Extrasynaptic Membrane Trafficking Regulated by GluR1 Serine 845 Phosphorylation Primes AMPA Receptors for Long-term Potentiation*

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Michael C. Oh, Victor A. Derkach, Eric S. Guire, and Thomas R. Soderling†
From the Vollum Institute, Oregon Health and Sciences University, Portland, Oregon 97239

Enhancement of synaptic transmission, as occurs in long-term potentiation (LTP), can result from several mechanisms that are regulated by phosphorylation of the AMPA-type glutamate receptor (AMPA). Using a quantitative assay of net serine 845 (Ser-845) phosphorylation in the GluR1 subunit of AMPARs, we investigated the relationship between phospho-Ser-845, GluR1 surface expression, and synaptic strength in hippocampal neurons. About 15% of surface AMPARs in cultured neurons were phosphorylated at Ser-845 basally, whereas chemical potentiation (forskolin/rolipram) treatment persistently increased this to 60% and chemical depression (N-methyl-D-aspartate) decreased it to 10%. These changes in Ser-845 phosphorylation were paralleled by corresponding changes in the surface expression of AMPARs in both cultured neurons and hippocampal slices. For every 1% increase in net phosphorylation of Ser-845, there was a 0.75% increase in the surface fraction of GluR1. Phosphorylation of Ser-845 correlated with a selective delivery of AMPARs to extrasynaptic sites, and their synaptic localization required coincident synaptic activity. Furthermore, increasing the extrasynaptic pool of AMPA receptors resulted in stronger theta burst LTP. Our results support a two-step model for delivery of GluR1-containing AMPARs to synapses during activity-dependent LTP, where Ser-845 phosphorylation can traffic AMPARs to extrasynaptic sites for subsequent delivery to synapses during LTP.

At central glutamatergic synapses, AMPAR function is dynamically regulated, resulting in bidirectional synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD). Phosphorylation can regulate AMPARs by two distinct mechanisms that affect synaptic transmission (1): modulation of channel properties (2–4) and trafficking of AMPARs to the surface membrane (5–9). Phosphorylation of Ser-831 in GluR1 C terminus by calcium/calmodulin-dependent protein kinase II (CaM-KII) (10, 11) potentiates single-channel conductance (2, 4), and phosphorylation of Ser-845 by cAMP-dependent protein kinase (PKA) (12) increases channel open probability (3). Importantly, Ser-845 phosphorylation can also regulate the surface expression of AMPARs by increasing the pool of GluR1 recycled back to the surface after their endocytosis (13). CaM-KII activity is also critical for synaptic incorporation of AMPARs but requires a serine at position 845, which is not phosphorylated by CaM-KII, in GluR1 (14), suggesting an important role of Ser-845 phosphorylation for trafficking of AMPARs in an activity-dependent manner.

Although these studies indicate that AMPAR phosphorylation and trafficking are coupled (13, 14), exactly how Ser-845 phosphorylation contributes to trafficking is unclear. Current understanding of the functional role of Ser-845 phosphorylation in GluR1 during synaptic plasticity is limited because previous studies have analyzed only relative changes in phospho-Ser-845. In fact, the functional significance of these changes is determined by the net level of phospho-Ser-845. In this report, we quantified the net Ser-845 phosphorylation and surface expression of GluR1 in hippocampal neurons during bidirectional synaptic plasticity. We observed that receptors phosphorylated at the Ser-845 trafficked specifically to extrasynaptic sites but not to synapses. However, extrasynaptic AMPARs can be incorporated into synapses by synaptic activity. Thus, Ser-845 phosphorylation serves as a “priming” step in enhancing synaptic strength during LTP by enhancing the extrasynaptic delivery of AMPARs.

MATERIALS AND METHODS

Cultured Hippocampal Neurons and Biochemistry—Hippocampal neurons were cultured from postnatal day 1–2 Sprague-Dawley rats as previously described (15, 16) and used on 13–15 days in vitro. For chemical stimulations, cultures were first incubated in ACSF for 30 min at room temperature (in mM): 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 33 d-glucose, and 25 HEPES (pH 7.3, 320 mosm final), followed by stimulation with 50 μM forskolin (Sigma) and 0.1 μM rolipram (Calbiochem) in ACSF (no MgCl2) or 50 μM NMDA. After 10 min of stimulation, neurons were replaced in regular ACSF and then subjected to surface biotinylation at indicated time points.

