THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 17, pp. 11445–11452, April 25, 2008

AKAP79 Selectively Enhances Protein Kinase C Regulation of GluR1 at a Ca^{2+}-Calmodulin-dependent Protein Kinase II/Protein Kinase C Site*

Received for publication, November 12, 2007, and in revised form, February 22, 2008 Published, JBC Papers in Press, February 27, 2008, DOI 10.1074/jbc.M709253200

Steven J. Tavalin[1]

From the Department of Pharmacology, University of Tennessee Health Science Center, Memphis, Tennessee 38163

Enhancement of AMPA receptor activity in response to synaptic plasticity inducing stimuli may arise, in part, through phosphorylation of the GluR1 AMPA receptor subunit at Ser-831. This site is a substrate for both Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). However, neuronal protein levels of CaMKII may exceed those of PKC by an order of magnitude. Thus, it is unclear how PKC could effectively regulate this common target site. The multivalent neuronal scaffold A-kinase-anchoring protein 79 (AKAP79) is known to bind PKC and is linked to GluR1 by synapse-associated protein 97 (SAP97). Here, biochemical studies demonstrate that AKAP79 localizes PKC activity near the receptor, thus accelerating Ser-831 phosphorylation. Complementary electrophysiological studies indicate that AKAP79 selectively shifts the dose-dependence for PKC modulation of GluR1 receptor currents ~20-fold, such that low concentrations of PKC are as effective as much higher CaMKII concentrations. By boosting PKC activity near a target substrate, AKAP79 provides a mechanism to overcome limitations in kinase abundance thereby ensuring faithful signal propagation and efficient modification of AMPA receptor-mediated responses.

While *in situ* determinations of kinase concentration may be difficult to ascertain, it is well established that the Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII)[2] is the most abundant kinase in brain and represents a major constituent of the postsynaptic density (PSD) (1). Like CaMKII, the Ca^{2+}/phospholipid-dependent protein kinase (PKC) is also highly abundant in the brain, albeit levels of this enzyme appear to be an order of magnitude lower as estimated via recent proteomic surveys of the PSD (2). Despite the apparent disparity in the abundance of these kinases, they perform remarkably similar postsynaptic functions. Indeed, infusion of constitutively active forms of either kinase into neurons potentiates AMPA receptor-mediated synaptic transmission and occludes long-term potentiation (LTP) (3, 4), a cellular model for learning and memory. Likewise, inhibitors of either kinase block LTP (5, 6), and in some cases both kinases may need to be inhibited to prevent LTP induction (7). Although several mechanisms encompassing multiple regulatory steps likely contribute to LTP (8), one common substrate for both kinases that has been linked to the enhancement of synaptic transmission during LTP and learning is Ser-831 of the GluR1 AMPA receptor subunit (9–16). Phosphorylation of this residue increases the single channel conductance of this ligand-gated ion channel (17) (but see Ref. 18) and may contribute to the enhanced synaptic conductance that has been observed following LTP (19). Although CaMKII and PKC share overlapping actions on synaptic transmission and a common substrate, the concentration of PKC needed to enhance synaptic transmission appears to be much lower than that for CaMKII to produce similar effects (3, 4). Thus, some mechanism may be in place to ensure that PKC can regulate common targets to a similar extent as CaMKII given the differences in abundance.

AKAP79 (AKAP150 in rodents) is a multivalent signaling scaffold that binds the cAMP-dependent protein kinase (PKA), the Ca^{2+}-dependent phosphatase calcineurin (CaN or PP2B), and PKC (20). AKAP79 is targeted to GluR1 via SAP97 thereby recruiting AKAP79-anchored PKA to GluR1 allowing for PKA phosphorylation of GluR1 at Ser-845 and Ca^{2+}/CaN-dependent down-regulation of GluR1 receptor currents (21–23). Thus, the goal of the present study was to determine whether AKAP79 also facilitates PKC regulation of GluR1 phosphorylation and function, as well as to compare the sensitivity of GluR1 to regulation by CaMKII and PKC.

EXPERIMENTAL PROCEDURES

Molecular Constructs and Production of CaMKII-(1–290)—$GluR1_{flip}$ in the pRK5 vector and the respective phospho-mutants were provided by Tom Soderling (Vollum Institute, OHSU, Portland, OR) and previously described (17). AKAP79-GFP and AKAP79-(75–427)-GFP (denoted as AKAP79ΔA) in pcDNA3 were previously described (24). CaMKII-(1–290) was provided by Michael S. Kapiloff (OHSU, Portland, OR), and was PCR-cloned into pET100/D-TOPO (Invitrogen, Carlsbad, CA)

* This work was supported by Grant NS46661 from the National Institutes of Health and by start-up funds provided by the University of Tennessee Health Science Center and the UTHSC Neurobiology of Brain Disease Center of Excellence. 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 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Pharmacology, University of Tennessee Health Science Center, 874 Union Ave., Memphis, TN 38163. Tel.: 901-448-3007; Fax: 901-448-7206; E-mail: stavalin@utmem.edu.

[2] The abbreviations used are: CaMKII, Ca^{2+}-calmodulin-dependent protein kinase II; LTP, long-term potentiation; PKA, cAMP-dependent kinase; PMA, phorbol 12-myristate 13-acetate; PSD, postsynaptic density; PDZ, PSD-95/discs large/zona occludens 1; HEK, human embryonic kidney cells; PKC, protein kinase C; BIS, bisindolylmaleimide; SAF, safingol; GFP, green fluorescent protein; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

in-frame to produce an N-terminal His tag, which was verified by automated sequencing. BL21 cells (Invitrogen) were transformed with the plasmid and grown in LB broth supplemented with ampicillin. Protein expression was induced by stimulation with isopropyl β-D-1-thiogalactopyranoside. Following that, the cells were pelleted by centrifugation, lysed, and purified by the B-PER 6× His tag fusion protein purification kit (Pierce). Contaminating salts were removed by dialysis, and the protein was concentrated (Millipore, Bedford, MA), then resuspended in phosphate-buffered saline (Invitrogen) supplemented with 10 mM dithiothreitol and 10% glycerol, and stored at −80 °C in convenient aliquots. The protein concentration was determined using the BCA protein assay (Pierce).

Kinase Activity Assays—Kinase activity was assessed using the Kinase-Glo Plus Luminescent Kinase Assay kit (Promega). CaMKII(1–290) (10 nM) was assayed using 100 μM autocamtide-2 (Biomol) as substrate and incubating in phosphorylation buffer (50 mM Tris, 10 mM $MgCl_2$, and 1 mM DTT and 10 μM ATP; pH 7.5) at 30 °C for 10 min. The catalytic fragment of PKC (PKM; 10 nM; Biomol) was independently assayed using 10 μM PKC[Ser-25]-(19–31) as substrate under identical conditions. The ability of CaMKII-(1–290) and PKM to phosphorylate GluR1 at Ser-831 was assessed using a peptide fragment of GluR1 encompassing residues 826–836 (100 μM; Tocris) with a 30-min incubation time at 30 °C. Activity is reported as the average from replicate assays.

