# Characterization of Multiple Phosphorylation Sites on the AMPA Receptor GluR1 Subunit

**Katherine W. Roche,* Richard J. O'Brien,† Andrew L. Mammen,* Jeffrey Bernhardt,* and Richard L. Huganir***

*Department of Neuroscience

†Department of Neurology

Howard Hughes Medical Institute

The Johns Hopkins University School of Medicine

Baltimore, Maryland 21205

## Summary

**We have characterized the phosphorylation of the glutamate receptor subunit GluR1, using biochemical and electrophysiological techniques. GluR1 is phosphorylated on multiple sites that are all located on the C-terminus of the protein. Cyclic AMP-dependent protein kinase specifically phosphorylates SER-845 of GluR1 in transfected HEK cells and in neurons in culture. Phosphorylation of this residue results in a 40% potentiation of the peak current through GluR1 homomeric channels. In addition, protein kinase C specifically phosphorylates Ser-831 of GluR1 in HEK-293 cells and in cultured neurons. These results are consistent with the recently proposed transmembrane topology models of glutamate receptors, in which the C-terminus is intracellular. In addition, the modulation of GluR1 by PKA phosphorylation of Ser-845 suggests that phosphorylation of this residue may underlie the PKA-induced potentiation of AMPA receptors in neurons.**

## Introduction

Ionotropic glutamate receptors are ligand-gated ion channels that mediate the majority of rapid excitatory neurotransmission in the mammalian CNS (Seeburg, 1993; Hollmann and Heinemann, 1994). In addition, glutamate receptors are critical for various forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), that are well-established paradigms for the study of the cellular basis of learning and memory (Bliss and Collingridge, 1993; Linden, 1994). Glutamate receptors have been classified based on their pharmacological and physiological properties as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors. Recent molecular cloning studies have shown that ionotropic glutamate receptors are oligomeric complexes of homologous subunits (Seeburg, 1993; Hollmann and Heinemann, 1994) that combine to form AMPA (GluR1–4 or A-D), kainate (GluR5–7; KA1–2), and NMDA (NR1; NR2A-D) receptors.

Protein phosphorylation is well accepted as a primary mechanism in the regulation of cellular function (Krebs, 1994) and recent studies have shown that protein phosphorylation modulates glutamate receptors and may underlie some forms of synaptic plasticity (Raymond et al., 1993b; Roche et al., 1994b; Nicoll and Malenka, 1995). Physiological studies have demonstrated that protein kinase and phosphatase activity in neurons regulates glutamate receptor ion channel function (Roche et al., 1994b). For example, intracellular perfusions of cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) have been shown to potentiate AMPA receptor currents in hippocampal neurons (Greengard et al., 1991; Wang et al., 1991; McGlade-McCulloh et al., 1993; Wang et al., 1994), while intracellular perfusion of PKC has been shown to potentiate NMDA receptors in spinal cord neurons (Chen and Huang, 1992). However, there are relatively few studies that demonstrate the direct phosphorylation and functional modulation of glutamate receptors by protein kinases.

Several studies have demonstrated direct phosphorylation of AMPA, kainate, and NMDA receptor subunits using biochemical techniques (Raymond et al., 1993a; Blackstone et al., 1994; Tingley et al., 1993; Tan et al., 1994). However, the identification of glutamate receptor phosphorylation sites has been difficult and controversial. Glutamate receptor subunits were originally proposed to have the same transmembrane topology as the subunits for other ligand-gated ion channels, such as the nicotinic acetylcholine receptor and the $GABA_A$ receptor, with a large N-terminal extracellular domain, four transmembrane domains (TM1-TM4), and an extracellular C-terminus. All of the phosphorylation sites on the nicotinic and $GABA_A$ receptor subunits have been localized to the major intracellular loop between TM3 and TM4 (Swope et al., 1992; Raymond et al., 1993b). Thus, most early studies mapping the phosphorylation sites on glutamate receptors concentrated on this region. Site-specific mutagenesis techniques combined with electrophysiological or biochemical techniques have identified phosphorylation sites on the major loop between TM3 and TM4 of kainate and AMPA receptor subunits (Raymond et al., 1993a; Wang et al., 1993; Yakel et al., 1995; Nakazawa et al., 1995). In contrast, the majority of phosphorylation sites on the NR1 subunit of the NMDA receptor were found on the C-terminal domain of the NR1 subunit (Tingley et al., 1993). Since regions containing phosphorylation sites are generally thought to be intracellular and accessible to protein kinases, this data was inconsistent with the originally proposed transmembrane topology model (Tingley et al., 1993). Immunocytochemical studies demonstrating that the C-terminus of GluR1 was intracellular (Molnar et al., 1993), as well as studies identifying glycosylation sites on the loop between TM3 and TM4 in GluR6 (Roche et al., 1994a; Taverna et al., 1994) also suggested that glutamate receptors are members of a distinct superfamily of ligand-gated ion channels. More recent studies using a variety of techniques have lead to the proposal of a new transmembrane topology model that is consistent with most of the available data (Wo and Oswald, 1994; Hollmann et al., 1994; Stern-Bach et al., 1994; Bennett and Dingledine, 1995; Wood et al., 1995). However, the experiments identifying phosphorylation sites on the

loop between TM3 and TM4 of kainate and AMPA receptor subunits are not consistent with this model (Raymond et al., 1993; Wang et al., 1993; Yakel et al., 1995; Nakazawa et al., 1995).

We have previously described the phosphorylation of the AMPA receptor subunit GluR1 in primary neuronal cultures, as well as in HEK-293 cells transiently expressing GluR1 (Blackstone et al., 1994). In neurons, GluR1 is basally phosphorylated on a single major phosphopeptide. Treatment of neuronal cultures with either forskolin and IBMX to activate PKA, or phorbol esters to activate PKC, regulated phosphorylation of the GluR1 protein (Blackstone et al., 1994). In addition, synaptic activity increased the phosphorylation of the GluR1 protein in a manner similar to PKC activation (Blackstone et al., 1994). In this study, a variety of biochemical techniques were used to further characterize the multiple sites of phosphorylation on the AMPA receptor GluR1 subunit, and the physiological effect of phosphorylation of these sites was examined. We have determined that the basal and regulated phosphorylation sites of the AMPA receptor GluR1 subunit are all present on the C-terminus. We were not able to detect phosphorylation of the loop region between TM3 and TM4, including Ser-627 and Ser-658, which have previously been reported to be sites of AMPA receptor phosphorylation (Yakel et al., 1995; Nakazawa et al., 1995). These data are consistent with the new transmembrane topology model for glutamate receptors, which proposes that the loop between TM3 and TM4 is extracellular and that the C-terminus is intracellular. In addition, our results demonstrated that PKA phosphorylation of Ser-845 on the C-terminus of GluR1 potentiates its response to glutamate, suggesting that phosphorylation of this residue may mediate PKA modulation of AMPA receptor function in neurons.

