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Protein Kinase Cγ Associates Directly with the GluR4 α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionate Receptor Subunit

EFFECT ON RECEPTOR PHOSPHORYLATION*

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Ionotropic glutamate receptors mediate the majority of excitatory synaptic transmission in the brain and are thought to be involved in learning and memory formation. The activity of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptors can be regulated by direct phosphorylation of their subunits, which affects the electrophysiological properties of the receptor, and the receptor association with numerous proteins that modulate membrane traffic and synaptic targeting of the receptor. In the present study we investigated the association of protein kinase C (PKC) γ isoform with the GluR4 AMPA receptor subunit. PKCγ was co-immunoprecipitated with GluR4 AMPA receptor subunit in rat cerebellum and in cultured chick retina cell extracts, and immunocytochemistry experiments showed co-localization of GluR4 and PKCγ in cultured chick retinal neurons. Pull-down assays showed that native PKCγ binds the GluR4 C-terminal membrane-proximal region, and recombinant PKCγ was retained by GST-GluR4 C-terminal fusion protein, suggesting that the kinase binds directly to GluR4. Furthermore, GST-GluR4 C-terminal protein was phosphorylated on GluR4 Ser-482 by bound kinases, retained by the fusion protein, including PKCγ. The GluR4 C-terminal segment that interacts with PKCγ, which lacks the PKC phosphorylation sites, inhibited histone H1 phosphorylation by PKC, to the same extent as the PKC pseudosubstrate peptide 19–31, indicating that PKCγ bound to GluR4 preferentially phosphorylates GluR4 to the detriment of other substrates. Additionally, PKCγ expression in GluR4 transfected human embryonic kidney 293T cells increased the amount of plasma membrane-associated GluR4. Our results suggest that PKCγ binds directly to GluR4, thereby modulating the function of GluR4-containing AMPA receptors.

Glutamate is the major excitatory neurotransmitter in the central nervous system. Ionotropic glutamate receptors are divided into three groups according to their pharmacological and electrophysiological characteristics: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)[1] receptors, kainate receptors, and *N*-methyl-D-aspartate receptors (reviewed in Ref. 1). AMPA-type glutamate receptors are oligomeric structures, formed by the assembly of four subunits (GluR1–4; Refs. 2–4), and their diversity is further increased by post-transcriptional modifications such as alternative splicing and RNA editing (reviewed in Ref. 1).

GluR4-containing AMPA receptors are specifically expressed in neurons and glia in several regions of the central nervous system (5–10), where they are responsible for signal transmission at high rates (11). In the rat hippocampus, GluR4 subunits are expressed early in development, and specifically mediate synaptic delivery of AMPA receptors at early stages (10). GluR4-containing AMPA receptors are delivered to hippocampal synapses by spontaneous activity, a mechanism that seems to be subunit specific (10). In fact, targeting of AMPA receptors to the post-synaptic membrane was described to be specifically mediated by the subunit composition of the receptors (12).

Targeting of AMPA receptors to the postsynaptic membrane of excitatory synapses is thought to be mediated through interaction of the C termini of AMPA receptor subunits with scaffolding proteins. The AMPA receptor-interacting proteins include PDZ (for PSD-95, Disc Large, and ZO–1) domain-containing proteins, like glutamate receptor-interacting protein (GRIP) or AMPA-binding protein, protein interacting with protein kinase C 1 (PICK1), syntenin and synapse-associated protein 97 (SAP97), and proteins lacking a PDZ domain, like stargazin and neuronal activity-regulated pentraxin (Narp; reviewed in Ref. 13). GluR4 AMPA receptor subunit was reported to associate with stargazin, GRIP, and syntenin (14, 15). The plasma membrane protein stargazin is believed to mediate AMPA receptor targeting to the membrane surface (14), whereas GRIP was proposed to play a role in receptor stabilization at synapses (16). The role of syntenin, which was also described to interact with syndecans (17), in addition to GluR1–3 and GluR2c, is not known. None of the interactions described for GluR4 so far can account for its specific delivery to hippocampal synapses following spontaneous synaptic activity.

AMPA receptors are known to be regulated by protein phosphorylation (reviewed in Ref. 18). AMPA receptor phosphorylation modulates channel conductance (19), peak open probability of the receptor (20), interaction with PDZ domain-containing proteins (21, 22), clustering (23), and synaptic

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[1] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; PKC, protein kinase C; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase; HEK, human embryonic kidney; GRIP, glutamate receptor-interacting protein; PKA, cAMP-dependent protein kinase.

delivery of the receptors (24). Several phosphorylation sites have been mapped in the C termini of AMPA receptor subunits, including GluR4. GluR4 can be phosphorylated on Ser-482 by PKA, PKC, and calcium/calmodulin-dependent protein kinase II and on Thr-830 by PKC (25). In hippocampal slices, PKA activation by spontaneous activity is necessary and sufficient for delivery of GluR4-containing receptors to postsynaptic sites (26). Additionally, PKC activation increases Ca^{2+} influx through activated AMPA receptor channels in cultured chick retinal neurons (27), where GluR4 is the main AMPA receptor subunit expressed (28).

PKC has been implicated in a variety of neuronal functions, including modulation of ion channel activity and synaptic transmission (reviewed in Ref. 29). PKCγ expression is developmentally regulated, and PKCγ is expressed postnatally in the rat brain (30), playing a role in both long term potentiation and in learning and memory (31–33).

Because GluR4 AMPA receptor subunit is phosphorylated and its phosphorylation may mediate synaptic delivery (26), early in development, we studied the biochemical interaction of GluR4 with PKCγ. Our work shows that PKCγ interacts with GluR4 AMPA receptor subunit, both in rat brain and in chick retina cultured neurons, and that bound PKCγ is able to preferentially phosphorylate GluR4 on Ser-482, relatively to other substrates. Furthermore, co-transfection of PKCγ with GluR4 in human embryonic kidney (HEK) cells increases GluR4 subunit surface expression. Together these results indicate that the association between PKCγ and GluR4 plays a role in regulating the function of GluR4-containing AMPA receptors.

