Cellular/Molecular

Phospholipase C Is Required for Changes in Postsynaptic Structure and Function Associated with NMDA Receptor-Dependent Long-Term Depression

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NMDA receptor (NMDAR)-dependent hippocampal synaptic plasticity underlying learning and memory coordinately regulates dendritic spine structure and AMPA receptor (AMPAR) postsynaptic strength through poorly understood mechanisms. Induction of long-term depression (LTD) activates protein phosphatase 2B/calcineurin (CaN), leading to dendritic spine shrinkage through actin depolymerization and AMPAR depression through receptor dephosphorylation and internalization. The scaffold proteins A-kinase-anchoring protein 79/150 (AKAP79/150) and postsynaptic density 95 (PSD95) form a complex that controls the opposing actions of the cAMP-dependent protein kinase (PKA) and CaN in regulation of AMPAR phosphorylation. The AKAP79/150–PSD95 complex is disrupted in hippocampal neurons during LTD coincident with internalization of AMPARs, decreases in PSD95 levels, and loss of AKAP79/150 and PKA from spines. AKAP79/150 is targeted to spines through binding F-actin and the phospholipid phosphatidylinositol-(4,5)-bisphosphate (PIP_2). Previous electrophysiological studies have demonstrated that inhibition of phospholipase C (PLC)-catalyzed hydrolysis of PIP_2 inhibits NMDAR-dependent LTD; however, the signaling mechanisms that link PLC activation to alterations in dendritic spine structure and AMPAR function in LTD are unknown. We show here that NMDAR stimulation of PLC in cultured hippocampal neurons is necessary for AKAP79/150 loss from spines and depolymerization of spine actin. Importantly, we demonstrate that NMDAR activation of PLC is also necessary for decreases in spine PSD95 levels and AMPAR internalization. Thus, PLC signaling is required for structural and functional changes in spine actin, PSD scaffolding, and AMPAR trafficking underlying postsynaptic expression of LTD.

Key words: **phospholipase C; LTD; actin; phosphatidylinositol-(4,5)-bisphosphate; A kinase-anchoring protein; PSD95; AMPA receptor**

Introduction

Most CNS excitatory synapses are located on small membrane protrusions called dendritic spines, in which a dynamic F-actin matrix is linked to postsynaptic density (PSD) scaffold proteins, signaling proteins, NMDA, and AMPA receptors (AMPARs) (Kim and Sheng, 2004; Tada and Sheng, 2006). Synaptic plasticity is characterized by coordinated regulation of spine actin, PSD structure, and AMPAR function in response to NMDA receptor (NMDAR) Ca^{2+} signals that activate kinases and phosphatases (Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002; Malenka and Bear, 2004). Long-term potentiation (LTP) is associated with spine formation and enlargement driven by actin polymerization, whereas long-term depression (LTD) causes actin depolymerization, spine shrinkage, and elimination (Muller et al., 2000; Fukazawa et al., 2003; Matsuzaki et al., 2004; Nagerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004). Numerous studies have shown that Ca^{2+}–calmodulin-activated protein kinase II and calcineurin (CaN) are upstream regulators of spine actin and AMPARs during LTP and LTD, respectively (Mulkey et al., 1994; Halpain et al., 1998; Beattie et al., 2000; Lee et al., 2000; Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004). However, little is known about other signaling molecules coordinating these plasticity mechanisms.

One PSD scaffold that may be central to LTD is A-kinase-anchoring protein 79/150 (AKAP79/150). AKAP79/150 binds cAMP-dependent protein kinase (PKA) (Carr et al., 1992) and CaN (Coghlan et al., 1995; Dell'Acqua et al., 2002) and is linked to AMPA and NMDARs through PSD95 scaffolds (Colledge et al., 2000). Activation of CaN in LTD results in dephosphorylation of PKA-phosphorylated Ser845 on AMPAR glutamate receptor 1 (GluR1) subunits (Lee et al., 1998, 2000, 2003; Ehlers, 2000) and AMPAR removal from synapses by endocytosis (Carroll et al., 1999a,b; Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000). Anchoring of PKA and CaN to AKAP79/150 is implicated in regulation of GluR1 Ser845 phosphorylation, AMPAR currents, and AMPAR internalization (Rosenmund et al., 1994; Colledge et al., 2000; Tavalin et al., 2002; Hoshi et al., 2005; Snyder et al., 2005). Importantly, NMDAR activation in LTD disrupts PSD95 binding

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and causes loss of AKAP79/150–PKA complexes from spines. This removal of AKAP–PKA complexes involves CaN activation and actin depolymerization and may prevent GluR1 rephosphorylation to promote a long-lasting reduction in AMPAR surface expression (Gomez et al., 2002; Snyder et al., 2005; Smith et al., 2006).

