PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity

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The regulated incorporation of AMPA receptors into synapses is important for synaptic plasticity. Here we examine the role of protein kinase A (PKA) in this process. We found that PKA phosphorylation of the AMPA receptor subunits GluR4 and GluR1 directly controlled the synaptic incorporation of AMPA receptors in organotypic slices from rat hippocampus. Activity-driven PKA phosphorylation of GluR4 was necessary and sufficient to relieve a retention interaction and drive receptors into synapses. In contrast, PKA phosphorylation of GluR1 and the activity of calcium/calmodulin-dependent kinase II (CaMKII) were both necessary for receptor incorporation. Thus, PKA phosphorylation of AMPA receptor subunits contributes to diverse mechanisms underlying synaptic plasticity.

Activity-dependent changes in synaptic strength are widely thought to underlie learning and memory[1,2]. In addition, synaptic plasticity is critical for the developmental maturation of neuronal circuits[3,4]. Thus, considerable effort is being directed toward understanding the molecular and cellular mechanisms responsible for long-lasting changes in synaptic strength.

A number of intracellular signaling pathways have been implicated in synaptic plasticity (for review, see refs. 5–7). However, the specific targets and mechanisms that directly mediate changes in synaptic strength in response to neuronal activity remain to be identified. Progress on this front has been delayed by the extensive cross-talk that exists between these pathways[8], the compensatory changes that occur when individual pathways are chronically perturbed[9] and the large number of potential downstream effectors[10].

The PKA signaling pathway has been implicated at different stages in the regulation of synaptic plasticity in the CA1 region of hippocampus. It has been proposed that PKA gates plasticity by modulating CaMKII activity[11,12]. In this manner, the threshold for plasticity such as long-term potentiation (LTP), which requires and is triggered by CaMKII activity, could be controlled by PKA. A number of studies indicate that PKA activity can control aspects of synaptic plasticity that depend on protein synthesis[13,14]. These processes could lead to the generation or stabilization of more synapses[15], and the specificity could be 'tagged' by local PKA activity[16]. In addition, in the CA3 hippocampus and cerebellum, PKA activity controls a separate form of plasticity that changes release probability[17,18]. Despite considerable study, the specific targets of PKA and their role in long-lasting changes in synaptic strength remain elusive.

One recently identified mechanism contributing to synaptic plasticity is the regulated trafficking of AMPA receptors in and out of synapses (for review, see refs 19–22). AMPA receptors are hetero-oligomeric proteins formed by different combinations of GluR1, GluR2, GluR3 and GluR4 subunits. These subunits contain either long (GluR1 and GluR4) or short (GluR2 and GluR3) cytoplasmic carboxyl termini that control their trafficking to synapses[23,24]. Receptors with only short cytoplasmic termini (GluR3/2) continuously cycle in and out of the synapse, while those with long cytoplasmic tails (GluR4/2 and GluR1/2) are driven into synapses by strong synaptic activity[23,25,26]. Interestingly, both GluR1 and GluR4 cytoplasmic termini contain PKA phosphorylation sites[27,28]. The state of phosphorylation at the GluR1 PKA site can control channel open time[29] and has been correlated with changes in synaptic strength[30]. In addition, GluR1 recycling between the plasma membrane and endosomal compartments is controlled by PKA phosphorylation in dissociated hippocampal neurons[31]. In this study, we tested the hypothesis that PKA phosphorylation of subunits GluR1 and GluR4 directly mediates synaptic plasticity by controlling AMPA receptor trafficking and targeting to synapses.

Results

We have examined the mechanisms of activity-dependent synaptic incorporation of AMPA receptors by acute expression of green fluorescent protein–tagged AMPA receptor subunits (GluR4–GFP or GluR1–GFP) in rat hippocampal organotypic slices[32,33]. These AMPA subunits form receptors that show rectification, which allows electrophysiological detection when they are incorporated into synapses[25].

PKA controls the synaptic delivery of GluR4

We have previously shown that spontaneous neuronal activity drives the synaptic incorporation of GluR4, but not GluR1, in