Surface Biotinylation—After F/R or NMDA stimulations, cultured neurons or slices were transferred to ice-cold ACSF for 2 min, followed by biotinylation in 1 mg/ml of biotin (EZ-Link Sulfo-NHS-SS-Biotin; Pierce) for 20 or 45 min with slow agitation for cultured neurons or slices, respectively. This results in complete biotinylation throughout 400-μm hippocampal slices (17). After three rinses in cold Tris-based ACSF (HEPES replaced with Tris) to quench free biotin, slices were snap frozen in liquid nitrogen. Cultures were immediately scraped without freezing in cold 1% Triton X-100 homogenization buffer (150 μl/35-mm well) (in mM: 50 NaCl, 10 EDTA, 10 EGTA, 1 Na2VO3, 50 NaF, 25 NaPi, 1 β-glycerophosphate, 1 phenylmethylsulfonyl fluoride, 0.001 micromycin, 1 protease inhibitor mixture tablet (Roche Applied Science), 1× phosphatase inhibitor mixture set I (Calbiochem), and 50 HEPES (pH 7.5). Solubilization was performed in 1% Triton X-100 because this protocol does not solubilize post synaptic densities

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1 To whom correspondence should be addressed: Vollum Inst., Oregon Health & Sciences University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239. Tel.: 503-494-4000; E-mail: soder@ohsu.edu.

2 The abbreviations used are: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate glutamate receptor; CaM-KII, calcium/calmodulin-dependent protein kinase II; CPS, consensus phosphorylation site; fEPSP, field excitatory postsynaptic potential; F/R, forskolin/rolipram; GluR1, AMPA-type glutamate receptor subunit 1; LTP, long-term potentiation; LTD, long-term depression; cLTP, chemical LTP; Chem-LTD, chemical LTD; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; GST, glutathione S-transferase.
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Ser-845 Phosphorylation Increases Surface GluR1 in Cultured Hippocampal Neurons—To quantify net Ser-845 phosphorylation in hippocampal neurons, we generated standards of GluR1 with known phosphorylation stoichiometries. This was accomplished by in vitro phosphorylation of purified GST fusions of the C-terminal 75 residues of GluR1 (GST-GluR1-CT) with purified protein kinases (11). Using [γ-32P]ATP, CaM-KII (10 nM) catalyzed phosphorylation of Ser-831 to a molar stoichiometry of ~1 (Fig. 1a). Surprisingly, PKA (10–200 nM) gave a stoichiometry of <0.1 for Ser-845 (Fig. 1b). Examination of the sequence around both Ser-831 and Ser-845 revealed that neither is a CPS for CaM-KII or PKA; respectively, since neither contains an arginine in the p-3 position (Fig. 1e). Therefore, the CPS GST-GluR1-CT fusion mutants P828R and P842R were expressed, purified, and examined for phosphorylation. CaM-KII phosphorylated the P828R mutant the same as wild-type (Fig. 1e), but now PKA catalyzed stoichiometric phosphorylation of the CPS P842R mutant (Fig. 1d). The results obtained by 32P incorporation were verified using phospho-specific antibodies for Ser-831 and Ser-845 (Fig. 1f). The CPS mutant P842R was stoichiometrically phosphorylated by PKA on Ser-845 and mixed with known amounts of non-phosphorylated GST-GluR1-CT to make calibration standards. These in vitro phospho-Ser-845 calibration standards were run on every SDS-PAGE (Fig. 2a) with extracts of cultured neurons or hippocampal slices and used for Western blotting with phospho-specific antibody to quantify the net Ser-845 phosphorylation levels of endogenous GluR1 in cultured hippocampal neurons.

To induce Ser-845 phosphorylation (12), we used a recently described chemical stimulation protocol of forskolin plus rolipram (F/R) that results in prolonged NMDA receptor (NMDAR)-dependent LTP (cLTP) (20) and recruits CaM-KII to dendritic spines (21). Treatment of hippocampal cultures with F/R (50 μM/0.1 μM) for 10 min persistently increased basal Ser-845 phosphorylation (10–20%) of surface GluR1 to ~60% at 30–40 min (Fig. 2b), which correlated with a significant increase in surface expression of GluR1 (Fig. 2c). Importantly, there was no net increase in Ser-831 phosphorylation during this same time period (Fig. 2e), suggesting that Ser-845 phosphorylation may play a specific role in GluR1 trafficking (13, 14). The Ser-845 phosphorylation stoichiometry did not differ between the surface and total GluR1 fractions (data not shown). Neither Ser-845 phosphorylation nor surface GluR1 delivery was blocked by the NMDAR antagonist d-2-amino-5-phosphonopentanoic acid (d-APV) (Fig. 2f, b and c) or tetrodotoxin (data not shown). The plot of net phospho-Ser-845 versus normalized surface GluR1 yielded a linear relationship with an R² value of 0.997 and slope of 0.75 (Fig. 2d). This tight relationship strongly implicates GluR1 Ser-845 phosphorylation in regulating surface expression of AMPARs.

