Mapping of Calmodulin and Gβγ Binding Domains within the C-terminal Region of the Metabotropic Glutamate Receptor 7A*

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Ca2+/calmodulin (Ca2+/CaM) and the βy subunits of heterotrimeric G-proteins (Gβγ) have recently been shown to interact in a mutually exclusive fashion with the intracellular C terminus of the presynaptic metabotropic glutamate receptor 7 (mGluR 7). Here, we further characterized the core CaM and Gβγ binding sequences. In contrast to a previous report, we find that the CaM binding motif localized in the N-terminal region of the cytoplasmic tail domain of mGluR 7 is conserved in the related group III mGluRs 4A and 8 and allows these receptors to also bind Ca2+/CaM. Mutational analysis of the Ca2+/CaM binding motif is consistent with group III receptors containing a conventional CaM binding site formed by an amphipathic α-helix. Substitutions adjacent to the core CaM target sequence selectively prevent Gβγ binding, suggesting that the CaM-dependent regulation of signal transduction involves determinants that overlap with but are different from those mediating Gβγ recruitment. In addition, we present evidence that Gβγ uses distinct nonoverlapping interfaces for interaction with the mGluR 7 C-terminal tail and the effector enzyme adenyl cyclase II, respectively. Although Gβγ-mediated signaling is abolished in receptors lacking the core CaM binding sequence, α subunit activation, as assayed by agonist-dependent GTPγS binding, was not affected. This suggests that Ca2+/CaM may alter the mode of group III mGluR signaling from mono- (α) to bidirectional (α and βγ) activation of downstream effector cascades.

Glutamate receptors mediate excitatory neurotransmission at most synapses in the central nervous system. Ionotropic glutamate receptors, such as AMPA, kainate, and NMDA receptors are involved in fast neurotransmission. In contrast, G protein-coupled metabotropic glutamate receptors (mGluRs)1 have been implicated in the short and long term modulation of synaptic transmission within many pathways of the brain. In particular, activation of mGluRs may regulate neuronal development and survival, transmitter release, electrical excitability, synaptic plasticity, and memory formation (1–4). At least eight different mGluR subtypes have been identified by molecular cloning. Based on sequence homology, pharmacology, and signal transduction mechanisms in heterologous expression systems, these receptors are classified into three groups. Group I receptors (mGluRs 1 and 5) are linked to phospholipase C, whereas members of the groups II (mGluRs 2 and 3) and III (mGluRs 4, 6, 7, and 8) couple to the cAMP signaling pathway. However, this effector coupling may not be stringent, since in cerebellar neurons mGluR 7-mediated inhibition of P/Q type calcium channels involves phospholipase C (5).

The different groups of mGluRs not only use distinct signaling pathways but also display different subcellular localizations. Group I mGluRs are predominantly postsynaptic receptors located at perisynaptic membrane areas. Group II receptors can be found pre- and postsynaptically but also display distribution over the surface of axons and dendrites. Members of group III mGluRs are primarily presynaptic and concentrated at or near active zones (6–8). This selective localization of mGluRs to discrete subcellular domains has an important impact on receptor signaling. First, restricting expression to the pre- or postsynaptic compartment will define the sites at which mGluRs regulate synaptic transmission. Second, receptor distance from release sites will determine how readily the receptors are confronted by activating concentrations of glutamate. Finally, their precise subcellular localization will regulate the receptors’ proximity to target ion channels, the transmitter release machinery, and/or enzyme cascades that regulate synaptic function.

The relevance of such precise topological arrangements for the synaptic function of mGluRs is highlighted by the group III receptor, mGluR 7. This low affinity receptor is selectively targeted to presynaptic transmitter release sites, enabling it to sense high levels of released glutamate. This in turn allows for rapid autoinhibition of the neighboring Ca2+ channels, whose opening triggers neurotransmitter release (7, 9). Axon targeting of mGluR 7 has been shown to involve the cytoplasmic C terminus of the receptor (10). Furthermore, the formation of presynaptic clusters of mGluR 7 is also mediated by this C-terminal tail, which provides a binding motif for PICK1 (protein interacting with C kinase; Refs. 11–13). Although the

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The abbreviations used are: mGluR, metabotropic glutamate receptor; CaM, calmodulin; 1-AP4, 1-amino-5-(+)-2-amino-4-phosphonobutyric acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; [125I]HPIA, ([125I]iodo-4-hydroxyphenyl-isopropyl)-adenosine; Gβγ, guanosine triphosphate binding protein βγ subunits; GST, glutathione S-transferase; GTPγS, guanosine 5′-O-3′-thiotriphosphate; MBP, maltose-binding protein; GST, glutathione S-transferase; MPPG, ((RS)-a-Methyl-4-phosphonophenylglycine).
interaction with PICK1 was found to be essential for clustering, but not for axonal targeting, of mGluR 7 (11). PICK1 binding did not influence effector regulation via this receptor (12).

