ct-mGluRs and GST alone (data not shown). Thus, among the known Ca²⁺-binding proteins modulating Ca²⁺ channels, I could detect specific binding of only CaM and CaBP1 to presynaptic group III mGluRs.

3.2. CaBP1 binding to CaM interaction domain in ct-mGluRs

To identify the CaBP1-interacting domain of the ct-mGluRs, I separated the ct-mGluRs into membrane-proximal and -distal portions at their conserved cysteine as described earlier [17], and asked which domain was responsible for the interaction with CaBP1 (Fig. 2). In the presence of Ca²⁺, CaBP1 specifically bound to the membrane-proximal portion of both ct-mGluR4 and 7, a conserved portion of the ct-mGluRs to which CaM is also known to bind [17]. Furthermore, in a competitive binding assay, the presence of a 10-fold molar excess of CaM completely disrupted the

binding between CaBP1 and ct-mGluR4 or 7 (Fig. 3). Thus, as in the case of Ca²⁺-binding proteins for the Ca²⁺ channel [10], CaM and CaBP1 seem to have bound to the same or overlapping portion of the ct-mGluRs.

3.3. Effect of PKC phosphorylation of ct-mGluRs on CaBP1 binding

We previously showed that PKC-mediated phosphorylation of the ct-mGluRs is inhibitory to CaM binding, which is a basis of signal cross-talk at the receptors [17]. Accordingly, I examined the effect of this phosphorylation on the CaBP1 binding. GST or GST fusion proteins were incubated with PKC in the presence or absence of ATP. The resultant GST fusion proteins were coupled to glutathione-Sepharose 4B beads and tested for their ability to bind to CaBP1 in the presence of Ca²⁺. Experiments with CaM were simultaneously conducted to confirm efficient phosphorylation by PKC [17]. The non-phosphorylated GST fusion proteins could bind to CaM or CaBP1; but once phosphorylated, they lost their ability to bind to these proteins (Fig. 4). These results indicate that PKC-mediated phosphorylation of presynaptic group III mGluRs is similarly inhibitory to the binding of CaM and CaBP1 to these receptors and that CaM and CaBP1 share a common binding site in the ct region of them.

4. Discussion

Based on the assumption that presynaptic group III mGluRs and Ca²⁺ channels share multiple EF-hand Ca²⁺-binding proteins in their signaling, I have identified CaBP1 as a binding protein of presynaptic group III mGluRs. The interaction between it and the receptors was dependent on Ca²⁺, blocked when the receptors had been phosphorylated by PKC, and competitive with CaM. Because the binding

properties of CaBP1 were similar to those of CaM, CaBP1 might be expected to take the place of CaM in many situations reported for CaM. However, CaBP1 and CaM have discrete and non-redundant roles despite their structural similarities, as was shown in the case of Ca²⁺ channel modulation [10]. Accordingly, CaBP1 may be considered as another participant in the neuronal activity-dependent mechanism by which presynaptic group III mGluRs regulate synaptic transmission.

There are 3 Ca²⁺-binding proteins, namely, CaM, CaBP1, and VILIP-2, that bind directly to Ca²⁺ channels [12, 13, 15]. Because the effects on facilitation and inactivation of Ca²⁺ channels by CaBP1 and VILIP-2 are strikingly different from those by CaM [10, 12, 13, 15], it may be predicted that differential expression of different Ca²⁺-binding proteins in different synapses would result in different ratios of synaptic facilitation and depression, which would provide a means of cell type-specific regulation of presynaptic Ca²⁺ channels and short-term synaptic plasticity [10]. Particularly, these EF-hand Ca²⁺-binding proteins have been argued to be sensors of the residual Ca²⁺ essential for the expression of synaptic plasticity [9, 10]. On the other hand, a role for CaM as a sensor of residual Ca²⁺ in synaptic plasticity involving presynaptic group III mGluRs has been proposed [8]. Thus, although I did not demonstrate a clear functional difference between CaM and CaBP1 in terms of binding to presynaptic mGluRs, the demonstrated competitive binding of CaM and CaBP1 to these mGluRs should hold functional significance, at least by regulating the availability of these sensors of residual Ca²⁺.