Dephosphorylation of Ser-845 Is Associated with a Decrease in Surface GluR1—Bath application of NMDA induces chemical LTD (Chem-LTD) and Ser-845 dephosphorylation in hippocampal slices (22, 23). We hypothesized that blocking Ser-845 phosphorylation during F/R stimulation may also block the surface delivery of new AMPARs. Bath application of NMDA (50 μM) to cultured hippocampal neurons resulted in a significant Ser-845 dephosphorylation of surface GluR1 and then subjected to surface biotinylation without microdissection at indicated time points.

Statistical Analysis—All statistics were performed by Student’s two-tailed, unpaired t-test compared with controls or basal (0-min time point) within each group. In all the figures, *, p <0.05; **, p <0.01; ***, p <0.001 All data are presented as mean ± S.E.
phosphorylation sequences for CaM-KII or PKA, respectively, with a proline rather than phosphorylation sites revealed that neither Ser-831 nor Ser-845 sites are consensus GluR1 antibodies of with a decrease in the surface pool of GluR1 (Fig. 3). These results suggest that the fraction of GluR1 delivered to the surface was closely correlated with net phospho-Ser-845 (13).

Ser-845-phosphorylated AMPARs Are Trafficked to Extrasynaptic Sites in Hippocampal Slices—The functional role of GluR1 Ser-845 phosphorylation was further investigated in acute hippocampal slices by testing whether F/R increases phospho-Ser-845, surface GluR1, and synaptic strength. Surface GluR1 was measured by biotinylation using conditions previously shown to penetrate the entire slice (17). Similar percentages of AMPARs (~30–35%) were found on the surface in both slices and cultured neurons, indicating that biotinylation was equally effective in these preparations (data not shown). A 10-min F/R (25 μM/0.1 mM) stimulation resulted in a robust Ser-845 phosphorylation from a basal value of 15 to ~50% that persisted for at least 1 h after the washout of drugs (Fig. 4a) that was associated with a significant increase in surface GluR1 fraction (Fig. 4c). Bath treatment with 25 μM NMDA for 3 min induced Chem-LTD (65 min after NMDA treatment, normalized fEPSP slope: Control = 0.94 ± 0.4, n = 6; NMDA = 0.75 ± 0.5, n = 12; *p < 0.05) (19) and completely blocked F/R-mediated Ser-845 phosphorylation (Fig. 4b) and surface delivery of GluR1 (Fig. 4c). Thus, pharmacological stimulations that either increased (F/R) or decreased (NMDA) net phospho-Ser-845 significantly increased or decreased the surface pool of GluR1, respectively, supporting the hypothesis that Ser-845 phosphorylation is involved in regulating the size of surface pool of GluR1 in hippocampal slices.
The major finding of this study was that GluR1 Ser-845 phosphorylation primes AMPARs for synaptic incorporation by trafficking.
AMPARs to extrasynaptic sites on the surface membrane. Previous studies (3, 14, 24) examined Ser-845 phosphorylation in GluR1 upon various stimulation paradigms, but only relative changes were analyzed, making it difficult to interpret the likely physiological significance of these changes. This report is the first investigation to quantify the net phosphorylation state of Ser-845 in endogenous hippocampal GluR1 during chemically induced bidirectional synaptic plasticity. This allowed analyses of functional effects in changes of net phospho-Ser-845 and surface GluR1 delivery during bidirectional synaptic plasticity by combining quantitative immunoblotting, surface biotinylation, and slice field recordings. Under basal conditions ~30–35% of GluR1 was on the surface membrane, and only 15% was phosphorylated at Ser-845. Induction of cLTP, in the absence of electrical stimulation, increased surface expression and Ser-845 phosphorylation without affecting Ser-831 phosphorylation, whereas Chem-LTD decreased surface expression and Ser-845 phosphorylation. Combined with a functional analysis of synaptic responses, we demonstrated that Ser-845 phosphorylation-regulated surface GluR1 delivery was to extrasynaptic sites. Results from this study indicate that Ser-845 phosphorylation can modulate the dynamic range of synaptic plasticity by regulating the pool of AMPARs available for synaptic incorporation. Thus, Ser-845 phosphorylation might be one of several mechanisms that neurons may use to regulate