AKAP79/150 spine localization requires an N-terminal targeting domain that binds phosphatidylinositol-(4,5)-bisphosphate (PIP_2), F-actin, and cadherin adhesion molecules (Dell'Acqua et al., 1998; Gomez et al., 2002; Gorski et al., 2005). Interestingly, electrophysiological studies have shown that postsynaptic inhibition of phospholipase C (PLC), which hydrolyzes PIP_2 to diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP_3), prevents induction of NMDAR-dependent LTD in hippocampus and visual cortex (Reyes-Harde and Stanton, 1998; Choi et al., 2005; Lee et al., 2005). However, signaling mechanisms downstream of PLC in NMDAR-LTD are poorly understood. Importantly, we show here that NMDAR activation associated with LTD induction in hippocampal neurons stimulates PLC hydrolysis of PIP_2, which is required for AKAP79/150 translocation, spine actin depolymerization, spine PSD95 decreases, and AMPAR internalization.

Materials and Methods

Mammalian cDNA expression vectors. The pEC/YFPN1 (Clontech, Palo Alto, CA) vectors encoding C-terminal cyan/yellow fluorescent protein (C/YFP) fusions of AKAP79WT, PLCδPH, and PSD95 were described previously (Gomez et al., 2002; Gorski et al., 2005). pEYFP–actin was purchased from Clontech.

Primary culture and transfection of rat hippocampal neurons. Neurons were cultured as described previously (Gomez et al., 2002; Smith et al., 2006). Briefly, the hippocampus was dissected from postnatal day 0 (P0) to P2 Sprague Dawley rats and dissociated by papain. Neurons were plated at low density (40,000 cells/ml) on glass coverslips coated with poly-D-lysine and laminin (Discovery Labware; BD Biosciences, Mountain View, CA), fed with glia-conditioned media, and used for immunostaining of endogenous proteins at 17–21 d *in vitro* (div). Neurons were transfected with C/YFP-tagged constructs at 0 div by Amaxa (Gaithersburg, MD) electroporation (Gorski et al., 2005; Smith et al., 2006). Transfected neurons were plated on poly-D-lysine/laminin-coated 35 mm glass-bottom dishes at medium density (300,000 cells/ml) and imaged live at 14–16 div.

Pharmacological treatments of cultured hippocampal neurons. Untransfected and transfected neurons were maintained in growth media during drug treatments. The various treatments are described in detail in Results and in the figure legends. Reagents were obtained from the following sources: NMDA, APV, wortmannin α-methyl-4-carboxyphenylglycine (MCPG), and 3,4-dihydroxyphenylglycol (DHPG) were from Tocris Bioscience (Saint Louis, MO); bisindolylmaleimide I (BIM), 1-[6-([17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-2,5-pyrrolidinedione (U73343), 1-[6-([17β-methoxyestra-1,3,5(10)-trien-17-yl]amino) hexyl]-1*H*-pyrrole-2,5-dione (U73122), xestospongin D, calphostin C (CalC), edelfosine (EMD Biosciences, La Jolla, CA). Other general chemicals and reagents were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Houston, TX).

Immunocytochemistry and AMPAR surface staining of low-density cultured hippocampal neurons. Low-density neuron cultures were labeled with antibodies as described previously (Smith et al., 2006). Briefly, after drug treatments, neurons were washed in PBS before fixing in 3.7% formaldehyde/PBS and permeabilized in 0.2% Triton X-100/PBS. Cells were blocked in PBS/10% BSA overnight at 4°C. Primary antibodies (1:1000 rabbit anti-AKAP150; 1:200 mouse anti-PSD95; Affinity BioReagents, Golden, CO) were incubated for 2 h at room temperature, followed by washing in PBS and incubation in fluorescent secondary antibody goat anti-mouse- or goat anti-rabbit Alexa 488 or Alexa 647 and Texas Red–phalloidin (Invitrogen, Eugene, OR). Coverslips were washed in PBS and water before mounting with ProLong (Invitrogen). Surface GluR2 subunits were labeled by live antibody feeding after drug treatments as described by Sossa et al. (2006). Briefly, after drug treatments, ice-cold growth media with antibodies that recognize the N-terminal domain of GluR2 (1:100; Millipore, Billerica, MA) were added for 30 min at 4°C. Neurons were then washed with cold PBS, fixed, blocked, and stained with fluorescent secondary antibodies.