## Results

To examine the phosphorylation of the recombinant AMPA receptor GluR1 subunit, HEK-293 cells were transfected with GluR1 cDNA, prelabelled with [$^{32}$P]-orthophosphate, and the GluR1 subunit immunoprecipitated with anti-GluR1 antibodies. As reported previously (Blackstone et al., 1994), the GluR1 subunit migrated on SDS-PAGE as a 106 kDa protein, and was basally phosphorylated (Figure 1A). The phosphorylation of GluR1 could be significantly increased by treatment of the cells with forskolin and IBMX (53%) or phorbol esters (16%) (Blackstone et al., 1994). To characterize this phosphorylation further, the GluR1 protein was excised from the gel, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping (Huganir et al., 1984). Tryptic phosphopeptide maps of GluR1 protein isolated from HEK-293 cells demonstrated that it was basally phosphorylated on at least five distinct reproducible tryptic phosphopeptides (phosphopeptides 1, 3, 4, 6, and 7, Figure 1B). Phosphorylation of phosphopeptide 2 was weak and highly variable. Treatment of the cells with forskolin induced phosphorylation of GluR1 on a novel phosphopeptide (phosphopeptide 5, Figure 1C), while TPA treatment resulted in a slight increase in phosphorylation of phosphopeptide 4 (Figure 1D). A schematic diagram of these phosphopeptides is shown in

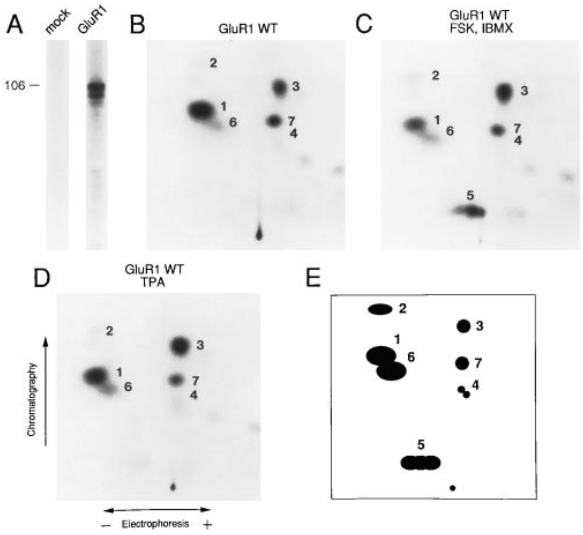


Figure 1. Phosphorylation of the GluR1 Subunit Transiently Expressed in HEK-293 Cells

HEK-293 cells transfected with GluR1 or mock transfected were prelabelled with 1 mCi/ml [$^{32}$P]-orthophosphate and then treated with 20 μM forskolin and 75 μM IBMX (C) or 100 nM TPA (D).

(A) The GluR1 subunit was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography.

(B–D) The immunoprecipitated GluR1 subunit was excised from the gel, digested with trypsin, resolved in two dimensions on TLC plates, and visualized by autoradiography.

(E) A schematic of the two dimensional phosphopeptide maps is shown.

Figure 1E. With the exception of phosphopeptide 7, all of these phosphopeptides have been observed previously in GluR1 isolated from cortical neurons in culture (Blackstone et al., 1994). Moreover, phosphorylation of phosphopeptide 5 was also regulated by forskolin in neurons. In contrast, phorbol esters barely increased the phosphorylation of phosphopeptides 3, 4, and 6 in HEK cells, while phorbol esters clearly increased phosphorylation of these sites in neurons (Blackstone et al., 1994). Apparently, phosphopeptides 3, 4 and 6 are basally phosphorylated in HEK-293 cells, which suggests that these peptides are phosphorylated by significant basal PKC activity (Tingley et al., 1993) or by another constitutively active kinase.

To identify the phosphorylation sites, we used site-directed mutagenesis to mutate potentially phosphorylated amino acid residues. However, our initial attempts to localize these sites were based on the originally proposed transmembrane topology models, and we therefore mutated several serine residues in the loop between TM3 and TM4, including S627A, S650A, and S658A. None of these mutations had any detectable effect on the phosphorylation of GluR1 in the presence of forskolin, IBMX, and TPA, as analyzed by phosphopeptide map analysis. Because of the evolving models of the transmembrane topology of glutamate receptors, we decided to generate chimeric proteins between AMPA receptor subunits to narrow down the intracellular domains that contained the phosphorylated residues of GluR1. Our early results demonstrated that the phosphopeptide maps of the AMPA receptor GluR1 and

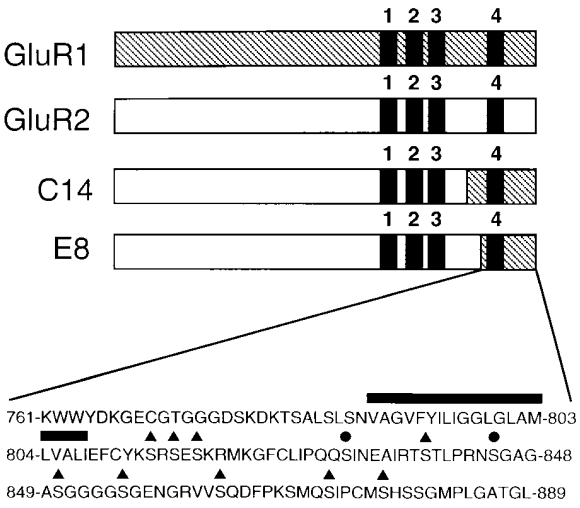


Figure 2. GluR2/GluR1 Subunit Chimeras

Schematic of the GluR2/GluR1 chimeras E8 and C14. Both of the chimeras consist of the N-terminus of GluR2 and the C-terminus of GluR1, with the majority of the protein being GluR2. The E8 chimera is almost entirely GluR2, with only the C-terminal 130 amino acids of GluR1. The expanded region of the C-terminus of the E8 chimera shows the amino acid sequence of the C-terminus of GluR1. The solid bar indicates TM4, the closed triangles indicate the serine residues mutated to alanine residues, and the closed circles indicate the identified phosphorylation sites.

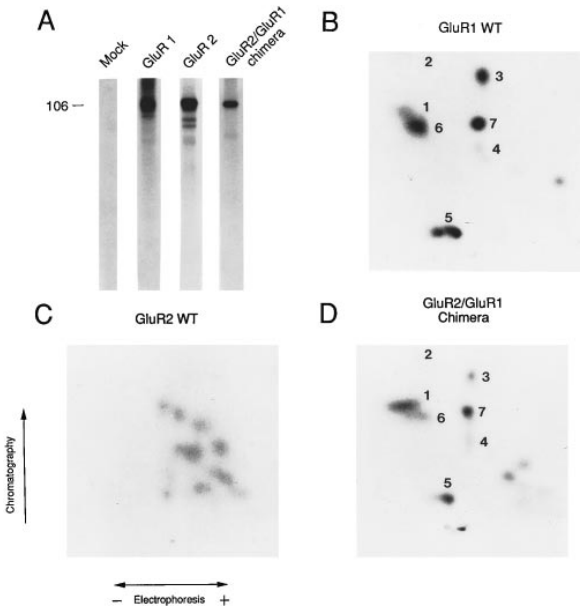


Figure 3. Phosphorylation of the GluR1, GluR2, and GluR2/GluR1 Subunits Transiently Expressed in HEK-293 Cells

HEK-293 cells transfected with GluR1, GluR2, or the GluR2/GluR1 chimera cDNA or mock transfected were prelabelled with 2 mCi/ml [$^{32}$P]-orthophosphate and then stimulated with 20 μM forskolin, 75 μM IBMX, and 100 nM TPA.

(A) The subunit of interest was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography. Mock, GluR1, and GluR2/GluR1 chimera-transfected cells were immunoprecipitated with GluR1 C-terminal antipeptide antibodies (lanes 1, 2, and 4). GluR2- transfected cells were immunoprecipitated with GluR2/3 C-terminal antipeptide antibodies (lane 3).

(B–D) The immunoprecipitated GluR1 (B), GluR2 (C), and GluR2/1 E8 chimera (D) subunits were excised from the gel, digested with trypsin, resolved in two dimensions on TLC plates, and visualized by autoradiography.

GluR2 subunits were distinct, enabling us to construct chimeric proteins of the GluR1 and GluR2 subunits to determine which region of GluR1 contained the characterized phosphorylation sites. Several GluR2/GluR1 chimeras were generated (Moore and Blakely, 1994) that consisted of the N-terminal region of GluR2 and the C-terminal region of GluR1, with switch points between GluR2 and GluR1 at various locations throughout the proteins. We analyzed the phosphorylation of two GluR2/GluR1 chimeras containing switch points within the loop between TM3 and TM4 (Figure 2). Specifically, the chimeras examined had the following switch points: C14 (Phe654; Phe655); E8 (Asn760;Lys761).

