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PKC phosphorylation of a conserved serine residue in the C-terminus of group III metabotropic glutamate receptors inhibits calmodulin binding

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Abstract Group III metabotropic glutamate receptors (mGluRs) serve as presynaptic receptors that mediate feedback inhibition of glutamate release via a Ca^{2+}/calmodulin (CaM)-dependent mechanism. In vitro phosphorylation of mGluR7A by protein kinase C (PKC) prevents its interaction with Ca^{2+}/CaM. In addition, activation of PKC leads to an inhibition of mGluR signaling. Here, we demonstrate that disrupting CaM binding to mGluR7A by PKC in vitro is due to phosphorylation of a highly conserved serine residue, S862. We propose charge neutralization of the CaM binding consensus sequence resulting from phosphorylation to constitute a general mechanism for the regulation of presynaptic mGluR signaling.

Key words: Metabotropic glutamate receptor; Protein kinase C; Calmodulin; Phosphorylation

1. Introduction

Glutamate serves as a neurotransmitter at the vast majority of excitatory synapses in the mammalian central nervous system by activating two different receptor families, the glutamate-gated cation channels forming 'ionotropic' glutamate receptors [1] and the G protein-coupled 'metabotropic' glutamate receptors (mGluRs) [2]. While ionotropic glutamate receptors drive fast neurotransmission, synaptic stimulation of mGluRs generates slower and longer lasting changes [2]. One of the most prominent roles of mGluRs is to serve as presynaptic auto-receptors that mediate feedback inhibition of glutamate release in a wide variety of brain regions, including the hippocampus, neocortex, spinal cord, cerebellum and olfactory bulb. This inhibition is thought to result from G protein-driven down-regulation of the voltage-activated Ca^{2+} channels that trigger the Ca^{2+} influx necessary for synaptic vesicle exocytosis [3–6].

At present, eight different members of the mGluR family have been identified [2,7]. Based on sequence homologies, pharmacological properties and intracellular transduction pathways, mGluRs are classified into three distinct groups. Groups I and II include mGluRs that are post- and presynaptic, whereas group III encompasses receptors (mGluRs 4, 6, 7 and 8 and their splice variants) that have been shown to be principally presynaptic [8]. In particular, mGluR7 was found to be highly enriched at active zones in terminals of hippocampal pyramidal cells [9]. This selective localization and its low affinity for glutamate have led to the hypothesis that mGluR7 functions as a low-pass filter that inhibits synapses that fire above certain frequencies [9]. Consistent with this view, mGluR7 knockout mice show epileptic seizures at >12 weeks of age in addition to an impairment of amygdala-dependent behavioral traits [10].

Many receptors including ion channels and G protein-coupled receptors are known to be modulated by phosphorylation. Such modulations can result in desensitization as shown for mGluR5 [11] or in a fine-tuning of receptor signaling as reported for mGluR1α [12]. For group III mGluRs, it has been shown that phorbol ester treatment of hippocampal slices reduces mGluR-mediated presynaptic inhibition [13]. Furthermore, we recently identified calmodulin (CaM) as an essential coregulator of mGluR7A [6], one of two known C-terminal splice variants of this receptor [14]. In vitro phosphorylation by protein kinase C (PKC) of the cytosolic C-terminal region of mGluR7A prevents its interaction with Ca^{2+}/CaM, and conversely this phosphorylation is inhibited by CaM binding [15]. These data provide functional and biochemical evidence that phosphorylation of the mGluR7A tail regulates Ca^{2+}-dependent CaM binding and G protein activation.

In this study, we identify serine 862 in the cytosolic C-terminus of mGluR7A as the only residue that is phosphorylated by PKC in vitro, and show that this phosphorylation is prevented by the presence of Ca^{2+}/CaM. In addition, we demonstrate that all group III mGluRs except mGluR4B and mGluR6 are similarly phosphorylated at a homologous site. Our data thus raise the intriguing possibility that most group III mGluRs are regulated in an antagonistic fashion by PKC and Ca^{2+}/CaM.

