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Dissociation of Protein Kinase-Mediated Regulation of Metabotropic Glutamate Receptor 7 (mGluR7) Interactions with Calmodulin and Regulation of mGluR7 Function

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ABSTRACT

Presynaptic metabotropic glutamate receptors (mGluRs) often act as feedback inhibitors of synaptic transmission and serve important roles in defining the activity of glutamatergic synapses. Recent investigations have begun to identify novel interactions of presynaptic mGluRs, especially mGluR7, with multiple protein kinases and putative regulatory proteins that probably serve to further shape the overall activity of glutamatergic synapses. In the present study, we report that in addition to protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) can inhibit calmodulin (CaM) interactions with the carboxyl-terminal tail of mGluR7. These actions are mediated by PKC-, PKA-, or PKG-dependent phosphorylation of mGluR7 at a single serine residue, Ser^{862}, in the carboxyl terminus of the receptor. Mutation of this residue inhibits kinase-mediated phosphorylation of the mGluR7 carboxyl terminus and reverses kinase-mediated inhibition of CaM binding to mGluR7. However, PKC-mediated inhibition of the functional coupling of mGluR7 to G protein-coupled inward rectifier potassium (GIRK) currents in a heterologous expression system is not affected by mutating Ser^{862}. Furthermore, mutation of Ser^{862} to glutamate to mimic receptor phosphorylation and inhibit CaM interactions with mGluR7 does not affect receptor function. These studies demonstrate that the ability of these second messenger-dependent kinases to inhibit mGluR7-mediated activation of GIRK current is not dependent on the phosphorylation of Ser^{862} or the regulation of CaM binding to mGluR7. Furthermore, our studies suggest that CaM binding is not required for mGluR7-mediated activation of GIRK current.

The majority of fast excitatory synaptic responses throughout the central nervous system are mediated by activation of glutamate-gated cation channels termed ionotropic glutamate receptors. Glutamate is able to modulate synaptic transmission and neuronal excitability at these same synapses by activation of metabotropic glutamate receptors (mGluR), which are seven transmembrane spanning receptors that elicit their effects on second-messenger systems and ion channels by the activation of intermediary G proteins. Eight mGluR subtypes have been identified and classified into three groups based on sequence homology, pharmacology, and second-messenger coupling. Group I mGluRs include mGluR1 and mGluR5, which couple primarily to phosphoinositide hydrolysis and activation of protein kinase C (PKC), whereas group II (mGluR2 and mGluR3) and group III (mGluRs 4, 6, 7, and 8) mGluRs couple to inhibition of adenylyl cyclase in expression systems. mGluRs serve diverse physiological roles in synaptic transmission and plasticity but a primary function of many mGluRs is to serve as presynaptic receptors that mediate feedback inhibition of glutamate release (Conn and Pin, 1997). In particular, mGluR7 has been shown to be localized at synaptic vesicle release sites in hippocampal neurons, where it is postulated

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; PICK, protein interacting with C kinase; ct-mGluR7, intracellular carboxyl terminus of mGluR7; CaM, calmodulin; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; GIRK, G protein coupled inward rectifier potassium channel; HEK, human embryonic kidney; PMA, phorbol 12-myristate 13-acetate; PKC-M, catalytic subunit of protein kinase C; WIN 55,2212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl methanone; L-AP4, L-2-amino-4-phosphonobutyrate; LY-341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

to act as a low-pass filter (Shigemoto et al., 1996). Pharmacological and immunocytochemical studies indicate that mGluR7 acts presynpatically to regulate neurotransmission in the hippocampus (Lanthorn et al., 1984; Gereau and Conn, 1995; Bradley et al., 1996; Shigemoto et al., 1997; Macek et al., 1998), and receptor knockout studies identify roles for these receptors in regulating amygdala-dependent learning and memory (Masugi et al., 1999).

Given the physiological roles that mGluR7 plays in modulating glutamatergic transmission, recent investigations have focused on the mechanisms that regulate the localization and function of these receptors (see Dev et al., 2001 for review). Several investigators have identified PICK (protein interacting with C kinase) as a mGluR7-interacting protein that regulates the presynaptic clustering and phosphorylation of this receptor (Boudin et al., 2000; Dev et al., 2000; El Far et al., 2000). In addition, investigators have used fusion proteins of the intracellular carboxyl terminus of mGluR7 (ct-mGluR7) to identify calmodulin (CaM) and G protein $\beta\gamma$ subunits as mGluR7 interacting proteins that may regulate signaling of these receptors. These studies have provided important insight into the regulation of mGluR7 signaling on a molecular level. Accordingly, CaM binding and $\beta\gamma$ binding were found to be mutually exclusive. Furthermore, disruption of CaM binding to mGluR7 by pharmacological agents or by deletion of a CaM binding domain in the mGluR7 carboxyl terminus inhibits $\beta\gamma$-mediated signaling by full-length mGluR7. These data support the hypothesis that interaction of CaM with ct-mGluR7 is required to allow for the dissociation of $\beta\gamma$ subunits from mGluR7 and for normal receptor signaling events (O'Connor et al., 1999; Dev et al., 2001). In addition, phosphorylation of mGluR7 by PKC has been shown to disrupt CaM binding to ct-mGluR7 (Nakajima et al., 1999). Because activation of PKC in hippocampal slices has been shown to inhibit the function of group III mGluRs (Macek et al., 1998), these data together suggest that PKC may regulate the function of mGluR7 by disrupting CaM interaction with the carboxyl terminus of the receptor and the subsequent release of G protein $\beta\gamma$ subunits from the receptor. However, a direct demonstration that the activity of second messenger-dependent kinases, such as PKC, can regulate mGluR7 function by disrupting the interaction of CaM with the receptor has not been reported. Furthermore, the activity of cAMP-dependent protein kinase (PKA) has also been shown to inhibit the function of mGluR7 in hippocampal slices (Cai et al., 2001). However, the roles that other second-messenger–dependent kinases, such as PKA and cGMP dependent protein kinase (PKG), may play in regulating mGluR7 phosphorylation and CaM binding have not been fully addressed.

In the present study, we have used a fusion protein of ct-mGluR7 to demonstrate roles for multiple second-messenger–dependent kinases in regulating CaM binding to ct-mGluR7. We have identified a single serine residue in ct-mGluR7 that is critical for both second-messenger–dependent, kinase-mediated phosphorylation of ct-mGluR7 and the regulation of CaM binding to the carboxyl terminus. We have also addressed the functional consequence of kinase-mediated regulation of CaM binding to mGluR7 by monitoring the coupling of overexpressed receptor to G protein-coupled inward rectifier potassium (GIRK) channels in human embryonic kidney (HEK) cells. Contrary to previous reports, our studies suggest that CaM binding is not required for mGluR7 coupling to GIRK channels. Furthermore, the ability of PKC to attenuate mGluR7 coupling to GIRK channels in HEK cells is not dependent on disruption of CaM binding to the receptor.

