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PKC Anchoring to GluR4 AMPA Receptor Subunit Modulates PKC-Driven Receptor Phosphorylation and Surface Expression

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Changes in the synaptic content of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)–type glutamate receptors lead to synaptic efficacy modifications, involved in synaptic plasticity mechanisms believed to underlie learning and memory formation. Early in development, GluR4 is highly expressed in the hippocampus, and GluR4-containing AMPA receptors are inserted into synapses. During synapse maturation, the number of AMPA receptors at the synapse is dynamically regulated, and both addition and removal of receptors from postsynaptic sites occur through regulated mechanisms. GluR4 delivery to synapses in rat hippocampal slices was shown to require protein kinase A (PKA)–mediated phosphorylation of GluR4 at serine 842 (Ser842). Protein kinase C (PKC) can also phosphorylate Ser842, and we have shown that PKCγ can associate with GluR4. Here we show that activation of PKC in retina neurons, or in human embryonic kidney 293 cells cotransfected with GluR4 and PKCγ, increases GluR4 surface expression and Ser842 phosphorylation. Moreover, mutation of amino acids R821A, K825A and R826A at the GluR4 C-terminal, within the interacting region of GluR4 with PKCγ, abolishes the interaction between PKCγ and GluR4 and prevents the stimulatory effect of PKCγ on GluR4 Ser842 phosphorylation and surface expression. These data argue for a role of anchored PKCγ in Ser842 phosphorylation and targeting to the plasma membrane. The triple GluR4 mutant is, however, phosphorylated by PKA, and it is targeted to the synapse in CA1 hippocampal neurons in organotypic rat hippocampal slices. The present findings show that the interaction between PKCγ and GluR4 is specifically required to assure PKC-driven phosphorylation and surface membrane expression of GluR4.

Key words: AMPA receptors, GluR4, phosphorylation, PKC, surface expression, synaptic delivery

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α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)–type ionotropic glutamate receptors mediate the majority of fast excitatory synaptic transmission in the brain and are believed to be involved in learning and memory formation. It has been shown that these receptors can be added to and removed from the postsynaptic membrane, resulting in changes in synaptic efficacy (1). These changes in synaptic strength are involved in synaptic plasticity mechanisms [long-term potentiation (LTP) and long-term depression (LTD)], which are believed to be the molecular basis of learning and memory (1,2).

The AMPA receptors are heterooligomeric structures formed by four subunits [GluR1-4; (3)]. The combination of different receptor subunits results in distinct trafficking properties of the AMPA receptors (4,5). GluR4-containing AMPA receptors exhibit fast currents and are expressed in several regions of the central nervous system (CNS) (6–11). In the hippocampus, GluR4 is expressed mainly in early postnatal development, and GluR4-containing AMPA receptors are delivered to the synapse by spontaneous activity (11). In this brain region, synaptic delivery of GluR4-containing AMPA receptors is dependent on protein kinase A (PKA) phosphorylation of the serine 842 (Ser842) residue located at the GluR4 C-terminal domain. The GluR4 phosphorylation at Ser842 is believed to relieve a retention interaction that blocks delivery of the receptor into synapses (12).

The details of the mechanism that regulates GluR4-containing AMPA receptor targeting to the synapse early in development, during synaptogenesis, is unknown; however, interactions with the C-termini of AMPA receptor subunits as well as protein phosphorylation are believed to play a role in receptor dynamics (13). GluR4 was described to interact with stargazin (14), 4.1N (15), protein kinase C (PKC) γ (16) and recently with α-actinin-1 and IQGAP-1 (17). The membrane protein stargazin is an AMPA receptor auxiliary subunit (18) and is believed to mediate synaptic trafficking of AMPA receptors by recruiting receptors from submembranous sites to the plasma membrane, and by associating with PDZ proteins to bring AMPA receptors to the synapse (14). Recently, stargazin was found to also modulate AMPA receptor kinetics (19,20). Stargazin is phosphorylated by Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and by PKC, and stargazin phosphorylation promotes synaptic trafficking of AMPA receptors and is required for LTP at hippocampal synapses (21). 4.1N, a protein that associates with the actin cytoskeleton, was shown to bind GluR4, and the association was

suggested to play a role in the receptor expression at the cell surface (15). α-Actinin-1 and IQGAP1 are also actin-binding proteins, which were shown to bind to the C-terminus of GluR4 at the region containing the Ser842 phosphorylation site (17). Phosphorylation of GluR4 disrupts the interaction with α-actinin-1, whereas the interaction with IQGAP-1 is preserved. The authors of the study suggest that α-actinin-1 retains GluR4 in intracellular pools, and that following synaptic activity and GluR4 phosphorylation, the interaction with α-actinin-1 is disrupted to release GluR4 to the synapse (17).

PKCγ is a CNS-specific PKC isoform shown to bind GluR4 AMPA receptor subunit at the C-terminal membrane-proximal domain (16). PKCγ was suggested to bind directly to GluR4, facilitating receptor phosphorylation at Ser842. Kinase targeting to specific subcellular microdomains is known to be necessary for signaling efficiency and specificity, and is, in many cases, accomplished by kinase interactions with protein partners. CaMKII interacts with various subunits of N-methyl-D-aspartate receptors (22); the interaction between CaMKII and NR2B can trap an active Ca^{2+}-independent form of CaMKII (23) and was recently shown to be required for different forms of synaptic enhancement (24). Moreover, A-kinase anchoring protein (AKAP)79/150-anchored PKA associates with GluR1 through the adaptor proteins SAP97 or PSD-95 and promotes phosphorylation of GluR1 at Ser845 (25). Disruption of the PKA–AKAP interaction is sufficient to cause a long-lasting reduction in synaptic AMPA receptors in cultured neurons and occludes synaptically induced LTD in hippocampal slices (26). To further clarify the role of PKCγ–GluR4 interaction in the delivery of GluR4-containing AMPA receptors to the plasma membrane, we studied the cell surface targeting of a mutated form of GluR4 protein, unable to bind PKCγ. Our results show that the PKCγ targeting to GluR4 is essential for PKCγ phosphorylation of Ser842 and GluR4 surface expression.