2. Materials and methods

2.1. Expression and mutant constructs

cDNAs corresponding to the C-terminal regions of mGluR4A, -4B, -7A, -7B, -8A, and -8B were generated by PCR on either mouse brain cDNA (mouse genomic DNA for mGluR6), or full-length mGluR4B cDNA (generous gift of S. Nakanishi), using procedures similar to those described previously [6]. cDNA fragments were subcloned into the *Eco*RI and *Sal*I sites of pGEX-5X1 (Pharmacia) or pMal-c2 (New England Biolabs) to generate glutathione *S*-transferase (GST) or maltose binding protein (MBP) fusion constructs. Mutations were intro-

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Abbreviations: CaM, calmodulin; GST, glutathione *S*-transferase; MBP, maltose binding protein; mGluR, metabotropic glutamate receptor; PKC, protein kinase C

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duced into these fusion proteins by PCR using a triple PCR method as described [16]. The constructs pGEX-7A-S1234A, pGEX-7A-S123A, pGEX-7A-S234A, pGEX-7A-S134A, pGEX-7A-S124A (numbers indicate the predicted phosphorylation sites S862, S873, S877 and S881, respectively), pGEX-7A-EEE (K864, A865 and V866 replaced by glutamates), pGEX-7A-S862A and pGEX-N25 were generated using as template pGEX-7A which harbors the cytosolic tail domain of mGluR7A [6]. The first 25 amino acids of the tail region of mGluR7A in pGEX-N25 served as template for creating pGEX-N25-S862A and pGEX-N25-S862E (see Fig. 2A for further details). All amplification products were verified by automated DNA sequencing.

2.2. Bacterial expression and affinity purification

Expression of GST and MBP fusion proteins in *Escherichia coli* BL21 (Stratagene) was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (Applichem) for 5 h. After passage through a French press, the $100\,000 \times g$ supernatants containing soluble protein were kept frozen (−80°C) until use.

2.3. CaM binding assay

Soluble fractions of GST or MBP fusion proteins (0.25 μg protein) were incubated with 20 μl of CaM-agarose beads (Sigma) for 2 h at 4°C in 0.5 ml of binding buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, protease inhibitor cocktail Complete® (Boehringer-Mannheim) and 0.2% (w/v) Triton X-100), containing either 2 mM $CaCl_2$ or 5 mM EGTA. The beads were then washed five times with 0.5 ml of the corresponding buffer. Bound fusion proteins were eluted with SDS sample buffer and resolved by 12% SDS–PAGE, followed by Coomassie staining.

2.4. Phosphorylation reactions

Soluble fractions of GST or MBP fusion proteins (0.25 μg protein) were incubated with 20 μl of glutathione-Sepharose beads for 2 h at 4°C followed by three washes, each in 0.5 ml of binding buffer. Bound proteins were then incubated in 100 μl phosphorylation buffer (50 mM 2-*N*-morpholinoethane-sulfonic acid (MES) pH 6.0, 12.5 mM $MgCl_2$, 1.25 mM EGTA, 0.125 mM [^{32}P]γ-ATP (3000 cpm/pmol)) containing 10 ng of PKM (Boehringer Mannheim), the catalytic fragment of PKC, at 30°C for 2 h. Phosphorylated proteins were eluted with SDS sample buffer and resolved by 12% SDS–PAGE. Dried gels were exposed to Kodak BioMax MR-1 film (Sigma) for 2–4 h.

3. Results

As shown in an alignment of the amino acid sequences of the group III mGluR cytoplasmic C-terminal regions (Fig. 1A), one or more predicted consensus sites for PKC phosphorylation are present in all group III mGluR tail domains. Notably, the serine residue corresponding to amino acid S862 in mGluR7A is conserved in all mGluRs with the exception of the highly divergent mGluR4B sequence.

To determine which of the receptor tails can be phosphorylated by PKC, we performed in vitro phosphorylation of all group III mGluR tails fused to GST or MBP using PKM. As shown in Fig. 1B, all fusion constructs except MBP-4B and GST-6 were phosphorylated under the conditions used. These phosphorylation events were mGluR tail-specific, since neither GST (Fig. 1B) nor MBP (not shown) displayed detectable ^{32}P incorporation under identical conditions. To elucidate which of the four serines predicted to be phosphorylated in mGluR7A (S862, S873, S877 and S881) are used in vitro, mutants in which all or only three of the predicted serines had been substituted by alanines (Fig. 2A) were also examined. Only wild-type GST-7A and the GST-7A-S234A mutant, which still contains serine 862, were phosphorylated. No ^{32}P radioactivity was detected in the mutants GST-7A-S1234A, GST-7A-S123A, GST-7A-S124A, and GST-7A-S134A (Fig. 2B). When serine 862 was mutated to alanine