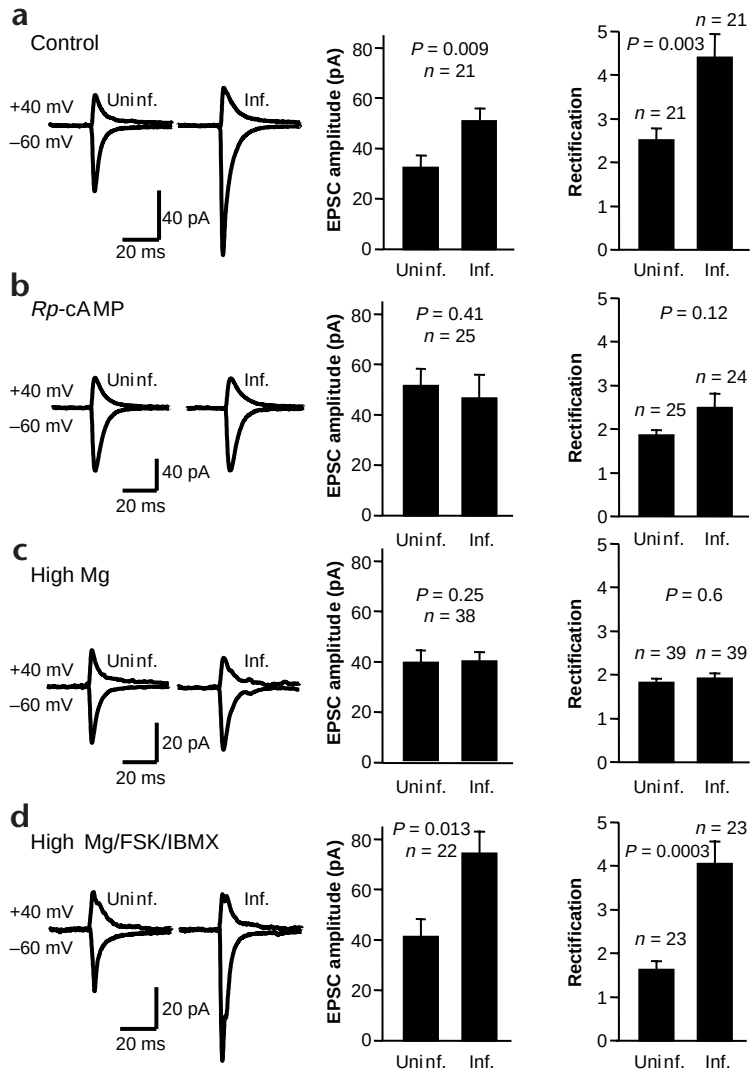

Fig. 1. PKA activity is necessary and sufficient for GluR4–GFP incorporation into synapses. (**a**–**d**) Left, sample trace of evoked AMPA receptor–mediated synaptic responses recorded at –60 mV and +40 mV from a neuron expressing GluR4–GFP (inf.) and neighboring control cell not expressing the recombinant protein (uninf.); middle, average current amplitudes at –60 mV from uninfected and infected cells (*n* represents the number of pathways from cell pairs; *P* is the probability value according to the Wilcoxon test); right, average rectification values ($I_{-60\ mV}/I_{+40\ mV}$) for uninfected and infected cells (*n* represents the number of pathways; *P* is the probability value according to the Student's *t*-test). (**a**) GluR4–GFP is incorporated into synapses in hippocampal CA1 pyramidal neurons from organotypic slice cultures. Average current amplitudes recorded at –60 mV from uninfected and infected cells were 33.0 ± 4.3 pA and 52.1 ± 4.8 pA, respectively; average rectification values for uninfected and infected cells were 2.5 ± 0.2 and 4.4 ± 0.5, respectively. (**b**) GluR4–GFP is not incorporated into synapses in the presence of the PKA inhibitor Rp-cAMP (0.2 mM). Average current amplitudes at –60 mV from uninfected and infected cells were 50.8 ± 6.6 pA and 46.0 ± 6.9 pA, respectively; average rectification values were 1.8 ± 0.1 and 2.5 ± 0.4, respectively. (**c**) GluR4–GFP is not incorporated into synapses in the presence of high Mg^{2+} concentrations (10 mM). Average current amplitudes at –60 mV from uninfected and infected cells were 38.0 ± 5.2 pA and 38.5 ± 4.2 pA, respectively; average rectification values were 1.8 ± 0.1 and 1.9 ± 0.2, respectively. (**d**) GluR4–GFP is incorporated into synapses in the presence of high Mg^{2+} concentration when cAMP levels are elevated (50 μM forskolin plus 10 μM IBMX). Average current amplitudes at –60 mV from uninfected and infected cells were 40.5 ± 7.2 pA and 72.8 ± 9.2 pA, respectively; average rectification values were 1.6 ± 0.2 and 4.1 ± 0.6, respectively.

hippocampal slices from early postnatal animals. The synaptic incorporation of GluR4 requires NMDA receptor activation but is independent of CaMKII activity[26]. The PKA signaling pathway can also be triggered by calcium entry through NMDA receptors, mediated by calcium-sensitive adenylyl cyclases[34,35]. Therefore, we tested whether PKA activity is required for synaptic incorporation of GluR4. We incubated slices for 36 hours with or without 0.2 mM Rp-cAMP, an inhibitor of PKA, soon after infection with a virus producing GluR4–GFP (see Methods). In slices maintained in the absence of inhibitor, synaptic transmission onto infected neurons showed the expected potentiation and increased rectification, indicating synaptic incorporation of receptor (**Fig. 1a**). However, transmission onto infected neurons from slices maintained in the PKA inhibitor showed no potentiation and no change in rectification (**Fig. 1b**). These results indicate that PKA activity is required for synaptic incorporation of GluR4-containing AMPA receptors. (Note that the amplitude of the synaptic responses obtained from an individual slice was arbitrary, as it depended on the particular stimulation strength used during the recording and was generally chosen to evoke responses ~30 pA. Therefore, absolute EPSC amplitudes should not be compared across slices, but only between uninfected and infected neurons.)

As previously shown, high Mg^{2+}, which reduces spontaneous neural activity, blocked activity-dependent receptor incorporation (**Fig. 1c** and ref. 26). To determine if PKA activity is sufficient to incorporate GluR4 receptors to synapses, we expressed GluR4–GFP in slices maintained in 12 mM Mg^{2+} with 10 μM IBMX and 50 μM forskolin—agents that increase the intracellular concentration of cyclic (cAMP). In these conditions, raising cAMP, and hence increasing PKA activity, induced GluR4 synaptic incorporation (**Fig. 1d**). These results indicate that PKA activation is necessary and sufficient to induce synaptic incorporation of GluR4-containing AMPA receptors.

To determine whether PKA activity can induce fast changes in synaptic strength by driving GluR4 incorporation, we expressed GluR4–GFP in slices kept in conditions with reduced neuronal activity (high Mg^{2+}), and acutely activated PKA with 10 μM IBMX and 50 μM forskolin. Synaptic transmission was nonrectifying in both control and GluR4–GFP-expressing cells before PKA activation (**Fig. 2a**), confirming that GluR4 is not incorporated into synapses when neuronal activity is reduced. A few minutes after PKA activation, however, AMPA receptor–mediated synaptic transmission was enhanced in both cells, with the potentiation being larger in the cell expressing GluR4–GFP (**Fig. 2a–c**). Notably, synaptic responses from the infected cell became rectifying after potentiation (**Fig. 2a** and **d**), indicating that the increase in transmission was at least in part due to the rapid incorporation of recombinant receptors into synapses.