EXPERIMENTAL PROCEDURES

Materials—Trypsin was purchased from Invitrogen (Paisley, Scotland), and basal Eagle's medium, penicillin, and streptomycin were obtained from Sigma (Madrid, Spain). PGEX4T-2 and pGEX-1λT vectors, glutathione-Sepharose 4B, protein A-Sepharose CL-4B, anti-GST antibody, alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies, and [γ-^{32}P]ATP, were all obtained from Amersham Biosciences (Uppsala, Sweden). Rabbit anti-goat alkaline phosphatase-conjugated antibody was from Zymed Laboratories Inc. (San Francisco, CA). Fetal calf serum was from Biochrom KG (Berlin, Germany), the pET23b vector was from Novagen (Madison, WI), the pBK-CMV vector was from Stratagene (Amsterdam, Netherlands), and nickel-nitrilotriacetic acid-agarose was from Qiagen (Hilden, Germany). Isopropyl-1-thio-β-galactopyranoside was from Promega (Madison, WI). Histone H1, phorbol 12-myristate 13-acetate (PMA), and the PKC pseudosubstrate peptide 19–31 were from Calbiochem (San Diego, CA). Complete Mini protease inhibitor mixture and microporous polyvinylidene difluoride membranes were obtained from Roche Diagnostics GmbH (Switzerland, Basel). UltraLink™ Plus immobilized streptavidin gel and EZ-Link™ Sulfo-NHS-SS-Biotin were from Pierce. The rabbit polyclonal anti-GluR4 antibody was purchased from Chemicon (Temecula, CA), and the mouse monoclonal anti-PKCγ, anti-PKCε, and anti-PKAcα antibodies were from Transduction Laboratories (Lexington, KY). The anti-rabbit secondary antibody conjugated to Alexa 488 and the Texas Red-conjugated anti-mouse antibody were from Molecular Probes (Leiden, Netherlands).

Chick Retinal Cultures—Monolayer primary cultures of chick retina amacrine-like cells were prepared from 8-day-old chick embryos as previously described (34, 35). After treatment with trypsin (0.1%, 15 min, 37 °C) in Hanks' balanced salt solution, the cells were plated on tissue culture dishes (at a density of 1.35×10^6 cells/cm^2) for cell extract preparation, or on glass coverslips (at a density of 0.75×10^6 cells/cm^2) for immunocytochemistry. Chick retina amacrine-like neurons were maintained in basal Eagle's medium, buffered with 25 mM HEPES and 10 mM $NaHCO_3$, pH 7.4, and supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were kept at 37 °C in a humidified incubator of 5% CO_2 and 95% air, for 5 days.

Recombinant Proteins—cDNA fragments coding for the intracellular C-terminal domain of GluR4, or fractions of the C-terminal domain, were amplified by PCR, using GluR4 cDNA as a template. PCR products were subcloned into the pGEX4T-2 vector (via *Bam*HI and *Eco*RI sites for the whole C-terminal length; via *Bam*HI and *Sal*I sites for the truncated constructs) or into the pGEX-1λT vector (*Bam*HI and *Eco*RI sites, for the constructs coding amino acids 815–828 and amino acids 829–882 of GluR4). The entire coding sequence of PKCγ was amplified by reverse transcription-PCR from total RNA isolated from rat brain cortex, using the specific primers 5′-agcagctagcatggcgggtct-3′ and 5′-ccgctcgagcatgacgggcacag-3′, which include the restriction sites for *Nhe*I and *Xho*I, respectively. PKCγ cDNA was subcloned into pBK-CMV vector restriction endonuclease sites *Nhe*I and *Xho*I (for HEK 293T cell transfection) or a His tag was added to the C terminus of PKCγ by subcloning PKCγ cDNA in frame into restriction endonuclease sites *Nhe*I and *Xho*I in the pET23b vector. Recombinant proteins were expressed in BL21 *Escherichia coli* transformed with the constructs described above. Bacteria grown to A_{600} = 0.8 were induced with isopropyl-1-thio-β-galactopyranoside (100 μM) for 2 h, and then lysed with phosphate-buffered saline (PBS) containing 1% Triton X-100 and a protease inhibitor mixture. The cells were sonicated and shaken for 30 min at 4 °C, and the insoluble fraction was then removed by centrifugation at 12,000 × *g*, for 10 min at 4 °C. GST fusion proteins were purified by glutathione-Sepharose affinity chromatography, and His-tagged PKCγ was purified using nickel-nitrilotriacetic acid-agarose according to the protocol of the manufacturers. Recombinant proteins were dialyzed overnight against Tris-buffered saline (TBS).

Immunocytochemistry—Chick retinal cells were kept in culture for 5 days and were then fixed with 2% paraformaldehyde for 30 min at room temperature. Cells were washed in PBS after fixation, permeabilized with 0.3% Triton X-100 for 10 min at 4 °C, and blocked in 0.2% bovine gelatin in PBS for 1 h at room temperature. Cells were incubated with a rabbit polyclonal anti-GluR4 antibody (10 μg/ml) and a mouse monoclonal anti-PKCγ (5 μg/ml) antibody for 1 h, at room temperature. Neurons were washed with 0.1% bovine gelatin in PBS with 0.1% Tween 20 and incubated with anti-rabbit secondary antibody conjugated with Alexa 488 and Texas Red-conjugated anti-mouse antibody. Images were obtained with a Zeiss confocal microscope.