Results

Effect of PKC on phosphorylation and surface expression of native GluR4-containing AMPA receptors

In a previous study, we have shown that PKA activation in primary cultured chick retinal neurons increases the phosphorylation and surface expression of native GluR4-containing AMPA receptors (27). Using the same preparation, which expresses high levels of GluR4 (28), we tested whether PKC activation can trigger GluR4 phosphorylation and surface expression. After 5 days in culture, cells were stimulated for 10 min with 200 nM phorbol 12-myristate 13-acetate (PMA), and the phosphorylation of GluR4 was analysed using a phosphospecific antibody against GluR4 phosphorylated at Ser842 (12). We found that PMA stimulation of cultured chick retina neurons significantly increased (25.7 ± 2.3%) GluR4 Ser842 phosphorylation, when compared with the control situation (Figure 1A). To test whether PKC activation can lead to GluR4 surface expression, cells were stimulated with 200 nM PMA, for 10 min, and were then incubated with a biotinylation reagent. Biotinylated proteins were purified with a streptavidin gel and analysed by immunoblotting in order to quantify plasma membrane GluR4. Activation of PKC with PMA in chick retinal neurons significantly increased (22.5 ± 1.3%) membrane surface GluR4, when compared with the control condition (Figure 1 B). Moreover, cell treatment with PMA increased the phosphorylation of GluR4 at the plasma membrane (41.9 ± 10.8%, Figure 1C), whereas the phosphorylation of intracellular GluR4 remained unchanged (Figure 1D). These results suggest that PKC activation leads to phosphorylation of GluR4 Ser842 and targeting of GluR4-containing AMPA receptors to the plasma membrane. Accordingly, it was previously described that Ser842 phosphorylation is necessary and sufficient for GluR4 delivery to synapses early in development in the rat hippocampus (12).

Mapping the interaction between GluR4 and PKCγ

We have previously found that GluR4 interacts with PKCγ through the membrane-proximal C-terminal amino acid segment E815–K828 in GluR4. This segment shows sequence homology to the PKCγ pseudosubstrate domain, suggesting that the catalytic domain of PKCγ may bind to GluR4 through the amino acid segment 815–828, thereby positioning the phosphorylation site Ser842 for preferential phosphorylation by PKCγ (16). In order to understand the role of this interaction in the regulation of GluR4 phosphorylation by PKC, we started by identifying the crucial amino acids for the interaction. Recombinant glutathione S-transferase (GST)-GluR4 C-terminal fusion protein with a triple point mutation at the amino acids R821A, K825A and R826A (Figure 2B) within the GluR4-PKCγ binding domain (GluR4AAA C-terminus) was produced. The mutated residues were selected in order to destroy the homology between this region of GluR4 and the PKCγ pseudosubstrate. The effect of these GluR4 point mutations on the interaction between GluR4 and PKCγ was tested by performing GST pull-down assays. Rat brain extract (RBE) was incubated with GST fused to GluR4WT C-terminus or GluR4AAA C-terminus, as indicated. Glutathione-Sepharose beads were used to pull down GST fusion proteins and their binding partners. PKCγ was copurified when the brain extract was incubated with GST-GluR4 C-terminus but not when incubated with GST-GluR4AAA C-terminus (Figure 2C). These data show that R821, K825 and R826 in GluR4 are critical amino acids for the interaction between GluR4 and PKCγ to occur and allow us to make constructs encoding a GluR4 form that does not bind PKCγ.

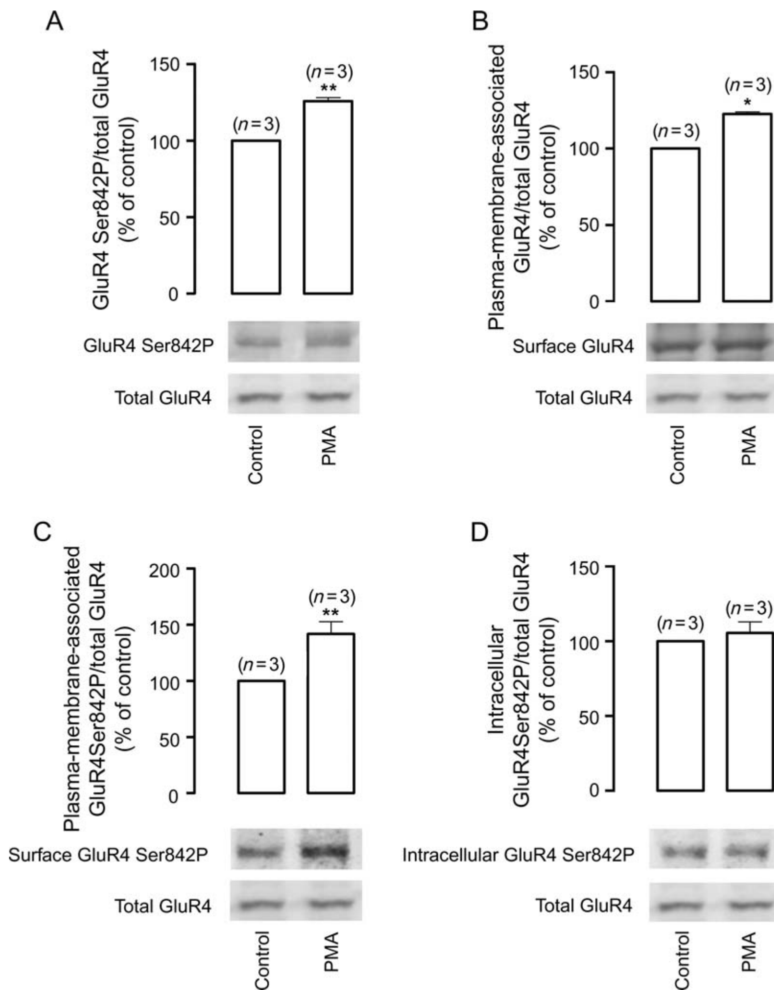


Figure 1: Effect of PKC on GluR4 phosphorylation and surface expression in primary cultures of retina amacrine-like neurons. A) Effect of PKC activation on GluR4 phosphorylation at Ser842. Cells were incubated with the PKC activator, PMA (200 nM) for 10 min. Cell extracts were prepared, subjected to SDS–PAGE and immunoblotted against phosphorylated GluR4 at Ser842 and total GluR4. The amount of phosphorylated GluR4 was normalized to the total amount of GluR4 in each lane. B) Effect of PKC activation on GluR4 surface expression. Biotinylation was performed as described in Materials and Methods. Streptavidin-retained protein complexes were collected and run on SDS–PAGE. Immunoblot was performed using an antibody against GluR4 C-terminal region. Surface GluR4 was normalized to the total amount of GluR4 in each condition. C, D) Effect of PKC activation on the phosphorylation of surface (C) or intracellular (D) GluR4. All data are expressed as percentage of control and plotted as the mean ± SEM for the indicated number of experiments performed in independent preparations (*$p < 0.05$, **$p < 0.01$, Bonferroni's test). Representative Western blots are shown.

Effect of blocking the PKCγ–GluR4 interaction on PKC-driven phosphorylation and plasma membrane expression of GluR4

Previous evidences suggest that PKCγ bound to GluR4 can phosphorylate Ser842 in GluR4, and cotransfection of PKCγ with GluR4, in human embryonic kidney (HEK) 293 cells, increased GluR4 subunit surface expression, following PKC activation with PMA (16), Figure 3). To examine the role of the association between PKCγ and GluR4 in the effect of PKC on GluR4 phosphorylation and AMPA receptor trafficking to the plasma membrane, we transfected HEK 293 cells with GluR4 or with the GluR4 triple point mutant (GluR4AAA), which is unable to bind PKCγ. Cells were cotransfected with PKCγ and stimulated with 200 nM PMA for 10 min (when indicated). Quantitative immunoblotting was used to compare phosphorylated versus total GluR4 (Figure 3A) and surface versus total GluR4 (Figure 3B).