Classically, presynaptic group III mGluRs modulate Ca²⁺ channels by ligand-dependent activation of heterotrimeric G proteins [1, 2]. Because the CaM-interacting domain in ct-mGluRs, together with their 2nd cytoplasmic loop, has

been shown to serve as a G protein-interacting domain [20, 21], the binding of CaBP1 to mGluRs may likewise affect this classical G protein-mediated signaling, as shown for CaM binding to other receptors [22, 23]. Furthermore, recent findings on G protein-mediated signaling indicate that presynaptic group III mGluRs inhibit Ca^{2+} channels tonically through ligand-independent mobilization of $\beta\gamma$ subunits, which is controlled by competition between Ca^{2+} /CaM and MacMARCKS for interaction with mGluRs [18, 24]. CaBP1 may also take part in this type of G protein-mediated signaling in place of CaM. On the other hand, the increase in intracellular Ca^{2+} caused by influx through Ca^{2+} channels activates CaM and CaBP1 or other intracellular signaling molecule such as PKC [25, 26], which activation modulates G protein-mediated signaling by presynaptic group III mGluRs [27]. Thus, presynaptic group III mGluRs and Ca^{2+} channels reciprocally regulate each other at the level of G protein-mediated signaling, in which CaM has been shown to play central roles. Because CaM and CaBP1 share a common binding site in the mGluRs and Ca^{2+} channels, and because they have different effects on Ca^{2+} channel modulation, CaBP1 would affect the G protein-mediated functional interaction between presynaptic group III mGluRs and Ca^{2+} channels in a different way than CaM.

In conclusion, my results indicate that presynaptic group III mGluRs and Ca^{2+} channels utilize CaBP1 in common to regulate synaptic transmission in at least 2 layers of activity-dependent mechanisms, namely, signaling by residual Ca^{2+} and by G protein. Considering the versatility and the differing properties of EF-hand Ca^{2+} -binding proteins [11], presynaptic group III mGluRs would utilize CaBP1 and CaM in many ways to bring about distinct fine-tuning of synaptic transmission.

Acknowledgments

This work was supported by research grants from the Ministry of Education, Science, and Culture of Japan. I thank S. Hori for critical discussions and reading of the manuscript.

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Figure Legends

Fig. 1. Ca^{2+} -dependent binding of CaBP1 to ct-mGluR4 and 7. The whole ct regions of group III mGluRs as indicated by numbers were fused to GST. The resultant GST fusion proteins or GST alone were immobilized on glutathione-Sepharose 4B beads and tested for their ability to bind to biotinylated CaBP1 in the presence of either 2 mM CaCl_2 or 2 mM EGTA. One-fifth amount of the bound proteins was separated by SDS-PAGE, and the bound CaBP1 was detected by use of horseradish peroxidase-conjugated NeutrAvidin (NA-HRP), as shown in the upper panel. CaBP1 (0.1 μg) was run as a control (control lane, C). Sizes of molecular mass markers (kDa) are shown on the left. The lower panel shows Coomassie Brilliant Blue R-250-stained GST or GST fusion proteins immobilized on glutathione-Sepharose 4B beads.

Fig. 2. CaBP1 binding to the membrane proximal domain in ct-mGluR4 and 7. In the upper panels, binding of CaBP1 to GST, GST-ct-mGluR, membrane-proximal (4-1, 7-1) and -distal (4-2, 7-2) domain of the corresponding ct-mGluR was analyzed in the presence of either 2 mM CaCl₂ or 2 mM EGTA (left panels, ct-mGluR4; right panels, ct-mGluR7, as indicated by numbers). In the lower panels, the amounts of GST or GST fusion proteins immobilized on the beads are shown. For other details, see the legend of Fig. 1.

Fig. 3. Disruption of Ca²⁺/CaBP1 binding to ct-mGluRs by Ca²⁺/CaM. In the presence of 2 mM CaCl₂, GST-ct-mGluR4, GST-ct-mGluR7 or GST alone was incubated with CaBP1 either in the presence or absence of a 10-fold molar excess of CaM (10 μM). CaM and CaBP1 (both 0.1 μg) were run as controls. Biotinylated CaBP1 and CaM were detected by using NA-HRP (upper panel). The lower panel shows comparable amounts of GST or GST fusion proteins immobilized on the beads. For other details, see the legend of Fig. 1.

Fig. 4. Inhibitory effect of PKC-mediated phosphorylation of ct-mGluR4 and 7 on Ca²⁺/CaBP1 binding to these proteins. GST-ct-mGluR4 (left panels), GST-ct-mGluR7 (right panels), and GST alone were subjected to phosphorylation by PKC in the presence or absence of ATP. The resultant GST fusion proteins or GST alone was immobilized on glutathione-Sepharose 4B beads, and each was tested for its ability to bind to CaBP1 in the presence of 2 mM CaCl₂ (upper panels). Ca²⁺/CaM binding to the GST fusion proteins was simultaneously monitored to confirm efficient PKC-mediated phosphorylation. The lower panels show the results of Coomassie Brilliant Blue R-250

staining, indicating no difference in retention of the phosphorylated and non-phosphorylated proteins on the glutathione beads. For other details, see the legend of Fig. 1.