Digital deconvolution fluorescence microscopy. Live-cell imaging of transfected neurons was performed on a Zeiss (Oberkochen, Germany) Axiovert 200M with 100× plan-apo/1.4 numerical aperture (NA) objective, 175 W xenon illumination, Coolsnap CCD camera, and Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO) as described by Gorski et al. (2005) and Smith et al. (2006). Live cells were maintained at 33°C and ~5% CO_2 during imaging. Immunocytochemical indirect fluorescence was detected using a Nikon (Tokyo, Japan) TE-300 inverted microscope (100× plan-apo; oil; 1.4 NA) equipped with Sensicam CCD camera and Slidebook 4.0 software. As described previously by Smith et al. (2006), three-dimensional *z*-stacks of *x–y* planes with 0.5 μm steps were collected and deconvolved to the nearest neighbor to generate confocal *x–y* sections. Two-dimensional maximum-intensity projection images were then generated to better represent a complete picture of dendrites and spines in quantitative mask analysis (see below). Images were exported from Slidebook as TIFF files and assembled using Adobe Photoshop 7.

Quantification of fluorescence images. Quantification of fluorescent images was performed in Slidebook 4.0 as described previously (Gomez et al., 2002; Smith et al., 2006) with minor changes. Each fluorescence channel was gated to produce puncta (or cluster) masks (2 × mean). Dendrite masks were obtained by hand drawing a mask around the outside of the dendrite and using the NOT function to overlay the dendrite and then combined using the OR function with all puncta masks. The dendrite mask and individual channel puncta masks were then combined with the AND function to obtain dendrite puncta masks for each channel from which additional colocalization masks were obtained by pairwise combinations using the AND function. AKAP150/PSD95 and actin/PSD95 colocalization measurements were obtained from these masks as described previously (Gomez et al., 2002). AKAP150/F-actin correlation values (*r*; $r = 1$ indicates perfect colocalization, 0 indicates random localization, and -1 indicates no overlap) were obtained as described (Smith et al., 2006). Mean fluorescence surface staining of GluR2 was measured from whole-dendrite masks. For time-lapse images, masks for shafts and spines were drawn by hand only for spines remaining in focus throughout imaging. Mean fluorescence of the spine and shaft masks was measured for each time point at 5 min intervals. A background mask was drawn outside the neuron, and this mean background fluorescence was subtracted from spine and shaft mask values before calculation of spine/shaft ratios. For most figures, experimental measurements were normalized to values obtained for appropriate control untreated neurons. However, for Figure 1, line scans of mean fluorescence intensity were quantified in Slidebook 4.0 by using the ruler tool to draw a line through dendritic spine heads, the plasma membrane of the dendrite shaft, and the cytosol of the dendrite shaft. The mean fluorescence intensities (in arbitrary fluorescence units) for each pixel along this line scan were then exported and graphed using Prism (GraphPad Software, San Diego, CA).

Statistical analysis. Analysis of data was performed in Prism using two-way repeated-measures or one-way ANOVA followed by Tukey's *post hoc* analysis for group data as indicated in figure legends. Pairwise analysis was performed using one-tailed Student's *t* tests where indicated. Data are expressed as mean ± SEM in all figures.