In cells expressing GluR4 or GluR4AAA, in the absence of cotransfected PKCγ, no significant differences between the two proteins were observed in terms of either phosphorylation or plasma membrane expression. When endogenous PKC is activated with PMA, there is increased GluR4 phosphorylation at Ser842 (Figure 3A) and surface expression (Figure 3B), which nevertheless fail to reach statistical significance, suggesting that the amount of endogenous PKC is limiting. In cells coexpressing GluR4 and PKCγ and stimulated with PMA, dramatic increases in both GluR4 phosphorylation (122.1 ± 16.4%, above control) and GluR4 delivery to the plasma membrane (59.9 ± 15.8%, above control) were observed. These data are in agreement with previous studies, showing that PKA phosphorylation of GluR4 at this site is necessary and sufficient for GluR4 surface and synaptic delivery (12,27). The PKC-stimulated GluR4 surface expression was also observed when the experiments were performed in cells incubated with 0.35 M sucrose to block endocytosis, suggesting that the effect of PKC is directly on the surface insertion of the receptors (data not shown). However, no significant changes in the phosphorylation or surface expression of GluR4AAA were observed upon coexpression and activation (with PMA) of PKCγ (Figure. 3A,B), indicating that the interaction between PKC and GluR4 is necessary for PKCγ-mediated GluR4 phosphorylation and receptor delivery to the cell surface.

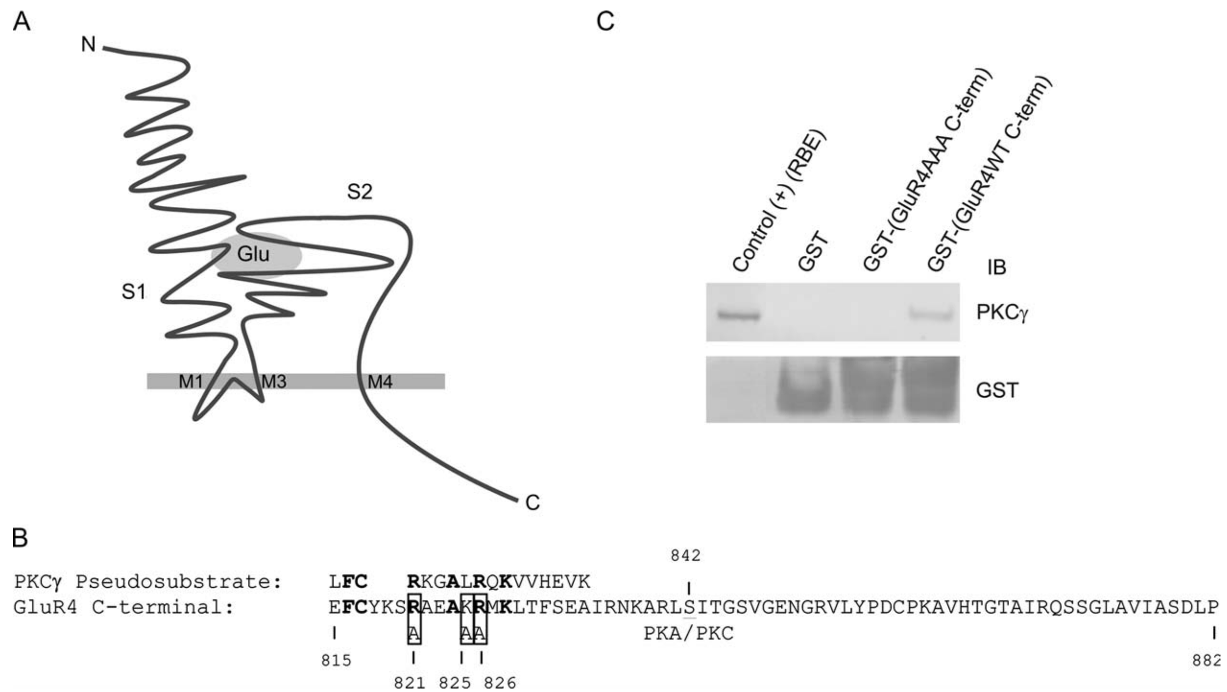


Figure 2: Mapping of the GluR4-PKCγ interaction motif. A) Topology of AMPA receptor subunits. B) The amino acid sequences of the PKCγ pseudosubstrate and of the GluR4 C-terminal region are compared, and the PKA/PKC phosphorylation site in GluR4 is indicated, as well as the triple point mutations R821A, K825A and R826A (GluR4AAA). C) The interaction motif with PKCγ at the GluR4 C-terminal sequence was identified using GST pull-down assays. RBE was incubated with GST fused to GluR4WT C-terminal or GluR4AAA C-terminal, as indicated. Glutathione-Sepharose beads were used to pull down GST fusion proteins, detected with anti-GST antibody. Pulled-down proteins were analysed with an anti-PKCγ antibody; PKCγ was present when the extract was incubated with GST-GluR4 C-terminal, but not when incubated with GST-GluR4 C-terminal triple mutant.

Effect of blocking the PKCγ–GluR4 interaction on PKA-driven phosphorylation and plasma membrane expression of GluR4

To determine whether the triple point mutations at the C-terminal membrane-proximal domain of GluR4 have an effect on protein folding that could impair GluR4 phosphorylation and surface expression, we tested whether GluR4-AAA can be phosphorylated by PKA and targeted to the membrane upon PKA activation in HEK 293 cells. The HEK 293 cells were transfected with GluR4 or GluR4AAA and stimulated with 10 μM forskolin (FSK) for 10 min (when indicated) followed by incubation with the biotinylation reagent. Cell extracts were collected as described in Materials and Methods and quantitative immunoblotting was used to compare phosphorylated versus total GluR4 (Figure 4A) and surface versus total GluR4 (Figure 4B). Stimulation by FSK induced a robust increase in Ser842 phosphorylation both in HEK 293 cells expressing GluR4 or GluR4AAA (159.7 ± 40.1% and 175.9 ± 45.0% above control, respectively; Figure 4A). Plasma membrane expression of GluR4 or GluR4AAA was also significantly increased in FSK-stimulated cells [GluR4 (59.6 ± 18.1%), GluR4AAA (68.1 ± 18.2%) above control; Figure 4B].