The wild-type GluR1 (GluR1 WT), wild-type GluR2 (GluR2 WT), or the GluR2/GluR1 chimeras were expressed in HEK-293 cells, prelabelled with [$^{32}$P]-orthophosphate, and treated with forskolin, IBMX, and TPA to activate PKA and PKC. The GluR1 WT, GluR2 WT, or the chimeric GluR2/GluR1 subunit (E8) was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography (Figure 3A). The GluR1 WT, GluR2 WT, and the GluR2/GluR1 chimera (E8) proteins were phosphorylated and migrated on SDS PAGE with apparent molecular weights of approximately 106 kDa. Mock transfected cells subjected to the same protocol have no phosphorylated proteins apparent on the autoradiogram. The phosphorylated GluR1 WT, GluR2 WT, and GluR2/GluR1 chimeric subunits were isolated from the gel and analyzed by tryptic phosphopeptide mapping. The phosphopeptide maps of the GluR1 WT and the GluR2 WT subunits are distinct with no apparent phosphopeptides in common (Figures 3B and 3C). The phosphopeptide map of the GluR2/GluR1 E8 chimera, including only the 130 C-terminal amino acid residues of GluR1, contained all of the phosphopeptides seen in the wild-type GluR1 phosphopeptide map (Figure 3D). Phosphopeptide maps of the other GluR2/GluR1 chimera (C14), with a switch point at a different position within the loop between TM3 and TM4, yielded similar results (data not shown).

**Phosphorylation of Ser-845**

Using the information that all of the GluR1 phosphorylation sites were contained within the GluR2/GluR1 chimera containing only TM4 and the C-terminus of GluR1, we proceeded to use site-directed mutagenesis to determine which residues in this region of GluR1 were phosphorylated. From previous work, we knew that most of GluR1 phosphorylation was on serine residues (Blackstone et al., 1994). The phosphorylation of the mutants was then examined in HEK-293 cells, as described above. Analysis of phosphopeptide maps of a mutant where Ser-845 was changed to an alanine residue (GluR1 S845A) demonstrated that this mutation specifically eliminated phosphopeptide 5 (Figures 4A and 4B). This same phosphopeptide, which is phosphorylated in the wild-type protein in response to forskolin, is also phosphorylated in primary neuronal cultures that

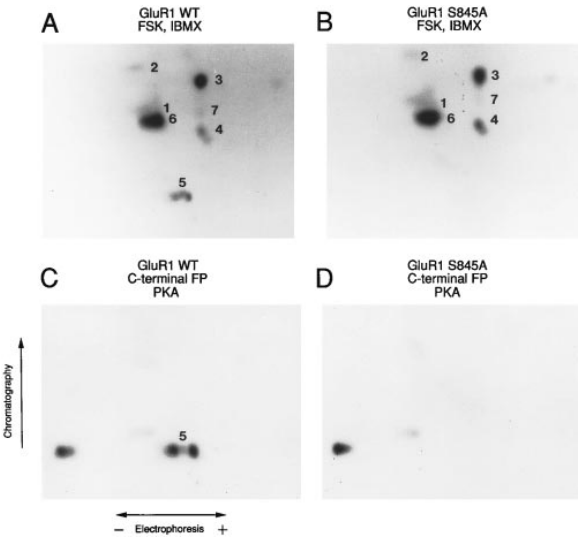


Figure 4. Identification of a Forskolin/IBMX-Stimulated Phosphorylation Site on the GluR1 Subunit Transiently Expressed in HEK-293 Cells

(A and B) HEK-293 cells transfected with wild-type GluR1, or GluR1 S845A cDNAs were prelabelled with 2 mCi/ml [$^{32}$P]-orthophosphate and then stimulated with 20 μM forskolin and 75 μM IBMX. The phosphorylated GluR WT (A) and GluR1 S845A mutant (B) subunits were immunoprecipitated, separated on SDS polyacrylamide gels, and analyzed by phosphopeptide mapping.

(C and D) PKA phosphorylation of GluR1 WT and GluR1 S845A C-terminal GST-fusion protein. GluR1 WT (C) and GluR1 S845A (D) C-terminal fusion proteins were phosphorylated with purified PKA in vitro and resolved by SDS-PAGE. The phosphorylated fusion protein was excised from the gel and digested with trypsin. The digested peptides were resolved in two dimensions on TLC plates, and the phosphopeptides were visualized by autoradiography.

have been stimulated with forskolin and IBMX (Blackstone et al., 1994).

To confirm that Ser-845 is directly phosphorylated by PKA, we constructed glutathione-S-transferase (GST) fusion proteins of the C-terminus of GluR1 WT and the GluR1 S845A mutant. Purified fusion proteins were phosphorylated in vitro with purified PKA and then analyzed by tryptic phosphopeptide mapping. The resulting phosphopeptide map of the GluR1 WT C-terminal fusion protein had two prominent phosphopeptides (Figure 4C). One of these phosphopeptides appeared identical to the forskolin-regulated phosphopeptide (phosphopeptide 5) seen in transfected HEK-293 cells or in neurons, while the other was not seen in situ. Mutation of Ser-845 to alanine within the fusion protein specifically eliminated phosphorylation of phosphopeptide 5, with no effect on the phosphorylation of the other site seen in vitro (Figure 4D), strongly suggesting that Ser-845 is directly phosphorylated by PKA in HEK-293 cells and neurons in situ.

The physiological effect of PKA phosphorylation of Ser-845 was investigated in transiently transfected HEK-293 cells, using whole-cell patch clamp recording techniques. Fast perfusion of glutamate onto the transfected HEK-293 cells resulted in the rapid activation of an inward current that desensitized in the continued presence of agonist with a time constant of approximately 9 msec, resulting in a small (5%–10%) steady-state plateau current (Figure 5A). This response had all of the characteristics of homomeric GluR1 AMPA receptors (data not shown). Under our recording conditions, in the presence of $Mg^{2+}$ and ATP the amplitude and kinetics of the glutamate-gated currents were stable for at least 30 min after establishing the whole-cell configuration.

To examine the functional effect of GluR1 phosphorylation by PKA, purified PKA was perfused into the recording pipette after stable baseline recordings were obtained. Intracellular perfusion of PKA resulted in a 40% potentiation of the peak amplitude of the whole-cell glutamate-gated current (Figures 5A and 5B). This potentiation developed over 15 min after introduction of PKA into the pipette, and then remained stable for the duration of the recording. No change in the rate of desensitization or in the steady state response was seen following the addition of PKA. The specificity of the PKA effect was demonstrated using PKI5–24 amide, a specific peptide inhibitor of PKA. No potentiation of the glutamate receptor response was observed when PKI was included with PKA in the perfusion solution (Figure 5B).

To determine whether the PKA potentiation of the GluR1-mediated response was due to phosphorylation of Ser-845, we examined the effect of PKA on the GluR1 S845A mutant receptor. The physiological properties of this mutant receptor were very similar to wild-type GluR1, both in terms of current amplitudes, desensitization rates, reversal potential, and plateau responses (data not shown). However, the mutant receptor was not potentiated by intracellular perfusion of PKA (Figures 5A and 5B). This result provides direct evidence that phosphorylation of Ser-845 on GluR1 by PKA potentiates its response to glutamate.