Different lines of evidence indicate that the C-terminal portion of mGluR 7 is essential for signal transduction. First, this region binds Gβγ subunits, which mediate Ca2+ channel closure and inhibition of neurotransmitter release (14, 15). Second, Ca2+/CaM binding to this region of the receptor promotes G protein-mediated signaling by displacing Gβγ subunits from the C-terminal tail (9). Third, binding of Ca2+/CaM prevents phosphorylation of the C terminus by protein kinase C (16), a reaction that may underlie receptor desensitization (17). In addition, protein kinase C-dependent phosphorylation of mGluR 7 may also be hindered by PICK1 binding (13). To further elucidate how the C terminus of mGluR 7 interacts with the various signaling components mentioned above, we investigated the determinants that mediate binding of the mGluR 7 C-tail to both Ca2+/CaM and Gβγ subunits. Previously, we and others have found that PICK1 interacts with the last three amino acids of the mGluR 7 C-tail (11–13). Here, we show that Ca2+/CaM and Gβγ bind to partially overlapping domains located at the N-terminal part of the mGluR 7 C-tail. This region is conserved in the related group III receptors, mGluR 4A and mGluR 8A and 8B, which are also shown to interact with Ca2+/CaM. Mutations that prevent CaM binding selectively inhibit mGluR 7 signaling through Gβγ subunits but do not affect trimeric G-protein receptor to the receptor. Our data are consistent with Ca2+/CaM causing group III mGlRs to switch from mono- (Gα) to bidirectional (Gα and Gβγ) signaling.

**EXPERIMENTAL PROCEDURES**

**Fusion Protein Expression Constructs—**EcoRI and SacI site flanked cDNA fragments encoding the C-terminal regions of mGluR 4A, 7A, 7B, 8A, and 8B, respectively, were generated by standard polymerase chain reactions on mouse brain cDNA using procedures similar to those described in Ref. 9. The polymerase chain reaction products were inserted in pGEX-5-X 1 (Amersham Pharmacia Biotech). The mGluR 4B and 6 C-terminal tail regions were amplified using mGluR 4 and mGluR 6 full-length cDNAs (kindly provided by Dr. Y. Seino, University of Kyoto, Japan). Due to insolubility of mGluR 4B as a GST fusion protein, the cDNA sequences encoding the mGluR 7A and mGluR 4B C-terminal tails were inserted into the pMAL-c2 vector (New England Biolabs) to produce soluble maltose-binding protein (MBP) fusion tails. The following truncated and mutated tail regions of mGluR 7A were also generated by polymerase chain reaction and fused to MBP: mGluR 7A-N25 (GST-7A-N25) (18), mGluR 7A-S862 (GST-7A-S862) (19) followed by a horseradish peroxidase-coupled anti-rabbit secondary antibody (Amersham Pharmacia Biotech).

**Adenylyl Cyclase II Stimulation Assay—**The short splice variant of recombinant Gαs (Gαs-s) was expressed in E. coli (BL21DE3) and purified from bacterial lysates. Where applicable, rGαs-s (10 μM) was preactivated for 60 min at 30 °C in buffer containing 50 mM HEPES/NaOH, pH 7.6, 1 mM EDTA, 10 mM MgSO4, 100 μM GTPγS, and 0.025% (w/v) Lubrol. The nucleotide was subsequently removed by gel filtration (20). S9 cell membranes expressing mammalian adenylyl cyclase type II (21) were a generous gift of C. Kless(2) (Free University, Berlin). For the determination of adenylyl cyclase activity, S9 membranes (10 μg assay) were incubated in a final volume of 50 μl containing 50 mM HEPES/NaOH, pH 7.6, 1 mM EDTA, 10 mM MgSO4, 100 μM GTPγS, and 0.025% (w/v) Lubrol; the nucleotide was subsequently removed by gel filtration (20). S9 cell membranes expressing mammalian adenylyl cyclase type II (21) were a generous gift of C. Kless(2) (Free University, Berlin). For the determination of adenylyl cyclase activity, S9 membranes (10 μg assay) were incubated in a final volume of 50 μl containing 50 mM HEPES/NaOH, pH 7.6, 1 mM EDTA, 10 mM MgSO4, 100 μM GTPγS, and 0.025% (w/v) Lubrol; the nucleotide was subsequently removed by gel filtration (20).
Calmodulin and G\(\beta\)\(\gamma\) Binding to mGluR 7

**RESULTS**

Mapping of the CaM and G\(\beta\)\(\gamma\) Binding Domains on mGluR 7—We have previously shown that Ca\(^{2+}\)/CaM and G\(\beta\)\(\gamma\) bind in a mutually exclusive manner to the C-terminal tail region of mGluR 7 (9). Since both known splice variants of mGluR 7, 7A and 7B, bind Ca\(^{2+}\)/CaM and since these variants differ only in their C-terminal half (Fig. 1A), we assigned the CaM binding site to the N-terminal portion of the tail regions. To confirm this idea and to exclude a contribution of the divergent sequences at the extreme C terminus, we used binary interaction assays with truncated receptor tail GST fusion constructs. Pull-down assays employing CaM immobilized on agarose beads proved superior to the previously used binding assay with purified CaM and G\(\beta\)\(\gamma\) proteins immobilized on glutathione-Sepharose. Fig. 1B shows that both of the N-terminal region constructs, GST-7A-N38 and GST-7A-N25, bound CaM-agarose in the presence of 1 mM Ca\(^{2+}\) but not EGTA, whereas the C-terminal GST-7A-C27 fusion protein failed to bind CaM even in Ca\(^{2+}\)-containing buffer. Ca\(^{2+}\)/CaM binding to mGluR 7 sequences was not a fusion protein artifact, since native mGluR 7 solubilized from hippocampal membranes with Triton X-100 also bound CaM-agarose in a Ca\(^{2+}\)-dependent manner (Fig. 1C).