Extract Preparation and Immunoprecipitation—Cultured chick retinal neurons were washed with ice-cold PBS, scraped with lysis buffer (20 mM Tris, 2 mM EGTA, 2 mM EDTA, and a protease inhibitor mixture) containing 1% Triton X-100, sonicated, and centrifuged at 1000 × *g*, for 10 min at 4 °C. The supernatants containing soluble proteins were used to immunoprecipitate the GluR4 AMPA receptor subunit. Rat cerebellum membranes were prepared according to the procedure described by Luo and colleagues (36). Membrane proteins were then diluted 1:1 with lysis buffer containing 1% Triton X-100 (for Triton X-100 extracts), or with lysis buffer containing 1% Triton X-100 and 2% SDS, and boiled for 30 s (for SDS extracts), to disassemble oligomeric receptor complexes. These samples were diluted 7 times with lysis buffer containing 1% Triton X-100 before immunoprecipitation. GluR4 and GluR1 immunoprecipitates were obtained by incubation of 2.5 μg of anti-GluR4 polyclonal antibody with chick retina extracts, or membranes obtained from rat cerebellum, and by incubation of 1 μl of GluR1 polyclonal antibody (a kind gift from Dr. Richard Huganir) with rat cerebellum membranes, overnight at 4 °C. These samples were then incubated for 90 min with 100 μl of protein A-Sepharose beads (50%) and extensively washed (twice with TBS containing 1% Triton X-100, twice with TBS containing 1% Triton X-100 and 0.5 M NaCl, and twice more with TBS). The immunoprecipitated proteins were eluted by boiling in 1× Laemmli sample buffer and were separated by SDS-PAGE.

GST Binding Assays—Extracts of cultured chick retina cells were prepared as described above. Whole rat brain extracts were prepared by homogenizing the tissue in lysis buffer with 1% Triton X-100 followed by centrifugation at 1000 × *g*, for 10 min at 4 °C. The resulting supernatant was re-centrifuged at 1000 × *g*, for 10 min at 4 °C, and the soluble brain proteins were used for binding studies. Ten μg of fusion protein was incubated with chick retinal extracts, rat brain homogenates, or recombinant His-tagged PKCγ overnight at 4 °C. The mixture was incubated with 50% glutathione-Sepharose beads for 30 min at 4 °C. Beads were washed extensively with TBS containing 1% Triton X-100 and 0.5 M NaCl, and binding proteins were eluted with 1× Laemmli sample buffer. Samples were analyzed by Western blotting.

Gel Electrophoresis and Immunoblotting—Samples were resolved by SDS-PAGE in 12% acrylamide gels. For immunoblot analysis, proteins were transferred onto a polyvinylidene difluoride membrane by electroblotting (40 V, overnight, at 10 °C). The membranes were blocked for 45 min with 5% (w/v) nonfat dry milk plus 0.1% (v/v) Tween 20 in TBS, and probed during 1 h at room temperature with anti-PKCγ (0.05 μg/ml), anti-PKCε (0.05 μg/ml), anti-GluR4 (0.5 μg/ml), anti-GluR1 (1:5000), anti-GST (1:2000), or anti-PKAcα (0.25 μg/ml) antibodies. Following several washes, the membranes were incubated for 1 h, at room temperature, with alkaline phosphatase-conjugated goat anti-mouse sec-

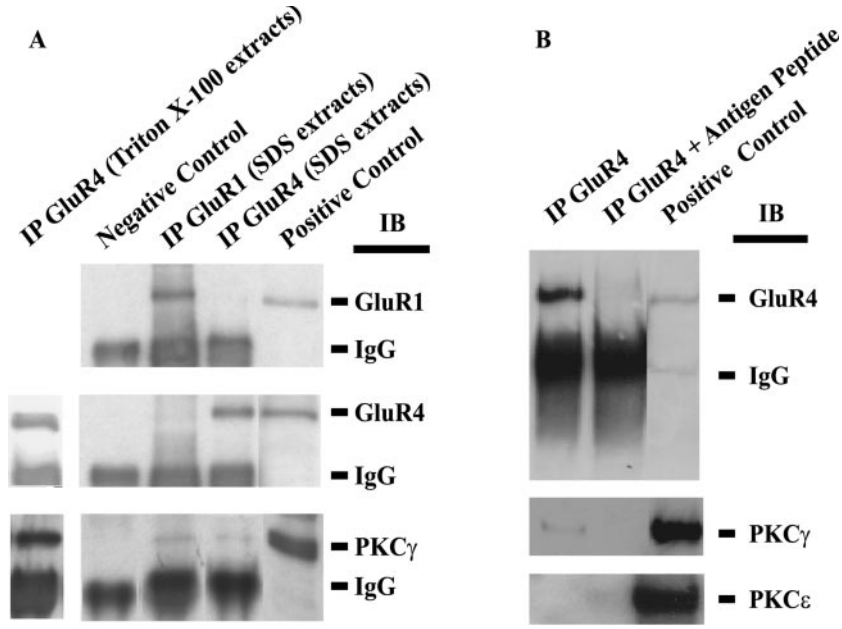

FIG. 1. **PKCγ associates with GluR4 *in vivo*.** *A*, rat cerebellum membrane preparation was solubilized with 0.5% Triton X-100 (Triton X-100 extracts) or with 0.5% Triton X-100 and 1% SDS (SDS extracts), and the extracts were used to immunoprecipitate GluR4 and GluR1. The immunoprecipitates were then analyzed for the presence of GluR1, GluR4, and PKCγ. *B*, total homogenates of cultured chick retina neurons were solubilized with 1% Triton X-100 and were immunoprecipitated either with anti-GluR4 antibody or with the same antibody with antigen pre-absorption. The immunoprecipitated complex was resolved by SDS-PAGE and probed with anti-GluR4, anti-PKCγ, and anti-PKCε antibodies. Whole rat brain lysates were used as a positive control in immunoblot staining (*A* and *B*).

ondary antibody for anti-PKCγ, anti-PKCε, and anti-PKAc staining, with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody for anti-GluR1 and anti-GluR4 antibodies, or with alkaline phosphatase-conjugated rabbit anti-goat secondary antibody for anti-GST staining. The blots were washed again, and immunostaining was visualized by the enhanced chemifluorescence method on a Storm 860 gel and blot imaging system (Amersham Biosciences).