**Phosphorylation of Ser-831**

Analysis of another mutant of GluR1 where Ser-831 was changed to an alanine residue (GluR1 S831A) demonstrated that this mutation resulted in the specific elimination of phosphopeptides 3, 4, and 6, which are normally present in GluR1 WT (Figures 6A and 6B). The fact that phosphorylation of Ser-831 of GluR1 results in an array of phosphopeptides on phosphopeptide maps of GluR1 is most likely due to partial tryptic digestion of the phosphorylated GluR1, as is commonly seen in tryptic phosphopeptides (Tingley et al., 1993). While these phosphopeptides appear to be highly phosphorylated when GluR1 WT is expressed in HEK-293 cells, previous results have shown that these same phosphopeptides are regulated by phorbol esters or by synaptic activity in cultured cortical neurons, suggesting that Ser-831 may be phosphorylated by PKC in neurons (Blackstone et al., 1994).

To examine whether PKC could directly phosphorylate this site, we examined in vitro phosphorylation of a C-terminal GST-fusion protein of GluR1 WT and the GluR1 S831A mutant by purified PKC. The phosphorylated fusion proteins were then analyzed by tryptic phosphopeptide mapping. The resulting phosphopeptide maps of wild-type fusion protein contained phosphopeptides corresponding to phosphopeptides 1, 3, 4, and

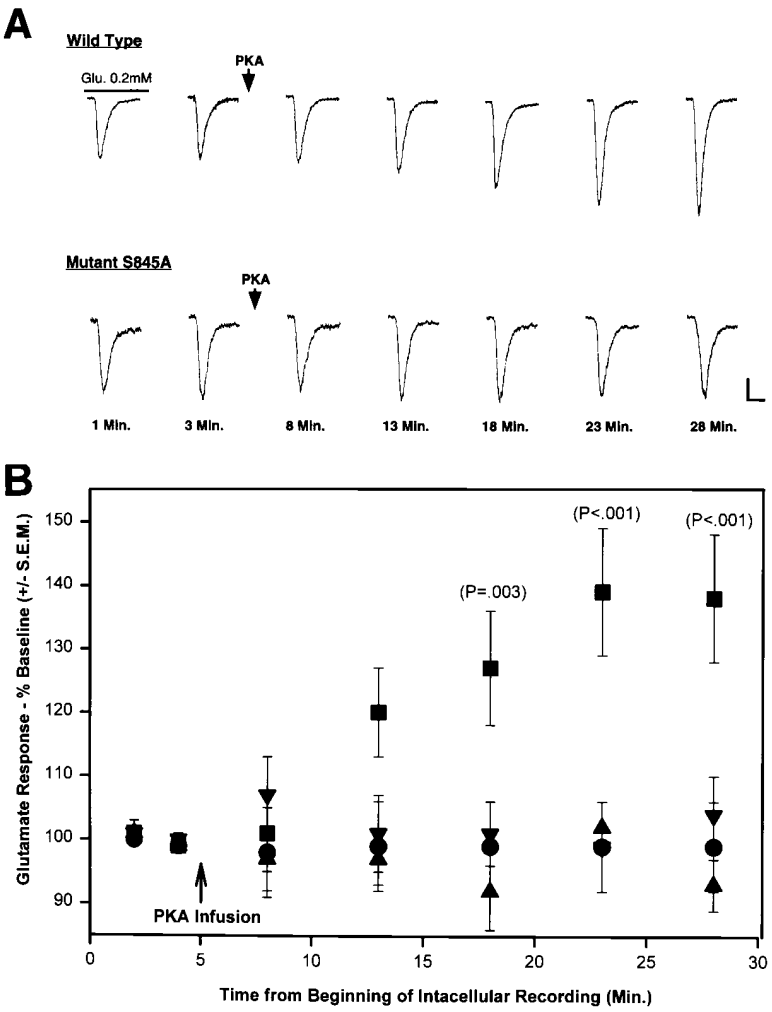


Figure 5. Effect of PKA on GluR1 Currents in Transfected HEK-293 Cells

HEK-293 cells transfected with either wild type, or S845A mutant GluR1 were voltage clamped at −60 mV in standard whole-cell clamp mode. At the times indicated, 0.2 mM glutamate was applied for 50 msec via a gravity-driven fast perfusion system. Purified PKA (20 μg/ml), control buffer, or PKA plus PKI (500 μg/ml) was infused via the patch pipette at the indicated time, and recordings were continued for an additional 23 min. (A) The two representative experiments of the effect of PKA perfusion on the glutamate-gated currents in cells transfected with wild-type (top) or S845A (bottom) GluR1 are shown. Calibration bars are 10 msec and 100 pA. (B) The mean ± SEM for a series of recordings similar to those depicted in (A) as a function of time after PKA perfusion. Closed square: wild-type GluR1 treated with PKA (n = 12); closed circle: wild-type GluR1 treated with control buffer (n = 11); upright closed triangle: wild-type GluR1 treated with PKA + PKI (n = 4); inverted closed triangle: S845A treated with PKA (n = 9). The P values represent a Student's t-test comparison of the peak currents elicited by glutamate in wild-type GluR1-transfected cells with a combination of the three controls at the indicated time points. Similar significance was seen with the raw data or with a log transformation of the data.

6 (Figure 6C), in addition to two prominent phosphopeptides that are not seen in situ. In contrast, the phosphopeptide map of the GluR1 S831A mutant C-terminal fusion protein was specifically missing phosphopeptides corresponding to phosphopeptides 3, 4, and 6 (Figure 6D). Although phosphorylation of phosphopeptide 1 and the two other fusion protein-specific phosphopeptides was less in the mutant fusion protein than in the wild-type fusion protein in this experiment, phosphopeptides 3, 4, and 6 were not detectable even in long overexposures of this autoradiogram. These results suggest that Ser-831 may be directly phosphorylated by PKC in neurons in situ.

We could not directly examine the physiological effect of phosphorylation at Ser-831, given its apparent high level of basal phosphorylation in transfected HEK-293 cells. However, when expressed in HEK-293 cells, the GluR1 S831A mutant responded to rapidly applied glutamate in a similar manner to wild-type GluR1, with the exception of an overall reduced peak current (Table 1). Other properties of the mutant receptor, such as desensitization rate, steady-state current, and current rundown in the absence of ATP were unchanged. Whether the difference in peak currents between wild-type GluR1 and the GluR1 S831A mutant were due to changes in receptor function or due to altered surface expression of the mutant receptor is not clear. We are presently attempting to characterize the endogenous kinase to further investigate this effect.

### Other Phosphorylation Sites

In spite of the fact that the chimera data demonstrated that all of the phosphorylation sites are on the C-terminus of GluR1, we have not yet been able to identify the sites of phosphorylation that correspond to phosphopeptides 1 and 7. In addition to the GluR1 S831A and GluR1 S845A mutants, we have constructed and screened many other GluR1 single point mutants on the C-terminus, including S814A, S816A, S818A, S839A, S850A, S855A, S863A, S872A, and S877A (Figure 2). The phosphorylation of each of these single point mutants was analyzed by phosphopeptide mapping, and the resulting phosphopeptide maps were indistinguishable from GluR1 WT peptide maps. Several of the predicted tryptic phosphopeptides of the C-terminus of GluR1 contain more than one serine residue; therefore, these peptides could be multiply phosphorylated and mutation of a single serine residue may not eliminate phosphorylation of these phosphopeptides. We are currently