Cell Cultures and Transfections—HEK293 cells were obtained from ATCC (Manassas, VA) at passage 36 and used for a maximum of 8 passages. Cell cultures were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (P/S). For biochemistry, HEK293 cells were plated at ~50% confluence on 6-well plates or 60-mm dishes. HEK293 cells were transfected by the calcium phosphate method. For these experiments, 1 or 2 μg (for 6-well plates or 60-mm dishes, respectively) of each construct was used. pEGFP (0.3 μg) was included under control conditions as all the AKAP constructs are GFP-tagged. Biochemical experiments were performed 24 h after transfection. For electrophysiology, cells were plated at low density (~50,000 cells/ml) on 15-mm round glass coverslips in 12-well plates. Cells were transfected with 1 μg of each construct per coverslip, and 0.3 μg of pEGFP was included for control cells as above. Green fluorescent protein epifluorescence was used to confirm the expression of the corresponding AKAP.

Cultured Hippocampal Neurons—60-mm dishes or 6-well plates were coated overnight with 100 μg/ml poly-D-lysine (Sigma) and 10 μg/ml laminin (Invitrogen). Hippocampi were removed from neonatal Sprague-Dawley rats (1–2 days old) following Institutional Animal Care and Use Committee-approved protocols. The tissue was dissociated by papain (20 units/ml; Sigma) treatment and trituration through pasteur pipettes and suspended in medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum and P/S. Cell were plated at a density of 150,000 cell/ml. Cells were washed with this medium 6 h after plating and exchanged for Neurobasal A Media with B27 supplement (Invitrogen), 0.5 mM L-glutamine, and P/S after 24 h. The medium was supplemented with 5-fluorouracil and uridine to prevent glial cell proliferation 3 days after plating. One-half of the medium was refreshed every 3 days. Biochemical experiments were performed between 12 and 14 days *in vitro*.

Cell Treatments and Immunoblotting—Cells were rinsed in extracellular solution containing 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and 1 μM cyclosporin A (CsA; LC Laboratories, Woburn, MA) (pH 7.4) and incubated for 10 min. Upon removal, cells were treated with extracellular solution supplemented with phorbol 12-myristate 13-acetate (PMA, LC laboratories), 4α-phorbol 12-myristate 13-acetate (4α-PMA, LC Laboratories), or ionomycin (Calbiochem, La Jolla, CA) for the indicated times at 20 °C. PKC inhibitors bisindolylmaleimide (BIS, LC Laboratories) or safingol (SAF, Sigma) were applied during the initial incubation and were maintained throughout the PMA treatment. Treatments were stopped by washing three times with ice-cold phosphate-buffered saline. Extracts were prepared by lysing cells in 200 μl (for 6-well plates) or 300 μl (for 60-mm dishes) lysis buffer (10 mM phosphate, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100) containing protease inhibitors (Sigma, 1:100 dilution) and phosphatase inhibitors (10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μM okadaic acid, 1 μM microcystin, and 1 μM cyclosporin A). Lysates were incubated on ice for 15 min and then centrifuged at 14,000 × *g* for 30 min at 4 °C. Supernatants were collected, 2× Laemmli sample buffer added and boiled for 5 min. 10–20 μg of extract were loaded per lane, and proteins were separated by SDS-PAGE on 4–12% gels (Cambrex, Rockland, ME), transferred to nitrocellulose, blocked overnight in PBS + 3% nonfat dry milk, and then immunoblotted using rabbit monoclonal or polyclonal antibodies directed against phospho-Ser-831 (1:1000 dilution; Millipore). Goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Millipore; 1:10,000) were used as a secondary antibody. Signals were visualized by enhanced chemiluminescence (Pierce). Data were digitally acquired and quantified using a Bio-Rad XRS Chemiluminescence Documentation System and Quantity One software. Blots were stripped and then re-probed with a rabbit polyclonal antibody directed against the C-terminal tail of GluR1 (Millipore: 0.5 μg/ml) and visualized as above. The ratio of phospho-Ser-831 to GluR1 was determined and normalized to the control condition for each experiment. Data are expressed as means ± S.E. and were subjected to statistical analysis using analysis of variance followed by Student's *t* test.

Electrophysiology and Data Analysis—Prior to recording, cells were pretreated (5–10 min) with cyclosporin A (LC laboratories; 1 μM) to prevent CaN-mediated down-regulation of the current (22). Whole cell recordings were made with an Axopatch 200B or Axoclamp 700A amplifier (Molecular Devices; Sunnyvale, CA). Patch pipettes (2 to 4 MΩ) contained 140 mM Cs methanesulfonate, 10 mM HEPES, 5 mM adenosine triphosphate (Na salt), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 10 mM BAPTA (pH 7.4). PKM and CaMKII(1–290) were added from frozen stocks to the concentration indicated. The extracellular solution was the same as used for biochemical experiments. Glutamate (1 mM) was added from frozen concentrated stock solutions. Cyclothiazide (Axxora, San Diego, CA or Ascent Scientific; 100 μM) was used to block AMPA receptor desensi-