Synaptic targeting of GluR4 in rat hippocampal CA1 neurons occurs early in development and has been shown to be driven by spontaneous synaptic activity and to depend on PKA activity (11,12). Therefore, we tested whether the GluR4 C-terminus triple mutant, which can be phosphorylated by PKA in HEK 293 cells, is phosphorylated and targeted to the synapse in CA1 neurons in hippocampal slice cultures. Green fluorescent protein (GFP)-tagged GluR4 or GFP-tagged GluR4AAA was expressed in CA1 neurons of rat hippocampal organotypic slices obtained from P5-6 animals, using the Sindbis virus expression system. Slices were then maintained in culture for approximately 36 h. The recombinant proteins showed homogeneous dendritic expression (Figure 4D). Delivery of GFP-GluR4 to synapses was monitored using the inward rectification properties of the overexpressed recombinant receptors (electrophysiological tagging, (4,11,12,29)). Recombinant GFP-GluR4 expressed in CA1 cells mainly forms homomeric receptors, which do not conduct outward currents. Hence, their delivery into synapses can be quantified as a decrease in the ratio of the evoked postsynaptic current at +40 mV relative to the current at −60 mV [rectification index (RI) = I_{+40}/I_{-60}]. The RI values obtained from GFP-GluR4AAA-infected cells were similar to those obtained from GFP-GluR4-infected cells, indicating that the PKA-driven synaptic incorporation of GluR4AAA is not compromised by the triple mutation (Figure 4C). Phosphorylation of GluR4 and GluR4AAA was

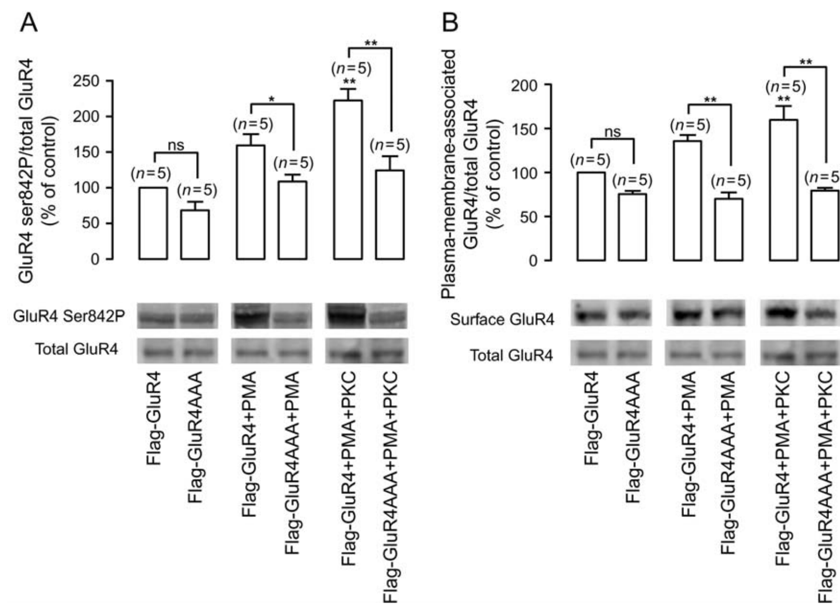


Figure 3: Effect of blocking the PKCγ–GluR4 interaction on PKC-driven phosphorylation and plasma membrane expression of GluR4. Cultured HEK 293 cells were transfected with N-terminally Flag-tagged GluR4 or Flag-tagged GluR4AAA, or cotransfected with Flag-tagged GluR4 or Flag-tagged GluR4AAA and PKCγ, as indicated. When indicated, cells were stimulated with PMA (200 nM for 10 min). A) Cell extracts were prepared, subjected to SDS–PAGE and immunoblotted against phosphorylated GluR4 at Ser842 and total Flag-GluR4. Phosphorylated GluR4 on Ser842 and total GluR4 were quantified, and the amount of phosphorylated GluR4 was normalized to the total amount of GluR4 in each condition. B) Plasma membrane proteins were biotinylated and purified. Streptavidin-retained protein complexes were collected and run on SDS–PAGE. Immunoblot was performed using an antibody against the Flag epitope. Plasma membrane GluR4 and total GluR4 were quantified, and the amount of surface GluR4 was normalized to the total amount of GluR4 in each condition. All data are expressed as percentage of control and plotted as the mean ± SEM for the indicated number of experiments performed in independent preparations (*$p < 0.05$; **$p < 0.01$, Bonferroni's test). Representative Western blots using antibodies against phosphorylated GluR4 at Ser842, surface GluR4 and total GluR4 are shown.

also compared in hippocampal slices. The GFP-tagged GluR4 recombinant proteins were immunoprecipitated with an anti-GFP antibody (Figure 4E, bottom panel). Both GFP-GluR4 and GFP-GluR4AAA were phosphorylated on Ser842 (Figure 4E, upper panel). No significant differences in phosphorylation were detected between wild-type GluR4 and the mutant in infected hippocampal slices.

These results indicate that the triple point mutation in GluR4AAA does not affect PKA phosphorylation and consequent plasma membrane or synaptic expression of GluR4, or its synaptic function, and exclude the possibility that the impairment in PKC-mediated phosphorylation and surface expression of GluR4AAA is the result of altered protein folding. Moreover, the results show that the interaction between PKCγ and GluR4 is specifically required to assure PKC-driven phosphorylation of GluR4.

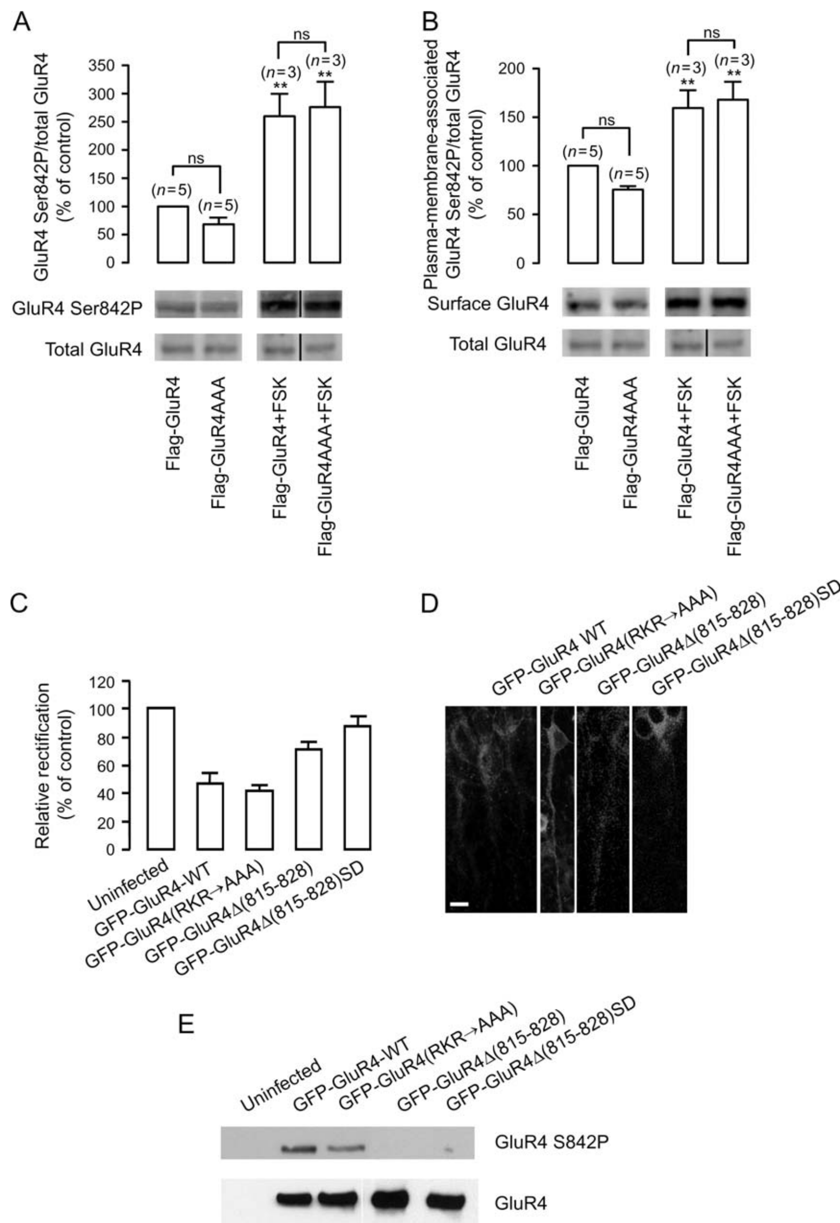
Experiments in hippocampal slice cultures were also performed using a deletion mutant of GluR4, which lacks a 14-amino-acid juxtamembrane region just past the fourth transmembrane domain of the receptor subunit (GluR4Δ815–828). The RI values obtained from neurons infected with GFP-GluR4Δ815–828 were significantly higher than those obtained from GFP-GluR4-infected cells (Figure 4C), indicating compromised synaptic incorporation of this mutant protein, although homogeneous dendritic expression was observed (Figure 4D). Moreover, the deletion mutant showed impaired phosphorylation at Ser842 (Figure 4E), but a serine to aspartate mutation at residue 842 to mimic GluR4 phosphorylation could not rescue GluR4 synaptic delivery (GFP-GluR4Δ815–828S842D, Figure 4C), suggesting that this membrane-proximal region of the receptor is necessary for synaptic delivery of GluR4.