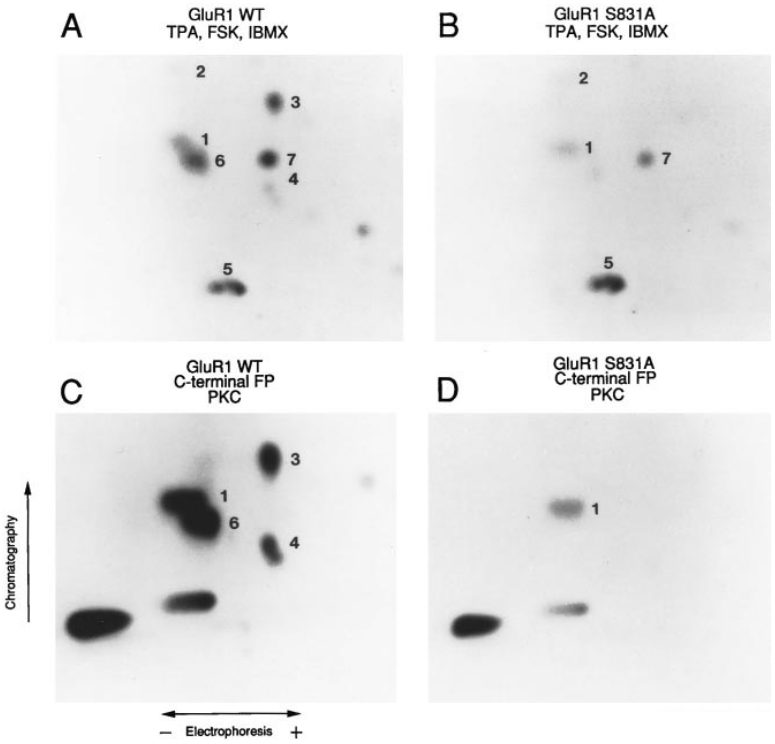


Figure 6. Identification of a Basal Phosphorylation Site on the GluR1 Subunit Transiently Expressed in HEK-293 Cells

(A and B) HEK-293 cells transfected with GluR1 WT or GluR1 S831A cDNAs were prelabelled with 1 mCi/ml [$^{32}$P]-orthophosphate and then stimulated with 20 μM forskolin, 75 μM IBMX, and 100 nM TPA. Wild-type (A) and mutant GluR1 (B) were isolated and analyzed by phosphopeptide mapping.

(C and D) Phosphorylation of GluR1 WT (C) and GluR1 S831A (D) C-terminal GST-fusion proteins. The fusion proteins were phosphorylated with purified PKC, resolved by SDS-PAGE, excised from the gel, and digested with trypsin. The digested peptides were resolved in two dimensions on TLC plates, and the phosphopeptides were visualized by audioradiography.

examining double and triple mutants to identify the serine residues contained within phosphopeptides 1 and 7.

## Discussion

Protein phosphorylation appears to be a central mechanism for the regulation of glutamate receptor function (Roche et al., 1994b). For example, there is substantial evidence that glutamate receptors are modulated by PKA phosphorylation. Early studies demonstrated that dopamine-mediated increases in cAMP lead to a potentiation of kainate responses in white perch retinal horizontal cells. Moreover, directly injecting PKA into these cells mimicked the effect of dopamine and cAMP (Knapp and Dowling, 1987; Liman et al., 1989). More recently, several studies have described an increase in the amplitude of AMPA receptor responses in cultured hippocampal neurons, upon the activation of PKA (Greengard et al., 1991; Wang et al., 1991; Rosenmund et al., 1994). In addition to experiments using primary neuronal cultures, PKA has been shown to potentiate AMPA currents in oocytes expressing recombinant receptors consisting of the GluR1 and GluR3 subunits (Keller et al., 1992). Although there is general agreement that PKA modulates AMPA receptors, there are conflicting reports concerning the biochemical evidence that PKA directly phosphorylates AMPA receptor subunits. Previous studies in our laboratory have shown that forskolin treatment of neuronal cultures or HEK-293 cells stimulates phosphorylation of the AMPA receptor subunit GluR1 (Blackstone et al., 1994). In contrast, another study reported no evidence of phosphorylation of GluR1 by purified preparations of PKA in vitro or by PKA in neurons in culture (McGlade-McCulloh et al., 1993; Tan et al., 1994). The reason for this discrepancy is not clear.

Recent studies have also provided evidence that PKC and CaM-KII may also directly phosphorylate and modulate AMPA receptors. CaM-KII and PKC directly phosphorylate the AMPA subunit GluR1 in vitro (McGlade-McCulloh et al., 1993; Tan et al., 1994) and appear to phosphorylate the GluR1 subunit in neurons in culture (Blackstone et al., 1994; Tan et al., 1994). In addition, intracellular perfusion of PKC and CaMKII potentiates AMPA responses in cultured hippocampal neurons (Wang et al., 1994; McGlade-McCulloh et al., 1993). CaM-KII has also been reported to potentiate the glutamate response of recombinant GluR1 subunits expressed in Xenopus oocytes (Yakel et al., 1995). Mutation of Ser-627 (S627A) contained within a CaM-KII consensus site in the major loop between TM3 and TM4 eliminated this potentiation (Yakel et al., 1995). This result provided evidence that the direct phosphorylation of Ser-627 in GluR1-modulated AMPA receptors. However,

Table 1. Comparison of Glutamate Currents in HEK-293 Cells Transfected with either Wild-Type GluR1 or with the GluR1 Mutant S831A.

| | Wild-Type GluR1 | Mutant S831A |
|---|---|---|
| Peak Current | 416 ± 81 pA | 278 ± 45 pA |
| Decay Time Constant | 9.3 ± 0.6 ms | 9.0 ± 1.0 ms |
| Plateau (Percent Peak) | 8 ± 1.1 | 8.1 ± 1.3 |

A series of three side-by-side transfections of HEK-293 with GluR1 wild-type and GluR1 S831 were performed. An alternating series of 10 cells of each type were voltage clamped at −60 mV and 0.2 mM glutamate applied by rapid perfusion for 50 msec. The characteristics of the response of each receptor to glutamate were quantitated and are displayed in Table 1 ± SEM. The difference in peak currents did not reach statistical significance at the .05 level.

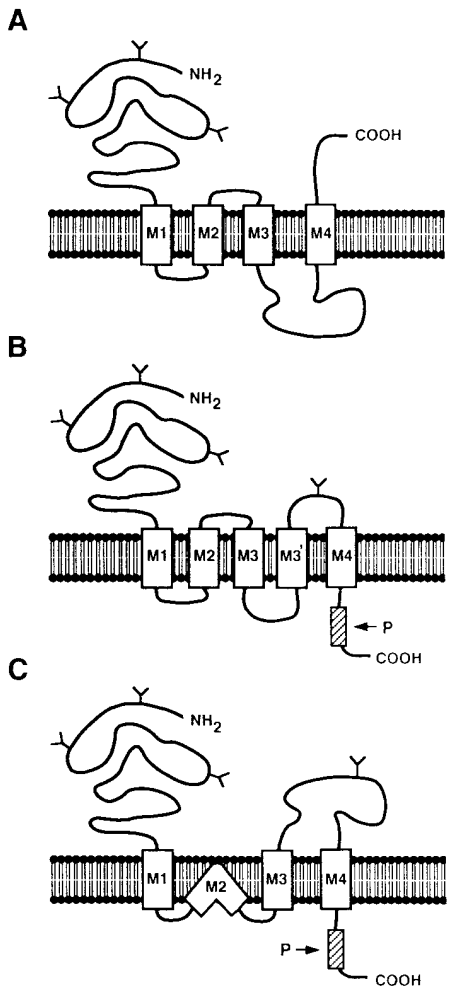


Figure 7. Proposed Transmembrane Topology Models for the Ionotropic Glutamate Receptor Subunits

(A) The originally proposed model proposed with a large extracellular N-terminal domain, four transmembrane domains, and an extracellular C-terminus (Hollman et al., 1989).

(B) A more recent model with an extracellular N-terminus, five transmembrane domains, and an intracellular C-terminus (Seeburg et al., 1993; Roche et al., 1994; Taverna et al., 1994).