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FIGURE 5. GluR1 Ser-845 phosphorylation primed AMPARs for synaptic incorporation. a, fEPSPs from hippocampal slices stimulated with F/R (black bar) with (open squares) and without (solid diamonds) basal stimulations (every min) are shown. Representative traces (a, b, and c time points indicated on graph) are shown on the right. Potentiation occurred only when F/R treatment was combined with basal stimulation (at 60 min after F/R; Control, 0.96 ± 0.02; F/R minus Basal Stim, 1.07 ± 0.05; F/R plus Basal Stim, 1.46 ± 0.10). For Control versus F/R plus Basal Stim, *p < 0.0001 at 60 min and p < 0.05 at 120 min (n = 9–12). b, after recordings, CA1 regions were microdissected from slices and subjected to quantitative Western blotting for net phospho-Ser-845. F/R stimulation resulted in significant increase in phospho-Ser-845, suggesting there was an increase in surface GluR1 with F/R stimulation regardless of basal stimulation (n = 5–7). c, F/R pretreatment (black bar, solid diamonds) without basal stimulation resulted in larger potentiation in response to triple theta bursts (arrow) (at 120 min after F/R; Control, 1.34 ± 0.04; n = 7; F/R, 1.63 ± 0.10; n = 9; p < 0.05. p was still <0.05 after normalization of fEPSP slope just before theta burst). Representative fEPSP traces are shown on the right. d, net phospho-Ser-845 was significantly elevated in the CA1 region from slices recorded in panel c (n = 9–13). e, Chem-LTD blocks Ser-845 phosphorylation-dependent priming. 10 min of F/R (black bar) followed by 3 min of NMDA (open bar) not only completely blocked the priming effect of Ser-845 phosphorylation on LTP induced with triple theta bursts but largely suppressed LTP itself. Representative fEPSP traces are shown on the right. f, NMDA significantly attenuated F/R-stimulated phospho-Ser-845 from 46.2 ± 5.4% (see panel d) to 19.4 ± 2.4% (p < 0.001), although phospho-Ser-845 was still slightly increased over the control group (n = 6–8). g, two-step model for synaptic delivery of AMPARs during LTP. Under basal conditions (left panel), GluR1 has a low phosphorylation at Ser-845. Constitutive recycling occurs between the surface and internal pools (1 in left panel) and the synaptic and extrasynaptic pools (2 in left panel) of AMPARs. Increasing Ser-845 phosphorylation (Step 1) stimulates trafficking of internal GluR1-containing AMPARs to extrasynaptic sites on the surface membrane, which primes AMPARs for synaptic incorporation (3 in middle panel). During strong synaptic activation (Step 2), synaptic NMDARs are activated, resulting in increased intracellular calcium (4 in right panel). Calcium triggers the activation of signaling cascades, likely involving CaM-KII (7, 14), which drives GluR1-containing AMPARs to synapses from extrasynaptic sites by lateral diffusion (5 in right panel). Thus, the two-step model for synaptic delivery of AMPARs consists of delivery of GluR1-containing AMPARs to extrasynaptic sites in a phospho-Ser-845-dependent manner (Step 1, the priming step), followed by synaptic incorporation of AMPARs, which requires synaptic NMDAR activation and calcium (Step 2). *p < 0.05; **p < 0.01; ***p < 0.001 for all the panels.
the amplitude of synaptic potentiation. Although there are likely other mechanisms for GluR1 delivery to the surface membrane during synaptic plasticity, the critical role of Ser-845 phosphorylation is in the enrichment of extrasynaptic AMPARs by shifting the equilibrium between the surface and internal pools of AMPARs. It is interesting that F/R treatment gave equivalent increases in Ser-845 phosphorylation of surface and total GluR1. A possible model to explain this observation is that in the absence of Ser-845 phosphorylation the equilibrium between surface and internal AMPARs strongly favors the internal pool (i.e. endocytosis). Upon Ser-845 phosphorylation, this equilibrium between surface insertion and endocytosis changes (13), such that approximately equal numbers of AMPAR may cycle between the surface and internal pools. Thus, F/R treatment could increase the pool of total Ser-845-phosphorylated AMPAR, thereby favoring surface expression by mass action.