Discussion

We have previously shown that PKCγ binds directly to the GluR4 membrane-proximal C-terminal domain and that GluR4 is phosphorylated on Ser842 by bound kinases, including PKCγ, suggesting that the interaction maintains the kinase in close proximity to GluR4, facilitating receptor phosphorylation at Ser842 (16). The present data show that PKC activation leads to GluR4 phosphorylation and surface expression in cultured retina neurons, and that PKCγ coexpression in GluR4-transfected HEK 293 cells increases GluR4 surface expression upon stimulation with PMA. These evidences argue for a role of PKC in GluR4 receptor subunit phosphorylation and targeting to the plasma membrane. Moreover, the presented results show that point mutations in GluR4 that disrupt the interaction site for

PKCγ in the GluR4 C-terminal membrane-proximal region impair PKC-driven GluR4 phosphorylation and surface expression in HEK 293 cells. These mutations do not alter protein folding or compromise the Ser842 phosphorylation site because the mutant protein is functional at synapses in hippocampal CA1 neurons, where synaptic targeting of GluR4 has been shown to depend on GluR4 phosphorylation by PKA and to be induced by spontaneous neuronal activity.

When we expressed a deletion mutant of GluR4 (GluR4Δ815–828) in organotypic rat hippocampal slices, impaired synaptic delivery and no protein phosphorylation at Ser842 were observed (Figure 4C,E). The C-terminal membrane-proximal region of GluR4 was previously implicated in the basal surface expression of GluR4 in HEK 293 cells and in dissociated hippocampal neurons in culture (15). The authors showed that deletion of the C-terminal domain up to the juxtamembrane region of GluR4 blocks receptor surface expression, which could be partially rescued if a C-terminal 14-amino-acid membrane-proximal segment, the same domain that PKCγ binds to in GluR4, was present (15). The correspondent sequence in GluR1 was previously shown to bind the 4.1N protein, the neuronal homologue of the erythrocyte membrane cytoskeletal protein 4.1 (30). Coleman et al. found that 4.1N can also associate to GluR4 and that a deletion of the C-terminal, membrane-proximal 14-amino-acid segment of GluR4, or mutation of the R821, K825 and R826 residues at



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this region, disrupts 4.1N binding and significantly reduces basal GluR4 surface expression (15). Our results obtained using infected hippocampal slices further show that synaptic delivery of GluR4 is blocked in the absence of the GluR4 juxtamembrane C-terminal domain (Figure 4C). Moreover, the deletion mutant is not phosphorylated in hippocampal slices (Figure 4E), indicating that the deletion may compromise the efficiency of GluR4 phosphorylation by several kinases, eventually by having an impact on the protein folding at the C-terminus. Early in the development of rat hippocampus, spontaneous activity drives GluR4-containing AMPA receptors to the synapse (11) by a mechanism mediated by PKA phosphorylation of GluR4 Ser842 (12). PKA phosphorylation of GluR4 Ser842 was suggested to relieve a retention interaction, driving receptors to synapses (12). Mutation of Ser842 to an aspartate was described to drive GluR4-containing AMPA receptors to synapses, bypassing the need for spontaneous activity (12). However, replacement of Ser842 for an aspartate on GluR4Δ(815–828) recombinant protein was unable to revert the effect of the GluR4 membrane-proximal segment deletion on GluR4 delivery to synapses in rat hippocampal slices (Figure 4C). Our data indicate that Ser842-phosphorylation-mediated targeting of GluR4 to synapses is blocked by deletion of the GluR4 membrane-proximal domain.

The GluR4 triple mutant (GluR4 R821A, K825A, R826A) was targeted to the synapse in CA1 neurons as efficiently as wild-type GluR4 (Figure 4C), and in HEK 293 cells, there was no significant difference between the basal surface expression of the wild-type and mutant forms of GluR4 (Figures 3B and 4B). Moreover, the increase in GluR4 surface expression in HEK 293 cells triggered by PKA activation was the same for wild-type GluR4 and the triple mutant (Figure 4B). Because the R821, K825 and R826 residues are critical for GluR4 binding to 4.1N (15), the GluR4 triple mutant (GluR4 R821A, K825A, R826A) is unable to bind 4.1N (data not shown); our results do not support the idea that GluR4 binding to 4.1N is required for GluR4 surface expression or synaptic targeting. However, our data and those of others suggest that other determinants exist at the membrane-proximal segment of the C-terminus of the receptor, apart from the R821, K825 and R826 residues, with a role in the surface expression of GluR4. A recent study identified C817 at the C-terminal region of GluR4 (and equivalent Cys residues at the C-terminus of the other AMPA receptor subunits) as a palmitoylation site and found that depalmitoylated AMPA receptors show a stronger association with 4.1N (31). Moreover, palmitoylation of this Cys residue at the C-terminus of AMPA receptors was shown to be necessary for agonist-induced internalization of the receptors, but not for their steady-state surface expression (31).