Experimental Procedures

Materials. Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol, and calmodulin were from Sigma (St. Louis, MO). Purified protein kinase C, a catalytic subunit of protein kinase C (PKC-M), a purified catalytic subunit of protein kinase A, recombinant protein kinase G (isoform 1α) isolated from *Spodoptera frugiperda*, and cGMP were obtained from Calbiochem (San Diego, CA). Monoclonal calmodulin antibody was purchased from Upstate Biotechnology (Lake Placid, NY). [γ-^{32}P]ATP (3000 Ci/mmol; 5 mCi/ml) was obtained from PerkinElmer Life Sciences (Boston, MA). The QuikChange site-directed mutagenesis kit and competent cells were purchased from Stratagene (La Jolla, CA). WIN 55,2212-2, L-2-amino-4-phosphonobutyric acid (L-AP4), and LY-341495 were purchased from Tocris Cookson (Ballwin, MO). The pTracer-EF/V5-His mammalian expression vector was obtained from Invitrogen (Carlsbad, CA). The glutathione *S*-transferase (GST) Gene Fusion System was purchased from Amersham Biosciences (Piscataway, NJ). FuGENE 6 transfection reagent was obtained from Roche Applied Science (Indianapolis, IN).

GST-Fusion Protein Generation and Purification. The carboxyl-terminal tail of rat mGluR7a was amplified by polymerase chain reaction (PCR) using directional primers engineered with restriction sites 5′ proximal to the end of the oligomer. The PCR product was then digested with *Eco*RI and *Not*I and subcloned in-frame into the polylinker region of pGex6P3 (Amersham Biosciences), a GST fusion protein bacterial expression vector. Point mutations were introduced into the fusion proteins using Stratagene's QuikChange site-directed mutagenesis system in accordance with the manufacturer's protocols. Mutations were confirmed by sequence analysis. GST fusion proteins containing the wild-type or mutant ct-mGluR7a were purified from bacterial lysates according to the manufacturer's protocols (Amersham Biosciences).

In Vitro Kinase Assays. For PKC assays, 1 μl (~0.05 units) of purified PKC was incubated with 2 μg of GST fusion protein in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA in a total volume of 50 μl. For PKG assays, ~300 units of purified PKG was incubated with 2 μg of GST fusion protein in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 10 mM cGMP in a total volume of 50 μl. For both assays, reactions were started by the addition of 10 μCi of [γ-^{32}P]ATP at 37°C for 30 min. Reactions were stopped by the addition of 6× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The phosphorylated GST fusion proteins were then separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue to visualize the fusion proteins and dried. Dried gels were exposed to a phosphoscreen or X-ray film and radioactivity was quantified with a Molecular Dynamics PhosphorImager.

Calmodulin Binding Assay. CaM binding to GST fusion protein of ct-mGluR7 was determined as described previously by Nakajima et al. (1999). In vitro kinase assays were carried out as described above in the presence or absence of nonlabeled ATP (2.5 mM) for 2 h at 37°C in a total volume of 30 μl with the exception that EGTA was not included in the PKC assay buffer. EGTA was excluded because CaM binding is Ca^{2+} dependent (Nakajima et al., 1999). In some experiments, phosphorylation of mGluR7 under these conditions was confirmed by the addition of [γ-^{32}P]ATP to the incubation mixture and exposure of dried gels to X-ray film as described above. PKA phosphorylation assays were performed with nonlabeled ATP as described for PKC with the exception that ~50 units of PKA was used. In some experiments, PKA-mediated phosphorylation of ct-mGluR7 was confirmed with [γ-^{32}P]ATP and gel electrophoresis.

Fusion proteins were immobilized on glutathione-Sepharose 4B

beads in the presence of 1 μg of CaM in 25 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 2 mM $CaCl_2$ for 2 h at 4°C. Beads were washed three times in the incubation buffer and bound proteins were eluted by the addition of SDS-PAGE sample buffer and heating to 60°C for 30 min. Proteins were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Bound CaM was detected by immunoblot analysis and bound fusion protein was visualized by staining of the membranes with Coomassie Brilliant Blue.

Preparation of Receptor Constructs. Rat mGluR7a in the pZEM229R vector (Saugstad et al., 1994) was digested with *Eco*RI and subcloned in-frame into the pTracer-EF/V5-His A vector. Point mutations were introduced using Stratagene's QuikChange site-directed mutagenesis system in accordance with the manufacturer's protocols and were confirmed by sequence analysis. The PKC-insensitive rat CB1 cannabinoid S317A mutant receptor (Garcia et al., 1998) was kindly provided by Dr. Ken Mackie (University of Washington, Seattle, WA) and subcloned in-frame into the pTracer-EF/V5-His vector using standard molecular biological techniques.

Transfection of Cultured Cells. HEK-293 cells stably transfected with GIRK 1 (Kir 3.1) and GIRK 2 (Kir 3.2) were kindly provided by Dr. Lilly Jan (University of San Francisco, San Francisco, CA). Cells were grown in Dulbecco's modified Eagle/Ham's F12 media supplemented with 10% fetal bovine serum, 1:100 penicillin/streptomycin and 750 μg/ml Geneticin (Invitrogen) in a humidified environment with 5% CO_2 at 37°C. Cells were plated onto coverslips in four-well dishes (1.9 cm^2/well) in 0.5 ml of the same media at 5 to 10% confluence 6 to 24 h before transfection. Individual wells were transfected with 0.5 μg of the appropriate cDNA complexed with 1.5 μl of FuGENE 6 transfection reagent in 25 μl of media according to manufacturer's direction (Roche Applied Science). Twenty-four hours after transfection, mGluR7 (wild-type or mutant) expressing cells were washed and treated with glutamine free Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 100 μM LY-341495, a glutamatergic antagonist, to prevent free glutamate from interacting with the expressed receptor. Whole-cell patch-clamp experiments were performed on single cells 40 to 48 h after transfection.

Whole-Cell Patch-Clamp Experiments with Cultured Cells. HEK cells stably expressing GIRK 1/2 were transiently transfected with receptor constructs in the pTracer-EF/V5-His A vector to allow for simultaneous expression of receptor and green fluorescent protein. Expression of green fluorescent protein allows for visual detection of transfected cells. Coverslips containing transfected cells were placed in a perfusion chamber attached to a fluorescence microscope where they were perfused at a rate of ~2 ml/min at room temperature in external buffer containing 40 mM KCl, 110 mM *N*-methyl-D-glucamine, 1 mM $CaCl_2$, 25 mM HEPES, and 10 mM glucose, pH 7.35. Individual fluorescent cells were identified and whole-cell patch-clamp experiments were performed as described previously by Garcia et al. (1998). Electrodes were pulled on a Flaming/Brown micropipette puller (Sutter Instruments, San Rafael, CA) from 1.15 mm capillary pipettes with filaments (Glass Type 8250; Garner Glass, Claremont, CA), fire-polished, and filled with internal solution containing 120 mM potassium gluconate, 10 mM HEPES, 5 mM EGTA, 3 mM $MgCl_2$, 3 mM Na_2ATP, and 0.3 mM NaGTP, pH 7.2. Electrode resistance ranged from 3 to 7 MΩ. The GIRK current was defined as the current elicited during the final 150 ms of a 250-ms pulse to −100mV from a holding potential of −45 mV. Currents were recorded in perfusion buffer alone before the application of perfusion buffer containing drug and/or phorbol as indicated. Solution reservoirs were selected by a digital modular valve positioner (Hamilton Company, Reno, NV). Currents were digitized at 1 kHz and data acquired by an Axoclamp amplifier and stored on an IBM Pentium clone using pClamp acquisition and analysis software (Axon Instruments, Union City, CA)

Statistical Analysis. Data comparisons were performed using one-way analysis of variance for multiple comparisons followed by post-testing using Dunnett's test to compare each condition to control. Where appropriate, either paired or unpaired Student's *t* test was performed.