The C-terminal tail of mGluR 7A also contains a binding site for G\(\beta\)\(\gamma\), and Ca\(^{2+}\)/CaM is known to inhibit binding of G\(\beta\)\(\gamma\) to GST-7A (9). We therefore examined whether the binding site for G\(\beta\)\(\gamma\) also resides in the N-terminal region of mGluR 7A by incubating GST-7A-N38, GST-7A-N25, and GST-7A-C27 with purified G\(\beta\)\(\gamma\). GST-7A-C27 did not retain G\(\beta\)\(\gamma\), while comparable amounts of G\(\beta\)\(\gamma\) were recovered with both GST-7A-N38 and GST-7A-N25 (Fig. 1D, top panel). When directly compared with GST-7A-N25 was as effective in retaining G\(\beta\)\(\gamma\) as the full-length tail region (see Fig. 5A). Finally, binding of G\(\beta\)\(\gamma\) to GST-7A-N25 was abolished in the presence of CaM (Fig. 1D, lower panel). We therefore conclude that the first 25 residues in the N-terminal region of the mGluR 7 tail domain suffice to support both Ca\(^{2+}\)/CaM and G\(\beta\)\(\gamma\) binding.

**GST-7A-N25 Contains the Determinants for High Affinity CaM Binding—**In order to obtain an estimate of the affinity of the C-terminal tail of mGluR 7A for Ca\(^{2+}\)/CaM, we incubated dansylated CaM (dansyl-CaM) with GST, GST-7A, or GST-7A-N25 fusion proteins and measured the changes in fluorescence...
emission induced by its interaction with the binding motif. In the absence of Ca$^{2+}$, fluorescence emission by dansyl-CaM was modest (Fig. 2A, trace 1). Binding of Ca$^{2+}$ induces a conformational change in CaM, which in dansyl-CaM reduces the exposure of the fluorophore to the aqueous environment and could be detected as an enhancement and a blue shift in the emitted fluorescence (Fig. 2A, trace 2). The addition of GST had no effect on the spectrum of dansyl-CaM in both the presence (Fig. 2A, trace 3) or absence of Ca$^{2+}$ (data not shown). In contrast, GST-7A (trace 4 in Fig. 2A) and GST-7A-N25 (trace 5 in Fig. 2A) caused substantial fluorescence enhancement; this increase in light emission probably reflects shielding of the fluorophore by binding of the C-terminal peptide. Because of an excellent signal/noise ratio, the concentration-dependent effects of GST-7A (and of GST-N25) were readily detected (Fig. 2B and data not shown). Notably, however, the apparent EC$_{50}$ values of GST-7A depended on the concentration of dansyl-CaM (see Fig. 2B); this was most readily evident when data were normalized to maximal fluorescence enhancement (Fig. 2C). This concentration dependence can be attributed to the depletion of free GST-7A, which is most pronounced at low concentrations of GST-7A. Depletion is most easily corrected for by determining EC$_{50}$ values over a reasonably large range of dansyl-CaM concentrations; for infinitely low concentrations of dansyl-CaM, depletion should be nonexistent, and the EC$_{50}$ value should hence be a true affinity estimate. Fig. 2D shows that the EC$_{50}$ estimates for both GST-7A or of GST-7A-N25 indeed depended in a linear manner on the dansyl-CaM concentration employed. The slope of the regression line was close to unity, indicating a 1:1 stoichiometry; the true affinity value calculated from the y intercept was in the range of 70 nM. Fig. 2D also indicates that the EC$_{50}$ values for GST-7A (closed symbols) and for GST-7A-N25 (open symbols) fell onto the same line. We therefore conclude that GST-7A-N25 comprises all residues required for high affinity binding of CaM.