Another essential finding of our study was that the surface delivery of AMPARs can be dissociated from synaptic targeting that required concomitant synaptic activity (Fig. 5n), suggesting that synaptic NMDAR activation is required (20). Ser-845 phosphorylation has previously been proposed to be important for regulating the amount of GluR1-containing AMPARs available for synaptic incorporation, whereas CaM-KII activity appears to be critical for synaptic incorporation (14). Our results provide a specific mechanism for this previous finding: Ser-845 phosphorylation-dependent delivery is selective to extrasynaptic sites. The increase in extrasynaptic pool of GluR1 resulted in larger LTP following triple theta burst stimulation (Fig. 5c), suggesting that synaptic NMDAR activity can drive GluR1-containing AMPARs into synapses from the extrasynaptic pools. This priming effect was suppressed by Chem-LTD, which blocked the F/R-mediated increase in phospho-Ser-845 and surface GluR1 (Fig. 3) (25). Therefore, our results support a two-step model for synaptic delivery of AMPARs (Fig. 5g), where GluR1-containing AMPARs are first delivered to the extrasynaptic sites in a Ser-845 phosphorylation-regulated manner. Activated synaptic NMDARs then cause an influx of calcium that likely triggers the signal transduction cascades necessary for anchoring AMPARs in synapses. Thus, Ser-845 phosphorylation primes the GluR1-containing AMPARs for synaptic incorporation.

The two-step model for synaptic delivery of AMPARs (Fig. 5g) is consistent with reports from other laboratories. For example, studies show that extrasynaptic AMPARs in hippocampal dendrites are increased nearly 2-fold during LTP (26) and extrasynaptic AMPARs are significantly depleted in GluR1 knock-out mice (27). Furthermore, recent data show that removal of GluR2-containing AMPARs also occurs first at extrasynaptic sites, immediately followed by a decrease in synaptic AMPARs (28). AMPARs have been shown to rapidly move in and out of synapses via lateral movement along the surface membrane, which may account for the increase in postsynaptic AMPAR responses during LTP (29–32). Elevating local intracellular calcium causes accumulation of AMPARs on the surface membrane, suggesting that calcium triggers the mechanism for synaptic anchoring of laterally diffusing AMPARs (30, 31). Whether calcium first increases the number of anchors at synapses or blocks the lateral diffusion of AMPARs, which then recruit the anchors, remains to be determined. Because AMPARs diffuse freely in extrasynaptic sites (31), it seems more likely that synaptic calcium recruits more anchors to postsynaptic densities, which then sequester laterally diffusing AMPARs. One potential component of this anchor might be postsynaptic density-95, which has been shown to be important for regulating the number of synaptic AMPARs (33–35). Our data do not exclude that AMPARs can be incorporated into synapses directly from the cytoplasm but do indicate that extrasynaptic AMPARs are important contributors to synaptic plasticity.

An important regulator of AMPAR trafficking is stargazin, which has been shown to be critical for the delivery of AMPARs to the surface membrane (36). Importantly, overexpression of stargazin significantly increases AMPA-mediated currents in CA1 pyramidal neurons but not their synaptic currents (36), mirroring present findings with F/R treatment and leading to similar conclusions on stargazin-mediated delivery of AMPARs selectively to extrasynaptic sites. Alternatively, direct regulation of AMPA receptor function by stargazin (37, 38) is also consistent with its potentiating effects. However, our data showed unambiguously, using surface biotinylation, that AMPARs can be trafficked to extrasynaptic sites. Interestingly, postsynaptic density-95 overexpression results in synaptic potentiation of AMPARs (33, 39). What might be the Ca2+-sensitive transducer that promotes anchoring of AMPARs at synapses? CaM-KII has been implicated in inserting AMPARs into synapses (7, 14), and F/R stimulation is known to recruit CaM-KII to dendritic spines (21). Thus, calcium influx through synaptic NMDARs can activate CaM-KII (40), which may then drive extrasynaptic AMPARs into synapses. The exact target of CaM-KII important for this delivery is unknown, as it does not appear to be Ser-831 in GluR1 (7). Intriguingly, stargazin has multiple phosphorylation sites, including CaM-KII and PKC sites that appear to be involved in regulating AMPAR trafficking (37). Clearly, additional studies are needed to elucidate the mechanisms of synaptic capture of AMPARs.

Note Added in Proof—Sun, X., Zhao, Y., and Wolf, M. E. (2005) J. Neurosci. 25, 7342–7351, show similar findings in prefrontal cortex neurons where stimulation of Ser-845 phosphorylation results in delivery of AMPARs to extrasynaptic sites followed by synaptic incorporation upon synaptic NMDAR activation.

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