Phosphorylation Assays—GST pull-down assays were performed using GluR4 C-terminal fragments fused to GST and whole rat brain extracts. GST fusion proteins and interacting proteins were pulled down with glutathione-Sepharose beads, which were washed extensively with TBS containing 1% Triton X-100 and 0.5 M NaCl, and incubated in a phosphorylation buffer containing 100 mM Hepes, 20 mM $MgCl_2$, 250 μM ATP, 5 μCi of [γ-^{32}P]ATP, 200 μM $CaCl_2$, and phosphatidylserine/diacylglycerol (50 μg/ml/5 μg/ml) for 30 min, at 30 °C. The bound proteins were eluted from beads with 1× Laemmli sample buffer and separated by SDS-PAGE in 12% acrylamide gels. Polyacrylamide gels were stained with Coomassie Blue R, destained, and then extensively washed with ultrapure H_2O and 10% glycerol. Gels were then dried for 45 min, at 80 °C, exposed to a storage Phosphor Screen (Eastman Kodak Co.) and analyzed on a Storm 860 gel and blot imaging system.

Histone Phosphorylation Assays—Histone H1 (20 μg) was incubated with purified protein kinase C from rat brain, in a phosphorylation buffer containing 100 mM Hepes, 20 mM $MgCl_2$, 250 μM ATP, 5 μCi of [γ-^{32}P]ATP, 200 μM $CaCl_2$, and phosphatidylserine/diacylglycerol (50 μg/ml/5 μg/ml), in the presence of equimolar amounts of GST (8.0 μg), GST fused to GluR4 C-terminal fragments corresponding to amino acids 815–828 (8.6 μg) or to amino acids 815–838 (9.0 μg), or in the presence of the PKC pseudosubstrate peptide 19–31 (RFARKGALRQKNV; 0.62 μg), for 30 min, at 30 °C. Reactions were stopped with Laemmli sample buffer, and the samples were separated by SDS-PAGE followed by autoradiography (as described above). Histone H1 phosphorylated bands were quantified using ImageQuant (Amersham Biosciences) software and plotted.

Transfection of Cultured HEK 293T Cells—HEK 293T cells maintained at 37 °C in a humidified incubator of 5% CO_2, 95% air were transiently transfected with 10 μg of cDNA (pBK-CMV- PKCγ and/or pGW1-GluR4), using the calcium phosphate coprecipitation method, as previously described (25).

Receptor Surface Expression—Cultured HEK 293T cells were stimulated with 200 nM PMA, for 10 min, 48 h after transfection. After stimulation the cells were washed with culture medium and then incubated at 37 °C in a humidified incubator of 5% CO_2, 95% air for 3 h. Cells were washed twice with PBS/Ca^{2+}/Mg^{2+} (PBS supplemented with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$) and incubated with 1 mg/ml EZ-Link™ Sulfo-NHS-SS-Biotin in PBS/Ca^{2+}/Mg^{2+} for 30 min at 4 °C. Cells were then washed twice with PBS/Ca^{2+}/Mg^{2+} supplemented with 0.1% bovine serum albumin, and once with PBS/Ca^{2+}/Mg^{2+} and scraped with lysis buffer containing 1% Triton X-100 and 0.1% SDS. The cells were then sonicated and centrifuged at 1000 × *g*, for 10 min at 4 °C. Aliquots of the supernatants containing soluble proteins (4% of total) were used to determine expression of total GluR4 and the remaining samples incubated with UltraLink™ Plus immobilized streptavidin gel for 2 h at 4 °C (according to the protocol of the manufacturers). Streptavidin beads were extensively washed, twice with TBS containing 1% Triton X-100, once with TBS containing 1% Triton X-100 and 0.5 M NaCl, and once more with TBS. The biotinylated proteins were eluted by boiling in 1× Laemmli sample buffer and were analyzed by immunoblotting. The digital images were quantified using ImageQuant software (Amersham Biosciences). Surface receptor expression was determined from the surface biotinylated/total receptor ratio.

RESULTS

PKCγ Associates with GluR4 in Vivo—To examine whether PKCγ is associated with GluR4 *in vivo*, the interaction of PKCγ with GluR4 was tested in rat cerebellum and in primary chick retina cultures, where GluR4 is highly expressed (11, 28, 37). Immunoprecipitation of GluR4 from rat cerebellum membrane extracts or from cultured chick retina cells resulted in specific co-immunoprecipitation of PKCγ (Fig. 1). Pre-absorption of the GluR4 antibody with its antigen blocked the co-immunoprecipitation of PKCγ in the retina cell extracts (Fig. 1*B*), confirming the specificity of the association. In contrast, PKCε, which is also expressed in this preparation (28), did not co-immunoprecipitate with GluR4 AMPA receptor subunit (Fig. 1*B*). Rat cerebellum membrane extracts prepared in 1% SDS and boiled, to ensure disassembly of tetrameric AMPA receptor complexes (2–4), were also tested. Immunoprecipitation of either GluR4 or GluR1 from rat cerebellum membranes, solubilized in SDS, also specifically co-immunoprecipitated PKCγ (Fig. 1*A*). Immunocytochemistry experiments using an antibody that specifically recognizes GluR4 showed a punctuate distribution of GluR4-containing AMPA receptors, along the dendrites, in cultured chick retina cells (Fig. 2). Double labeling with anti-GluR4 and anti-PKCγ antibodies revealed that some of the GluR4 punctuate distribution is co-localized with PKCγ (Fig. 2).