Ca$^{2+}$/CaM Interacts with Several Group III mGluRs—To unravel whether other members of group III mGluRs also might interact with Ca$^{2+}$/CaM, we tested the CaM binding of fusion proteins of all known group III mGluR tail sequences to CaM-agarose. Fig. 3A shows that GST fusion proteins encoding the C-terminal tails of mGluR 4A, 8A, and 8B (GST-4A, -8A, -8B) bound CaM-agarose in the presence of Ca$^{2+}$, but not of EGTA. Notably, GST-6 failed to interact with CaM agarose even in the presence of Ca$^{2+}$ (not shown). To confirm this observation by a different approach, we immobilized GST-6 on glutathione-Sepharose and incubated the resulting matrix with purified CaM. Again, no CaM binding was detected (Fig. 3C). This result is consistent with several substitutions in the putative CaM binding region of mGluR 6 (Fig. 1A). A high intrinsic insolubility of GST-4B precluded assays in the CaM binding potential (data not shown). To overcome this problem, we generated an MBP fusion construct encoding the C-terminal tail of mGluR 4B. The resulting fusion protein (MBP-4B) showed improved solubility upon expression in bacterial cells. As a positive control, the mGluR 7A tail region was also fused to MBP. Fig. 3A demonstrates that MBP-7A but neither MBP nor MBP-4B interacted with CaM-agarose in the presence of Ca$^{2+}$. This again is consistent with the predicted CaM binding consensus sequence highlighted in Fig. 1A that is conserved in all group III mGluRs except mGluR 4B and mGluR 6. Confirming our assignment of the CaM binding region, a previously described fusion protein lacking amino acids 864–876 within the CaM binding region of GST-7A (GST-7A-$\Delta$CaM; see Ref. 9) also failed to bind to CaM agarose (data not shown). Since this fusion protein was very sensitive to proteolysis, we also immobilized GST-7A-$\Delta$CaM on glutathione-Sepharose. Again, the resulting matrix displayed no detectable CaM binding (Fig. 3C).

Our results contrast with previous observations showing that CaM binding is a unique feature of mGluR 7 (16). To corroborate that indeed native group III mGluRs other than mGluR 7 bind Ca$^{2+}$/CaM, we also performed CaM affinity adsorption of mGluR 4A solubilized from cerebellar membranes. Native mGluR 4A receptors were specifically retained on CaM-agarose in the presence of Ca$^{2+}$ but not EGTA. This result corroborates the pull-down results with GST fusion proteins, which show that the highly conserved 25 N-terminal amino acids of the C-tails of mGluR 4A, 7A, 7B, 8A, and 8B provide a CaM binding site found in most group III mGluRs.

Determinants of CaM Binding—To delineate residues that are important for CaM binding, we generated point mutations in the CaM binding region of constructs GST-7A and GST-7A-N25. Several of these mutations were designed on the basis of determinants that have been identified in previously described Ca$^{2+}$/CaM binding domains (Se$_{862}^{5}$, Phe$_{863}^{5}$, and Met$_{872}^{5}$). Surprisingly, substitution of the polar glutamine residue 857 by alanine (GST-N25-Q857A) did not affect binding to immobilized Ca$^{2+}$/CaM. Ca$^{2+}$/CaM also bound mutant tails in which arginine 859 (GST-7A-R859E) or arginine 861 (GST-7A-R861P) were substituted by glutamate and proline, respectively. However, replacing Phe$_{863}^{5}$ by alanine significantly reduced CaM binding, whereas replacing Met$_{872}^{5}$ by alanine or glutamic acid did not result in altered CaM binding. In contrast, introducing negative charges into the CaM binding sequence weakened or abolished this interaction. While a lysine to glutamic acid substitution at position 860 (GST-N25-K860E) reduced binding to CaM-agarose only to some extent, replacement of a single serine at position 862 by glutamic acid (GST-N25-S862E) produced a fusion protein that bound only weakly as compared with wild-type GST-7A-N25 (26). The importance of the hydrophobic domain that follows the polar cluster QKRKR was investigated by introducing multiple glutamate residues to this domain. Accordingly, GST-7A-3E carrying three adjacent glutamate residues (K864E, A865E, and V866E) (26) and two mutants harboring four adjacent or closely separated glutamate substitutions, i.e. GST-7A-4E (K863E, K864E, A865E, and V866E) and GST-7A-4X (S862E, T868E/T871E, and S873E), all showed a complete loss of CaM binding (Fig. 3B).

The CaM Binding Motif Is Not Required for Go Signaling—To examine whether deletion of the CaM binding region impairs trimeric G protein coupling to mGluR 7A, we performed [$^{[35]}$S]GTP$\gamma$S binding experiments with both the wild type mGluR 7A and the mGluR 7A-$\Delta$CaM mutant full-length receptors in the presence of either the selective group III agonist t-AP4 or the respective antagonist MPPG. As shown in Fig. 4, t-AP4 increased the binding of GTP$\gamma$S to membranes prepared from HEK293 cells transiently expressing either the wild-type mGluR 7A (Fig. 4A) or mGluR-7A-$\Delta$CaM (Fig. 4B) in a similar fashion. This indicates that G$\gamma$ coupling was not affected by deletion of the CaM binding motif. The agonist-promoted increase in binding was most pronounced at the first time point examined (i.e. after 2.5 min of agonist exposure). This is to be expected for receptor-mediated guanine-nucleotide exchange, because the supply of G proteins accessible to an individual receptor is limited by the small internal volume of the membrane vesicle. Receptor-induced [$^{[35]}$S]GTP$\gamma$S binding therefore rapidly reaches equilibrium, while basal binding progressively increases at later time points; the difference between receptor-driven and basal guanine nucleotide exchange therefore disappears as basal binding proceeds to steady state.