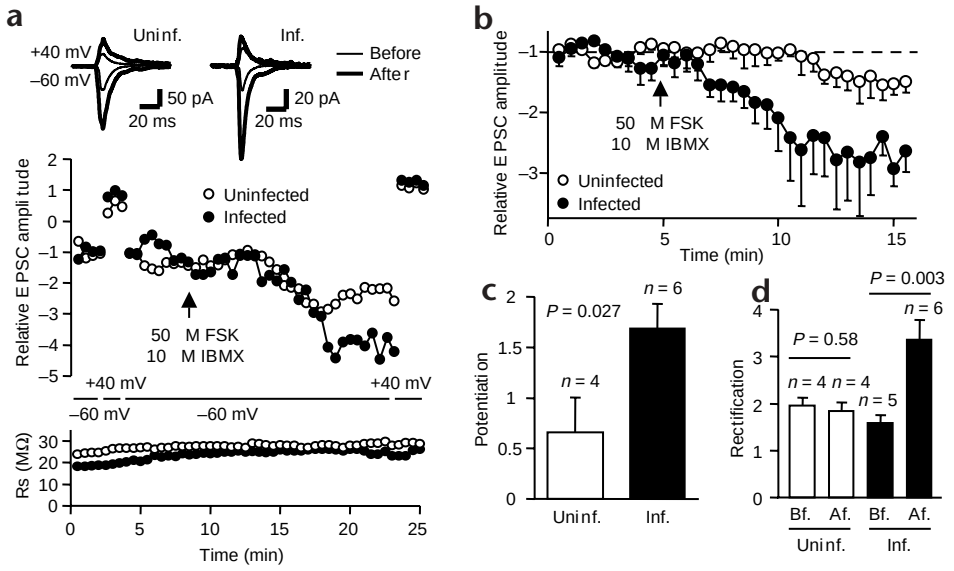

Fig. 2. GluR4–GFP is driven into synapses rapidly upon acute PKA activation. (**a**) Top, sample recordings at –60 and +40 mV from a control cell (uninfected) and a cell expressing GluR4–GFP (infected) before (thin line) and after (thick line) raising cAMP levels with forskolin and IBMX. Middle, time course of normalized AMPA receptor–mediated responses recorded from the cell pair shown in the top panel (open circles, uninfected cell; filled circles, infected cell). Forskolin (50 μM) and IBMX (10 μM) were added in the perfusion system at the time indicated by an arrow. Bottom, series resistance values measured from the uninfected (open circles) and infected (filled circles) cell during the recording shown in the middle panel. (**b**) Average time course of PKA-induced potentiation. Synaptic responses were normalized to the average response size before adding forskolin and IBMX. The average time course was calculated from four uninfected cells (open circles) and six cells expressing GluR4–GFP (filled circles). (**c**) Average synaptic potentiation after PKA activation for uninfected and infected cells. Potentiation was calculated from the ratio between the average current amplitude from 5–10 min after adding forskolin and IBMX, and the average amplitude 5 min before adding the drugs. Potentiation equals this ratio minus 1. (**d**) Average rectification values before (Bf) and after (Af) adding forskolin and IBMX for uninfected and infected cells (*n*, number of cells; *P*, probability value according to a Student's *t*-test).

PKA phosphorylation of GluR4 is activity-dependent

How does PKA regulate the synaptic incorporation of GluR4-containing AMPA receptors? As mentioned above, GluR4 can be phosphorylated by PKA at Ser842 (**Fig. 3a** and ref. 28). Therefore, neuronal activity might trigger GluR4 synaptic incorporation by inducing PKA phosphorylation of Ser842. To examine the regulation of PKA phosphorylation of GluR4 in slices, we used a phosphorylation site–specific antibody for Ser842 (see **Supplementary Fig. 1** online for the characterization of this phospho-specific antibody). Slices maintained in a medium that promotes neural activity showed significantly more GluR4 phosphorylated at Ser842 (**Fig. 3b** and **c**). This indicates that neural activity leads to phosphorylation of GluR4 at its PKA phosphorylation site.

To test if PKA phosphorylation of GluR4 at Ser842 is sufficient for synaptic incorporation, we mutated this residue from serine (S) to aspartate (D). An aspartate residue contains a negative charge and therefore may mimic phosphorylation. Transmission onto neurons expressing GluR4(S842D) and maintained in high Mg^{2+} showed potentiated transmission and increased rectification (**Fig. 4a**), indicating synaptic incorporation of the mutant receptor in the absence of neuronal activity.

PKA phosphorylation of GluR4 relieves retention

To determine the role of Ser842 in the synaptic incorporation of GluR4, we examined several GluR4 mutants. We found that replacement of Ser842 to alanine allowed GluR4 receptors to go to the synapse (**Fig. 4b**), even when neuronal activity was blocked (**Fig. 4c**). In addition, this incorporation was now independent of PKA activity (**Fig. 4d**). One possible explanation is that the hydroxyl group of Ser842 is necessary for a retention interaction that restricts the receptor from being incorporated into synapses. Phosphorylation by PKA or mutation to aspartate or alanine could prevent this interaction, leading to the synaptic incorporation of the receptor. To test if this region on the GluR4 tail was required for an interaction that prevented synaptic incorporation, we generated a GluR4 mutant with a deletion of 13 amino acids around the PKA phosphorylation site: GluR4(Δ838–850). This receptor was incorporated into synapses in an activity-independent manner (**Fig. 4e**), indicating that this region is critical for a retention interaction that is relieved by activity through PKA phosphorylation of Ser842.

GluR1 PKA phosphorylation and synaptic incorporation

As animals age, GluR4 expression in the hippocampus decreases, whereas the expression of the homologous subunit GluR1 increases[26]. We thus investigated the possibility that synaptic incorporation of GluR1 was also controlled by PKA activity. We have previously shown that CaMKII drives the synaptic incorporation of GluR1-containing AMPA receptors (**Fig. 5a** and ref. 25). Therefore, we first examined whether PKA activity is required for CaMKII to drive GluR1 into synapses. We infected slices (see Methods) with a virus coexpressing GluR1–GFP and a constitutively active CaMKII (tCaMKII), and incubated them for 36 hours with the PKA inhibitor Rp-cAMP (0.2 mM). Blockade of PKA activity prevented incorporation of GluR1 by tCaMKII (**Fig. 5b**), indicating that PKA activity is necessary for the synaptic incorporation of GluR1 by CaMKII. In fact, synaptic transmission was depressed when we coexpressed GluR1 and tCaMKII in the presence of the PKA inhibitor. This is similar to the effects of coexpression of tCaMKII with a mutant of GluR1 lacking its PDZ-binding motif[25].