Results

Multiple Second-Messenger–Dependent Protein Kinases Inhibit mGluR7 Binding to CaM. Although the role that PKC plays in regulating the phosphorylation and CaM binding of the mGluR7 carboxyl terminus has been well documented (Nakajima et al., 1999; Airas et al., 2001), recent studies indicate that other second-messenger–dependent kinases, such as PKA, can phosphorylate the carboxyl termini of multiple mGluRs, including mGluR7 (Schaffhauser et al., 2000; Cai et al., 2001). Therefore, we assessed the role that these kinases may play in regulating the ability of CaM to bind ct-mGluR7. Fusion proteins of the mGluR7 carboxyl terminus were generated and used in CaM binding assays. Fusion proteins were incubated with the indicated kinase in the presence or absence of ATP, immobilized with glutathione-Sepharose beads and tested for their ability to bind CaM. As reported previously (Nakajima et al., 1999; O'Connor et al., 1999), nonphosphorylated ct-mGluR7 fusion proteins readily bound to CaM; this interaction was disrupted by PKC-mediated phosphorylation of the fusion protein. The present results indicate that both PKA and PKG can regulate CaM binding to ct-mGluR7 in an analogous manner. Accordingly, upon phosphorylation of the ct-mGluR7 by either PKC, PKA, or PKG, CaM binding was inhibited almost completely (Fig. 1).

PKC and PKG Phosphorylate the Carboxy Terminal Tail of mGluR7 at Ser^{862}. The ability of multiple second-messenger–dependent kinases to regulate CaM binding to the ct-mGluR7 raises the possibility that these kinases may phosphorylate ct-mGluR7 at a conserved site. Recent studies indicate that a single serine residue at position 862 (Ser^{862}) in the mGluR7 carboxyl terminus is phosphorylated by PKA (Cai et al., 2001). We therefore tested the ability of both PKC and PKG to directly phosphorylate the ct-mGluR7 and used site-directed mutagenesis to identify the site(s) required for phosphorylation by these kinases. Several PKC and PKG consensus sites are present in the carboxyl terminus of mGluR7. However, previous results demonstrate that a truncation mutant of the mGluR7 carboxyl terminus that does not contain serine 909 is still phosphorylated by PKC (Nakajima et al., 1999). These studies indicate that serine 909 is not a site of PKC phosphorylation and this site was not further considered for the present study. Site-directed mutagenesis was performed to individually mutate each of the remaining PKC and PKG consensus sites present in the carboxyl terminus of mGluR7 (serine 862, serine 873, serine 877, and serine 881) from serine to alanine. Whereas PKC phosphorylated the S873A, S877A, and S881A mutants as effectively as wild-type carboxyl-terminal fusion protein, phosphorylation of the S862A construct was markedly reduced (Fig. 2, A and B). PKG also readily phosphorylated the carboxyl-terminal fusion protein. Although the level of phosphorylation of the S873A, S877A, and S881A constructs by PKG were similar to the level of phosphorylation of wild-type ct-mGluR7, phosphorylation of the S862A ct-mGluR7 construct by PKG was markedly reduced (Fig. 2, C and D). Together, these studies indicate that Ser^{862} serves as an

important determinant for PKA-, PKC-, and PKG-mediated phosphorylation of ct-mGluR7.

Mutation of Ser^{862} Reverses PKC-, PKA-, and PKG-Dependent Inhibition of CaM Binding to the Carboxyl Terminus of mGluR7. To confirm that Ser^{862} regulates the second-messenger–dependent kinase-mediated effects on ct-mGluR7 interaction with CaM, the same serine-to-alanine point mutants were used in CaM binding assays. Wild-type or mutant fusion proteins were subjected to in vitro kinase assays with PKC, PKA, or PKG in the presence or absence of ATP. The ability of these fusion proteins to interact with CaM was then determined. For all kinases studied, mutation of serine 873, serine 877, or serine 881 had no effect on kinase-mediated inhibition of CaM binding compared with wild-type controls. However, mutation of Ser^{862} completely reverses the kinase-dependent inhibition of CaM binding (Fig. 3). These studies indicate that Ser^{862} is a key determinant for PKC-, PKA-, and PKG-mediated regulation of CaM/mGluR7 interaction. In agreement with these results, Airas et al. (2001) have also identified Ser^{862} as a site for PKC-mediated phosphorylation of ct-mGluR7. Furthermore, these investigators have demonstrated that introduction of a negatively charged residue, such as glutamate, into the CaM binding region of ct-mGluR7, thereby mimicking the phos-

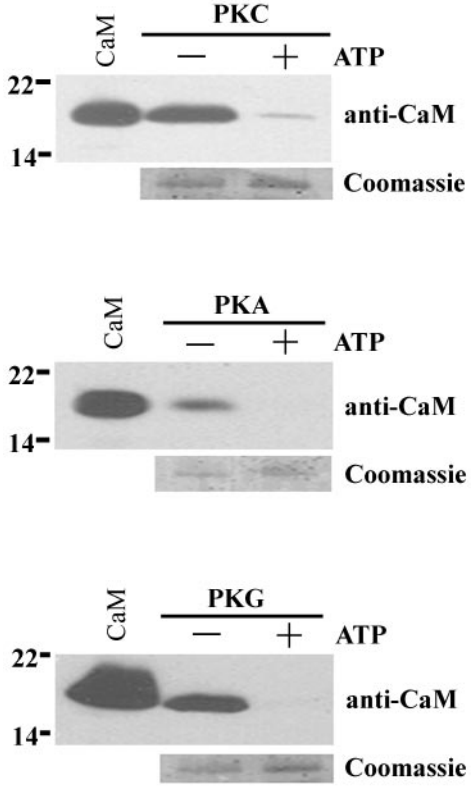

Fig. 1. PKC-, PKA-, or PKG-mediated phosphorylation of mGluR7 carboxyl terminus inhibits calmodulin binding. GST fusion protein of mGluR7 carboxyl terminus was subjected to phosphorylation by either PKC, PKA, or PKG in the presence or absence of ATP as shown. Reactions were stopped by the addition of ice-cold buffer. Fusion protein was then immobilized with glutathione-Sepharose 4B beads in the presence of purified calmodulin and 2 mM $CaCl_2$. Bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Bound CaM was detected by immunoblotting with CaM antibody (anti-CaM). Size of molecular mass markers are indicated by numbers to the left. Purified calmodulin (CaM) (0.5 μg) was run as a standard. Fusion proteins on the membrane were detected by Coomassie Brilliant Blue staining to insure that equivalent amounts of fusion proteins were retained by the beads and loaded onto gels. Blots shown are representative of three independent experiments.