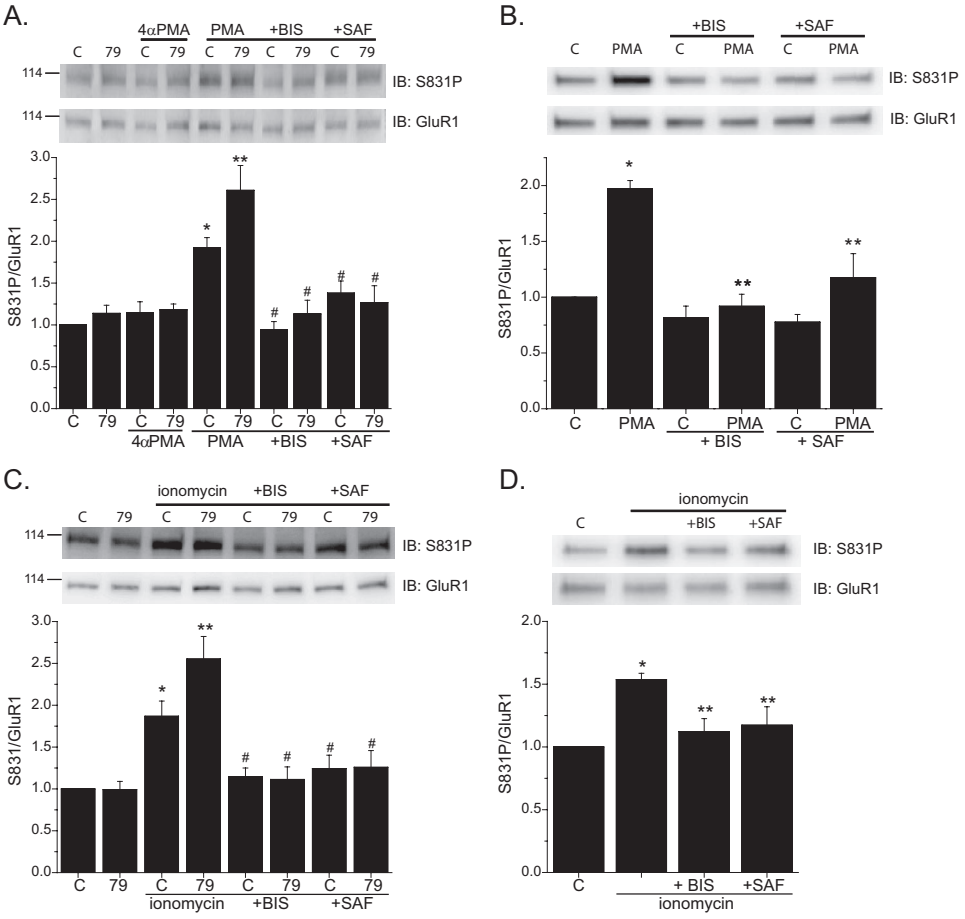

FIGURE 1. **AKAP79 enhances PKC and Ca^{2+}-induced phosphorylation of GluR1 at Ser-831.** *A*, HEK293 cells were transfected with GluR1 ± AKAP79. Cells were untreated or were exposed to PMA (200 nM) for 3 min in the presence or absence of PKC inhibitors BIS and SAF or an inactive phorbol ester. GluR1 Ser-831 phosphorylation was assessed by Western blotting with phosphospecific antibodies to Ser-831 or to GluR1. *Top panels* show a representative experiment. Extracts were immunoblotted (*IB*) for pSer-831 (S831P) or GluR1. *C* denotes GluR1 alone (control) while *79* denotes GluR1 + AKAP79. *Bottom panel* show bar graph summarizing data. *, $p < 0.01$ compared with untreated controls; **, $p < 0.05$ compared with PMA-treated controls, #, $p < 0.01$ compared with corresponding PMA-alone treatment. *B*, representative experiment and summary for similarly treated hippocampal neurons. *, $p < 0.01$ compared with untreated controls; **, $p < 0.05$ compared with PMA alone. *C*, representative experiment and summary bar graph for experimental treatments in HEK293 cells similar to *A* except using ionomycin (1 μM, 3 min) as a stimulus. *, $p < 0.01$ compared with untreated controls; **, $p < 0.05$ compared with ionomycin-treated controls; #, $p < 0.01$ compared with corresponding ionomycin-alone treatment. *D*, representative experiment and summary for similarly treated hippocampal neurons. *, $p < 0.01$ compared with untreated control; **, $p < 0.05$ compared with ionomycin alone.

tization. Solution exchanges were accomplished through a series of flow pipes, which were controlled by solenoid valves (Warner, Hamden, CT) and moved into position by a piezoelectric bimorph. HEK293 cells were lifted off the coverslip to speed the solution exchange time. Currents were digitized at 5 kHz and filtered at 1 kHz and acquired with a Digidata 1322A board and Clampex 9 software (Molecular Devices). Series resistance (90–95%) and whole cell capacitance compensation were employed. Series resistance was monitored throughout the experiments by 10-mV hyperpolarizing jumps prior to each application of glutamate. Only cells with series resistance of <6 mΩ and which were stable throughout the recording were included for analysis. All experiments were initiated within 1 min of establishing the whole cell configuration and were performed at a holding potential of −60 mV at 20 °C. Currents were normalized to the amplitude of current from the initial agonist application for each experiment. All data are expressed as means ± S.E. and were subjected to statistical analysis using a one-way analysis of variance followed by Student's *t* test.

RESULTS

AKAP79 Facilitates PKC-mediated Phosphorylation of GluR1—HEK293 cells were used as a simplified system to study the regulation of recombinant GluR1 receptors. In contrast to the enhancement of basal phosphorylation of GluR1 at Ser-845 by AKAP79-anchored PKA described previously (21, 22), AKAP79 did not affect basal phosphorylation of GluR1 at Ser-831 (Fig. 1). Instead, stimulation of PKC activity via brief (3 min) application of the phorbol ester PMA (200 nM) induced a marked increase in Ser-831 phosphorylation that was greater in AKAP79-expressing cells (control: 92.1 ± 12.2%, $n = 12$ *versus* AKAP79 160.2 ± 29.7%, $n = 12$ increase over untreated controls, $p < 0.05$ compared with PMA-treated controls; Fig. 1*A*). Ser-831 phosphorylation, however, was unaffected by the inactive phorbol ester 4α-PMA (control 14.8 ± 12.9%, $n = 4$; AKAP79: 18.2 ± 6.8%, $n = 4$ change from untreated controls). The phorbol ester-induced phosphorylation of the receptor was largely blocked by both bisindolylmaleimide (BIS; 500 nM) and safingol (SAF; 10 μM) inhibitors at the catalytic site and the phospholipid-activating site of PKC, respectively (control (BIS): −5.5 ± 9.6%, $n = 4$; AKAP79 (BIS): 13.1 ± 16.4%, $n = 4$; control (SAF): 37.9 ± 14.6%, $n = 3$; AKAP79 (SAF): 26.7 ± 20.2%, $n = 3$; Fig. 1*A*). This pharmacological profile for recombinant GluR1 Ser-831 phosphorylation largely matches that observed using similarly treated hippocampal cultures (Fig. 1*B*). Importantly, stimulation of PKC activity by application of the Ca^{2+} ionophore, ionomycin (1 μM; 3min), enhanced Ser-831 phosphorylation in AKAP79-expressing cells to a greater extent than those transfected with GluR1 alone (control: 86.8 ± 18.2%; $n = 8$ *versus* AKAP79: 155.2 ± 27.0%, $n = 8$ over untreated controls, $p < 0.01$; Fig. 1*C*). Ser-831 phosphorylation in response to ionomycin was also largely blocked in both control and AKAP79-expressing cells by both PKC inhibitors (control (BIS): 14.6 ± 10.5%, $n = 6$; AKAP79 (BIS): 11.3 ± 15.0%, $n = 6$; control (SAF): 24.3 ± 16.0%, $n = 6$; AKAP79 (SAF): 25.8 ± 20.0%, $n = 6$; Fig. 1*C*). This sensitivity of ionomy-

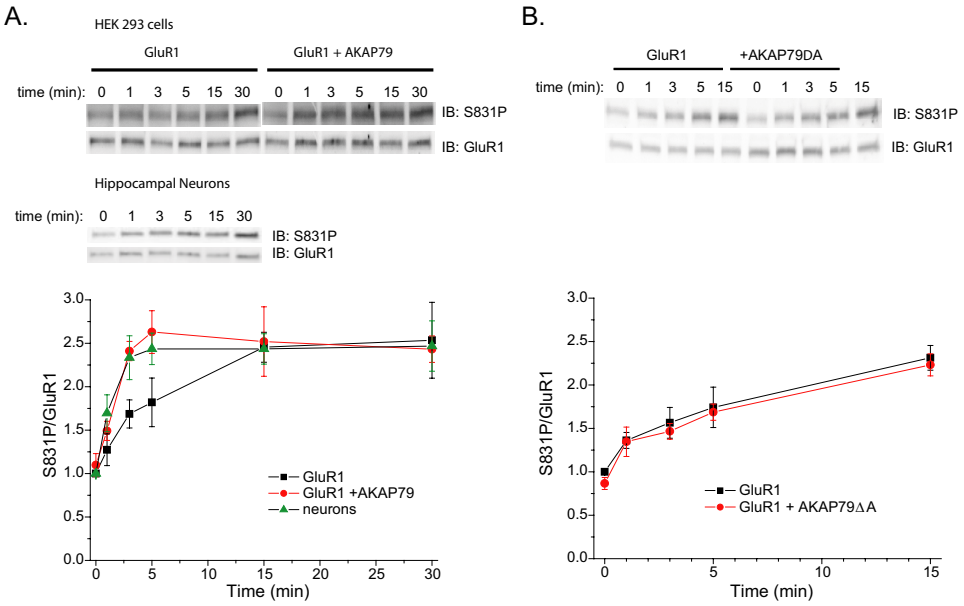

FIGURE 2. **AKAP79-anchored PKC accelerates phosphorylation of recombinant GluR1 matching the early time course for regulation of native GluR1.** *A, top panels*, representative time course experiment for phorbol ester-induced Ser-831 phosphorylation in HEK293 cells transfected with GluR1 ± AKAP79 and for cultured hippocampal neurons. *Bottom panel*, summary of data from multiple experiments is shown ($n = 4$–10 experiments for each time point for HEK293 cells, $n = 3$–6 for each time point for hippocampal neurons). *B, top panels* show representative time course experiment for HEK293 cells transfected with GluR1 ± AKAP79ΔA (a mutant form of AKAP79 unable to bind to PKC). *Right panel* shows summary of four experiments.