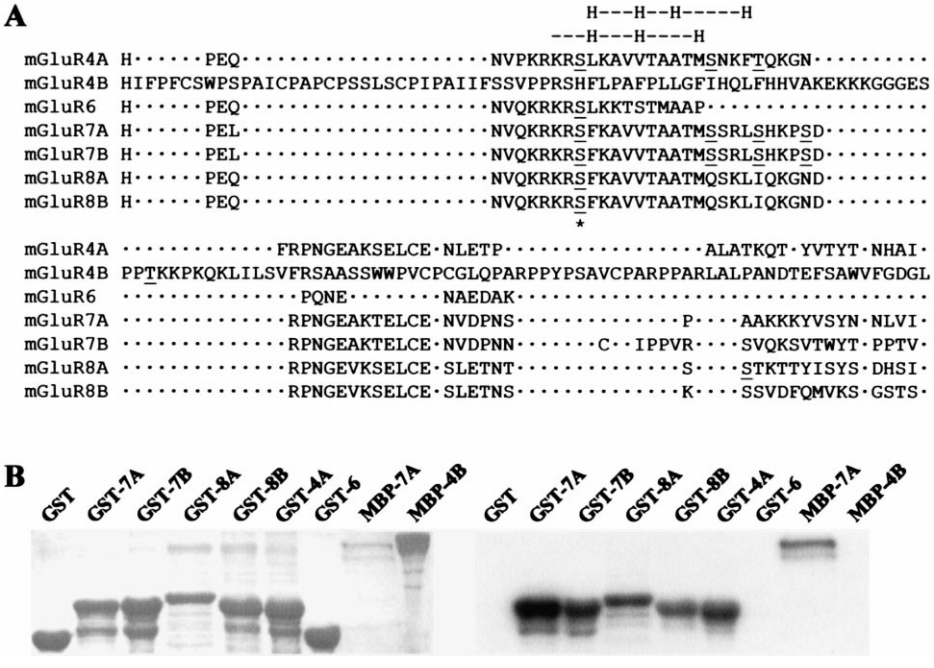

Fig. 1. A: Alignment of the C-terminal tail regions of group III mGluRs. The sequences shown include the following amino acid residues: mGluR4A: 848–912; mGluR4B: 848–983; mGluR6: 840–871; mGluR7A: 851–916; mGluR7B: 851–923; mGluR8A: 844–908; and mGluR8B: 844–908. Two overlapping consensus sites for Ca^{2+}-dependent CaM binding are indicated: a 1-5-10 motif (---H---H----H) and a 1-8-14 motif (H------H-----H), where H indicates hydrophobic amino acid side chains. Predicted PKC phosphorylation sites are underlined. Serine residues homologous to S862 in mGluR7A are marked with an asterisk. B: Phosphorylation of group III mGluR tails. Coomassie staining (left) and ^{32}P incorporation revealed by autoradiography (right) are shown. GST and MBP fusion proteins of the C-tails of group III mGluRs were immobilized on CaM-agarose and in vitro phosphorylated with PKM. Proteins were eluted with SDS sample buffer and analyzed by 12% SDS–PAGE.

(GST-7A-S862A) or its phosphorylation consensus sequence was modified (GST-7A-EEE), PKM also failed to phosphorylate the GST-7A C-terminal tail domain (Fig. 2B). This result indicates that serine 862 is the only residue in the mGluR7A tail that is modified by PKC in vitro. Notably, the homologous serine residue is not precisely conserved in mGluR4B, consistent with the lack of phosphorylation seen with the corresponding tail fusion protein (Fig. 1B). In mGluR6, a serine predicted to be phosphorylated by PKC is found at a position homologous to that of S862 in mGluR7A; however, its C-terminally flanking residues differ significantly from those found in the other group III mGluRs (Fig. 1A). Secondary structure analysis predicts this variation to cause a loss of α-helical content within this region (data not shown); we propose this structural change to abolish recognition by PKC.