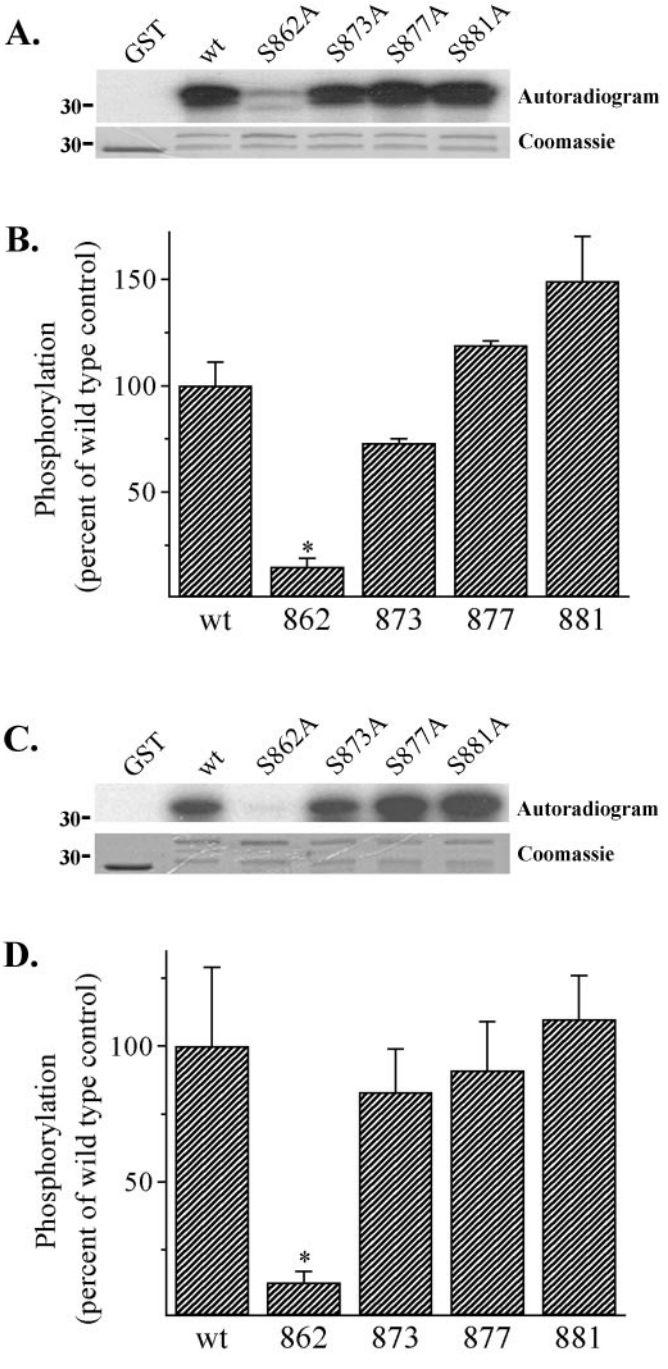

Fig. 2. In vitro phosphorylation of mGluR7 carboxyl terminus by PKC and PKG. GST fusion proteins of wild-type (wt) mGluR7 carboxyl terminus or the indicated serine-to-alanine mutants were subjected to phosphorylation by purified PKC (A, B) or PKG (C, D) in the presence of [γ-^{32}P]ATP. A and C, the peptides were separated by SDS-PAGE and analyzed by autoradiography. Coomassie staining was used to insure equal loading of fusion proteins. The molecular mass of GST alone is 28 kDa and the calculated molecular mass of the ct-mGluR7 GST fusion protein is 35 kDa, which corresponds with the upper band on Coomassie staining. B and D, the amount of radioactivity incorporated into the fusion proteins was quantified by PhosphorImager analysis. Data are reported as the percentage of radioactivity incorporated into wild-type fusion protein and are mean ± S.E. of three separate experiments. *, $p < 0.05$, different from wild-type control.

phorylated state of the receptor, almost completely inhibits CaM binding to the receptor (Airas et al., 2001). Together, these data identify Ser^{862} as a key factor in the phosphorylation-dependent regulation of CaM binding to ct-mGluR7.

Protein kinase C Inhibits mGluR7-Mediated Activation of GIRK Current. We next sought to address the functional consequence of second-messenger–dependent kinase-mediated inhibition of CaM binding to mGluR7. For these studies, the activity of transiently expressed mGluR7 was monitored by whole-cell patch-clamp experiments as an agonist-induced increase in inward current in HEK cells stably expressing Kir 3.1 and Kir 3.2 (GIRK1 and GIRK2) and incubated in a high potassium external buffer. mGluR7 has been shown to couple to GIRK when both components are expressed in *Xenopus laevis* oocytes or HEK cells (Saugstad et al., 1996; O'Connor et al., 1999). The ability of mGluR7 to couple to GIRK in HEK cells was confirmed by the application of a glutamatergic agonist, L-AP4, which elicited inward currents in mGluR7-expressing cells (Fig. 4A). These inward currents rectified at positive potentials (Fig. 4B) and were sensitive to 1 mM Ba^{2+} (Fig. 6A), characteristic of GIRK currents. L-AP4 did not elicit any detectable current in untransfected cells (data not shown).

To determine whether PKC disrupted the coupling of mGluR7 to GIRK activation, either 4α-phorbol, an inactive phorbol ester, or PMA, a direct activator of PKC, were bath applied to cells during repeated application of L-AP4. Cells were first treated with L-AP4 alone to determine the agonist-induced current for each individual cell before repeated application of L-AP4 at 5-min intervals in the presence or absence of phorbol. Repeated application of L-AP4 to cells transfected with mGluR7 resulted in an approximately 15 to 20% reduction in current from the initial application of L-AP4 (data not shown). If the perfusion buffer was switched after the initial application of L-AP4 from standard recording buffer to one containing 4α-phorbol, a similar 15 to 20% reduction in current with subsequent applications of L-AP4 occurred (Fig. 5A) that was not different from standard perfusion buffer. Conversely, if the perfusion buffer was switched from standard recording buffer to one containing PMA after the initial application of L-AP4, then the subsequent activation of GIRK by mGluR7 was significantly reduced (Fig. 5B). The effect of PMA was maximal after 12 min and was significantly different from 4α-phorbol treated cells (65 $\pm$ 5% of control response versus 85 $\pm$ 4% of control response for PMA and 4α-phorbol treated cells, respectively; $p < 0.05$; $n = 9$ to 11) (Fig. 5C).

To ensure that the effect of PMA on mGluR7-mediated currents was not caused by nonspecific effects on drug washout mediated by differences in the flow rates from different solution reservoirs, the effect of PMA on mGluR7 signaling was alternatively monitored by pretreatment of cells with either 4α-phorbol or PMA (1 μM) for 10 min before the

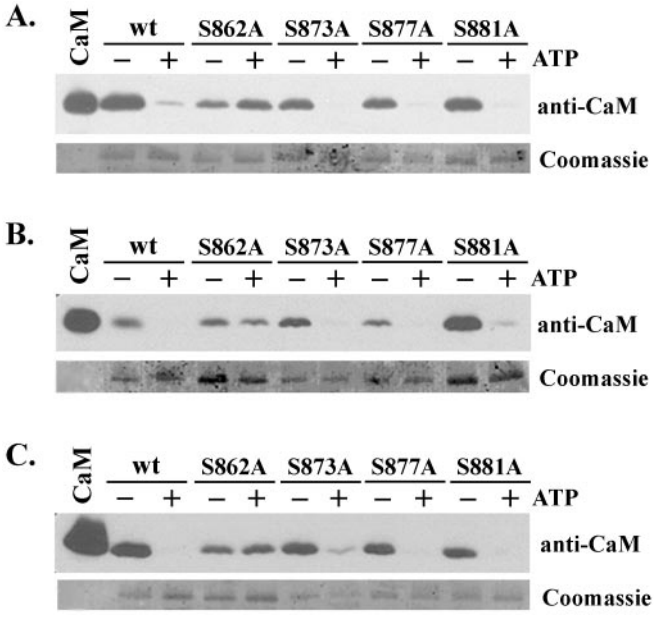

Fig. 3. Effect of protein kinases on calmodulin binding to mGluR7 mutants. GST fusion proteins of either wild-type (wt) mGluR7 carboxyl terminus or the indicated serine-to-alanine mutation were subjected to phosphorylation by either PKC (A), PKA (B), or PKG (C) in the presence or absence of ATP as shown. Fusion protein was then immobilized with glutathione-Sepharose 4B beads in the presence of purified CaM and 2 mM $CaCl_2$. Bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE and transferred to polyvinylidene fluoride. Bound calmodulin was detected by immunoblotting with calmodulin antibody (anti-CaM). Purified calmodulin (CaM) (0.5 μg) was run as a standard. Fusion proteins on the membrane were detected by Coomassie Brilliant Blue staining to insure that comparable amounts of fusion proteins were retained by the beads and loaded onto gels. Blots shown are representative of three independent experiments.