cin-induced Ser-831 phosphorylation to PKC inhibitors was also matched in similarly treated hippocampal cultures (Fig. 1*D*). Collectively, these results parallel recent work (25) that suggests that GluR1 phosphorylation at Ser-831 in both native and heterologous expression systems is dependent on PKC regardless of the mechanism by which PKC is activated. Moreover, these results are consistent with the idea AKAP79 facilitates PKC-mediated phosphorylation of Ser-831.

AKAP79-anchored PKC Accelerates Phosphorylation of GluR1—The AKAP79-mediated enhancement of phorbol ester-induced phosphorylation of GluR1 could arise by either expanding the phosphorylatable pool of receptors or by speeding phosphorylation of a fixed pool of receptors or both. The time course of GluR1 phosphorylation in response to phorbol ester stimulation was determined in control and AKAP79-expressing cells to discriminate between these possibilities. Ser-831 phosphorylation was enhanced in AKAP79-expressing cells compared with controls upon 3–5-min treatment of PMA but not with longer treatments (Fig. 2*A*). Exponential fits to the data indicated that AKAP79 shifted the time constant for phorbol ester-induced phosphorylation of GluR1 ~4-fold, from 6.4 to 1.8 min in agreement with the idea that AKAP79 predominantly accelerated PKC phosphorylation of GluR1. Interestingly, GluR1 phosphorylation in the presence of AKAP79 virtually matched the phosphorylation time course for hippocampal neurons ($\tau \sim 1.6$ min, Fig. 2*A*) suggesting that an "AKAP79-like" mechanism may control phosphorylation of native GluR1, which is consistent with the targeting of AKAP150 in rat hippocampal neurons to GluR1 (21). Phosphorylation of GluR1, however, was not different from controls when AKAP79ΔA, an N-terminal deletion mutant of AKAP79 unable to bind PKC (24, 26), was used (Fig. 2*B*). Aside from the loss of the principal binding determinants for PKC and Ca^{2+}/CaM, which both bind to this region (27), this mutant is not sufficient to compromise other known AKAP79 interactions (23, 28, 29). In light of the above pharmacological studies, these data suggests that AKAP79 speeds phosphorylation by providing a local pool of PKC to GluR1.

AKAP79 Concentrates PKC to Facilitate Regulation of GluR1 Receptor Currents—Whole cell patch clamp recordings were performed to test whether AKAP79 also facilitates PKC regulation of GluR1 function. In cells expressing GluR1 alone (control), infusion of the catalytically active fragment of PKC (PKM; 4 nM) via the whole cell recording pipette led to only a modest increase of glutamate-evoked currents during a 15-min recording period (7.2 ± 7.3%, $n = 7$; Fig. 3*A*). In contrast, in cells expressing AKAP79, GluR1 receptor currents were dramatically enhanced by PKM (45.1 ± 9.1%, $n = 7$, $p < 0.01$; Fig. 3*A*). This PKM induced increase is consistent with the ability of AKAP79 to bind PKM (26). Indeed, this PKM-induced increase in current was absent in cells transfected with the AKAP79ΔA construct (−2.9 ± 8.2%; $n = 5$; Fig. 3*A*), suggesting that AKAP79 provides a platform to recruit PKC to the receptor for functional modulation of channel activity. Given that these experiments were performed in the presence of high concentrations of the Ca^{2+} chelator BAPTA (10 mM), these data further exclude the likelihood that the interaction of AKAP79 with Ca^{2+}/CaM contributes to the regulation described here.

Interestingly, the augmentation of GluR1 receptor currents by PKM was similar in magnitude to that which had been previously observed with much higher concentrations of CaMKII (9). Although AKAP79 facilitated PKC regulation of GluR1 receptor currents, it was unclear if AKAP79 would influence CaMKII modulation of GluR1. Thus parallel experiments were performed with CaMKII-(1–290) within the pipette. CaMKII(1–290) is a Ca^{2+}/CaM-independent constitutively active form of CaMKII that retains full activity (30), and therefore is analogous to PKM. As was observed for PKM, infusion of 4 nM CaMKII(1–290) into control cells led to a relatively modest increase in current (4.5 ± 4.4%, $n = 5$; Fig. 3*B*). AKAP79, though, failed to modify the responsiveness of the currents to the kinase (1.2 ± 5.3%, $n = 5$; Fig. 3*B*), indicating that AKAP79 selectively facilitated PKC regulation of GluR1 receptor currents. Previous studies examining CaMKII regulation of GluR1 activity, though, had applied 400 nM autophosphorylated CaMKII within the pipette solution (9). Autophosphorylated CaMKII is also constitutively active but only retains ~20–25% of full activity (30). Therefore, to ensure that CaMKII(1–290) could effectively modulate GluR1, the kinase was included in