Analysis of the PKCγ Binding Domain in GluR4—To map the amino acid segment in GluR4 AMPA receptor subunit that binds PKCγ, GST fusion proteins with the C-terminal domain of GluR4 were produced, because this region is the main intracellular domain of the receptor subunit (see Fig. 3*D*; Refs. 1, 38, and 39). GST fused to full-length GluR4 C terminus or to partial segments of GluR4 C terminus were used to pull down

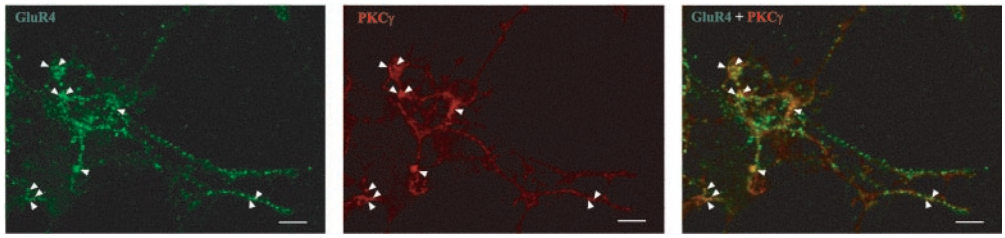

FIG. 2. **Co-localization of GluR4-containing AMPA receptors with PKCγ in cultured neurons.** Cultured chick retina cells were double-labeled with rabbit anti-GluR4 and mouse anti-PKCγ antibodies, followed by Alexa 488-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG. Merged images show a partial co-localization of GluR4 punctuate staining and PKCγ immunoreactivity. *Arrowheads* indicate areas of co-localization, and the *scale bar* represents 5 μm.

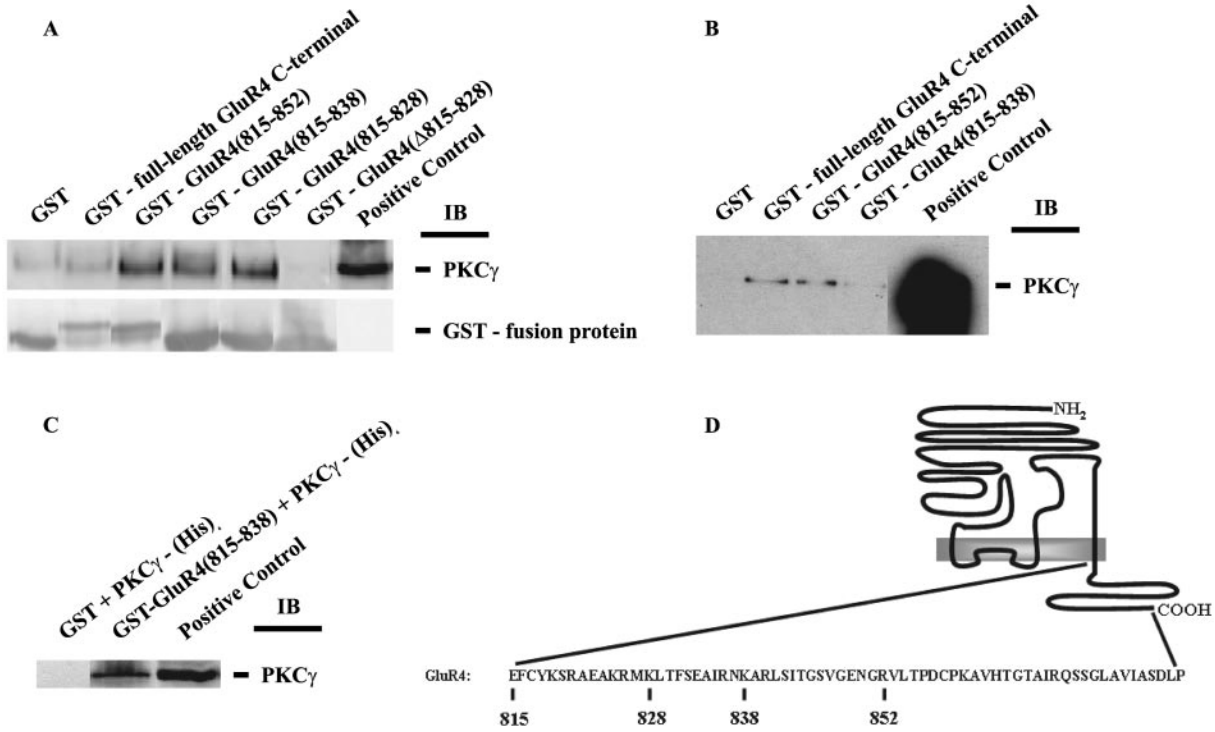

FIG. 3. **PKCγ interacts with GluR4 C-terminal sequence.** A rat brain extract (*A*), an extract of cultured chick retina neurons (*B*), or purified recombinant PKCγ (*C*) was incubated with GST fused to GluR4 C-terminal peptides (GST-full-length GluR4 C terminus or GST fused to GluR4 C-terminal amino acids 815–852 (GST-GluR4 (815–852)), 815–838 (GST-GluR4 (815–838)), 815–828 (GST-GluR4 (815–828)), or 829–882 (GST-GluR4(Δ815–828)), as indicated. Glutathione-Sepharose beads were used to pull down GST fusion proteins (detected with anti-GST antibody; Fig. 1*A*). Analysis of the pull-down samples with an anti-PKCγ antibody showed that PKCγ was present when the extracts were incubated with GST fusion proteins containing GluR4 C-terminal amino acids 815–828 (*A* and *B*). Experiments with recombinant PKCγ show direct interaction of recombinant PKCγ with GluR4 C-terminal fragment (*C*). *D*, topology and C-terminal sequence of GluR4 AMPA receptor subunit. Whole rat brain lysates were used as a positive control in immunoblot staining (*A–C*).