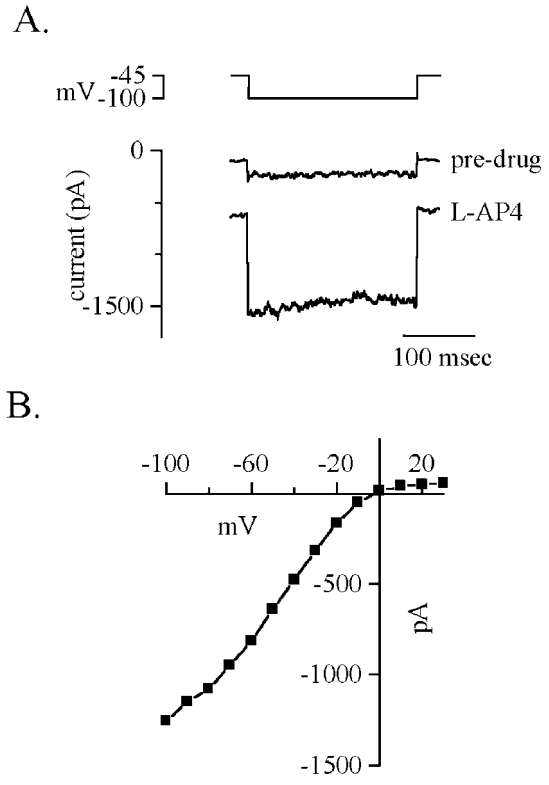

Fig. 4. Activation of mGluR7 mediates an increase in inward current through GIRK channels. HEK cells stably expressing GIRK 1/2 were transiently transfected with mGluR7a cDNA and whole-cell patch-clamp of individual cells was performed. A, a 250-ms step to −100 mV from a holding potential of −45 mV was given either before the application of L-AP4 (predrug) or after the addition of 1 mM L-AP4. Individual current traces from a representative cell are shown. B, voltage steps (10 mV) from −100 mV to +30 mV were given for the same representative cell in either buffer alone or in the presence of 1 mM L-AP4. Currents were averaged and those obtained before L-AP4 were subtracted from those obtained in the presence of L-AP4 and plotted as current versus voltage.

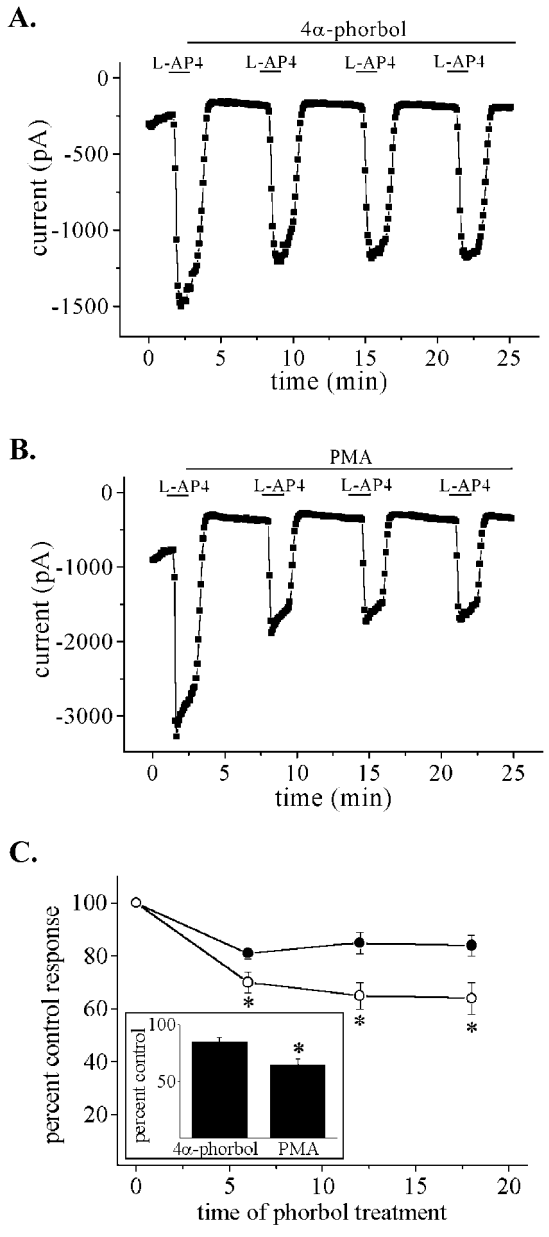

Fig. 5. Stimulation of protein kinase C attenuates L-AP4 induced currents in individual mGluR7-expressing HEK cells. HEK cells stably expressing GIRK 1/2 were transiently transfected with mGluR7 cDNA and whole-cell patch-clamp of individual cells was performed. Mean current activated by a 250-ms step to −100 mV from a holding potential of −45 mV is plotted (■). Voltage steps to −100 mV were performed every 5 s. Baseline current was recorded in buffer alone for 1 min before the application of buffer plus 1 mM L-AP4 for 1.5 min. Cells were then treated in buffer plus the indicated phorbol (10 nM) before repeated applications of L-AP4 at 5 min intervals. Data from one representative 4α-phorbol-treated cell is shown in A and one representative PMA-treated cell is shown in B. C, the maximal L-AP4 induced current was calculated for each drug application. Baseline current for each L-AP4 application was calculated as the average of the currents obtained in the five trials immediately preceding each drug application. Responses in the presence of phorbol ester were calculated as a percentage of the initial response obtained in perfusion buffer alone (control) for the same cell. Combined data are reported as a percentage of the control response for 4α-phorbol–treated cells (●) and for PMA-treated cells (○) and are mean ± S.E. Inset, comparison of responses for 4α-phorbol–treated cells versus PMA-treated cells after 12 min of phorbol treatment. Data are depicted as percentage of response obtained in perfusion buffer alone (control) for the same cell. Control responses were 1418 ± 223 pA for 4α-phorbol–treated cells and 1339 ± 174 pA for PMA-treated cells. *, $p < 0.05$, different from time-matched responses from 4α-phorbol–treated cells; $n = 9$ to 11 for each condition.

experiment. As shown in Fig. 6, pretreatment of cells with PMA inhibited L-AP4–induced current by 40 to 45% compared with 4α-phorbol–treated control cells. To further confirm the effects of PKC on mGluR7 signaling, the effect of a catalytic subunit of PKC, or PKC-M, on mGluR7 activation of GIRK was monitored. Similar to the results obtained with PMA, the inclusion of PKC-M in the internal patch solution inhibited L-AP4-induced currents by 40 to 45% (data not shown). Together, these results demonstrate that the ability of mGluR7 to activate GIRK currents is attenuated by the activity of PKC.