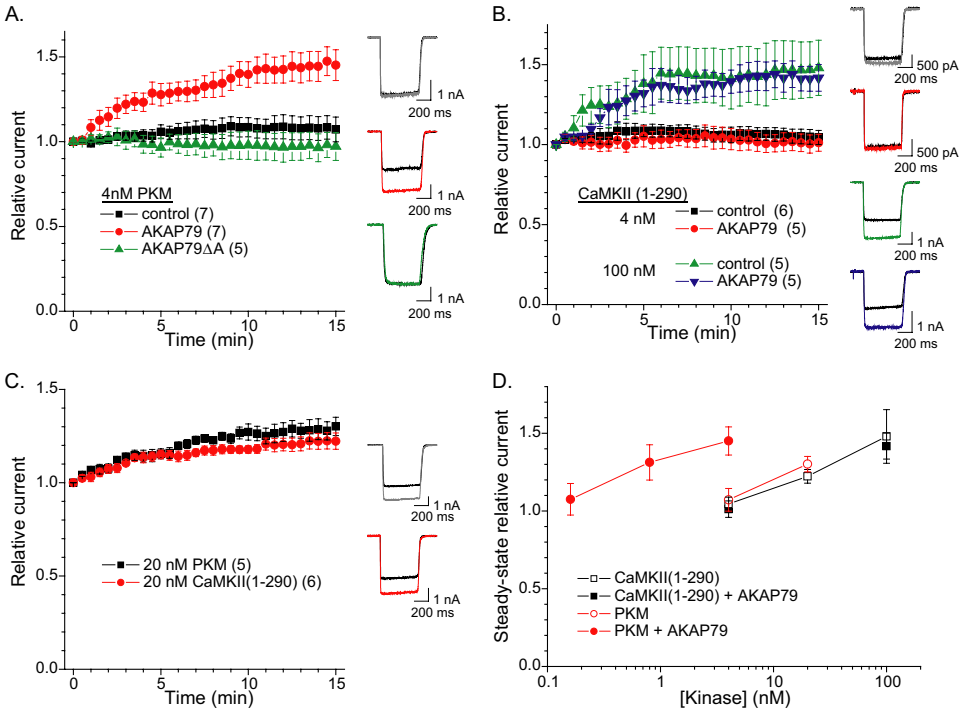

FIGURE 3. **AKAP79 selectively enhances PKC regulation of GluR1 receptor currents.** Time course of whole cell recordings of peak GluR1 receptor currents evoked by glutamate (1 mM) in the presence of cyclothiazide (100 μM). *Side panels* show representative *first* and *last traces* for each condition. *Numbers* in parentheses denote number of observations for each condition. *A,* PKC regulation of GluR1 receptor current is facilitated by association with AKAP79. HEK293 cells were transfected with GluR1 alone (control) or with AKAP79 or with AKAP79ΔA. PKM (4 nM) was included in the whole cell patch pipette solution. *B,* AKAP79 does not alter CaMKII regulation of GluR1 at either low or high concentrations of the kinase. *C,* application of the same concentration of constitutively active PKC and CaMKII via the patch pipette leads to a similar augmentation of GluR1 receptor currents. *D,* summary graph demonstrating the percent increase in GluR1 receptor currents in response to infusion of various concentrations of CaMKII and PKM into the cells.

the pipette solution at a concentration of 100 nM to approximate the levels of kinase activity used in earlier studies. Under these conditions, CaMKII(1–290) did effectively augment GluR1 receptor currents to the same extent seen previously (48.0 ± 17.3%, $n = 5$; Fig. 3*B*). However, this regulation was unaffected by the presence of AKAP79 (41.7 ± 8.4%, $n = 5$; Fig. 3*B*), suggesting that AKAP79 does not restrict CaMKII access to the receptor.

It has been noted that the sequence surrounding GluR1 at Ser-831 does not conform to a consensus phosphorylation site for either PKC or CaMKII (10, 11, 31). Consistent with this idea, both PKM and CaMKII-(1–290) exhibited negligible activity toward a small peptide substrate containing GluR1 residues 826–836 (data not shown), although both kinases demonstrated similar levels of activity toward their respective prototypical substrates PKC[Ser-25](19–31) and autocamtide-2 (PKM: 1102 nmol/min/mg; CaMKII(1–290): 882 nmol/min/mg). However, previous studies have found both PKC and CaMKII can phosphorylate GluR1 at Ser-831 when the entire intracellular C terminus of GluR1 is used as substrate (9, 11, 31). This suggests that additional determinants outside the region of residues 826–836 and which exist within the native protein improve the ability of GluR1 to serve as an *in vitro* substrate. Indeed, application of either kinase at an intermediate concentration (20 nM) led to a similar augmentation of GluR1 receptor currents (GluR1 + PKM: 30.2 ± 8.2%; $n = 5$, GluR1 + CaMK(1–290): 22.3 ± 4.4%; $n = 6$; Fig. 3*C*), indicating both kinases may possess similar activity toward the receptor as substrate *in situ* in the absence of AKAP79. Further examination of the responsiveness of GluR1 to varying PKC concentrations indicate that the presence of AKAP79 shifts the sensitivity of GluR1 to PKC by ~20-fold (Fig. 3*D*), thus providing an *in situ* estimate of the ability of AKAP79 to locally enhance PKC activity.

Regulation of GluR1 Receptor Currents by AKAP79 Requires Ser-831 and Relies on a PSD-95/Discs Large/Zona Occludens 1 (PDZ) Interaction at the GluR1 C-terminal Tail—CaMKII regulation of recombinant GluR1 receptors selectively requires Ser-831 as a substrate (9, 11, 17). Consistent with Ser-831 also serving as a substrate for PKC (9, 11), mutation of Ser-831 to alanine prevented the PKM-induced augmentation of the current in AKAP79-expressing cells (S831A: −3.8% ± 9.4%, $n = 5$ *versus* S831A + AKAP79: 0.9 ± 8.4%, $n = 6$; Fig. 4*A*). In contrast, mutation of the PKA site, Ser-845 to alanine, did not affect the ability of PKM to augment the current in AKAP79-expressing cells (S845A: 1.0 ± 7.1%, $n = 6$ *versus* S845A + AKAP79: 31.4 ± 7.0%, $n = 6$, $p < 0.05$; Fig. 4*B*). Thus, AKAP79 selectively facilitates PKC regulation of GluR1 currents at the CaMKII/PKC site.

Previous studies indicated that SAP97 serves to link AKAP79 to GluR1 (21). The C-terminal PDZ ligand of GluR1 interacts with SAP97 (32), which in turn binds to AKAP79 (21). SAP97 is endogenously expressed within HEK293 cells, giving rise to a GluR1/SAP97/AKAP79 complex upon expression of AKAP79 and GluR1 (22). Formation of this complex is necessary for effective regulation of GluR1 by AKAP79-anchored PKA and CaN (21, 22). In order to test whether regulation of GluR1 by AKAP79-anchored PKC also relied on this complex, the PDZ interaction between GluR1 and SAP97 was disrupted. GluR1 T887A (21, 22), a mutant form of GluR1 unable to bind to SAP97, was not effectively modulated by PKM in control or AKAP79-expressing cells (T887A: −8.4 ± 2.1%, $n = 5$ *versus* T887A + AKAP79: −6.9 ± 2.9%, $n = 6$; Fig. 4*C*). These data suggest that AKAP79 needs to be directed to GluR1 for effective PKC modulation of channel activity.

DISCUSSION

Confining signaling enzymes with appropriate physiological targets via scaffolds and/or anchoring proteins has long been proposed to improve the efficiency and speed of signal transduction (33). This is largely thought to occur by increasing the