2 V. O’Connor, unpublished data.
agonist-induced increment over basal binding was compared at the earliest (2.5 min) time point, there was no appreciable difference between wild-type full-length mGluR 7A (Fig. 4A) and mGluR-7A-ΔCaM (Fig. 4B). As an internal control, the human D2-dopamine receptor, another G\(_i\)/\(G_\alpha\)-coupled receptor, was simultaneously coexpressed with each of the two mGluR 7A versions. The increment in [\(^{35}\)S]GTP\(^\gamma\)S binding induced by activating the human D2-dopamine receptor was comparable with that caused by agonist stimulation of wild-type mGluR 7A and mGluR-7A-ΔCaM. This further supports the interpretation that the ability of mGluR-7A-ΔCaM to activate G proteins is within the range expected for a G\(_i\)/\(G_\alpha\)-coupled receptor transiently expressed in HEK 293 cells.

Mapping of the G\(\beta\)\(\gamma\) Binding Site of mGluR 7—CaM and G\(\beta\)\(\gamma\) both bind to the N-terminal 25 residues of the mGluR 7A tail. However, removal of residues that are crucial for binding of CaM did not impair the coupling of the receptor to G proteins (Fig. 4A). Furthermore, G\(\beta\)\(\gamma\) has been shown to bind to GST-7A-ΔCaM, which lacks residues 864–876 and does not bind Ca\(^{2+}\)/CaM (9). CaM and G\(\beta\)\(\gamma\) subunits may therefore use distinct but overlapping binding sites within mGluR 7A-N25. To test this hypothesis, we evaluated the importance of Ser\(^{862}\). This residue is found immediately after the QKRKR motif in all group III mGluRs (with the notable exception of mGluR 4B; see Fig. 1A), is important for binding of CaM (26), and precedes the sequence that is dispensable for binding of G\(\beta\)\(\gamma\) (residues 864–876 in GST-7A-ΔCaM; see Ref. 9). Substitution of serine 862 by a negatively charged glutamate residue greatly impaired the ability of the resulting mutant GST-N25-S862E to retain G\(\beta\)\(\gamma\) (Fig. 5A, top). In addition, GST-N25-K860E, which displayed a decreased binding to Ca\(^{2+}\)/CaM, also interacted only weakly with G\(\beta\)\(\gamma\) (Fig. 5A, middle). Thus, Ser\(^{862}\) and Lys\(^{865}\) define a site where the G\(\beta\)\(\gamma\)-binding region and the CaM binding region appear to overlap. Notably, the QKRKR motif that precedes Ser\(^{862}\) is reminiscent of the consensus motif for G\(\beta\)\(\gamma\) interaction found in some adenyl cyclases and other effectors of G\(\beta\)\(\gamma\) (QXXER; see Ref. 27). Substitution of the most N-terminal residue within this motif, Gln\(^{857}\), by alanine (GST-N25-Q857A) did not affect G\(\beta\)\(\gamma\) binding (Fig. 5A, middle), although the corresponding mutation in adenyl cyclases II abolishes binding to and activation by G\(\beta\)\(\gamma\) (27). Also, mGluR 4A differs from mGluR 7A only by a single amino acid in its N25 segment with proline at the position corresponding to Gln\(^{857}\) of mGluR 7A. Despite this nonconservative substitution, GST-4A retained G\(\beta\)\(\gamma\) to an extent that was indistinguishable from that of GST-7A (Fig. 5A, bottom). This result and the presence of a lysine rather than a glutamate at the QKRKR sequence of G\(\beta\)\(\gamma\) defines a site where the G\(\beta\)\(\gamma\)-binding region and the CaM binding region appear to overlap. Notably, the QKRKR motif that precedes Ser\(^{862}\) is reminiscent of the consensus motif for G\(\beta\)\(\gamma\) interaction found in some adenyl cyclases and other effectors of G\(\beta\)\(\gamma\) (QXXER; see Ref. 27). Substitution of the most N-terminal residue within this motif, Gln\(^{857}\), by alanine (GST-N25-Q857A) did not affect G\(\beta\)\(\gamma\) binding (Fig. 5A, middle), although the corresponding mutation in adenyl cyclases II abolishes binding to and activation by G\(\beta\)\(\gamma\) (27). Also, mGluR 4A differs from mGluR 7A only by a single amino acid in its N25 segment with proline at the position corresponding to Gln\(^{857}\) of mGluR 7A. Despite this nonconservative substitution, GST-4A retained G\(\beta\)\(\gamma\) to an extent that was indistinguishable from that of GST-7A (Fig. 5A, bottom). This result and the presence of a lysine rather than a glutamate at the QKRKR sequence of mGluR 7 suggests fundamental differences in the G\(\beta\)\(\gamma\) binding properties of mGluRs and adenyl cyclase II related interaction domains.