In order to unravel whether phosphorylation of residue 862 in mGluR7A is sufficient to inhibit its interaction with CaM, we mimicked phosphorylation of this amino acid by replacing it by glutamate. CaM binding of the resulting mutant GST-N25-S862E was strongly decreased as compared to the wild-type GST-N25 fusion protein (Fig. 3A). To verify whether S862 constitutes a residue important for CaM binding, we replaced it by alanine (GST-N25-S862A). As shown in Fig. 3A, the binding of Ca^{2+}/CaM was not significantly affected by this mutation but comparable to binding to the wild-type GST-N25 tail. In contrast, increasing the number of negative charges by introducing three glutamates in the vicinity of S862 in mutant GST-7A-EEE completely abolished CaM binding (Fig. 3A). A complete loss of CaM binding was also seen when S862, T868, T871 and S873 in GST-N25 were replaced by glutamate, or when replacing amino acids F863–V866 by four glutamates (data not shown). Apparently, introduction of negative charges in the N-terminal region of the mGluR7A tail impairs CaM binding.

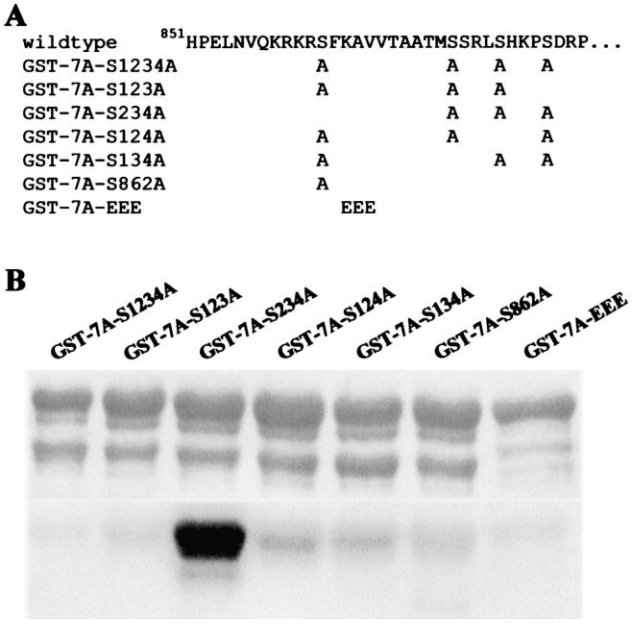

Fig. 2. A: A scheme illustrating the mutations introduced into the mGluR7A tail region. The numbers in the first five constructs indicate the four predicted phosphorylation sites S862, S873, S877 and S881. In GST-7A-EEE, amino acids K864, A865 and V866 were replaced by glutamates. B: Phosphorylation of GST-7A mutants. Phosphorylation of the wild-type GST-7A is shown in Fig. 1. Coomassie staining (top) and ^{32}P incorporation revealed by autoradiography (bottom) are shown. Fusion proteins were processed and in vitro phosphorylated as described in the legend to Fig. 1.

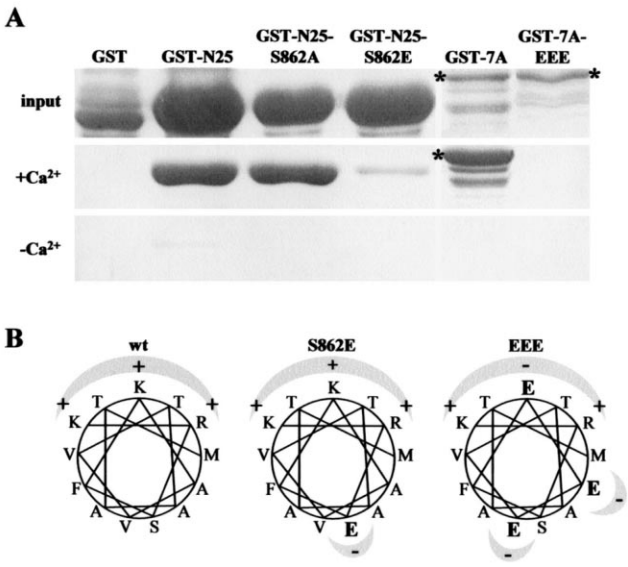

Fig. 3. A: Ca^{2+}-dependent CaM binding of mGluR7A mutants. Fusion proteins were immobilized on CaM-agarose in the presence of either 2 mM $CaCl_2$ or 5 mM EGTA. Bound material was eluted with SDS sample buffer, analyzed on 12% SDS–PAGE and revealed by Coomassie staining. Note the drastic reduction, or complete loss, of CaM binding with GST-N25-S862E and GST-7A-EEE. The bands marked with asterisks in the GST-7A and GST-7A-EEE lanes represent the full-length proteins; the more rapidly migrating polypeptide species correspond to degradation products observed previously [6]. B: Amphipathic α-helical wheel projections demonstrating loss of the amphipathic character of the putative CaM binding helix (residues 860–872) upon glutamate substitutions.