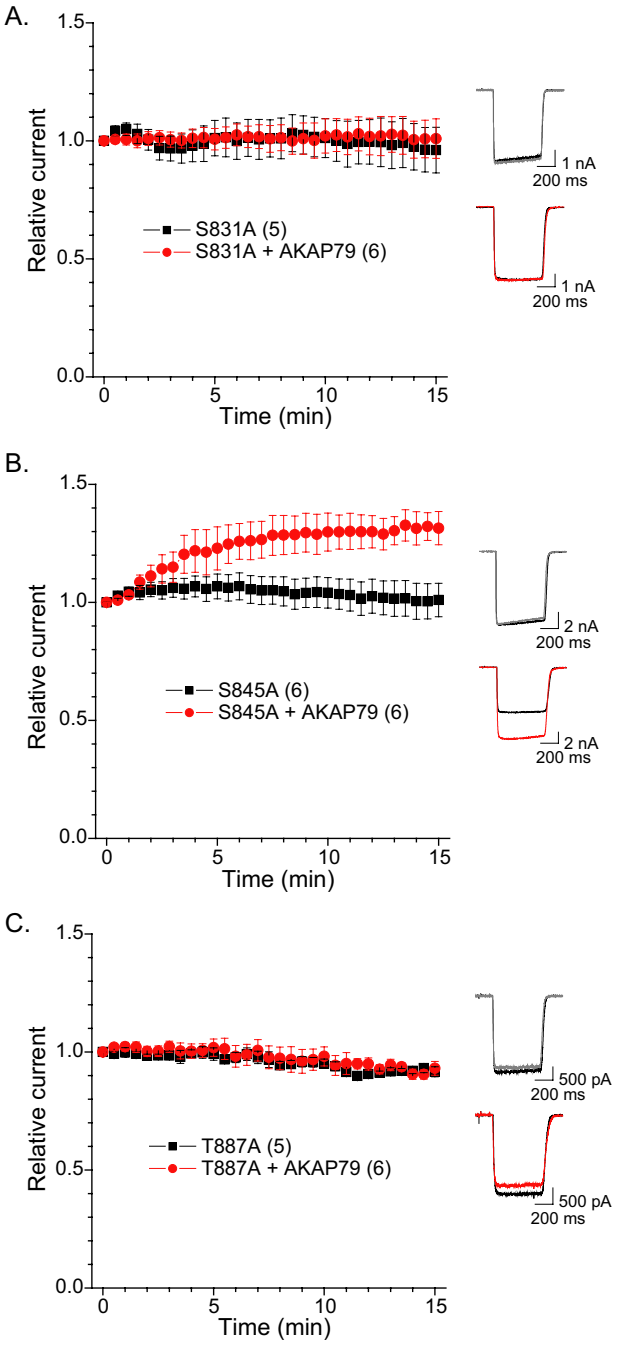

FIGURE 4. **Regulation of GluR1 by AKAP79-anchored PKC occurs at the CaMKII/PKC site and requires a PDZ-domain interaction at C-terminal of GluR1.** Time courses of whole cell recordings from HEK293 cells transfected with various GluR1 alanine mutants ± AKAP79 are shown. PKM (4 nM) was included in the patch pipette. Representative traces are shown in *side panels*, and the number of observations for each condition is indicated in *parentheses*. *A*, regulation of GluR1 by AKAP79-anchored PKC occurs at Ser-831 as an alanine mutant (S831A) of this site blocks the AKAP79-mediated enhancement of GluR1 receptor currents. *B*, regulation of GluR1 by AKAP79-anchored PKC is unaffected by mutation of the PKA site, Ser-845, to alanine (S845A). *C*, PDZ-domain interaction between GluR1 and endogenous SAP97 is required for enhancement of GluR1 currents by AKAP79-anchored PKC as disruption of the C-terminal PDZ ligand by mutation of Thr-887 to alanine (T887A) prevented the enhancement of the current by PKM.

effective local concentration and/or orientation of signaling enzymes toward their respective targets. However, quantitative estimates of the degree to which the participants are concentrated have remained unclear. Here, based on biochemical measures of the rate of PKC phosphorylation of GluR1 the apparent PKC activity near the receptor is estimated to be increased by ~4-fold due to interaction with AKAP79 (Fig. 2*A*). Parallel electrophysiological assessment of the sensitivity of GluR1 to varying concentrations of PKM suggest that AKAP79 may increase the local apparent activity of the enzyme by ~20-fold (Fig. 3*D*). This may be sufficient to overcome the limited abundance of PKC relative to CaMKII that is thought to exist within neurons and may provide one explanation as to why PKC appears more potent than CaMKII at augmenting AMPA receptor-mediated synaptic transmission.

Previous *in vitro* biochemical measures indicated that AKAP79 partially suppresses PKC activity toward a prototypical PKC substrate (26, 27). In contrast, the *in situ* studies described here demonstrate that within cellular contexts, AKAP79 is facilitative toward a physiologically relevant substrate. This finding is similar in many regards to the analogous situation described for CaN association with AKAP79 and regulation of GluR1 (22, 23). These differences between *in vitro* and *in situ* analysis presumably reflect not only the different experimental environments but also the specific orientation of PKC toward Ser-831 provided by a GluR1/SAP97/AKAP79/PKC complex. Previous studies have collectively provided clear evidence for the existence of each of these individual interactions (20, 21, 26, 27) and are further supported functionally by the cellular data provided here. Whether such a complex actually alters enzymatic parameters such as the V_{max} and K_m of PKC for Ser-831 within a cellular context is presently unknown. Changes in either or both of these parameters could suffice to explain the data presented here. In the absence of this type of data, the most parsimonious description of the ability of AKAP79 to both accelerate PKC-mediated phosphorylation of GluR1 at Ser-831 and to shift the concentration dependence of PKC modulation of GluR1 currents contingent on its ability to bind PKC is that AKAP79 locally concentrates PKC activity.

It is important to emphasize that the electrophysiologically based estimates provided above are limited to the apparent PKM concentration at the receptor relative to the bulk cytoplasmic concentration, which is assumed to be dictated by the level of PKM supplied via the pipette solution. Because PKM lacks membrane association domains this provides a more direct estimate of the ability of AKAP79 to target PKC to GluR1. In contrast, endogenous PKC is endowed with a mechanism to increase its local concentration near potential targets, such as GluR1, because of the enzyme ability to associate with the plasma membrane upon activation (34). Thus membrane association of PKC may limit the relative extent by which AKAP79 locally enhances PKC activity. Indeed, AKAP79 accelerates GluR1 phosphorylation by endogenous PKC by a smaller, yet still substantial ~4-fold factor (Fig. 2). These data are consistent with the ability of AKAP79 to localize, orient, and/or retain PKC near an appropriate substrate and enhance the likelihood of successful signal transduction. Thus, AKAP79 provides an additional mechanism to elevate PKC activity near substrates. Whether PKC is recruited to AKAP79 directly from a cytosolic pool upon activation or requires an initial translocation to the membrane followed by subsequent sequestering

within an AKAP79-organized nanodomain remains unclear. However, previous biochemical studies have indicated that PKC associates with AKAP79 in an activation state-independent manner (26) suggesting that both routes are possible.

Similar to the regulation of GluR1 described here, AKAP79/150-anchored PKC has also been demonstrated to contribute to effective receptor-mediated closure of native M-current and recombinant KCNQ2/KCNQ3 channels (35). For M-current, disruption of AKAP79/150-mediated PKC anchoring shifts the concentration-response curve for muscarinic agonists rightward 3–4 fold (35). Interestingly, this shift is in line with the lower level estimates by which AKAP79 elevates PKC activity near GluR1. However, a recent study suggests that the interaction between AKAP79 and PKC is dispensable for regulation of GluR1 receptor currents (36). This discrepancy, with the data presented here, likely stems from the manner in which GluR1 regulation was examined. Specifically, only the contribution of AKAP79-anchored PKC toward the rundown of GluR1 receptor currents upon PKA inhibition had been examined (36). However, earlier work indicated that Ser-831 on GluR1 is not involved in CaN-mediated down-regulation of GluR1 receptor currents (22). Instead, this form of regulation relies on the PKA phosphorylation state of the receptor at Ser-845 (22). Thus, disabling AKAP79-mediated PKC anchoring would not be expected to affect this mode of regulation. Therefore, the data presented here expands the number of AKAP79 signaling components that have been demonstrated to regulate a single target protein.