To corroborate that G\(\beta\)\(\gamma\) binding to mGluR 7A might represent interaction site distinct from that defined by AC II, we tested whether GST-7A affected G\(\beta\)\(\gamma\) stimulation of AC II (Fig. 5B). AC II expressed in S99 cells (21) was stimulated by Gln\(^{857}\), as expected, cAMP production was stimulated further by adding purified G\(\beta\)\(\gamma\) (28). The addition of GST-7A-N25 did not inhibit the G\(\beta\)\(\gamma\)-dependent stimulation of catalysis to any appreciable extent (Fig. 5B). It should be noted that the concentration of G\(\beta\)\(\gamma\) was not saturating (but below the EC\(_{50}\)) for stimulation of adenyl cyclase type II. Furthermore, the concentration of GST-7A (and of the control GST) was 100 \(\mu\)M. Under comparable conditions, 100 \(\mu\)M of the QHEA-peptide (that comprises the interaction domain of ACII) markedly suppressed the stimulatory effect of \(\beta\)\(\gamma\) dimers on ACII (27). We therefore

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3 E. Bofill-Cardona, unpublished data.
FIG. 3. CaM binding of different group III mGluR tail fusion proteins and for GST-7A mutants to CaM-agarose. GST (or MBP) and GST (or MBP) fusion proteins of the indicated constructs were incubated in binding buffer containing either 1 mM CaCl₂ or 5 mM EGTA with CaM-agarose beads (A and B) or immobilized on glutathione-Sepharose 4B beads and incubated with purified CaM (C). After repeated washing of the beads with the corresponding buffer, bound proteins were eluted with SDS sample buffer. One-fifth of the eluates (top panels in A and B) as well as 5% of the input (top panels in A and B) were separated by SDS-polyacrylamide gel electrophoresis and stained by Coomassie Blue. C, one-fifth of the input and the eluates were analyzed. An asterisk indicates the position of CaM.

FIG. 4. [³²P]GTPγS binding to wild type and Δ-CaM full-length mGluR 7A. [³²P]GTPγS binding to membranes prepared from HEK293 cells transiently expressing wild-type mGluR 7A (A) or mGluR 7A-ΔCaM (B). Aliquots of the membrane fractions were incubated in the presence of L-AP4 (300 μM) (closed circles) or MPPG (100 μM) (open circles). Binding was started by adding 10 μl of binding buffer containing 50 μM GDP and 100 nM [³²P]GTPγS (specific activity ~1600 cpm/ fmol). The reaction was quenched at the indicated time points by adding 1 ml of ice-cold stop buffer, and the bound radioactivity was determined. Data represent arithmetic means ± S.D. (not shown if not exceeding the symbols) from three experiments carried out in duplicate. Variance analysis using an analysis of variance statistical test indicated that the means of all values determined in the presence of l-AP4 (n = 9) were significantly different from those of the values obtained in the presence of MPPG (p < 0.01).

conclude that the domain of Gβγ that interacts with ACII must be different from and nonoverlapping with the region that mediates binding to the mGluR 7A C-terminal tail. This result points to the existence of multiple facets of Gβγ binding to its effectors. This is consistent with recent findings (29) in which a new “hot spot” site that binds PLC β2 was identified on Gβγ.

Our previous studies on wild-type and mutant mGluR 7 have provided evidence that a mutually exclusive interaction between CaM and Gβγ subunits controls downstream signaling at the level of the receptor. To corroborate this conclusion, we relied on an assay that more directly reflects receptor-G-protein interactions. Upon activation by agonists, G protein-coupled receptors associate with their cognate trimeric G protein(s). This results in the formation of a ternary complex, in which the agonist is bound with high affinity. Compounds that bind to G protein subunits and thereby inhibit receptor-G protein coupling suppress this high affinity agonist binding (reviewed in Ref. 30). To test whether the interaction between the C-terminal tail of mGluR 7A and Gβγ may affect receptor-G protein coupling, we incubated GST-7A with membranes from HEK 293 cells that stably expressed the human A₁-adenosine receptor. Like group III mGluRs, the A₁ receptor also couples to G₁ and G₉ (31), but its G protein coupling (25) and effector signaling (9) is not affected by CaM. Binding of the A₁-adenosine receptor agonist [¹²⁵I]HPIA was inhibited in a concentration-dependent manner by GST-7A, reaching a maximum inhibition of 40–50% at 30–70 μM GST-7A (Fig. 5C). The S862E mutant (which bound Gβγ poorly; see Fig. 5A) had a more modest effect on high affinity agonist binding (Fig. 5C). In contrast, the Q857A mutant (which still efficiently retained Gβγ; see Fig. 5A) significantly suppressed [¹²⁵I]HPIA binding. The inhibitory effect of GST-7A was not affected by Ca²⁺ (Fig. 5C), but the addition of both CaM and Ca²⁺ reduced the inhibitory effect of GST-7A (Fig. 5C). The inhibition by GST-7A could not be attributed to direct effects on the ligand binding pocket of the A₁-adenosine receptor, because high affinity binding of the radiolabeled A₁ antagonist H₁-[¹²⁵]labeled 8-cyclopentyl-1,3-dipropylxanthine was not inhibited by GST-7A in both the absence and presence of Ca²⁺/CaM.³