Our data point to a clear role for the basic R821, K825 and R826 residues at the C-terminus of GluR4 in receptor binding to PKCγ, in GluR4 phosphorylation by PKC and in PKC-driven surface expression of GluR4 (Figure 5). When PKCγ was coexpressed with GluR4, there was a striking difference between surface expression of GluR4 and GluR4AAA (Figure 3B). This suggests that the interaction of GluR4 with PKCγ through the C-terminal juxtamembrane domain of GluR4 is necessary for surface delivery promoted by PKC activation. Moreover, the triple mutation impairs efficient GluR4 phosphorylation by PKC in HEK 293 cells (Figure 3A), indicating that the kinase anchoring through the 815–828 GluR4 region is crucial for receptor phosphorylation by this kinase. Interestingly, the sequence in GluR1 (RSE**S**KR) homologous to the PKC interaction site on GluR4 (RAEAKR) contains a PKC phosphorylation site, which controls synaptic incorporation of GluR1 during LTP (32).

Taken together, these results point to a dual role for the membrane-proximal region of the C-terminus of GluR4:

Figure 4: Effect of blocking the PKCγ–GluR4 interaction on PKA-driven phosphorylation, plasma membrane expression and synaptic delivery of GluR4. A) Cultured HEK 293 cells were transfected with N-terminally Flag-tagged GluR4 or Flag-tagged GluR4AAA. When indicated, cells were stimulated with FSK (10 μM for 10 min). Cell extracts were prepared, subjected to SDS–PAGE and immunoblotted against phosphorylated GluR4 at Ser842 and total Flag-GluR4. The amount of phosphorylated GluR4 was normalized to the total amount of GluR4 in each condition. B) Plasma-membrane-associated GluR4 and total GluR4 were quantified in transfected HEK 293 cells, and the amount of surface GluR4 was normalized to the total amount of GluR4 in each condition. All data are expressed as percentage of control and plotted as the mean ± SEM for the indicated number of experiments performed in independent preparations (**$p < 0.01$, Bonferroni's test). Representative Western blots using antibodies against phosphorylated GluR4 at Ser842, surface GluR4 and total GluR4 are shown. C) GluR4 expression and delivery to synapses in CA1 neurons of rat hippocampal slices. Cultured rat hippocampal slices expressing GFP-GluR4, GFP-GluR4AAA, GFP-GluR4Δ(815–828) or GluR4Δ(815–828)S842D were used to collect electrophysiological data. GFP-GluR4 is delivered to synapses when expressed in rat hippocampal slices [normalized average rectification value (I_{+40mV}/I_{-60mV}) was 46.4% ± 7.1% for GluR4 infected]. GFP-GluR4AAA delivery to synapses is not significantly different when compared with GFP-GluR4 (average rectification value was 41.1% ± 3.7% of control uninfected cells). GFP-GluR4Δ(815–828) or GFP-GluR4Δ(815–828)S842D delivery to synapses is impaired when compared with GFP-GluR4. Normalized average rectification values were 70.8% ± 5.4% for GFP-GluR4Δ(815–828) and 87.1% ± 6.3% for GFP-GluR4Δ(815–828)S842D. Results are presented as mean ± SEM, and statistical significance was determined by the *t*-test (assuming unequal variances). D) GFP fluorescence shows homogeneous dendritic expression of GFP-GluR4, GFP-GluR4AAA, GFP-GluR4(815–828) and GFP-GluR4(815–828)S842D. Scale bar represents 10 μM. E) Phosphorylation of GFP-GluR4, GFP-GluR4AAA, GFP-GluR4Δ(815–828) or GFP-GluR4Δ(815–828)S842D in rat hippocampal slices. Cell extracts from cultured rat hippocampal slices expressing GFP-GluR4 or GFP-GluR4-AAA, GFP-GluR4Δ(815–828) or GFP-GluR4Δ(815–828)S842D were prepared and used for immunoprecipitation with an anti-GFP antibody. Immunoprecipitated proteins were analysed by Western blotting with antibodies against phosphorylated GluR4 at Ser842 and total GluR4.

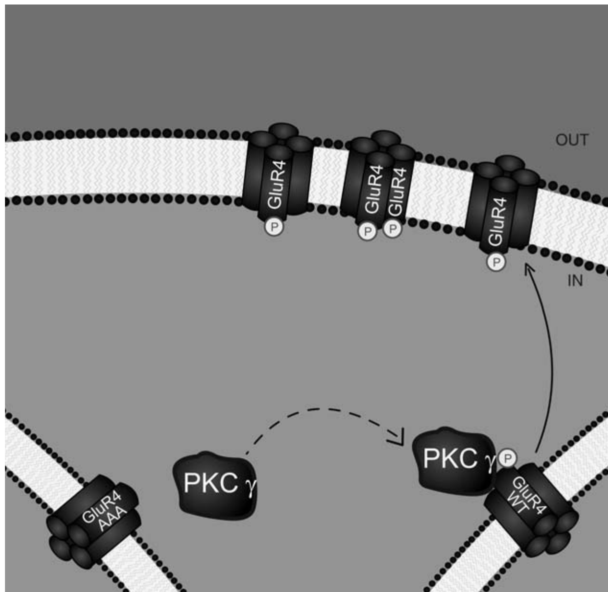

Figure 5: Model for the mechanism of GluR4 AMPA receptor trafficking and how it depends on the interaction between GluR4 and PKC.

(i) on the one hand, the R821, K825 and R826 residues present in this region are necessary for the Ser842 residue in GluR4 to be phosphorylated by PKC and (ii) on the other hand, other determinants at the membrane-proximal segment of the C-terminus are required for localization of Ser842-phosphorylated GluR4 at the synapse or the plasma membrane. The interaction of GluR4 with PKCγ plays a role in receptor phosphorylation, whereas the interaction of this region of GluR4 with other proteins could be important in stabilizing the receptor at synapses and at the plasma membrane, after the phosphorylated receptor has been driven to the membrane, eventually regulating receptor internalization. In fact, members of the 4.1N protein family have been involved in linking plasma-membrane-associated proteins to the actin cytoskeleton (33), and polymerized actin was previously shown to be important for immobilization and clustering of AMPA receptors (34,35). The phosphatidylinositol 3-kinase (PI3K) was also reported to be clustered with AMPA receptors at synapses and was shown to bind directly to GluR1 and GluR2 (36). The 21 amino acids at the GluR2 juxtamembrane C-terminal domain are responsible for the interaction of PI3K (36), and the first 14 amino acids of this segment in GluR2 are common to GluR4. It is possible, therefore, that PI3K interacts with GluR4. Man et al. (36) suggested that the accumulation of PI3K products, like PtdIns(3,4,5)P_3, near AMPA-receptor-containing vesicles may facilitate the fusion of these vesicles with the postsynaptic membrane.