Recently, it has been proposed that AKAP79 may use distinct subsets of its signaling components to regulate different target proteins as a mechanism to promote signaling diversity (36). Yet, based on simple permutations, the number of signaling outcomes dictated via a scaffold (such as AKAP79) toward an associated target would be expected to be maximized by equipping the scaffold with all its signaling components. However, competition among signaling components for binding sites on AKAP79 exists (21, 27), suggesting that AKAP79 can exist in states in which only subsets of components are associated. This would be expected to limit the number of potential regulatory events that can be transduced via the complex at any one time. Subsequent realignment of the composition of the complex may then set into motion another set of specific signaling events. In fact, a recent study supports the idea that the localization and composition of the AKAP79 signaling complex is activity-dependent (37). This could allow multivalent signaling complexes, like AKAP79, to coordinate specific signaling sequences and/or enable state-dependent contingencies in signaling. This degree of orchestration may be especially important for AKAP79/150-regulated substrates like GluR1, whose phosphorylation state during bidirectional plasticity appears to be exquisitely controlled based on recent synaptic history (12).

Similar to AKAP79-mediated targeting of PKC to GluR1, synaptic translocation and targeting of CaMKII to NMDA receptors has also been proposed as a mechanism to place CaMKII near GluR1 to facilitate phosphorylation of Ser-831 (38). Indeed, within recombinant systems targeting of CaMKII to the plasma membrane via interaction with the NMDA NR2B subunit appears sufficient to enhance Ser-831 phosphorylation (39). At present, the relative degree to which each of these kinase targeting strategies control GluR1 phosphorylation at Ser-831 and the conditions which would favor one over the other is unclear. However, as AKAP79 targets both PKA and PKC to GluR1, allowing for phosphorylation of Ser-845 and Ser-831, respectively, as well as CaN-mediated dephosphorylation of Ser-845, the AKAP79 signaling complex may define the most proximal physiologically relevant pathways controlling GluR1 phosphorylation and dephosphorylation at these sites.

CaMKII phosphorylation of GluR1 at Ser-831 increases the single channel conductance of homomeric GluR1 receptors (17), consistent with the increased conductance that is observed for a subset of potentiated synapses (19, 40, 41). Given that Ser-831 is also a target for PKC it might be expected that PKC would also lead to similar changes. However, an increase in AMPA receptor number appears to be the predominant change following postsynaptic infusion of the constitutively active PKC isoform PKMζ. One possibility for this difference between kinases is the presence of additional regulatory phosphorylation sites on GluR1. In fact, two additional PKC sites have been detected in GluR1, with at least one which appears important for synaptic incorporation of GluR1 during LTP (42, 43). However, it is unlikely that these sites are sufficient to account for the regulation described here, as mutation of Ser-831 to alanine was sufficient to ablate the AKAP79-anchored PKC-induced increase in GluR1 receptor currents Whether AKAP79 facilitates PKC phosphorylation at these alternative PKC sites remains to be determined. Alternatively, the contribution of these kinases to LTP and/or the mechanism by which they augment potentiation may be developmentally regulated (7, 44).

The majority of hippocampal AMPA receptors are thought to be heteromers of GluR1 and GluR2 (45). Given that Ser-831 phosphorylation by CaMKII does not cause an increase in currents when examined in the context of heteromeric GluR1/GluR2 AMPA receptors (18), it is quite plausible that AKAP79/150-anchored PKC is focused to regulate homomeric GluR1 AMPA receptors. Although, a role for GluR2-lacking AMPA receptors during early LTP has been proposed (46), this idea has not been supported by some studies (47, 48). However, a recent study suggests that distinct developmental periods and the intensity of LTP-inducing protocols are critical determinants as to whether GluR2-lacking AMPA receptors contribute to the early expression of LTP (49). Moreover, these developmental switches in the contribution of GluR2-lacking AMPA receptors to LTP are also accompanied by similar requirements for AKAP150 associated-PKA signaling to GluR1 during LTP (49). Thus, it is possible that AKAP150-anchored PKC may participate in a similar manner.

Although this study has focused on the regulation of the CaMKII/PKC site on GluR1 by AKAP79, it is worth noting that the GluR2 is also a PKC substrate (Ser-880) (50). In contrast to postsynaptic enhancement of AMPA receptor activity associated with PKC activation in hippocampal neurons (4, 51, 52) and of GluR1 described here, phosphorylation of GluR2 at Ser-880 site is associated with endocytosis of GluR2 receptors and down-regulation of synaptic function (53). Given the opposing direction by which these sites appear to operate, it is tempting to speculate that the net result would be the selective enhancement of GluR2-lack-

ing AMPA receptor-mediated transmission. Indeed, recent studies have shown that some forms of synaptic plasticity are associated with an increase in the presence and/or activity of Ca^{2+}-permeable, GluR2-lacking AMPA receptors, which are presumably GluR1 homomers (46, 54–57). Interestingly, GluR2, like GluR1, appears to have its own dedicated pool of PKC targeted to it via the synaptic scaffold protein interacting with protein kinase C 1 (PICK1) (50). Whether AKAP79 and PICK1 work in a complementary fashion to control AMPA receptor composition is unclear. Therefore, future studies will need to determine how the combined activity of PKC at GluR1 and GluR2 contributes to the modulation of heteromeric receptors and how their respective PKC-affiliated scaffolds influence this regulation particularly during synaptic plasticity.

Acknowledgments—I thank Tom Soderling and Michael Kapiloff for providing plasmids used in this study. I also thank Alex Dopico, Angela Cantrell, and Ian Brooks for helpful comments.