DISCUSSION

Ca²⁺/CaM, Gβγ subunits, and PICK1 all mediate signaling through and presynaptic clustering of mGluR 7 by binding to its C-terminal tail region (9, 11). Our previous (9) and the present results demonstrate that Ca²⁺/CaM and Gβγ share some common binding determinants that reside in the N-terminal region of the mGluR 7 C-tail that follows the last transmembrane segment. PICK1, in contrast, uses a remote binding site at the extreme C-terminal end of mGluR 7 (11–13). The use of dansyl-CaM allowed us to spectrofluorometrically follow the interaction of CaM with its target sequence. A fusion protein harboring only the 25 amino acids that directly follow the seventh transmembrane helix of mGluR 7A bound to Ca²⁺/CaM-agarose with the same affinity as the entire carboxyl terminus. This indicates that the complete CaM binding domain is located within these residues, which are highly conserved among all known members of group III mGluRs with the notable exception of mGluR 4B and mGluR 6. The stretch from histidine 851 to methionine 872 (mGluR 7A numbering) is very conserved in mGluR 7A, mGluR 7B, mGluR 8A, mGluR 8B, and mGluR 4A (Fig. 1A). Despite this conservation, an earlier report has suggested that, among all group III mGluRs, binding of CaM was a feature unique to mGluR 7 (16). In contrast to that conclusion but in accordance with the sequence identity seen, the present results reveal that Ca²⁺/CaM binds to the C-tails of all group III mGluRs but mGluR 4B and mGluR 6.

Comparable binding of Ca²⁺/CaM to the C-tails of the splice variants of mGluRs 7 and 8 and of mGluR 4A suggests that the residue at which these receptor proteins differ (glutamine 857 in mGluR 7 and the respective proline residue in mGluR 4A) do...
not lie within the binding domain for CaM. Furthermore, point mutations in the mGluR 7A sequence between Gln\textsuperscript{657} and Ser\textsuperscript{662} did not prevent CaM binding, whereas this interaction was lost in the ΔCaM deletion mutant (9). These results indicate that residues within the stretch Ser\textsuperscript{662}–Leu\textsuperscript{678} are essential for CaM binding. This region is identical for the two splice variants of mGluR 7 and mGluR 8, respectively, and varies only by up to four conservative substitutions between the subtypes mGluR 7, 8, and 4A (Fig. 1A). In contrast, the carboxyl terminus of mGluR 4B does not contain any sequence element related to this motif, and the sequence in mGluR 6 diverges after Lys\textsuperscript{664} such that the hydrophobic nature of the core CaM binding domain is altered. This may explain why fusion proteins containing the C-tails of either mGluR 4B or mGluR 6 did not interact with CaM, whereas the carboxyl termini of the other members of the group III family did bind CaM.

While point mutations in the polar stretch QKRRK preceding Ser\textsuperscript{662} had little or no effect on CaM binding, the introduction of multiple negative charges at and after Phe\textsuperscript{663} (K864E/A865E/V866E, F863E/K864E/A865E/V866E, and S862E/T868E/T871E/S873E) completely abolished CaM binding to the N25 tail region. This result reinforces the notion that hydrophobic side chains constitute key determinants of Ca\textsuperscript{2+}/CaM binding. Recently, x-ray crystallography and NMR spectroscopy have provided convincing evidence that hydrophobic interactions mediated by bulky amino acid residues represent contact points for Ca\textsuperscript{2+}/CaM binding. Three canonical CaM target peptides from CaM-dependent protein kinase II α, smooth muscle myosin light chain kinase, and CaM-dependent protein kinase kinase have been resolved in their Ca\textsuperscript{2+}/CaM-bound form, and their corresponding structures have been compared (32, 33). This revealed that different modes of CaM interactions may result from differences in the relative spacing of hydrophobic residues. Alignment of the putative CaM binding sequence within the carboxyterminal tails of group III mGluRs with that of CaM-dependent protein kinase II α reveals an equidistant positioning of hydrophobic key residues (Phe\textsuperscript{662} and Met\textsuperscript{672} in mGluR 7A and Leu\textsuperscript{299} and Leu\textsuperscript{308} in CaM-dependent protein kinase II). Residues Leu\textsuperscript{299} and Leu\textsuperscript{308} both have been shown to be crucial for the interaction of CaM with CaM-dependent protein kinase II α. In mGluR 7, Phe\textsuperscript{662} and Met\textsuperscript{672} provide the only suitable hydrophobic side chains with a spacing of 8 amino acids that recapitulate this binding motif in CaM-dependent protein kinase II α. Indeed, the alanine substitution in GST-N25-F863A exhibited a dramatic reduction in Ca\textsuperscript{2+}/CaM binding. Surprisingly, however, replacement of Met\textsuperscript{672} by alanine or even glutamic acid did not affect CaM binding. Apparently, different contact sites contribute differentially to CaM interaction in different target sequences. Taken together, our data corroborate the idea that the C-tail of group III mGluRs harbors a canonical binding site located on an amphipathic helix whose hydrophobic surface provides the major determinants of CaM binding. Moreover, substitution of Ser\textsuperscript{662} done in duplicate. C, membranes (15–25 μg) from 293 cells stably expressing the human A\textsubscript{1}-adenosine receptor were resuspended in buffer containing [\textsuperscript{125}I]HPA and the indicated concentrations of GST-7A fusion proteins. Incubations were carried out in the absence or presence of 2 mM Ca\textsuperscript{2+} or of both 2 mM Ca\textsuperscript{2+} and 20 μM CaM, respectively. Control binding reactions (100% values) were done in the presence of corresponding GST concentrations. Specific binding was not affected by the presence of Ca\textsuperscript{2+} or of both Ca\textsuperscript{2+} and CaM, and it corresponded to 115 ± 21 (control), 122 ± 16 (+Ca\textsuperscript{2+}), and 109 ± 18 (+Ca\textsuperscript{2+}/CaM) fmol/mg protein, respectively. Data are means ± S.D. from 4–5 separate experiments carried out in duplicate. *, significantly different from GST-7A-N25-Q857A and GST-7A (p < 0.05; analysis of variance); values obtained in the presence of Ca\textsuperscript{2+}/CaM were significantly different from those in the absence of Ca\textsuperscript{2+} or those in the presence of Ca\textsuperscript{2+} alone (p < 0.05; paired Student’s t test).
by glutamate also is sufficient to significantly decrease CaM binding (26). These results support the idea that a change in the electrostatic potential of the otherwise hydrophobic motif may regulate CaM binding. Such changes may be provided, for instance, by phosphorylation of serine or threonine residues, as described for various other Ca\textsuperscript{2+}/CaM binding sequences (34). Indeed, phosphorylation of the C-terminal tail of mGluR 7 by protein kinase C has been reported to prevent Ca\textsuperscript{2+}/CaM binding (16, 26).