GluR1 contains a PKA phosphorylation site (Ser845) in a similar region as that of GluR4 (**Fig. 3a** and ref. 27). Thus, to prevent phosphorylation by PKA, we generated a GluR1 mutant with Ser845 replaced by alanine: GluR1(S845A)–GFP. When coexpressed with constitutively active CaMKII (tCaMKII), GluR1(S845A)–GFP was not driven into synapses (**Fig. 5c**). These

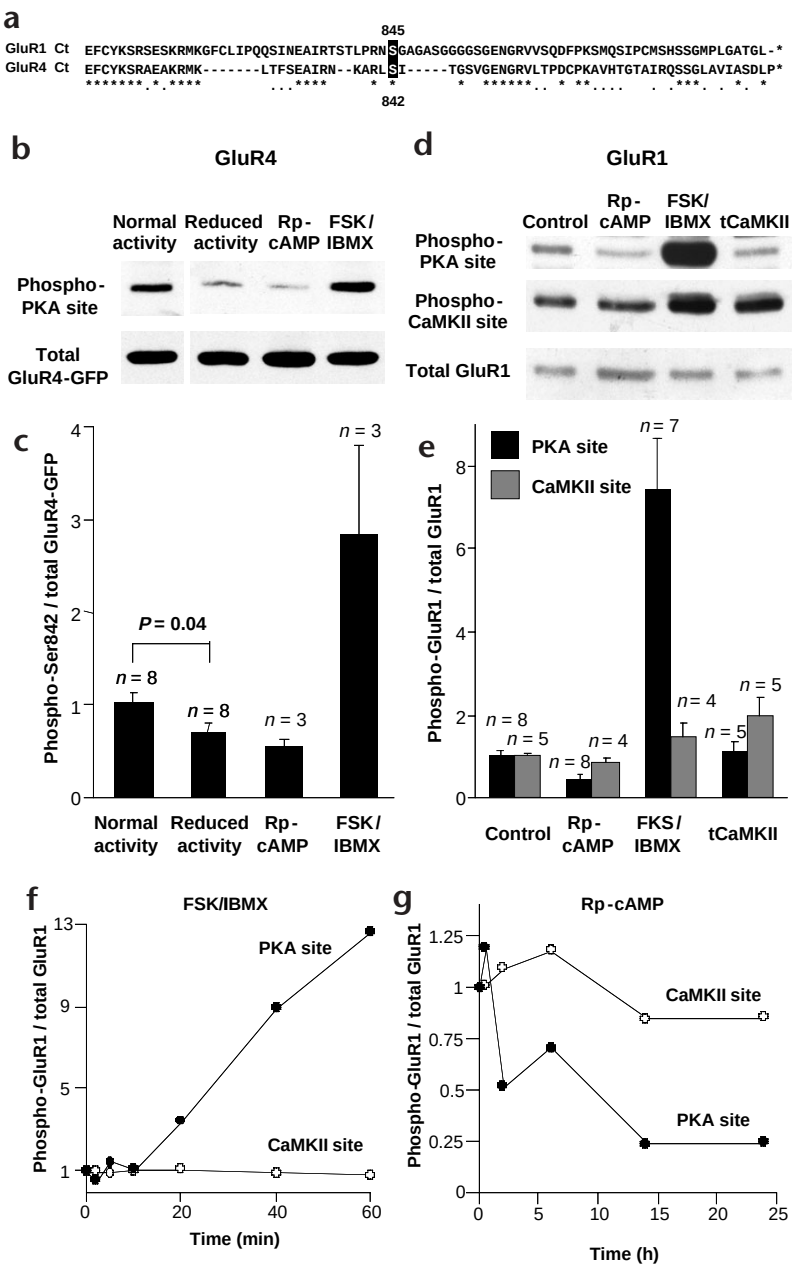

Fig. 3. Regulated phosphorylation of GluR4 and GluR1 by PKA and CaMKII. (**a**) Alignment of GluR1 and GluR4 cytoplasmic C-termini showing the corresponding serine residues phosphorylated by PKA[27,28]. Amino acid similarity is indicated by (.); identical residues as (*). (**b**) PKA phosphorylation of GluR4 is enhanced by neuronal activity. Western blot analysis of the amount of phosphorylated Ser842 in GluR4–GFP from slices kept in conditions of normal neuronal activity, reduced activity (10 mM Mg^{2+}), blockade of PKA activity (0.2 mM Rp-cAMP) or enhanced PKA activity (50 μM forskolin plus 10 μM IBMX). Total amount of GluR4–GFP was assayed with an antibody insensitive to serine 842 phosphorylation. (**c**) Quantification of several experiments as the one shown in (**b**); the amount of phosphorylated receptor was normalized by its total amount. (**d**) PKA phosphorylation of GluR1 is not affected by CaMKII activity. Western blot analysis of the amount of phosphorylated Ser845 (PKA site) and Ser831 (CaMKII site) in GluR1 from slices kept in normal medium, in the presence of the PKA inhibitor Rp-cAMP (0.2 mM), or the PKA activators forskolin and IBMX (10 μM each), or from slices infected with a virus expressing a constitutively active form of CaMKII (tCKII)[25]. The total amount of GluR1 was assayed with a phospho-insensitive antibody. (**e**) Quantification of PKA and CaMKII phosphorylation of GluR1 from several experiments as the one shown in (**d**). Phospho-specific signal was normalized by the total amount of GluR1 (*n* represents number of independent experiments; *P* is the probability value according to a Student's *t*-test). (**f**) Time course of PKA and CaMKII phosphorylation of GluR1 upon PKA activation. Western blot quantification of the amount of phosphorylated Ser845 (PKA site) and Ser831 (CaMKII site) in GluR1 from slices treated acutely with the PKA activators forskolin and IBMX (10 μM each) for different periods of time. The total amount of GluR1 was assayed with a phospho-insensitive antibody. Each time point corresponds to four slices treated in parallel and pooled together for western blot analysis. (**g**) Time course of GluR1 dephosphorylation after blocking PKA activity. Western blot quantification of the amount of phosphorylated Ser845 (PKA site) and Ser831 (CaMKII site) in GluR1 from slices kept in the presence of the PKA inhibitor Rp-cAMP (0.2 mM) for different periods of time. The total amount of GluR1 was assayed with a phospho-insensitive antibody. Each time point corresponds to four slices treated in parallel and pooled together for western blot analysis.

results indicate that PKA phosphorylation of GluR1 at Ser845 is required for incorporation of GluR1 by CaMKII.