interacting proteins, present either in Triton X-100 solubilized rat brain homogenates (Fig. 3*A*) or cultured chick embryo retinal amacrine-like neurons (Fig. 3*B*). We found that PKCγ present both in the rat brain (Fig. 3*A*) and in the chick retina culture extracts (Fig. 3*B*) binds full-length GluR4 C terminus and the C-terminal fragments corresponding to amino acids 815–852 and 815–838. GST fused to the protein segment corresponding to amino acids 815–828 of GluR4 also bound PKCγ in GST pull-down assays using rat brain homogenates (Fig. 3*A*). However, the fusion protein lacking amino acids 815–828 was unable to bind PKCγ (Fig. 3*A*). This result suggests that the membrane-proximal region between amino acids 815 and 828 in the C terminus of GluR4 is important for the interaction with PKCγ.

Because there are several scaffolding proteins described to interact with glutamate receptors (reviewed in Ref. 40) and, in some cases, known to mediate the interaction of AMPA receptors with kinases and phosphatases (41), we investigated whether PKCγ can directly bind to the C terminus of GluR4 AMPA receptor subunit or whether an adaptor protein is required. Purified recombinant PKCγ with a C-terminal His tag was incubated with GST fused to the GluR4 C-terminal segment containing amino acids 815–838. Again, glutathione-Sepharose was used to pull down GST fusion proteins and the presence of PKCγ in these samples was analyzed by immunoblotting with a monoclonal anti-PKCγ antibody. As shown in Fig. 3*C*, recombinant PKCγ was detected in pull-down samples, indicating that it directly binds the C-terminal domain of GluR4.

Phosphorylation within GluR4-PKCγ Complexes—Previous work (25) showed that the GluR4 AMPA receptor subunit is phosphorylated by PKC at the C-terminal domain, mainly on Ser-482. Our results demonstrating that PKCγ binds directly to the GluR4 AMPA receptor subunit suggest that bound PKCγ may facilitate GluR4 AMPA receptor phosphorylation. To test this hypothesis, we performed GST pull-down assays from rat brain extracts and, after extensively washing beads, incubated immobilized GST fused to GluR4 C-terminal segments and