(C) The most recently proposed model with an extracellular N-terminus, three transmembrane domains, and an intracellular C-terminus (Wo and Oswald, 1994; Hollman et al., 1994; Sternbach et al., 1994; Bennett and Dingledine, 1995; Wood et al., 1995). P indicates location of phosphorylation sites on GluR1.

no biochemical analysis was performed to confirm this conclusion.

The identification of phosphorylation sites on glutamate receptor subunits has been difficult, since many mutagenesis studies have been based on the originally proposed transmembrane topology (Figure 7A). In fact, studies demonstrating that the C-terminus of the NR1 protein was phosphorylated by PKC suggested that this region was intracellular (Tingley et al., 1993). This result, combined with experiments mapping glycosylation sites in the kainate receptor GluR6 subunit, lead to the proposal of an alternative model with an intracellular C-terminus and part of the loop between TM3 and TM4 outside of the cell (Figure 7B) (Roche et al., 1994a; Taverna et al., 1994). Most recently, several groups using a variety of techniques have proposed a new transmembrane topology model with an extracellular N-terminus, three transmembrane domains, and an intracellular C-terminus (Figure 7C) (Wo and Oswald, 1994; Hollman et al., 1994; Stern-Bach et al., 1994; Bennett and Dingledine, 1995; Wood et al., 1995). This model places the entire loop between TM3 and TM4 on the extracellular side of the membrane. Studies identifying phosphorylation sites on the loop between the TM3 and TM4 of AMPA (Yakel et al., 1995; Nakazawa et al., 1995) and kainate (Raymond et al., 1993a; Wang et al., 1993) receptors are inconsistent with this model.

Owing to the lack of clarity concerning the transmembrane topology of ionotropic glutamate receptors, we initially used chimeric receptors to define the region of GluR1 that is phosphorylated, and then followed this analysis with site-specific mutagenesis. Using GluR2/GluR1 chimeras, we demonstrated that all GluR1 phosphorylation sites characterized in neurons and in transfected HEK-293 cells are located on the final 130 amino acids of GluR1. Using site-directed mutagenesis, we determined that PKA phosphorylated Ser-845 on the C-terminus of GluR1. We also demonstrated that Ser-831 on the C-terminus of GluR1 is phosphorylated by a constitutively active kinase in HEK-293 cells. We have previously shown that phosphorylation of this site is increased by phorbol ester treatment of neurons or by synaptic activity (Blackstone et al., 1995). To confirm the results obtained from GluR2/GluR1 chimeras and GluR1 point mutants expressed in HEK-293 cells, GST-fusion proteins were constructed of the C-termini of GluR1 WT and the mutant GluR1 subunits. Phosphorylation of these fusion proteins confirmed that PKA and PKC phosphorylate Ser845 and Ser831, respectively, in vitro.

The identification of phosphorylation sites on the C-terminus of the AMPA receptor GluR1 subunit further confirms the intracellular location of the C-terminus of AMPA receptor subunits, consistent with the two recently proposed transmembrane topology models (Figures 7B and 7C). Although our data does not definitively differentiate between these two proposed models, none of the sites that we have characterized on GluR1 are located on the loop between TM3 and TM4. Recent studies have provided evidence that GluR1 is phosphorylated on Ser-627 and Ser-658, which are both contained within this loop (Yakel et al., 1995; Mc Kanza et al., 1995). However, in the experiments described here, site-specific mutagenesis of Ser-627 or Ser-658 had no apparent effect on the phosphopeptide maps of GluR1. In addition, although Ser-627 has been suggested to be phosphorylated by CaMKII, coexpression of either wild-type or a constitutively active form of CaM Kinase II with GluR1 in HEK-293 cells had no detectable effect on phosphorylation of GluR1 analyzed by phosphopeptide maps (K. W. R., A. M., and R. L. H., unpublished data).

Finally, physiological recordings from transfected HEK-293 cells provided direct evidence that PKA phosphorylation of Ser-845 potentiates the peak response of GluR1 to its agonist. Given the widespread distribution of GluR1 at post-synaptic endings and in neuronal

dendrites in hippocampal neurons (Craig et al., 1993; Eshhar et al., 1993; Roche and Huganir, 1995), phosphorylation of Ser-845 may mediate the potentiation of AMPA receptors by PKA previously reported (Greengard et al., 1991; Wang et al., 1991; Rosenmund et al., 1994). Although the physiological significance of phosphorylation of Ser-831 is unclear, considering the proximity of Ser-831 to Ser-845, phosphorylation of this Ser may also modulate channel function. PKC has been implicated in the induction and maintenance of LTP (Roche et al., 1994b) and it is possible that PKC phosphorylation of Ser-831 is involved in the maintenance of increased synaptic transmission during LTP. Taken together, these studies support the hypothesis that protein phosphorylation of glutamate receptors plays an important role in the regulation of their function, and may mediate certain forms of synaptic plasticity (Raymond et al., 1993b; Roche et al., 1994b).

## Experimental Procedures

### Materials

Aprotinin was purchased from Mobay Biochemical. Restriction enzymes were purchased from New England Biolaboratories. Cellulose TLC plates were from Kodak. Radioisotopes were obtained from New England Nuclear. Forskolin (Coleus forskohlii), phorbol 12-myristate, 13-acetate (TPA), and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Calbiochem. All other chemicals were obtained from Sigma Chemical Company, unless otherwise indicated.

### GluR2/GluR1 Chimeras

GluR2/GluR1 chimeras were constructed according to a previously published protocol (Moore and Blakely, 1994). Xho I and Xba I linkers were ligated to cDNAs encoding GluR1 and GluR2, respectively, and the resulting inserts ligated into either Xho I or Xba I sites of pBluescript SK- (pBS). The final construct consisted of GluR2 and GluR1 both in pBS in a head-to-tail tandem configuration. The resulting construct was digested with Sal I and Hind III, resulting in linearization of the plasmid at a site in between the GluR1 and GluR2 inserts. The linearized DNA was transformed into bacteria and the resulting colonies were screened for chimeras. The resulting chimeras all consist of GluR2 at the 5′ end and GluR1 at the 3′ end (see Figure 2), with the switch points of each chimera occurring at a region of high sequence identity between GluR1 and GluR2. The chimeras generated have switch points at various points in the GluR1 sequence. The exact mechanism underlying the chimera formation is unknown. For more technical details, see Moore and Blakely, 1994. The switchpoint of the chimeras were identified by DNA sequencing and are Phe654;Phe655 for C14 and Asn 760;Lys761 for E8. The numbering of the amino acids is based on the mature protein sequence (Hollmann et al., 1989).

### Site-Directed Mutagenesis

Mutagenesis was performed using single-stranded DNA mutagenesis techniques (Kunkel et al., 1987). The following primers were used to produce the following mutants: GluR1 S845A (5′-TCCTGCCCCAG CGTTCCGGGG-3′) and GluR1 S831A (5′-TTCATTGATGGCTTGCTG TGG-3′).

### Transient Transfections of GluR1 cDNA and Other Constructs

The wild-type GluR1, mutant GluR1, and the GluR2/GluR1 chimera cDNAs in CMV-driven vectors were transiently transfected into human embryonic kidney (HEK) 293 cells (American Type Culture Collection CRL 1573) using the calcium phosphate coprecipitation method with 20 μg of DNA/10 cm culture dish, as described previously (Blackstone et al., 1992b). The cells were used for biochemical analysis 48 hr following transfection.