REFERENCES

1. Kennedy, M. B., Bennett, M. K., and Erondu, N. E. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 7357–7361
2. Peng, J., Kim, M. J., Cheng, D., Duong, D. M., Gygi, S. P., and Sheng, M. (2004) *J. Biol. Chem.* **279,** 21003–21011
3. Lledo, P. M., Hjelmstad, G. O., Mukherji, S., Soderling, T. R., Malenka, R. C., and Nicoll, R. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11175–11179
4. Ling, D. S., Benardo, L. S., Serrano, P. A., Blace, N., Kelly, M. T., Crary, J. F., and Sacktor, T. C. (2002) *Nat. Neurosci.* **5,** 295–296
5. Malinow, R., Schulman, H., and Tsien, R. W. (1989) *Science* **245,** 862–866
6. Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., and Waxham, M. N. (1989) *Nature* **340,** 554–557
7. Wikstrom, M. A., Matthews, P., Roberts, D., Collingridge, G. L., and Bortolotto, Z. A. (2003) *Neuropharmacology* **45,** 828–836
8. Malenka, R. C., and Bear, M. F. (2004) *Neuron* **44,** 5–21
9. Barria, A., Derkach, V., and Soderling, T. (1997) *J. Biol. Chem.* **272,** 32727–32730
10. Barria, A., Muller, D., Derkach, V., Griffith, L. C., and Soderling, T. R. (1997) *Science* **276,** 2042–2045
11. Mammen, A. L., Kameyama, K., Roche, K. W., and Huganir, R. L. (1997) *J. Biol. Chem.* **272,** 32528–32533
12. Lee, H. K., Barbarosie, M., Kameyama, K., Bear, M. F., and Huganir, R. L. (2000) *Nature* **405,** 955–959
13. Lee, H. K., Takamiya, K., Han, J. S., Man, H., Kim, C. H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R. S., Wenthold, R. J., Gallagher, M., and Huganir, R. L. (2003) *Cell* **112,** 631–643
14. Whitlock, J. R., Heynen, A. J., Shuler, M. G., and Bear, M. F. (2006) *Science* **313,** 1093–1097
15. Shukla, K., Kim, J., Blundell, J., and Powell, C. M. (2007) *J. Biol. Chem.* **282,** 18100–18107
16. Hu, X. D., Huang, Q., Yang, X., and Xia, H. (2007) *J. Neurosci.* **27,** 4674–4686
17. Derkach, V., Barria, A., and Soderling, T. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3269–3274
18. Oh, M. C., and Derkach, V. A. (2005) *Nat. Neurosci.* **8,** 853–854
19. Benke, T. A., Luthi, A., Isaac, J. T., and Collingridge, G. L. (1998) *Nature* **393,** 793–797
20. Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996) *Science* **271,** 1589–1592
21. Colledge, M., Dean, R. A., Scott, G. K., Langeberg, L. K., Huganir, R. L., and Scott, J. D. (2000) *Neuron* **27,** 107–119
22. Tavalin, S. J., Colledge, M., Hell, J. W., Langeberg, L. K., Huganir, R. L., and Scott, J. D. (2002) *J. Neurosci.* **22,** 3044–3051
23. Dell'Acqua, M. L., Dodge, K. L., Tavalin, S. J., and Scott, J. D. (2002) *J. Biol. Chem.* **277,** 48796–48802
24. Dell'Acqua, M. L., Faux, M. C., Thorburn, J., Thorburn, A., and Scott, J. D. (1998) *EMBO J.* **17,** 2246–2260
25. Tsui, J., and Malenka, R. C. (2006) *J. Biol. Chem.* **281,** 13794–13804
26. Faux, M. C., Rollins, E. N., Edwards, A. S., Langeberg, L. K., Newton, A. C., and Scott, J. D. (1999) *Biochem. J.* **343,** 443–452
27. Faux, M. C., and Scott, J. D. (1997) *J. Biol. Chem.* **272,** 17038–17044
28. Gomez, L. L., Alam, S., Smith, K. E., Horne, E., and Dell'Acqua, M. L. (2002) *J. Neurosci.* **22,** 7027–7044
29. Gorski, J. A., Gomez, L. L., Scott, J. D., and Dell'Acqua, M. L. (2005) *Mol. Biol. Cell* **16,** 3574–3590
30. Hanson, P. I., Kapiloff, M. S., Lou, L. L., Rosenfeld, M. G., and Schulman, H. (1989) *Neuron* **3,** 59–70
31. Oh, M. C., Derkach, V. A., Guire, E. S., and Soderling, T. R. (2006) *J. Biol. Chem.* **281,** 752–758
32. Leonard, A. S., Davare, M. A., Horne, M. C., Garner, C. C., and Hell, J. W. (1998) *J. Biol. Chem.* **273,** 19518–19524
33. Wong, W., and Scott, J. D. (2004) *Nat. Rev. Mol. Cell. Biol.* **5,** 959–970
34. Newton, A. C. (2003) *Biochem. J.* **370,** 361–371
35. Hoshi, N., Zhang, J. S., Omaki, M., Takeuchi, T., Yokoyama, S., Wanaverbecq, N., Langeberg, L. K., Yoneda, Y., Scott, J. D., Brown, D. A., and Higashida, H. (2003) *Nat. Neurosci.* **6,** 564–571
36. Hoshi, N., Langeberg, L. K., and Scott, J. D. (2005) *Nat. Cell Biol.* **7,** 1066–1073
37. Smith, K. E., Gibson, E. S., and Dell'Acqua, M. L. (2006) *J. Neurosci.* **26,** 2391–2402
38. Leonard, A. S., Lim, I. A., Hemsworth, D. E., Horne, M. C., and Hell, J. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3239–3244
39. Tsui, J., Inagaki, M., and Schulman, H. (2005) *J. Biol. Chem.* **280,** 9210–9216
40. Luthi, A., Wikstrom, M. A., Palmer, M. J., Matthews, P., Benke, T. A., Isaac, J. T., and Collingridge, G. L. (2004) *BMC Neurosci.* **5,** 44
41. Poncer, J. C., Esteban, J. A., and Malinow, R. (2002) *J. Neurosci.* **22,** 4406–4411
42. Boehm, J., Kang, M. G., Johnson, R. C., Esteban, J., Huganir, R. L., and Malinow, R. (2006) *Neuron* **51,** 213–225
43. Lee, H. K., Takamiya, K., Kameyama, K., He, K., Yu, S., Rossetti, L., Wilen, D., and Huganir, R. L. (2007) *Mol. Cell Neurosci.* **36,** 86–94
44. Palmer, M. J., Isaac, J. T., and Collingridge, G. L. (2004) *J. Neurosci.* **24,** 4903–4911
45. Wenthold, R. J., Petralia, R. S., Blahos, J., II, and Niedzielski, A. S. (1996) *J. Neurosci.* **16,** 1982–1989
46. Plant, K., Pelkey, K. A., Bortolotto, Z. A., Morita, D., Terashima, A., McBain, C. J., Collingridge, G. L., and Isaac, J. T. (2006) *Nat. Neurosci.* **9,** 602–604
47. Adesnik, H., and Nicoll, R. A. (2007) *J. Neurosci.* **27,** 4598–4602
48. Gray, E. E., Fink, A. E., Sarinana, J., Vissel, B., and O'Dell T, J. (2007) *J. Neurophysiol.* **98,** 2488–2492
49. Lu, Y., Allen, M., Halt, A. R., Weisenhaus, M., Dallapiazza, R. F., Hall, D. D., Usachev, Y. M., McKnight, G. S., and Hell, J. W. (2007) *EMBO J.*
50. Chung, H. J., Xia, J., Scannevin, R. H., Zhang, X., and Huganir, R. L. (2000) *J. Neurosci.* **20,** 7258–7267
51. Wang, L. Y., Dudek, E. M., Browning, M. D., and MacDonald, J. F. (1994) *J. Physiol.* **475,** 431–437
52. Carroll, R. C., Nicoll, R. A., and Malenka, R. C. (1998) *J. Neurophysiol.* **80,** 2797–2800
53. Seidenman, K. J., Steinberg, J. P., Huganir, R., and Malinow, R. (2003) *J. Neurosci.* **23,** 9220–9228
54. Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T. J., Adams, S. R., Garner, C. C., Tsien, R. Y., Ellisman, M. H., and Malenka, R. C. (2004) *Nat. Neurosci.* **7,** 244–253
55. Thiagarajan, T. C., Lindskog, M., and Tsien, R. W. (2005) *Neuron* **47,** 725–737
56. Sutton, M. A., Ito, H. T., Cressy, P., Kempf, C., Woo, J. C., and Schuman, E. M. (2006) *Cell* **125,** 785–799
57. Caldeira, M. V., Melo, C. V., Pereira, D. B., Carvalho, R., Correia, S. S., Backos, D. S., Carvalho, A. L., Esteban, J. A., and Duarte, C. B. (2007) *J. Biol. Chem.* **282,** 12619–12628