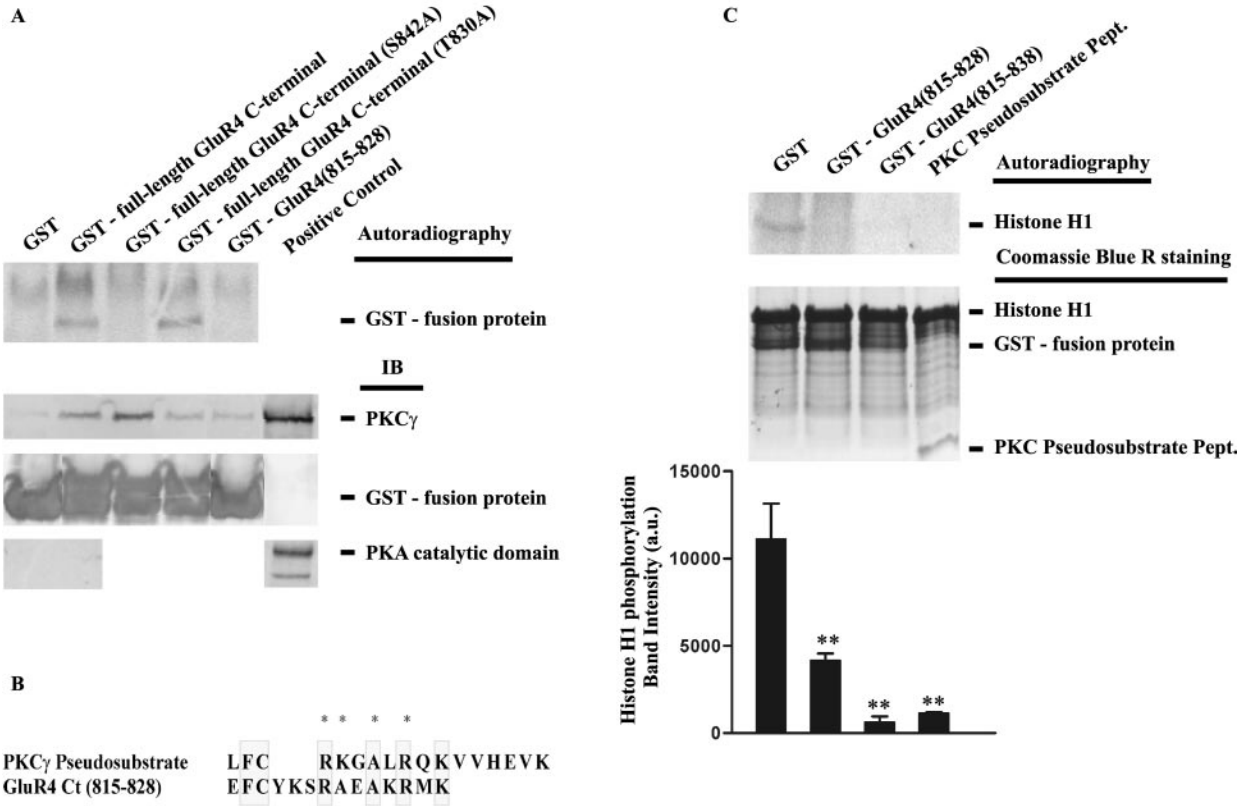

FIG. 4. **Phosphorylation within PKCγ-GluR4 complexes.** A rat brain extract was incubated with GST-GluR4 C-terminal proteins, and the fusion proteins, together with interacting proteins, were pulled down with glutathione-Sepharose beads. *A*, pull-down samples were incubated with [γ-^{32}P]ATP for 30 min at 30 °C in PKC phosphorylation buffer. Phosphorylated GST-GluR4 proteins are shown in the *upper panel*. PKCγ present in the pull-down samples was detected by Western blotting, and total GST fusion protein was labeled with an anti-GST antibody (*middle panels*). Western blotting analysis using a monoclonal antibody that recognizes the catalytic domain of PKA (PKAcα) showed that PKAcα was not present in GluR4 C-terminal complexes (*lower panel*). Whole rat brain lysates were used as a positive control. *B*, sequence alignment of PKCγ pseudosubstrate domain and GluR4 C-terminal amino acids 815–828. Homologous amino acids (*box*) and conserved pseudosubstrate amino acids (*) are indicated. *C*, histone H1 was incubated with [γ-^{32}P]ATP and purified protein kinase C, for 30 min at 30 °C, in the absence or presence of GST-GluR4 C-terminal proteins or the PKC pseudosubstrate peptide 19–31, as indicated. Samples were subjected to SDS-PAGE, and the gels were stained with Coomassie Blue R (*middle panel*) and autoradiographed (*top panel*), to detect histone phosphorylation. Histone H1 phosphorylated bands were quantified using ImageQuant software (Amersham Biosciences) and plotted (*bottom graphic*). Results are presented as means ± S.E. of three independent experiments. Statistical significance was determined by analysis of variance followed by Dunnett's test.

interacting proteins, including bound PKCγ (Fig. 4*A*, *middle blot*), in the presence of [γ-^{32}P]ATP. Eluted samples were then analyzed by SDS-PAGE, and autoradiography. GluR4 C-terminal fusion protein was phosphorylated by bound kinases (Fig. 4*A*, *upper panel*). The S842A GluR4 C-terminal fusion protein, which lacks the major GluR4 phosphorylation site, was not phosphorylated by bound kinases, but binds PKCγ (Fig. 4*A*). T830A GluR4-GST protein, lacking a minor PKC phosphorylation site on GluR4, was still phosphorylated in pull-down samples. Deletion of GluR4 C terminus after amino acid 828 did not disrupt its binding to PKCγ, but eliminated the phosphorylation sites (Thr-830 and Ser-482) on GluR4 and therefore prevented its phosphorylation (Fig. 4*A*). Our results suggest that PKCγ bound to GluR4 phosphorylates Ser-482 in GluR4.

PKA was previously described to phosphorylate GluR4 on Ser-482 (25), and it has been reported to interact with GluR1, through A kinase anchor protein, via SAP97 (41). Therefore, to exclude the hypothesis of GluR4 phosphorylation by PKA eventually present in GluR4 complexes, we looked for the presence of PKA in pull-down samples. As shown in Fig. 4*A*, no PKA immunoreactivity was detected in GluR4 C-terminal complexes using an antibody against the catalytic fragment of PKAα (an ubiquitously expressed PKA isoform).

The GluR4 C-terminal segment that interacts with PKCγ is homologous to PKCγ pseudosubstrate sequence (Fig. 4*B*), and its interaction with PKCγ may mimic the pseudosubstrate interaction. In the activated state of the kinase, the pseudosubstrate is displaced from the catalytic groove that enables it to interact with substrates. GluR4, through its C-terminal membrane-proximal segment, which presents homology to the pseudosubstrate, might preferentially occupy the vacated catalytic site. To test this hypothesis, we tested the effect of PKC interaction with GluR4 C terminus on the kinase activity on another substrate. GluR4 C-terminal amino acids 815–828 and 815–838, which were shown to bind PKCγ, inhibited histone H1 phosphorylation by purified protein kinase C from rat brain to the same extent as the PKC pseudosubstrate peptide (Fig. 4*C*). This result supports the hypothesis that PKCγ bound to GluR4 C terminus preferentially phosphorylates GluR4, to the detriment of other substrates.

PKCγ Activation Increases GluR4 Surface Expression—It was recently shown that, early in development, rat hippocampal GluR4 Ser-482 phosphorylation by PKA is necessary and sufficient for GluR4-containing AMPA receptors delivery to synapses (26). Additionally, stimulation of GluR4-expressing HEK cells with PMA resulted in Ser-482 phosphorylation (25). To evaluate the contribution of GluR4 Ser-482 phosphorylation by PKCγ to the expression of GluR4-containing AMPA receptor at the plasma membrane, we transfected HEK 293T cells with GluR4, or co-transfected cells with GluR4 and PKCγ. Cells were then stimulated with 200 nM PMA for 10 min. Three

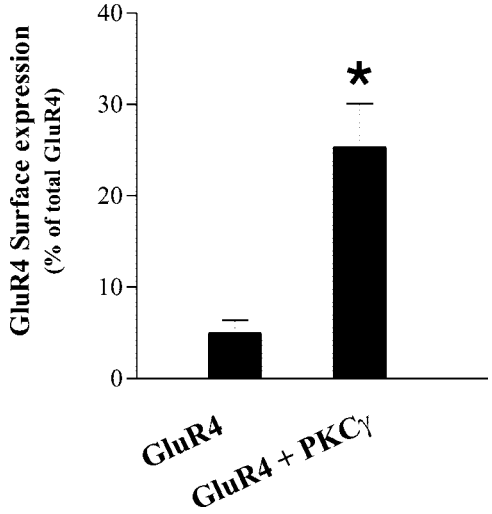

FIG. 5. **GluR4 surface expression in transfected HEK 293T cells.** Cultured HEK cells were transfected with GluR4 or co-transfected with GluR4 and PKCγ, as indicated. Cells were biotinylated following stimulation with 200 nM PMA, for 10 min, and surface *versus* total GluR4 bands were quantified using the ImageQuant software (Amersham Biosciences) and plotted. Results are presented as means ± S.E. of three independent experiments. Statistical significance was determined by the *t* test.

hours after the stimulus, cells were incubated with a biotinylation reagent that reacts with the extracellular domains of membrane proteins. Proteins extracted from HEK 293T cells were then incubated with streptavidin gel, which allowed the purification of biotinylated proteins. Surface and total GluR4 were compared using quantitative immunoblotting. Co-transfection of PKCγ with GluR4 resulted in increased GluR4 subunit surface expression following stimulation with PMA (Fig. 5), indicating that phosphorylation by PKC targets GluR4 to the plasma membrane.