As observed with CaM, the first 25 amino acids of the tail region bound G\textbeta\gamma with similar efficiency as the entire carboxyl terminus of mGluR 7A. Furthermore, the binding of G\textbeta\gamma and CaM to this sequence was mutually exclusive, as previously shown for the full-length mGluR 7A C-tail (9). These results suggest that G\textbeta\gamma and CaM share a common binding site at this receptor. This conclusion is supported by the finding that a point mutation at position Ser862 affected both binding of G\textbeta\gamma and of CaM, whereas a point mutation at Gln857 left the binding of both unaltered. Nevertheless, the regions of CaM and G\textbeta\gamma binding are not identical but only partially overlapping, since our previous experiments revealed that fusion proteins containing the dCafM-deletion mutant of the mGluR 7A C-tail bound G\textbeta\gamma but not CaM (9). Indeed, here the full-length dCafM-deletion mutant mediated G protein activation, as revealed by GTP\textgammaS binding, but failed to support G\textbeta\gamma-mediated signaling. Probably G\textbeta\gamma, but not CaM, became trapped at the C-terminal binding motif due to a lack of Ca\textsuperscript{2+}/CaM displacement.

The sequence preceding the core CaM binding domain is highly conserved in all mGluR group III members but mGluR 4B. There are only two substitutions: (i) L854 in mGluR 7A and mGluR 7B is replaced by glutamine in mGluR 6, mGluR 4A, and both mGluR 8 splice variants; (ii) only in mGluR 4A, Gln807 of mGluR 7A is substituted by proline. Thus, mGluR 4A and mGluR 7A differ by two residues, but the respective C-tail fusion proteins did not differ to any appreciable extent in their ability to bind G\textbeta\gamma. Accordingly, we conclude that, apart from mGluR 4B, all group III metabotropic glutamate receptors possess the capacity to bind G\textbeta\gamma within their N-terminal tail regions.

PICK1 has been reported to also bind mGluR 4A and both splice variants of mGluR 8 and 7 (12), although its binding domain is formed by the extreme C-terminal residues of the receptors. Thus, Ca\textsuperscript{2+}/CaM, G\textbeta\gamma, and PICK1 bind to the same subset of group III mGluRs. In neurons, mGluR 7 is clustered at presynaptic sites through its interaction with PICK1 (11, 12). In addition, PICK1 recruits protein kinase Ca to colocalize with mGluR 7 (16). Thus, the carboxyl termini of mGluRs 7, 8, and 4A integrate various Ca\textsuperscript{2+}-dependent signals in order to precisely determine the amount of excitatory transmitter being released. In the presence of synaptically released glutamate, the receptor mediates autoinhibition of transmitter release provided that Ca\textsuperscript{2+} influx activates CaM to release G\textbeta\gamma from the carboxyl terminus (9). In addition, Ca\textsuperscript{2+} influx may facilitate protein kinase C activation, which then results in phosphorylation of the same subdomain of the mGluR 7 C-tail. This, in turn, prevents binding of CaM (16) and thereby reduces the feedback inhibition of glutamate release (17). In conclusion, it appears that the various protein binding sites on the carboxyl terminus of mGluR 7 and probably other members of the group III family) render this receptor an important component in learning processes as well as epileptogenesis (35).

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Mapping of Calmodulin and \( G_{\beta\gamma} \) Binding Domains within the C-terminal Region of the Metabotropic Glutamate Receptor 7A

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