The ability of PKC to inhibit mGluR7 activation of GIRK channels could occur at multiple levels within the signal transduction cascade including the receptor, the G-protein, and the channel itself. To confirm that GIRK channels can be activated in the presence of PMA, a PKC-insensitive cannabinoid receptor (CB1 S317A) was transfected into the HEK cells. This receptor, similar to mGluR7, couples to effectors

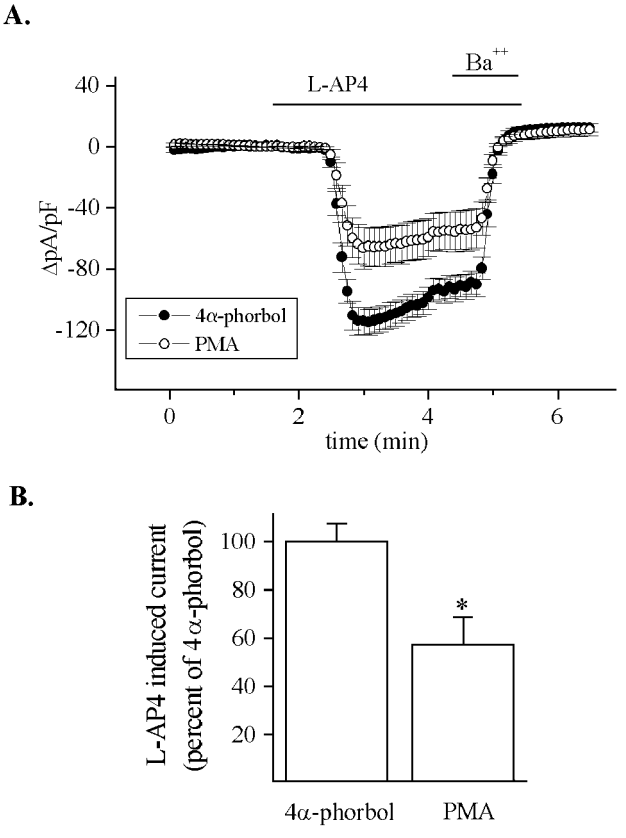

Fig. 6. PMA pretreatment of HEK cells inhibits mGluR7-mediated activation of GIRK current. HEK cells stably expressing GIRK 1/2 were transiently transfected with mGluR7 cDNA and treated with either 4α-phorbol (1 μM) or PMA (1 μM) in perfusion buffer for 10 min, then washed before being placed in the recording chamber. Mean current activated by a 250-ms step to −100 mV from a holding potential of −45 mV was recorded from individual cells every 5 s. A, baseline current was obtained for 1 min before the application of L-AP4 (1 mM) and $BaCl_2$ (1 mM) as indicated. Recordings from either 4α-phorbol– (●) or PMA- (○) pretreated cells were combined and averaged. Mean current was normalized to cell capacitance to correct for differences in cell size. Aggregate data are mean ± S.E. from six cells for each condition and current is plotted as the change in pA/pF compared with baseline current. B, data obtained as in A are plotted as the percentage of response obtained in the presence of 4α-phorbol and are mean ± S.E. *, $p < 0.05$, different from 4α-phorbol–treated cells, $n = 6$ for each condition.

primarily through activation of $G_{i/o}$. Application of PMA to CB1 S317A-transfected HEK cells had no effect on the ability of a cannabinoid agonist to activate GIRK channels (Fig. 7). These experiments demonstrate that PKC inhibits mGluR7-mediated activation of GIRK current in HEK cells by direct actions at the level of the receptor and does not directly inhibit the GIRK channels themselves.

Point Mutants of Ser^{862} That Disrupt mGluR7 Phosphorylation and CaM Binding Do Not Affect mGluR7 Activation of GIRK. To address the role that Ser^{862} may play in the protein kinase-mediated regulation of mGluR7 function, full-length receptor constructs containing a mutation of serine 862 to alanine were made. Mutant receptor constructs were transfected into HEK cells that stably express GIRK channels. Similar control responses to L-AP4 alone were seen for mutant receptors compared with wild-type receptors (1339 ± 174 pA for wild-type; 1527 ± 97 pA for the S862A mutant; n = 9 to 11 for each). Upon repeated application of L-AP4 after treatment with PMA, the inhibition of GIRK current activation was similar for the S862A receptor and wild-type receptor (Figs. 5B and 8A). As seen for the wild-type receptor, the maximal effect of PMA on the S862A mutant receptor occurred after 12 min of PMA application (65 ± 5 and 67 ± 6% of control responses for wild-type and S862A receptors, respectively, n = 9 to 11) and the inhibition of the mGluR7 S862A mutant receptor by PMA was significantly different from the effect of 4α-phorbol (Fig. 8B). These studies demonstrate that phosphorylation mGluR7 at Ser^{862} is not required for PKC-mediated regulation of mGluR7 function. In addition, these studies indicate that the ability of PKC to regulate mGluR7 function is unrelated to its ability to disrupt CaM binding at a site including Ser^{862}.

To further assess the role that CaM plays in regulating mGluR7 function, we created a mutant receptor in which Ser^{862} was mutated to glutamate. This mutation mimics the phosphorylated state of the receptor and markedly reduces CaM binding to ct-mGluR7 (Airas et al., 2001). As shown in Fig. 8C, application of L-AP4 to cells expressing the S862E mutant of mGluR7 activates GIRK current, and the signaling of this mutant is indistinguishable from signaling mediated by the wild-type receptor (maximal responses for wild-type and S862E mutant receptors are 128 ± 17 pA/pF and 131 ± 19 pA/pF, respectively, n = 9 to 10 for each). These studies indicate that the CaM interaction with mGluR7 that is disrupted by mutation of Ser^{862} is not required for the coupling of this receptor to GIRK channels.

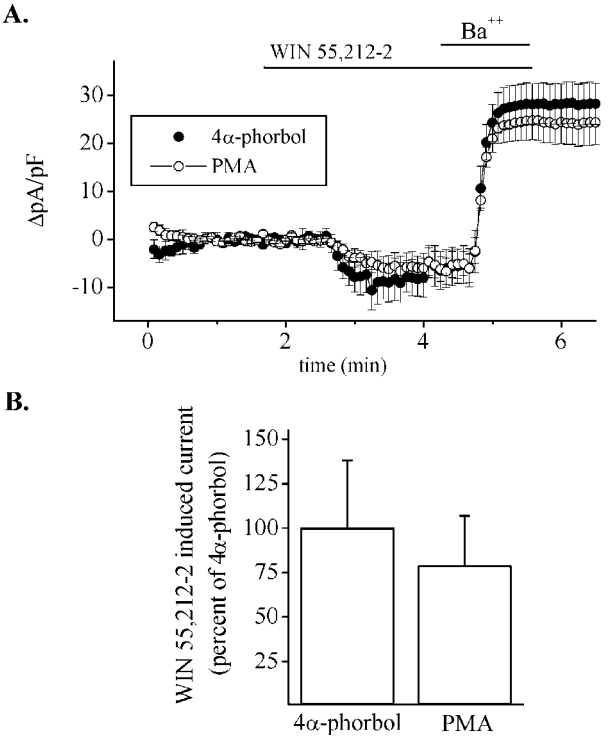

Fig. 7. Activation of GIRK channels by a PKC-insensitive CB1 cannabinoid receptor is unaffected by PMA. HEK cells stably expressing GIRK 1/2 were transiently transfected with CB1 S317A receptor cDNA and treated with 4α-phorbol or PMA as described in the legend to Fig. 6. A, baseline current was obtained for 1 min before the application of WIN 55,212-2 (100 nM) and $BaCl_2$ (1 mM) as indicated. Recordings from either 4α-phorbol– (●) or PMA- (○) pretreated cells were combined and averaged. B, data obtained as in A are plotted as the percentage of response obtained in the presence of 4α-phorbol and are mean ± S.E. n = 6 to 7 for each condition.