We have previously shown that PKC up-regulates AMPA receptor activity in chick embryo retinal cultures (37). Activation of PKC with PMA in cultured chick embryo retinal neurons significantly increased plasma membrane GluR4 expression (Figure 1A) and GluR4 Ser842 phosphorylation (Figure 1B,C). Previous work showed that PKA phosphorylation of Ser842 on GluR4 is necessary and sufficient for GluR4 delivery to synapses (12); however, PKC can also phosphorylate GluR4 Ser842 (38). Our results show a role of PKC phosphorylation of GluR4 Ser842 in GluR4 targeting to the plasma membrane, in retinal neurons, and suggest that phosphorylation-regulated delivery of GluR4 could be mediated by PKA or PKC in different regions of the brain. The PMA-induced increase in the surface expression of GluR4 in retina neurons indicates that PKC regulates the targeting of GluR4-containing AMPA receptors to the plasma membrane in neurons. Moreover, several physiological stimuli could result in increased PKC activity in amacrine neurons. Group I metabotropic glutamate receptors, as well as metabotropic acetylcholine receptors, are expressed and functional in these cells and elicit inositol phospholipids hydrolysis and $[Ca^{2+}]_i$ elevations (39–41).

In conclusion, our results support the involvement of GluR4-anchored PKCγ in Ser842 phosphorylation and in the PKC-regulated delivery of GluR4 to the plasma membrane.

Materials and Methods

Materials

Trypsin was purchased from Life Technologies Ltd (Paisley, UK), and fetal calf serum was from Biochrom KG (Berlin, Germany) or from Biowhittaker (Walkersville, MD, USA). The PMA was from Calbiochem (San Diego, CA, USA). Forskolin was obtained from BIOMOL Research Laboratories, Inc (Plymouth Meeting, PA, USA). Complete Mini protease inhibitor cocktail, microporous polyvinylidene difluoride (PVDF) membranes and the mouse anti-GFP antibody were obtained from Roche Diagnostics GmbH (Basel, Switzerland). EZ-link Sulfo-NHS-SS-biotin, UltraLink Plus Immobilized Streptavidin Gel and the bicinchoninic acid (BCA) protein assay reagent kit were from Pierce (Rockford, IL, USA). pGEX4T-2 vector, glutathione-Sepharose 4B, goat polyclonal anti-GST antibody, alkaline-phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies, and the Enhanced Chemifluorescence (ECF) immunodetection substrate were all obtained from Amersham Biosciences (Uppsala, Sweden). Anti-goat alkaline-phosphatase-conjugated antibody and mouse monoclonal anti-transferrin receptor antibody were from Zymed Laboratories Inc (San Francisco, CA, USA). The mouse monoclonal anti-PKCγ antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Isopropyl-1-thio-β-galactopyranoside was from Promega (Madison, WI, USA). The rabbit polyclonal anti-GluR4 antibody was purchased from Chemicon (Temecula, CA, USA), and the rabbit polyclonal anti-GluR4 phosphorylated at Ser842 antibody was a gift from Dr Richard L. Huganir. This antibody was produced against the chemically phosphorylated peptide (RNKARL**S^P**ITGSV) corresponding to rat GluR4 C-terminal 12 amino acids (836–847) phosphorylated at Ser842 (12). The expression construct Flag-tagged GluR4 was a kind gift from Dr Kari Keinanen (15), and the Quick Change mutagenesis kit was purchased from Stratagene (Amsterdam, the Netherlands). All the other reagents were obtained from Sigma (St Louis, MO, USA) or from Merck (Darmstadt, Germany).

Embryonic chick retina cell culture

Monolayer cultures of chick retina amacrine-like cells were prepared as previously described (39,42). Briefly, the retinas from 8-day-old chick embryos (white leghorn) were dissected and digested with 0.1% trypsin in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution for 15 min at 37°C.

The cells were cultured on poly-D-lysine tissue culture dishes in basal medium of Eagle, buffered with 25 mM HEPES and 10 mM $NaHCO_3$, pH 7.4, and supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2, and used after 5 days in culture.

Recombinant proteins expressed in Escherichia coli

Glutathione S-transferase fused to the GluR4 C-terminal [GST-(GluR4 C-term)] construct was cloned in pGEX4T-2 vector as described in Correia et al. (16). The triple mutant GST-(GluR4AAA C-term), with the R821A, K825A and R826A substitutions (Figure 2B), was prepared with the Quick Change mutagenesis kit, using GST-(GluR4 C-term) construct as a template and the primers 5′-ttctgttacaagtccgctgcagaggcggcggcaatgaagctgact-3′ and 5′-agtcagcttcattgccgccgcctctgcagcggacttgtaacagaa-3′.

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 (500 μM) for 30 min at 30°C and then lysed with PBS containing 1% Triton X-100 and supplemented with protease inhibitors. 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. Glutathione S-transferase fusion proteins were purified by glutathione-Sepharose affinity chromatography. Protein concentration was measured using the BCA protein assay reagent kit.

Glutathione S-transferase binding assays

Whole RBE were prepared by homogenizing the tissue in lysis buffer [20 mM Tris, 2 mM EGTA, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton-X-100 and supplemented with protease inhibitors] followed by centrifugation at 1000 × ***g*** for 10 min at 4°C. The resulting supernatant was recentrifuged at 1000 × ***g*** for 10 min at 4°C, and the soluble brain proteins were used for binding studies. Fifteen micrograms of fusion protein was incubated with rat brain homogenates overnight at 4°C. The mixture was incubated with 50% glutathione-Sepharose beads for 30 min at 4°C. Beads were washed extensively with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS; pH 7.5) supplemented to 0.5 M NaCl, and binding proteins were eluted with SDS–PAGE sample buffer [0.125 M Tris, 2% (w/v) SDS, 5% (v/v) glycerol, 5% (v/v) β-mercaptoethanol] and boiled for 10 min. Samples were analysed by Western blotting.

Recombinant proteins expressed in mammalian cells

The construct encoding N-terminally Flag-tagged GluR4 was a kind gift from Dr Kari Keinanen (15), and Flag-tagged GluR4AAA (construct coding for the Flag-GluR4 sequence with C-terminal triple mutations R821A, K825A and R826A, Figure 2B) was prepared with the Quick Change mutagenesis kit, using the Flag-tagged GluR4 construct as a template and the primers 5′-ttctgttacaagtccgctgcagaggcggcggcaatgaagctgact-3′ and 5′-agtcagcttcattgccgccgcctctgcagcggacttgtaacagaa-3′. The pBK-CMV-PKCγ construct was prepared as previously described (16). Cultured HEK 293 cells were transfected with Flag-GluR4 or Flag-GluR4AAA, or cotransfected with Flag-GluR4 or Flag-GluR4AAA and pBK-CMV-PKCγ by the calcium phosphate method (38).