4. Discussion

Here, we demonstrate that PKC phosphorylates the cytosolic C-terminal regions of most group III mGluRs. All group III mGluRs except mGluR4B display high homology (> 70% identity) in the N-terminal portion of their cytosolic C-termini. In particular, the first 26 residues of the mGluR4A, -7 and -8 tails are nearly identical (Fig. 1A). Divergence of mGluR4B is due to a different splicing event as compared to the other group III mGluR members [14,17,18]. Although all group III mGluRs are predicted to be phosphorylated by PKC due to the existence of one or multiple PKC phosphorylation consensus sites ((S/T)-X-(K/R)) [19,20], only the tails sharing high homology in their juxta-membrane region could be phosphorylated in vitro. No ^{32}P incorporation was detectable with the mGluR4B or mGluR6 cytosolic domains. These results are consistent with a potential in vivo regulation of CaM binding to group III mGluR tails by PKC phosphorylation.

As only the serine residue corresponding to S862 in mGluR7A is conserved in the homologous C-tails, we performed additional experiments using alanine substitutions in the mGluR7A tail region to examine the relevance of this serine for PKC phosphorylation. Only mutants in which S862 was not substituted showed ^{32}P incorporation. Also, a GST-7A-S862A construct was not phosphorylated, and mutation of the consensus sequence for PKC phosphorylation

around residue 862 in GST-7A-EEE abolished serine modification. Thus, the serine residue adjacent to the seventh transmembrane region that is conserved in most group III mGluRs constitutes their principal site of PKC phosphorylation.

S862 lies within a region of the mGluR7A C-terminal domain known to mediate Ca^{2+}/CaM binding [6], and PKC phosphorylation of the mGluR7A tail has been found to prevent the CaM interaction [15]. Notably, S862 is embedded into positively charged amino acids interspersed among hydrophobic residues; such sequences have the potential to form a helical amphipathic structure, with positive charges on one side of the helix and hydrophobic ones on the other (Fig. 3B) [21]. Such structures are characteristic for many Ca^{2+}/CaM binding proteins. Ca^{2+}-dependent CaM interaction motifs have been grouped into two major classes, designated motifs 1-8-14 and 1-5-10 [21]. Based on the different positions of conserved hydrophobic residues, the first and last residues of these motifs seem to be particularly important for CaM binding. Most of the high-affinity Ca^{2+}/CaM binding proteins have a 1-8-14 motif, whose net charge varies from +3 to +6. The net charge of the 1-5-10 motif, the other Ca^{2+}-dependent CaM binding motif found in a number of proteins like CaM-KII, MARCKS and synapsin, is only +2 to +3 [21,22]. All mGluRs except mGluR6 contain both consensus motifs. In fact, mGluR4A, -7A, -7B, -8A, and -8B possess a net charge of +3, characteristic of the 1-5-10 motif, but not the high value associated with the 1-8-14 motif (Figs. 1A and 3B). PKC phosphorylation of the conserved serine is predicted to reduce the net charge of the 1-5-10 motif. Indeed, mimicking phosphorylation by replacing serine 862 with glutamate in GST-7A-S862E reduced CaM binding drastically, whereas alanine substitution in GST-7A-S862A showed no major effect. Also, introducing three consecutive negative charges into the 1-5-10 motif by generating GST-7A-EEE completely abolished CaM binding (Fig. 3A). This is consistent with the loss of CaM binding resulting from neutralization of the positive net charge of the amphipathic α-helix (Fig. 3B). Secondary structure predictions indicate that the probability of residues 860–872 to form an α-helix is not altered in the mutants used (data not shown). We therefore conclude that PKC phosphorylation controls CaM binding by a simple electrostatic mechanism.

PKC activation has been shown to reduce inhibition of forskolin-stimulated cAMP accumulation by group II and group III mGluRs [23] and to regulate neurotransmission at major synapses in the hippocampal formation [13]. These findings suggest that PKC-induced inhibition of mGluR signaling may be mediated by the inhibition of coupling of mGluRs to G proteins. Such reduced coupling might reflect an impaired CaM regulation of Gβγ-dependent group III mGluR signaling [6]. However, a direct effect of PKC phosphorylation on G protein coupling to group III mGluRs remains to be demonstrated.

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