Discussion

Recent investigations into the molecular mechanisms involved in regulation of mGluR7 function have led to important insights into the role that the carboxyl terminus of mGluR7 plays in interacting with a number of proteins. In particular, GST fusion protein constructs of ct-mGluR7 have revealed that mGluR7 interacts with CaM. Furthermore, PKC activity, which is known to inhibit mGluR7 function in hippocampal slices (Macek et al., 1998), disrupts CaM binding to this fusion protein (Nakajima et al., 1999). Although PKA has also been shown to phosphorylate the carboxyl-terminal tail of mGluR7 and to regulate the function of mGluR7 in hippocampal slices (Cai et al., 2001), a role for kinases other than PKC in regulating CaM/mGluR7 interaction has not been shown. In the present study, we found that in addition to PKC, both PKA and PKG can phosphorylate ct-mGluR7 and regulate CaM/mGluR7 interactions. To gain further insight into the molecular mechanisms that regulate CaM binding to ct-mGluR7, we attempted to define the site at which these kinases phosphorylate mGluR7. Although multiple PKC, PKG, and PKA consensus sequence sites are found within the mGluR7 carboxyl terminus, our data combined with previous studies indicate that each of these kinases phosphorylates the ct-mGluR7 fusion protein at a single serine residue, Ser^{862} (Fig. 2) (Airas et al., 2001; Cai et al., 2001). Furthermore, the present results show that phosphorylation of Ser^{862} is required for PKC-, PKA-, and PKG-mediated inhibition of CaM binding (Fig. 3). Therefore, Ser^{862} seems to be an important determinant, not only for second-messenger kinase-mediated phosphorylation of ct-mGluR7 but also for regulation of CaM binding to mGluR7. Indeed, a recent report demonstrates that mutation of this serine residue to glutamate, which mimics the phosphorylation state, markedly reduces CaM binding, whereas mutation of the same residue to alanine does not affect CaM binding (Airas et al., 2001). Together, these studies suggest that inhibition of CaM binding by phosphorylation of Ser^{862} may represent a general mechanism for second-messenger–dependent kinases to regulate mGluR7 function.

We next sought to directly address the functional conse-

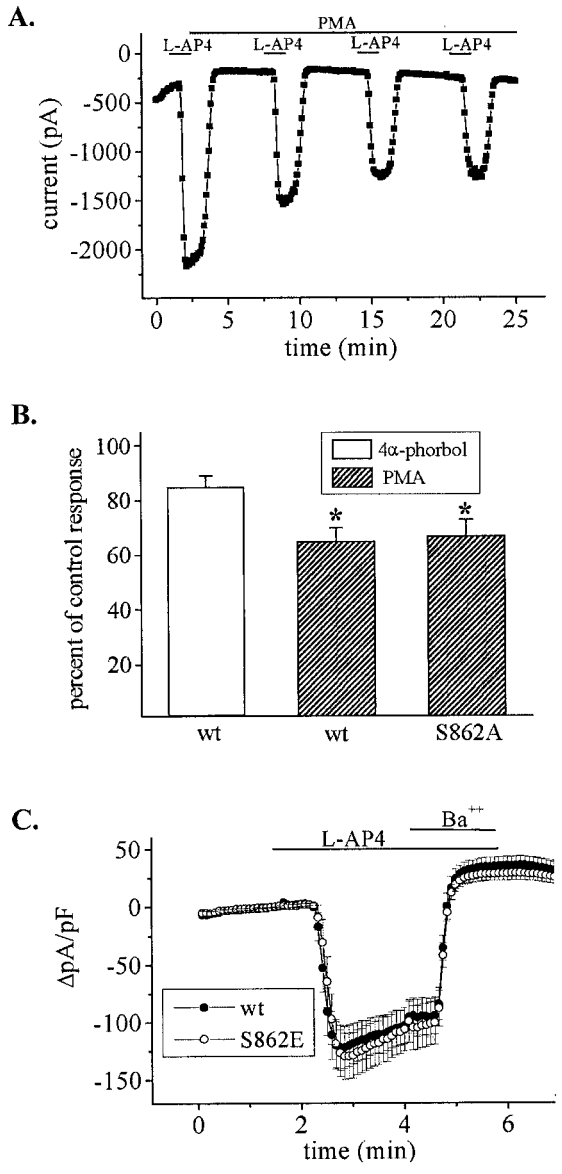

Fig. 8. Mutation of serine 862 has no effect on PMA-mediated attenuation of mGluR7 signaling. A, mutant full-length mGluR7 containing a single S862A point mutation was transfected into HEK cells stably expressing GIRK 1/2. Whole-cell patch-clamp of individual cells was performed as described in the legend to Fig. 5. Baseline current was recorded in buffer alone for 1 min before the application of buffer plus 1 mM L-AP4 for 1.5 min. Cells were then treated in buffer plus PMA (10 nM) before repeated applications of L-AP4 at 5 min intervals. Data from one representative cell are shown. B, the maximal L-AP4 induced current was calculated for each drug application and responses in the presence of the indicated phorbol were calculated as a percentage of the response obtained in perfusion buffer alone (control). Data represent the effect of phorbol after 12 min of phorbol treatment. Combined data are reported as a percentage of the control response for the indicated wild-type (wt) or mutant receptor and are mean ± S.E. Control responses were 1418 ± 223 pA for 4α-phorbol–treated wild-type receptor, 1339 ± 174 pA for the PMA-treated wild-type receptor, and 1527 ± 97 pA for the S862A mutant receptor. *, $p < 0.05$, different from 4α-phorbol effect on wild-type mGluR7, $n = 9$ to 11 for each condition. C, HEK cells stably expressing GIRK 1/2 were transiently transfected with either wild-type mGluR7 or mGluR7 S862E cDNA. Mean current activated by a 250-ms step to −100 mV from a holding potential of −45 mV was recorded from individual cells every 5 s. Baseline current was obtained for 1 min before the application of L-AP4 (1 mM) and $BaCl_2$ (1 mM) as indicated. Recordings from either wild-type (wt) (●) or S862E (○) transfected cells were combined and are mean ± S.E. from eight cells for each condition. Current is plotted as the change in pA/pF compared with baseline current.

quence of second-messenger–dependent kinase-mediated inhibition of CaM binding to mGluR7. For these studies, we employed two strategies. In the first, the ability of PKC to regulate the signaling of both a wild-type mGluR7 and mGluR7 S862A was determined. Although PKC inhibits CaM binding to wild-type mGluR7, it has no effect on the ability of mGluR7 S862A to interact with CaM (Fig. 3). Therefore, these studies directly address the role that CaM binding plays in the ability of a second-messenger–dependent kinase to inhibit mGluR7 coupling to GIRK channels. The second strategy used was to directly compare the functional response of a mutant mGluR7 that mimics the phosphorylated state and does not bind CaM, mGluR7 S862E, to wild-type mGluR7. The results of these studies indicate that second-messenger–dependent kinase-mediated regulation of mGluR7 coupling to GIRK channels is not dependent on the regulation of CaM biding to mGluR7 and suggest that CaM binding is not a requirement for mGluR7 coupling to GIRK channels.