### Phosphorylation and Immunoprecipitation of Wild-Type and Mutant GluR1 Expressed in HEK-293 Cells

HEK-293 cells transiently expressing GluR1 WT, GluR2 WT, GluR2/GluR1 chimeras, or point mutant GluR1s, were prelabelled with [$^{32}$P]-orthophosphate (2 mCi/ml) for 3–4 hr in phosphate-free DMEM (GIBCO). The cells were treated with forskolin (20 μM), TPA (100 nM), and IBMX (75 μM) for 20–30 min. Cells were then scraped into IP buffer containing 10 mM sodium phosphate (pH 7.0), 150 mm NaCl, 5 mM EDTA, 5 mM EGTA, 10 u/ml Aprotinin, 50 mM NaF, 10 mM sodium pyrophosphate, and 1 mM sodium vanadate, then sonicated. The membranes were collected by centrifugation at 14,000 × g for 20 min in a refrigerated microcentrifuge. The membrane pellet was resuspended in 150 μl of 1% SDS in the buffer described above, and then brought up in 750 μl of 1% Triton X-100 in IP buffer and any residual particulate fraction removed by centrifugation at 14,000 × g for 15 min in a refrigerated microcentrifuge. This lysate was incubated for 2 hr at 4°C, with protein A Sepharose (blocked with 10 mg/ml bovine serum albumin) and the GluR1 antipeptide antibody (10 μg). The beads were washed two times with IP buffer with 1% Triton X-100, two times with IP buffer with 1% Triton X-100, and high salt (650 mM NaCl), and finally twice with IP buffer alone. The immunoprecipitate was eluted from the protein A Sepharose by boiling the beads in SDS-PAGE sample buffer for 3′. The sample was then analyzed on an 8% polyacrylamide gel and visualized by autoradiography.

### Fusion Proteins

Glutathione-S-transferase (GST) fusion proteins consisting of the C-termini of wild-type and mutant GluR1 were constructed by PCR amplification of the C-termini using the following primers: 5′ sense strand (5′-GCGGATCCGAGTTCTGCTACAAATCC-3′ ) and the 3′ antisense primer (5′-ACCATGAATTCCGGCACTGAAGGGCTTGGG-3′). The PCR primers had Bam HI and Eco RI sites incorporated at the 5′ and 3′ ends, respectively, thereby allowing subcloning into the Bam HI and Eco RI sites of the fusion protein vector pGEX 2T (Pharmacia). The fusion proteins were purified essentially following the protocol of the manufacturer (Pharmacia). Briefly, the vectors were transformed into bacteria, and 100 ml cultures were allowed to grow for 1.5 hr at which point they were then induced with 0.5 mg/ml IPTG. The cells were allowed to grow for 4 hr following induction, and then lysed in PBS containing: 5 mM EDTA, 1 mM DTT, and 10 u/ml Aprotinin, sonicated, and Triton X-100 added to a final concentration of 1%. The lysate was then shaken on ice for 30–60 min, and the insoluble fraction was removed by centrifugation for 20 min at 6000 rpms in a Sorvall HS-4 rotor. The solubilized sample was adsorbed to glutathione agarose (Pharmacia) for 1 hr at 4°C, washed with the remaining lysis buffer five times, then eluted with 10 mM reduced glutathione. All the eluted fractions were dialyzed overnight against TBS. The protein concentration was estimated by analysis of aliquots on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

### Fusion Protein Phosphorylation

Phosphorylation of fusion proteins by PKA was performed in 10 mM Hepes (pH 7.0), 20 mM $MgCl_2$, 50mM ATP (20,000 cpm/pmol) with 45 ng of purified PKA (Reimann and Beham, 1983) in a final volume of 100 μl. Phosphorylation of the fusion proteins by PKC was performed in 10 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 800 μM $CaCl_2$, 500 μg/ml phosphatidyl Ser, 5 μg/ml diolein, 50 mM ATP (20,000 cpm/pmol) with 6 ng of purified PKC (Woodgett and Hunter, 1987) in a final volume of 100 μl. All in vitro phosphorylation reactions included approximately 1 μg of fusion protein substrate. The reactions were allowed to proceed at 30°C for 30 min, and were stopped by the addition of SDS-PAGE sample buffer. The phosphorylated fusion protein was resolved by SDS-PAGE and then visualized by autoradiography. The phosphorylated proteins were excised and subjected to phosphopeptide mapping, as described below.

### Phosphopeptide Mapping

After the immunoprecipitate was resolved by SDS-PAGE and visualized by autoradiography, the protein of interest was excised from the gel, digested with trypsin (0.3 mg/ml), and spotted onto a thin layer chromatography (TLC) cellulose plate (Huganir et al., 1984).

These peptide fragments were then resolved in two dimensions, by electrophoresis in the first dimension in pyridine/acetic acid/$H_2O$, 1:19:89 (vol/vol) and ascending chromatography in the second dimension in pyridine/butanol/acetic acid/$H_2O$, 15:10:3:12 (vol/vol) (Huganir et al., 1984). The TLC plate was then exposed to X-ray film and the resulting pattern of phosphopeptides visualized.

**Physiology**

Whole-cell patch clamp recordings were performed at 25 degrees in HEK-293 cells transfected 48 hr earlier with cDNA, encoding either GluR1 (flip) or one of the two mutants GluR S845A or GluR1 S831A. Cells were continuously bathed in a solution containing 135 mM NaCl, 5.4 mM KCl, 2mM $CaCl_2$, 15 mM HEPES, 15 mM glucose and 1 mM $MgCl_2$. The osmolarity was adjusted to 310 and the pH to 7.3. Glutamate (0.2 mM) was applied by a two solonoid fast perfusion system, as described by Raymond et al. (1993). Cells were lifted off the dish and positioned in front of the perfusion system for optimum kinetics. There were two baseline recordings obtained, one at 1 min and another at 3 min prior to addition of enzyme. At each time point, two 50 msec pulses of glutamate were applied, separated by 15 s. The first pulse served to clear any dead space accumulation in the agonist pipet. The second pulse was taken as the actual response for the given time point. The system was then turned off until the next time point. Patch electrodes were filled with a solution containing 140 mM KCl, 4 mM MgATP, 11 mM EGTA, 1mM $CaCl_2$, 10 mM HEPES and 2 mM TEA. After two baseline recordings were obtained, 15 μl of a solution containing either PKA (20 μg/ml), PKA (20 μg/ml) plus $PKI_{5-24}$ amide (200 μg/ml), or control buffer solution was infused intracellularly through a second capillary positioned within 300 μM of tip of the patch pipet and controlled by a manually operated hydraulic system. The control solution contained a 1:10 dilution of the buffer in which PKA was stored. All solutions except those initially present in the patch pipet contained 0.1 mg/ml creatine phosphokinase as carrier, and were pH and osmotically matched to the standard intracellular solution. Only stable cells in which the full 28 min of recording were obtained were analyzed. During the experiment, series resistance (typically 5–10 M-ohm) was continuously monitored and cells with any abrupt or sustained changes in series resistance were eliminated.

**Acknowledgments**

We thank Drs. Lonny Levin and Randy Reed for advice on the construction of the chimeras, and Cindy Finch for help in the preparation of the manuscript. This work was supported by a training grant from the National Institutes of Health (1T32NS07368) to K. W. R., an MSTP fellowship from the National Institutes of Health to A. L. M., a CIDA grant from the National Institutes of Health to R. J. O., and a grant from the Howard Hughes Medical Institute to R. L. H.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received February 22, 1996; revised May 2, 1996.

**References**

Bennett, J.A., and Dingledine, R. (1995). Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. Neuron *14,* 373–384.

Blackstone, C.D., Levey, A.I., Martin, L.J., Price, D.L., and Huganir, R.L. (1992a). Immunological detection of glutamate receptor subtypes in human central nervous system. Ann. Neurol. *31*, 680–683.

Blackstone, C.D., Moss, S.J., Martin, S.J., Levey, A.I., Price, D.L., and Huganir, R.L. (1992b). Biochemical characterization and localization of a non-*N*-methyl-D-aspartate glutamate receptor in rat brain. J. Neurochem. *58,* 1118–1126.