We also examined whether PKA activity is sufficient to incorporate GluR1 into synapses. Activation of PKA alone by increasing intracellular cAMP levels pharmacologically did produce phosphorylation at Ser845 (**Fig. 3d–f**), but was not sufficient for the synaptic incorporation of GluR1 (**Fig. 5d**). As a further contrast to GluR4, the mutation of Ser845 to neither alanine nor aspartate led to synaptic incorporation (rectification values for neurons expressing versus non-infected: GluR1(S845A), 2.4 ± 0.2 versus 2.3 ± 0.2, $P > 0.5$; GluR1(S845D), 2.3 ± 0.1 versus 2.0 ± 0.1, $P > 0.07$). We conclude that phosphorylation of GluR1 by PKA is necessary but not sufficient for its synaptic incorporation.

GluR1 Ser845 phosphorylation required for plasticity

We have previously shown that GluR1 is incorporated into synapses during LTP[25]. To determine which phosphorylation events on GluR1 are important for LTP, we examined cells expressing GluR1–GFP, GluR1(S831A)–GFP or GluR1(S845A)–GFP (investigator was blind to which construct was expressed). Similar and robust potentiation was obtained from cells expressing GluR1–GFP or GluR1(S831A)–GFP (**Fig. 6**). These results are in agreement with previous observations showing that active CaMKII incorporates GluR1(S831A) into synapses[25]. In contrast, LTP was significantly diminished in cells expressing GluR1(S845A). These results indicate that PKA phosphorylation of GluR1 S845 is necessary for efficient LTP.

PKA and CaMKII act in parallel

Is PKA phosphorylation of GluR1 Ser845 downstream of CaMKII activity, or do these kinases act in parallel? To examine this, we used two phosphospecific antibodies that recognize