Using a similar functional paradigm (mGluR7 coupling to GIRK in transfected HEK cells), other investigators have reported that CaM binding is required for mGluR7 function. These investigators designed a mutant mGluR7 in which amino acids 864 to 876 were deleted from the carboxyl terminus to disrupt CaM binding. Although guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding experiments indicated that the coupling of this mutant to G protein α subunits was similar to wild-type receptor (Dev et al., 2001), the ability of the mutant receptor to activate GIRK currents was markedly reduced (O'Connor et al., 1999). These findings, coupled with the demonstration that CaM binding to the ct-mGluR7 promotes the dissociation of purified G protein βγ subunits from ct-mGluR7, indicated that CaM binding specifically regulated the release of βγ from mGluR7 and allowed for βγ-mediated activation of GIRK currents (O'Connor et al., 1999; Dev et al., 2001). However, the deletion of a 13-amino acid segment in the mGluR7 carboxyl terminus could mediate disruption of mGluR7 interaction with multiple other unidentified effectors or regulators of mGluR7 signaling besides CaM. In the present study, mutation of a single serine residue (S862E) to disrupt CaM binding may allow for a more specific targeting of mGluR7/CaM interactions, thus providing a different view of the functional relevance of CaM binding to mGluR7 in HEK cells. Recent studies indicate that the mGluR7 S862E point mutant also inhibits βγ binding (El Far et al., 2001), which in theory could allow for enhanced βγ release upon GTP binding to G protein α subunits that offsets the effect of this mutation to disrupt CaM binding. However, our conclusion that CaM binding is not the only determinant for mGluR7 to activate GIRK currents is supported by studies with PKC. Under conditions in which CaM binding to mGluR7 is not disrupted by PKC (by mutation of serine 862 to alanine), PKC is able to attenuate the coupling of mGluR7 to GIRK channels. These studies demonstrate that a strict relationship between CaM binding and mGluR7-mediated activation of GIRK channels does not exist in HEK cells and support the conclusion that CaM interaction with mGluR7 is not the only determinant for this functional response.

Given these conclusions, the functional significance of CaM binding to mGluR7 remains unclear. However, the present studies are limited to the examination of CaM binding and phosphorylation of mGluR7 fusion proteins in vitro and func-

tional responses in a heterologous expression system. These conditions probably do not fully recapitulate the signaling events as they occur in neurons. Therefore, these studies do not fully address the role of receptor phosphorylation and CaM binding in the regulation of mGluR7 coupling to effectors in vivo. Given the role that mGluR7 plays in regulating synaptic transmission and the changes in intracellular calcium concentrations that occur during synaptic transmission, CaM interaction with mGluR7 is likely to have an important role in shaping the overall activity of glutamatergic synapses. A growing appreciation of the multiplicity of effectors regulated by the G protein-coupled receptor family (Hall et al., 1999) raises the possibility that CaM binding may regulate other as-yet-unknown mGluR7 signaling pathways. Indeed, recent evidence suggests that in addition to the known inhibition of adenylyl cyclase, mGluR7 can couple to the activation of phospholipase C as well (Perroy et al., 2000). Further study may reveal novel signaling pathways for mGluR7 and roles for CaM in the activation or regulation of these pathways. Interestingly, a similar interaction of CaM with the carboxyl terminus of other group III mGluRs (El Far et al., 2001) as well as mGluR5 has previously been reported. For mGluR7 and mGluR5, the interaction with CaM is regulated by the activity of PKC (Minakami et al., 1997). Although speculative, these parallels between interaction with a group I mGluR and a group III mGluR raise the possibility that CaM activates or regulates a common, unidentified pathway shared by both mGluR5 and mGluR7. As future studies further define the signaling pathways used by mGluR5 and mGluR7, it will be important to determine the role that CaM plays in regulating these functions.

These studies also indicate that the ability of second-messenger–dependent kinases to regulate mGluR7 coupling to GIRK channels is not dependent on phosphorylation of mGluR7 at Ser^{862}. Although potential PKC-mediated effects directly at the level of the G protein or the GIRK channel itself provides a plausible explanation for the inhibition of mGluR7 (S862A) signaling, several lines of evidence argue against this. A similar functional paradigm has been used to demonstrate that a mutation of a single serine residue in the CB1 cannabinoid receptor can reverse PKC-mediated inhibition of CB1 receptor coupling to GIRK currents in AtT-20 cells, which express GIRK1 and GIRK2, the same GIRK subunits used in this study (Garcia et al., 1998). Furthermore, when this same PKC-insensitive CB1 receptor is overexpressed in the HEK cells used in the present study, PMA has no effect on the ability of this receptor to couple to the activation of GIRK current (Fig. 7). Together, these findings suggest that PKC-mediated regulation of mGluR7 signaling occurs at the level of the receptor but the molecular determinants for PKC-mediated inhibition of mGluR7 function remain to be defined.

The present findings, coupled with previous reports (Airas et al., 2001; Cai et al., 2001), demonstrate that multiple kinases are able to phosphorylate mGluR7a at Ser^{862}. PKC has also been shown to phosphorylate the carboxyl-terminal tails of mGluR7b, mGluR8a, mGluR8b, and mGluR4a. All of these receptors contain a conserved serine corresponding to position 862 in mGluR7a (Airas et al., 2001). In addition, mutational and biochemical analysis demonstrates that PKA phosphorylates mGluR7a, mGluR4a, and mGluR8a at the same conserved serine residue corresponding to serine 862 of mGluR7a. PKA also phosphorylates mGluR2 and mGluR3 at a single serine residue in the same region of the carboxyl-terminal tails of these receptors (Cai et al., 2001; Schaffhauser et al., 2000). Together, these studies implicate an important functional role for this serine in the regulation of mGluR function by second-messenger–dependent kinases. Indeed, for mGluR2, mutation of the receptor at this single serine, Ser^{843}, almost completely reverses the PKA-mediated inhibition of receptor function (Schaffhauser et al., 2000). However, the present results indicate that Ser^{862} of mGluR7 is not the sole determinant for second-messenger–dependent kinase-mediated regulation of mGluR7 activation of GIRK. These findings raise the possibility that phosphorylation of other serine residue(s), either alone or in concert with Ser^{862}, is required for kinase-mediated inhibition of mGluR7 function. Importantly, phosphorylation of serine or threonine residues present in the intracellular loops of mGluR7 has not been addressed and could potentially contribute to the overall effect of second-messenger–dependent kinases to regulate mGluR7 as has been shown for PKC-mediated regulation of mGluR5 (Gereau and Heinemann, 1998). In summary, further studies to define the site(s) that regulate second-messenger–dependent kinase-mediated inhibition of mGluR7 signaling will provide important insights into the function of this receptor as well as the interrelationship between second-messenger–dependent kinases, CaM and mGluR7.

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