Blackstone, C., Murphy, T.H., Moss, S.J., Baraban, J.M., and Huganir, R.L. (1994). Cyclic AMP and synaptic activity-dependent phosphorylation of AMPA-preferring glutamate receptors. J. Neurosci. *14,* 7585–7593.

Bliss, T.V.P., and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361,* 31–39.

Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C., and Heinemann, S. (1990). Molecular cloning and functional expression of glutamate receptor subunit genes. Science *249*, 1033–1037.

Chen, L., and Huang, L-Y.M. (1992). Protein kinase C reduces $Mg^{2+}$ block of NMDA-receptor channels as a mechanism of modulation. Nature *356*, 521–523.

Craig, A.M., Blackstone, C.D., Huganir, R.L., and Banker, G. (1993). The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. Neuron *10,*1055–1068.

Eshhar, N., Petralia, R.S., Winters, C.A., Niedzielski, A.S., and Wenthold, R.J. (1993). The segregation and expression of glutamate receptor subunits in cultured hippocampal neurons. Neuroscience *57*, 943–964.

Greengard, P., Jen, J., Nairn, A.C., and Stevens, C.F. (1991). Enhancement of the glutamate response by cAMP dependent protein kinase in hippocampal neurons. Science *253*, 1135–1138.

Hollmann, M., and Heinemann, S. (1994). Cloned glutamate receptors. Annu. Rev. Neurosci. *17*, 31–108.

Hollmann, M., O'Shea-Greenfield, A., Rogers, S.W., and Heinemann, S. (1989). Cloning by functional expression of a member of the glutamate receptor family. Nature *342,* 643–648.

Hollmann, M., Maron, C., and Heinemann, S. (1994). N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. Neuron *13,* 1331–1343.

Huganir, R.L., Miles, K., and Greengard, P. (1984). Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. Proc. Natl. Acad. Sci. USA *81*, 6968–6972.

Keller, B.U., Hollmann, M., Heinemann S., and Konnerth, A. (1992). Calcium influx through subunits GluR1/GluR3 of kainate/AMPA receptor channels is regulated by cAMP dependent protein kinase. EMBO J. *11,* 891–896.

Knapp, A.G., and Dowling, J.E. (1987). Dopamine enhances excitatory amino acid-gated conductances in cultured retinal horizontal cells. Nature *325,* 437–439.

Krebs, E.G. (1994). The growth of research on protein phosphorylation. Trends Biochem. Sci. *19*, 439.

Liman, E.R., Knapp, A.G., and Dowling, J.E. (1989). Enhancement of kainate-gated currents in retinal horizontal cells by cyclic AMP-dependent protein kinase. Brain Res. *481*, 399–402.

Linden, D.J. (1994). Long-term synaptic depression in the mammalian brain. Neuron *12,* 457–472.

McGlade-McCulloh, E., Yamamoto, H., Tan, S.-E., Brickey, D.A., and Soderling, T.R. (1993). Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. Nature *362*, 640–642.

Molnár, E., Baude, A., Richmond, S.A., Patel, P.B., Somogyi, P., and McIlhinney, R.A.J. (1993). Biochemical and immunocytochemical characterization of antipeptide antibodies to a cloned GluR1 glutamate receptor subunit: cellular and subcellular distribution in the rat forebrain. Neuroscience *53,* 307–326.

Moore, K.R., and Blakely, R.D. (1994). Restriction site-independent formation of chimeras from homologous neurotransmitter-transporter cDNAs. Biotechniques *17*, 130–135.

Moss, S.J., Blackstone, C.D., and Huganir, R.L. (1993). Phosphorylation of recombinant non-NMDA glutamate receptors on serine and tyrosine residues. Neurochem. Res. *18,* 105–110.

Nakazawa, K., Mikawa, S., Hashikawa, T., and Ito, M. (1995). Transient and persistent phosphorylation of AMPA-type glutamate receptor subunits in cerebellar Purkinje cells. Neuron *15*, 697–709.

Nicoll, R.A., and Malenka, R.C. (1995). Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature *377*, 115–118.

Raymond, L.A., Blackstone, C.D., and Huganir, R.L. (1993a). Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. Nature *361*, 637–641.

Raymond, L.A., Blackstone, C.D., and Huganir, R.L. (1993b). Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. Trends Neurosci. *16,* 147–153.

Reimann, E.M., and Beham, R.A. (1983). Catalytic subunit of cAMP-dependent protein kinase. Methods Enzymol. *99*, 51–55.

Roche, K.W., Raymond, L.A., Blackstone, C.D., and Huganir, R.L. (1994a). Transmembrane topology of the glutamate receptor subunit GluR6. J. Biol. Chem. *269*, 11679–11682.

Roche, K.W., Tingley, W.G., and Huganir, R.L. (1994b). Glutamate receptor phosphorylation and synaptic plasticity. Curr. Opin. Neurol. *4,* 383–388.

Roche, K.W., and Huganir, R.L. (1995). Synaptic expression of the high-affinity kainate receptor subunit KA2 in hippocampal cultures. Neuroscience *69,* 383–393.

Rosenmund, C., Carr, D.W., Bergeson, S.E., Nilaver, G., Scott, J.D., and Westbrook, G.L. (1994). Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. Nature *368,* 853–856.

Seeburg, P.H. (1993). The TIPS/TINS lecture: the molecular biology of mammalian glutamate receptor channels. Trends Pharmacol. Sci. *14*, 297–303.

Stern-Bach, Y., Bettler, B., Hartley, M., Sheppard, P.O., O'Hara, P.J., and Heinemann, S.F. (1994). Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. Neuron *13,* 1345–1357.

Swope, S.L., Moss, S.J., Blackstone, C.D., and Huganir, R. L. (1991). Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. FASEB J. *6*, 2514–2523.

Tan, S-E., Wenthold, R.J., and Soderling, T.R. (1994). Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. J. Neurosci. *14*, 1123–1129.

Taverna, F.A., Wang, L., MacDonald, J.F., and Hampson, D.R. (1994). A transmembrane model for an ionotropic glutamate receptor predicted on the basis of the location of asparagine-linked oligosaccharides. J. Biol. Chem. *269*, 14159–14164.

Tingley, W.G., Roche, K.W., Thompson, A.K., and Huganir, R.L. (1993). Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. Nature *364*, 70–73.

Wang, L.-Y., Dudek, E.M., Browning, M.D., and MacDonald, J.F. (1994). Protein kinase C modulates AMPA/kainate receptors in cultured murine hippocampal neurons. J. Physiol. (Lond.) *475,* 431–437.

Wang, L.-Y., Salter, M.W., and MacDonald, J.F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. Science *253,* 1132–1135.

Wang, L.-Y., Taverna, F., Huang, X.-P., MacDonald, J., and Hampson, D. (1993). Phosphorylation and modulation of a kainate receptor (GluR6) by cAMP-dependent protein kinase. Science *259*, 1173–1175.

Wo, Z.G., and Oswald, R.E. (1994). Transmembrane topology of two kainate receptor subunits revealed by N-glycosylation. Proc. Natl. Acad. Sci. USA *91,* 7154–7158.

Wood, M.W., VanDongen, H.M., and VanDongen, A.M. (1995). Structural conservation of ion conduction pathways in K channels and glutamate receptors. Proc. Natl. Acad. Sci. USA *92*, 4882–4886.

Woodgett, J.R., and Hunter, T. (1987). Isolation and characterization of two distinct forms of protein kinase C. J. Biol. Chem. *262*, 4836–4843.

Yakel, J.L., Vissavajjhala, P., Derkach, V.A., Brickey, D.A., and Soderling, T.R. (1995). Identification of a $Ca^{2+}$/calmodulin-dependent protein kinase II regulatory phosphorylation site in non-N-methyl-D-aspartate glutamate receptors. Proc. Natl. Acad. Sci. USA *92,* 1376–1380.