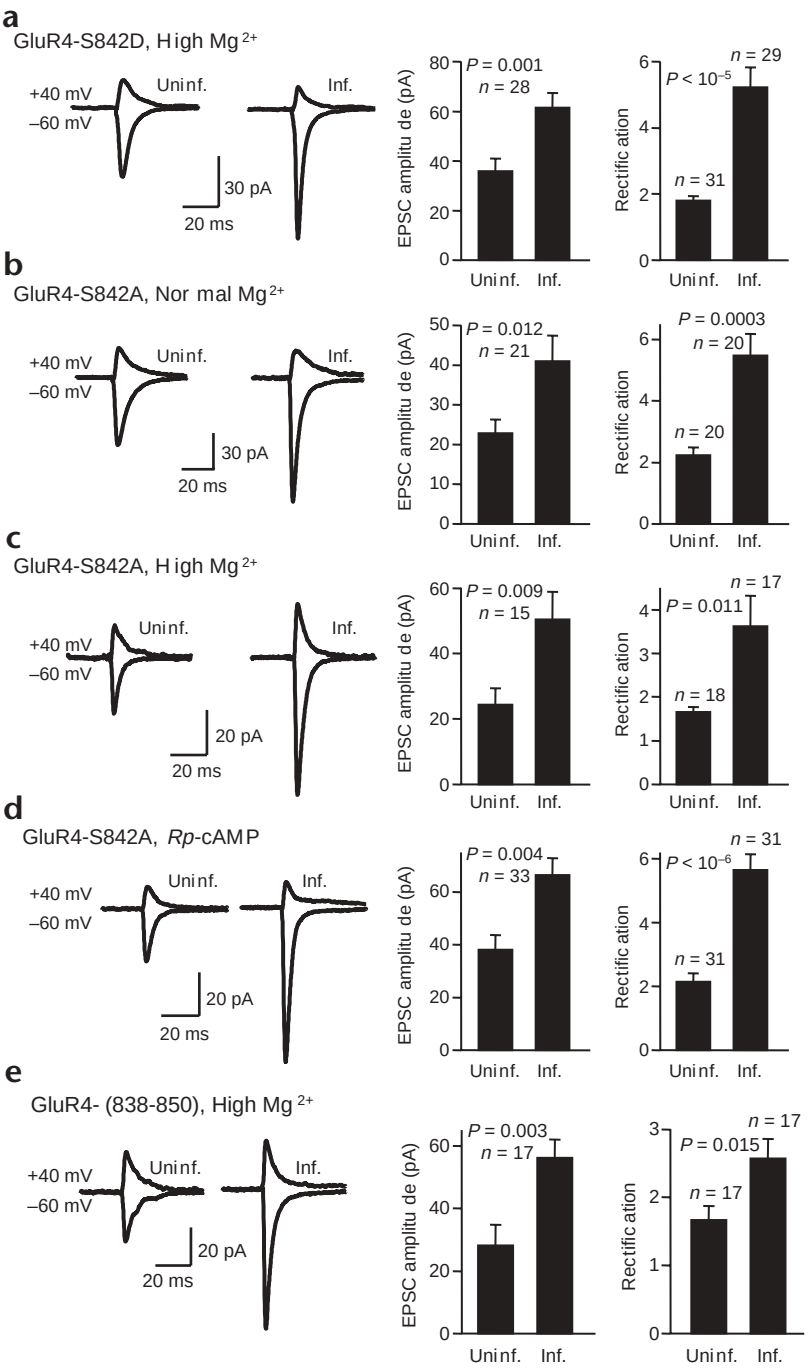

Fig. 4. GluR4 Ser842 controls a retention interaction. (**a**–**e**) Left, sample trace; middle, average current amplitude at –60 mV from uninfected and infected cells; right, average rectification values (I_{-60mV}/I_{+40mV}) for uninfected and infected cells. (**a**) GluR4(S842D) is incorporated into synapses in the presence of high Mg^{2+} concentration (10 mM). Average current amplitudes at –60 mV from uninfected and infected cells were 36.0 ± 5.0 pA and 61.6 ± 5.9 pA, respectively; average rectification values were 1.8 ± 0.1 and 5.2 ± 0.6, respectively. (**b**) GluR4(S842A) is incorporated into synapses in normal culture conditions. Average current amplitudes at –60 mV for uninfected and infected cells were 22.9 ± 3.5 pA and 41.0 ± 6.4 pA, respectively; average rectification values were 2.2 ± 0.3 and 5.5 ± 0.7, respectively. (**c**) GluR4(S842A) is incorporated into synapses in the presence of high Mg^{2+} concentration (10 mM). Average current amplitudes at –60 mV from uninfected and infected cells were 24.3 ± 5.0 pA and 50.3 ± 8.4 pA, respectively; average rectification values were 1.7 ± 0.1 and 3.6 ± 0.7, respectively. (**d**) GluR4(S842A) is incorporated into synapses in the presence of the PKA inhibitor Rp-cAMP (0.2 mM). Average current amplitudes at –60 mV from uninfected and infected cells were 38.2 ± 5.4 pA and 66.4 ± 6.4 pA, respectively; average rectification values were 2.1 ± 0.3 and 5.6 ± 0.5, respectively. (**e**) GluR4(Δ838–850) is incorporated into synapses in the presence of high Mg^{2+} concentration (10 mM). Average current amplitudes at –60 mV from uninfected and infected cells were 28.2 ± 6.6 pA and 56.2 ± 5.8 pA, respectively; average rectification values were 1.7 ± 0.2 and 2.6 ± 0.3, respectively.

phosphorylation of GluR1 at either its CaMKII site (Ser831) or PKA site (Ser845). Slices infected with tCaMKII showed increased phosphorylation of GluR1 at its Ser831 site, but not its PKA site (**Fig. 3d** and **e**). This suggests that PKA phosphorylation of GluR1 is not downstream of CaMKII activity. As a control, we pharmacologically increased cAMP levels, which did lead to the phosphorylation of GluR1 at Ser845 (**Fig. 3d–f**). Indeed, the large increase in phospho-Ser845 signal upon PKA activation indicated that only a small fraction of GluR1 is phosphorylated at Ser845 in basal conditions or after CaMKII activation.

We also observed that pharmacological activation of PKA led to a modest increase in the CaMKII phosphorylation of GluR1 (**Fig. 3d** and **e**). To investigate the relation between CaMKII and PKA phosphorylation of GluR1, we looked at the time course of these two phosphorylation events upon modification of PKA activity. Acute activation of PKA led to a rapid phosphorylation of S845 (more than ten-fold increase in phosphorylation after 1 hour incubation with forskolin and IBMX) without any significant effect on S831 phosphorylation over the same period of time (**Fig. 3f**). This result suggests that the increased phosphorylation of S831 upon PKA activation (**Fig. 3d** and **e**) was a secondary effect due to a prolonged (more than 24 hours) pharmacological enhancement of PKA activity. Additionally, blockade of PKA activity resulted in a slow decrease in phospho-S845, which reached a plateau after 12 hours of incubation with Rp-cAMP. No significant effect on S831 phosphorylation was observed over the same period of time (**Fig. 3g**). These results taken together indicate that CaMKII activity and PKA phosphorylation of GluR1 at Ser845 are largely independent processes and that both are required for the synaptic incorporation of GluR1-containing AMPA receptors.

Discussion

Here we have shown that PKA is directly involved in synaptic plasticity by controlling the incorporation of AMPA receptors