DISCUSSION

Phosphorylation of AMPA receptors is critical in the control of synaptic function and plasticity (reviewed in Ref. 18). In this study we found that PKCγ associates with GluR4 AMPA receptor subunit *in vivo* and *in vitro*. Our results further suggest that this interaction localizes the kinase in close proximity to GluR4, facilitating receptor phosphorylation, and that phosphorylation by PKC targets the receptor to the plasma membrane.

Co-immunoprecipitation experiments showed that the AMPA receptor subunit GluR4 associates with the γ conventional isoform of PKC, both in rat cerebellum and in chick retinal cultures (Fig. 1, *A* and *B*). Additionally, PKCγ and GluR4 showed partial overlapping distributions in chick embryo retina cultures (Fig. 2). Recombinant and native PKCγ were retained by GST-GluR4 C terminus, and deletion analysis showed that the membrane-proximal region of GluR4 C terminus (conserved among AMPA receptor subunits), from amino acids 815–828, is crucial for the binding of GluR4 to PKCγ (Fig. 3, *A–C*). The corresponding sequence in GluR1 was described to interact with the protein 4.1N, the neuronal homologue of the erythrocyte membrane cytoskeletal protein 4.1 (42). PKCγ binding to the GluR4 C-terminal sequence may bring close together the kinase and its substrate, thereby influencing receptor phosphorylation.

In contrast to the direct interaction between GluR4 and PKCγ, reported here, AMPA receptors have been shown to associate with protein kinases mainly through adaptor proteins (reviewed in Ref. 18). PICK1, which interacts with PKCα, is now known to associate with GluR2/3 and GluR4c (43). PICK1 is co-localized with PKCα and AMPA receptors at excitatory synapses and was described to homo-oligomerize through its PDZ domain. In an heterologous expression system, PICK1 was shown to induce AMPA receptor clustering (44). PICK1 dimers may target PKCα to AMPA receptors, thus providing a mechanism for selective phosphorylation of AMPA receptors. GluR2 was determined to be phosphorylated on Ser-880 by PKC (22), and activation of this kinase increases phosphorylation of GluR2 Ser-880 and induces long term depression in the cerebellum (21, 45). Moreover, long term depression induction in cultured Purkinje cells resulted in Ser-880 phosphorylation and in a long lasting disruption of GluR2 clusters (23). PICK1 was also reported to form a complex with the mGluR7a metabotropic glutamate receptors and PKCα. In this case, PICK1 was shown to play an inhibitory role on PKCα phosphorylation of mGluR7a (46).

SAP97 binds GluR1 AMPA receptor subunit and was reported to be important for recruitment of PKA, PKC, and protein phosphatase PP2B through AKAP79/150. This protein forms a complex with SAP97 that directs PKA (41) or protein phosphatase PP2B (47) to GluR1, facilitating GluR1 Ser-845 phosphorylation or dephosphorylation. However, it has recently been reported that the interaction between GluR1 and SAP97 occurs predominantly in the biosynthetic and secretory pathway (48), raising the question of whether the kinase and phosphatase in the complex can regulate receptor activity at synapses.

Having established that GluR4 directly assembles with PKCγ, we searched for functional consequences of this interaction. Previous work showed that GluR4 is phosphorylated on Ser-482 by PKA, PKC, and calcium/calmodulin-dependent protein kinase II (25). Our results showed that GST fused to GluR4 C terminus pulled down PKCγ, and that there was phosphorylation of GluR4 Ser-482 within those complexes (Fig. 4*A*). In addition, incubation of the GluR4 C-terminal domain, which binds PKCγ, with histone H1 (1:3 molar ratio) and purified PKC inhibited histone phosphorylation (Fig. 4*C*). Sequence alignment of PKCγ pseudosubstrate domain and GluR4 C-terminal amino acids 815–828, the crucial peptide for PKCγ binding, shows sequence homology (Fig. 4*B*). This suggests that interaction of PKC with the GluR4 C-terminal region, through the catalytic domain of the kinase, may prevent histone H1 phosphorylation. PKC pseudosubstrate domain contains several basic residues, and it was suggested that this domain binds to an acidic sequence of the PKC catalytic domain (49, 50), distinct from the ATP-binding core and the phosphate transfer region. GluR4 may bind this region in PKC through its membrane-proximal segment, analogous to the pseudosubstrate, thereby positioning the phosphorylation site for preferential phosphorylation by PKC.

We have previously found that PKC up-regulates AMPA receptor activity in chick embryo retinal cultures, where GluR4 is the main AMPA receptor subunit expressed (27, 28). Recent work showed that in the rat hippocampus GluR4 Ser-482 phosphorylation by PKA, activated by spontaneous activity early in development, is necessary and sufficient for GluR4-containing AMPA receptors delivery to synapses (26). PKC also phosphorylates GluR4 Ser-482 in transfected HEK 293T (25). Our results show that PKCγ expression in GluR4 transfected HEK 293T cells increase GluR4 surface expression upon stimulation with PMA, when compared with PKCγ-deficient cells (Fig. 5). Our results argue for a role for anchored PKCγ in GluR4 receptor subunit phosphorylation and targeting to the plasma membrane.

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