To produce Sindbis virus containing GFP-GluR4Δ(815–828), GFP-GluR4Δ(815–828)S842D, the C-terminal coding sequence of GluR4 with a deletion of the 42 bp coding for the 14 juxtamembrane amino acids was amplified using the primers 5′-ggcatctctatcatgatc-3′ and 5′-cgacgggccct-tatggtaggtccgatgcaatgacagc-3′ (which include the restriction sites for bcl*I* and Apa*I*, respectively) and the pGW1-GluR4Δ(815–828) and pGW1-GluR4Δ(815–828)S-842D constructs as a template. To obtain Sindbis virus expressing the GFP-tagged GluR4 with the C-terminal triple mutations R821A, K825A and R826A, the C-terminal sequence of Flag-tagged GluR4-AAA was amplified using the same set of primers as above. The amplified complementary DNAs were subcloned into the pSinRep5-GFP-GluR4 construct (11) previously digested with bcl*I* and Apa*I* endonucleases to remove the C-terminal segment of GluR4.

Organotypic cultures of hippocampal slices [made as described by Zhu et al. (11)] were prepared from P5-6 rats and infected with Sindbis virus expressing GFP-tagged GluR4 or its mutants within 12 h of their preparation to mimic the expression profile of endogenous GluR4 and kept in culture for 36 h.

Biotinylation of plasma-membrane-associated proteins

Transfected HEK 293 cells and chick retina amacrine-like neurons in culture were subjected to a membrane surface biotinylation assay, as previously described (27). After stimulation, cells were washed twice with PBS with calcium and magnesium (PBS/Ca^{2+}/Mg^{2+}: 137 mM NaCl; 2.7 mM KCl; 1.8 mM KH_2PO_4; 10 mM Na_2HPO_4; plus 0.5 mM $MgCl_2$; 1 mM $CaCl_2$; pH 7.4) and incubated with 1 mg/mL NHS-SS-Biotin for 30 min at 4°C under mild shaking. Cells were then rinsed three times with PBS/Ca^{2+}/Mg^{2+} supplemented with glycine (100 mM) and a fourth time with PBS supplemented with protease inhibitors. Cells were then lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (10 mM Na_4PO_7; 50 mM NaF; 1 mM Na_3VO_4), scraped off the plates and centrifuged at 14 000 × ***g*** for 10 min at 4°C. The supernatants were transferred to clean tubes. A sample was collected corresponding to the input, which was used as a control for transfection efficiency and to determine the level of GluR4 Ser842 phosphorylation in each experimental condition. UltraLink Streptavidin Plus was added to equal amounts of supernatant and incubated for 2 h at 4°C with mild shaking (orbital shaker). Complexes were centrifuged (2500 × ***g***, 3 min) and washed further with RIPA buffer for four times. Proteins were eluted from streptavidin beads by boiling for 10 min in SDS–PAGE sample buffer. The efficiency of purification of biotinylated proteins was compared among different samples by performing Western blot against the transferrin receptor.

SDS–PAGE and immunoblotting

The extracts obtained were resolved by SDS–PAGE in 10% polyacrylamide (43). This was followed by overnight electrotransfer to PVDF membranes at 40 V complemented by 30 min at 200 V. Membranes were then blocked for 1 h with 1% (w/v) BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and probed for 1 h with the primary anti-GluR4 (1:400), anti-GluR4 phosphorylated at Ser842 (1:2,500), anti-Flag (1:1000), anti-transferrin (1:1000) receptor, anti-PKCγ (1:1000) or anti-GST (1:2500) antibodies. Following five washes (5 min) in 1% BSA/TBS-T, the membranes were incubated for 1 h with alkaline-phosphatase-conjugated secondary anti-rabbit, anti-mouse or anti-goat (1:20 000) antibodies. The membranes were then washed again five times (5 min), incubated with ECF for 5 min and scanned with the Storm™ 860 scanner (Amersham Biosciences). The scanned digital images were quantified using the ImageQuant 5 software (Amersham Biosciences).

Electrophysiology

Organotypic cultures of rat hippocampal slices infected with the Sindbis virus expressing recombinant GluR4 or mutants tagged with GFP (as indicated) were used to collect electrophysiological data. Voltage-clamp whole-cell recordings were obtained from nearby infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. External solution contained 119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 11 mM glucose, 26 mM $NaHCO_3$, 4 mM $MgCl_2$, 4 mM $CaCl_2$, 100 μM picrotoxin, 2 μM 2-chloroadenosine and 100 μM DL-2-amino-5-phosphonopentanoic acid (APV), pH 7.4, and was gassed with 95% O_2 and 5% CO_2. Patch recording pipettes (4–7 MΩ) were filled with internal solution containing 115 mM $CsMeSO_3$, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2-ATP, 0.4 mM Na-GTP, 10 mM sodium phosphocreatine, 0.6 mM EGTA and 0.1 mM spermine, pH 7.25. Bipolar stimulating electrodes were placed over Schaffer collateral fibres between 250 and 300 μM from the CA1 recorded cell, and synaptic responses were evoked with single-voltage pulses (200 μs, up to 30 V). Whole-cell recordings were made with a Multiclamp 700A amplifier (Axon Instruments, Union City, CA, USA). Synaptic AMPA-receptor-mediated responses at −60 mV and +40 mV

were averaged from 50 to 70 trials, and their ratio was used as an index of rectification.

Hippocampal slices protein extract preparation and immunoprecipitation

Organotypic cultures of hippocampal slices infected with Sindbis virus expressing GFP-tagged GluR4 or its mutants were kept in culture for 36 h and were then homogenized in 10 mM HEPES, pH 7.4, 500 mM NaCl, 10 mM NaF, 1 μM microcystin LR, 0.5 μM calyculin A, 10 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 2 μg/mL CLAP (cocktail of chymostatin, leupeptin, pepstatin A and antipain) and 1% Triton X-100. GFP immunoprecipitates were obtained by incubation of 4 μg of anti-GFP monoclonal antibody with 40 μl of protein G-sepharose beads (50%) and the rat hippocampal homogenates for 4 h at 4°C. These samples were then washed, and immunoprecipitated proteins were eluted by boiling in 1× Laemmli sample buffer.

Immunohistochemistry for confocal microscopy

Cultured rat hippocampal slices infected with Sindbis virus expressing GluR4, GluR4Δ(815–828) or GluR4Δ(815–828)S842D recombinant proteins tagged to GFP were kept in culture for 36 h and were then fixed with 4% paraformaldehyde and 4% sucrose for 2 h at 4°C. Cells were washed in PBS after fixation and were then incubated with 2% goat serum and 0.3% Triton X-100 in PBS (for blocking and permeabilization of neurons, respectively) for 1 h at room temperature. Cells were incubated with a rabbit polyclonal anti-GluR4 antibody overnight at 4°C. Neurons were then incubated with biotinylated anti-rabbit secondary antibody for 1 h at room temperature and then labelled with avidin-Alexa594 for 1 h. Antibody excess was washed with PBS. Images were obtained with a Zeiss confocal microscope.

Statistical analysis

Results are presented as means ± SEM of the indicated number of experiments carried out in different preparations. Statistical significance was determined by one-way ANOVA followed by Bonferroni's test, or in the electrophysiology experiments by the *t*-test (assuming unequal variances).

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