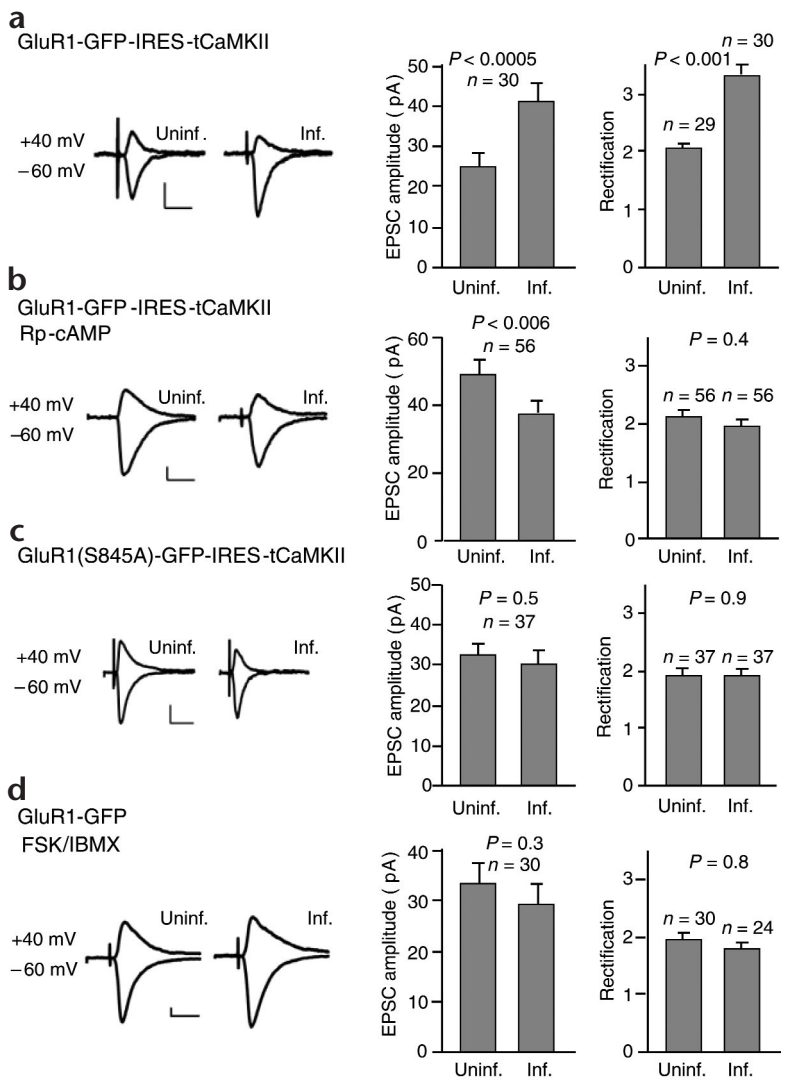

Fig. 5. PKA activity is necessary but not sufficient for the CaMKII-driven incorporation of GluR1 into synapses. (**a**–**d**) Left, sample trace; middle, average current amplitudes at –60 mV from uninfected and infected cells; right, average rectification values (I_{-60mV}/I_{+40mV}) for uninfected and infected cells. (**a**) GluR1 is incorporated into synapses when coexpressed with a constitutively active form of CaMKII (tCaMKII). For uninfected and infected cells, average current amplitudes at –60 mV were 24.6 ± 3.6 pA and 40.6 ± 5.0 pA, respectively; average rectification values were 2.0 ± 0.1 and 3.3 ± 0.2, respectively. (**b**) GluR1–GFP coexpressed with constitutively active CaMKII is not incorporated into synapses in the presence of the PKA inhibitor Rp-cAMP (0.2 mM). For uninfected and infected cells, average current amplitudes at –60 mV were 49.0 ± 3.6 pA and 37.5 ± 3.6 pA, respectively; average rectification values were 2.1 ± 0.1 and 1.9 ± 0.1, respectively. (**c**) The PKA phosphorylation mutant GluR1(S845A) is not incorporated into synapses when coexpressed with constitutively active CaMKII. For uninfected and infected cells, average current amplitudes at –60 mV were 32.3 ± 3.0 pA and 29.8 ± 3.3 pA, respectively; average rectification values were 1.9 ± 0.1 and 1.9 ± 0.1, respectively. (**d**) Activation of PKA with forskolin and IBMX (10 μM each) is not sufficient to incorporate GluR1. For uninfected and infected cells, average current amplitudes at –60 mV were 33.1 ± 4.0 pA and 29.1 ± 4.0 pA, respectively; average rectification values were 1.9 ± 0.1 and 1.8 ± 0.1, respectively. Scale bar, 10 pA, 25 ms.

into synapses. We have previously reported that the synaptic incorporation of GluR4-containing receptors is dependent on spontaneous neuronal activity[26]. Now, we find that neuronal activity leads to phosphorylation of GluR4 at Ser842 by PKA. From a combination of pharmacological manipulations and site-directed mutagenesis, we conclude that this phosphorylation event is necessary and sufficient for receptor incorporation. In addition, our mutagenesis studies indicate that receptor incorporation is controlled by a retention interaction that keeps the unphosphorylated receptor away from synapses. Taken together, our results support a model in which neuronal activity leads to PKA activation and phosphorylation of GluR4 at Ser842. This phosphorylation releases a retention interaction, leading to the incorporation of the receptor into synapses. It is notable that phosphorylation of this single site on GluR4, with the concomitant release from retention, is the dominant target for activity-dependent regulation of these AMPA receptors. Other aspects of receptor incorporation, localization to precise synaptic loci, and subsequent stabilization, seem to occur in the absence of neuronal activity. Controlling synaptic accumulation of receptors by regulating their non-synaptic retention may be a general mechanism; it may also be a mechanism for control of GluR2-containing receptors during LTD[36,37].

PKA activity is also critical for regulating the synaptic incorporation of GluR1-containing AMPA receptors. By combining pharmacology with site-directed mutagenesis, we have shown that PKA phosphorylation of GluR1 at Ser845 is required for synaptic incorporation and LTP. These results are consistent with a deficiency in LTP in mutant mice lacking the PKA and CaMKII phosphorylation sites (R.H., unpub. data). The residual LTP observed in these mutant mice could be explained by the regulated delivery of other AMPA receptor subunits such as GluR2-long[38]. In contrast to GluR4, PKA phosphorylation is not sufficient for incorporation of GluR1-containing receptors. In this case, additional signals that depend on CaMKII activation are also required. Notably, phosphorylation of GluR1 at Ser831 by CaMKII is not required for receptor incorporation[25] or LTP (present finding). We have also determined that CaMKII activation does not lead to phosphorylation of GluR1 at Ser845. This result is in agreement with previous data showing that PKA phosphorylation of GluR1 at Ser845 occurs before CaMKII phosphorylation of Ser831 during synaptic potentiation[30]. Taking these results together, we propose that PKA phosphorylation at Ser845 in GluR1 sets the number of AMPA receptors that are available for incorporation and/or stabilizes synaptically delivered receptors, whereas CaMKII triggers the cellular machinery, mediated by Ras signaling[38], that results in the synaptic insertion of the receptor.

The precise molecular mechanism that is mediated by PKA phosphorylation of GluR1 at Ser845 remains to be elucidated. As alanine substitution of this residue blocks synaptic delivery, it seems that PKA phosphorylation is required either to facilitate a protein–protein interaction or to 'push off' a retention interaction; mere release, as in the case with an alanine substitution

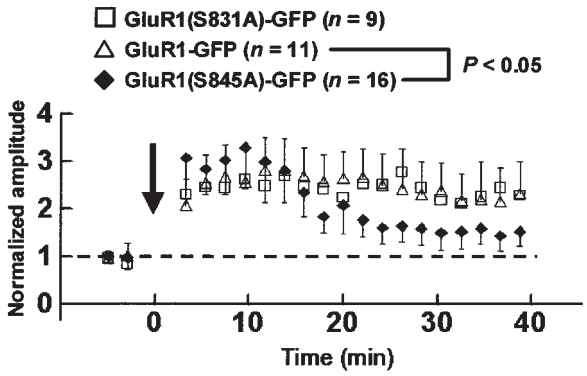

Fig. 6. Phosphorylation of PKA target S845, but not CaMKII target S831, is required for stable LTP. Organotypic slice cultures were infected with virus expressing either GluR1–GFP, GluR1(S831A)–GFP or GluR1(S845A)–GFP. Whole-cell recordings were established from neurons expressing the recombinant proteins, and LTP was induced by pairing as previously described[25]. Experiments were done blind with respect to which construct was expressed.

for GluR4, is insufficient. The fact that the aspartate mutation of GluR1 Ser845 also blocks delivery (data not shown) suggests that either the unphosphorylated serine residue is required at some step during the delivery process, or the aspartate negative charge does not fully mimic the properties of a phospho-serine residue in this particular case. It is notable that after blockade of PKA activity, phosphorylation at Ser845 persists for hours (**Fig. 3g**). This suggests that phosphorylation of GluR1 by PKA can occur hours before activation of CaMKII-induced signaling and still permit synaptic incorporation of the receptor. It will be important to identify physiological stimuli that engage the molecular machinery (for example, AKAP and SAP-97)[39] and drive PKA phosphoryaltion of GluR1. These events seem to be critical to some forms of learning[40].

Several studies indicate that PKA activity can serve as a gate for neural plasticity by controlling phosphatase activity[11,12]. It has also been shown that dopaminergic[41,42] and noradrenergic[43] activities can facilitate synaptic plasticity through PKA-dependent mechanisms. Furthermore, dopaminergic activation increases GluR1 phosphorylation at the PKA site[44–47]. While there may be several sites where PKA can modulate plasticity, our results show that PKA acts as a gate for plasticity directly by phosphorylating GluR1 at Ser845, and by making AMPA receptors available for stable synaptic incorporation.

GluR4 is expressed in early postnatal development of the hippocampus, whereas GluR1 is mainly expressed at later stages[26]. Here we show that PKA phosphorylation is sufficient for GluR4 synaptic incorporation, but it is only one of the requirements for GluR1 incorporation. Thus early in development, PKA phosphorylation of AMPA receptors may be the primary mediator of receptor incorporation and, at later developmental stages, AMPA receptor phosphorylation by PKA may become a gate for incorporation as other signaling pathways become required. It is tempting to speculate that the number of signaling pathways required for plasticity increases during development, thus imposing a higher threshold for plasticity and imparting greater specificity with age. A recent study[48] confirms our previous findings[26] that CaMKII does not control plasticity in early postnatal development and agrees with findings in this study indicating a critical role for PKA.

Methods

Constructs of recombinant receptors and expression. The GFP-tagged AMPA receptor subunits (GluR1–GFP and GluR4–GFP) were made as previously described[26,33]. Point mutants and deletion mutants were made by PCR. All constructs were re-cloned into pSinRep5 for expression using Sindbis virus[32]. Recombinant proteins were expressed in hippocampal CA1 pyramidal neurons from organotypic slice cultures using the Sindbis expression system[32]. All biosafety procedures and animal care protocols were approved by Cold Spring Harbor Laboratories. Slices were prepared from postnatal day 5–7. To mimic the expression profile of the endogenous AMPA receptor subunits[26], slices were infected with GluR4–GFP virus within 12 h of their preparation, and with GluR1–GFP after slices had been maintained one week in culture. Previous control experiments[26] indicate that the different trafficking of GluR1 and GluR4 is due to their sequences and not to the age when they are expressed. Expression of the recombinant proteins was always for 36 h. For pharmacological experiments, the drugs were added to the culture medium immediately after infecting with the Sindbis virus, and they were maintained in the medium during the expression time. Different treatments were: 0.2 mM Rp-8-Br-cAMPS (Calbiochem, San Diego, California; abbreviated as Rp-cAMP in the figures and text), 10 μM (GluR1 experiments) or 50 μM (GluR4 experiments) forskolin (Calbiochem), 10 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, Missouri), 10 mM $MgCl_2$ (high Mg^{2+}). Drugs included during the slice incubation period were omitted during recordings.

Electrophysiology. Simultaneous double whole-cell recordings were obtained from nearby pairs of infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. The recording chamber was perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 0.1 mM picrotoxin, 10 μM bicuculline, 0.1 mM DL-APV and 2 μM 2-chloroadenosine, at pH 7.4, gassed with 5% CO_2/95% O_2. In the case of the experiment shown in **Fig. 2**, the perfusion solution also contained 5 mM $MgCl_2$; 50 μM forskolin and 10 μM IBMX were added during the recording at the indicated time. Patch recording pipettes (3–6 MΩ) were filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine, 0.6 mM EGTA and 0.1 mM spermine, at pH 7.25. Whole-cell recordings were carried out with two Axopatch 1D amplifiers (Axon Instruments, Union City, California). Synaptic responses were evoked with two bipolar electrodes with single voltage pulses (200 μs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 μm and 500 μm from the recorded cells. Synaptic AMPA receptor–mediated responses at –60 mV and +40 mV were averaged over 50–100 trials, and their ratio was used as an index of rectification. LTP experiments were carried out as previously described[25]. In this study, we used data from two pathways in approximately one-half of the cell pairs and one pathway in the remainder (when reliable transmission was not obtained on the second pathway). The two pathways provided independent information (see below), so they were counted as independent observations and included in the value of *n*. The two pathways were tested for providing independent information in the following manner. A potentiation index (PI = amplitude from infected cell / amplitude from non-infected cell) was calculated for each pathway. For each cell pair, we plotted PI of one pathway against PI of the other pathway. If pathways are correlated (that is, they do not provide independent information), then there should be a positive correlation. However, for 64 two-pathway cells, we found a correlation coefficient of 0.004, indicating independent information in the two pathways (only cell pairs from treatments that, on the average, increased EPSC size and rectification, were used for this analysis). For LTP experiments, each cell was counted as $n = 1$.

Biochemistry. Hippocampal slice extracts were prepared in homogenization buffer containing phosphatase inhibitors as previously

described[25]. Expression of AMPA receptor subunits and level of phosphorylation was analyzed by western blot with the following antibodies: anti-GluR1 (Chemicon, Temecula, California), anti-GluR4 (Chemicon), anti-phospho-serine 842 in GluR4 (characterization shown in **Supplementary Fig. 1**), anti-phospho-serine 831 in GluR1 (Upstate Biotechnology, Lake Placid, New York) and anti-phospho-serine 845 in GluR1 (Upstate Biotechnology). Quantifications were done by chemoluminescence and densitometric scanning of the films under linear exposure conditions.

Note: Supplementary information is available on the Nature Neuroscience website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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