Results

NMDA receptor activation leads to a rapid decrease in PIP_2 before AKAP79 loss from spines

Activation of NMDARs or group I metabotropic glutamate receptors (mGluRs) by agonists or low-frequency stimulation (LFS) can induce forms of LTD in the hippocampus and other brain regions that involve AMPAR receptor internalization and can involve either entirely distinct or partially overlapping downstream signaling pathways depending on the neuron type and

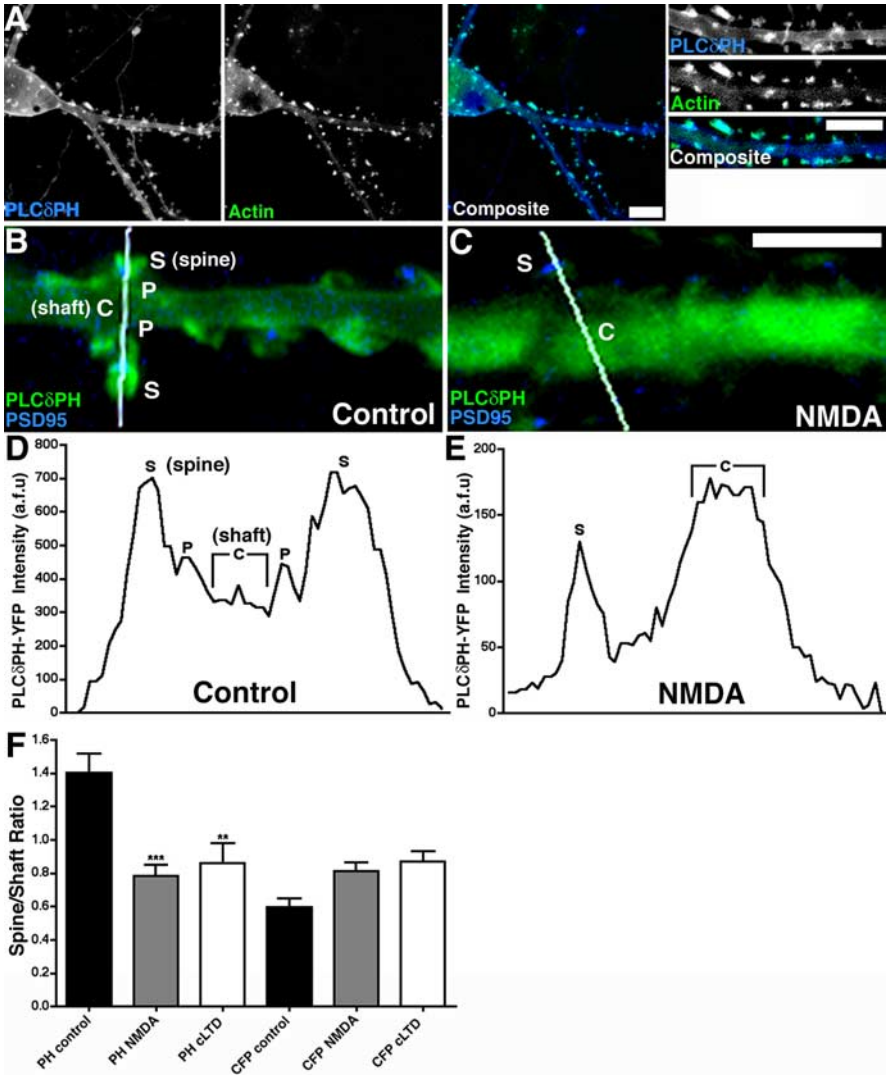

Figure 1. PIP_2 is enriched in dendritic spines and decreases in response to NMDA receptor activation. ***A***, The PIP_2 marker PLCδPH–CFP colocalizes with actin–YFP in dendritic spines of hippocampal neurons. ***B***, Line-scan analysis (see ***D***) of enlarged images of dendrites from neurons cotransfected with PLCδPH–YFP and PSD95–CFP shows an enrichment of PLCδPH–YFP in spine (S) heads and the plasma membrane (P) of the dendritic shaft over the interior cytoplasm (C) of the dendritic shaft in untreated neurons. ***C***, Thirty minutes after 50 μM NMDA treatment, line-scan analysis (see ***E***) shows a shift of PLCδPH–YFP from dendritic spines to the cytoplasm of the dendritic shaft. ***D***, ***E***, Graph of PLCδPH–YFP mean intensity [arbitrary fluorescence units (a.f.u)] from dendritic line scans of the untreated neuron in ***B*** (***D***) and the 50 μM NMDA-treated neuron in ***C*** (***E***). ***F***, Graph of the ratio of spine head to shaft cytoplasm fluorescence (spine/shaft ratio) of neurons cotransfected with PLCδPH–YFP and CFP as a cytoplasmic filling agent show a significant decrease in the spine/shaft ratio of PLCδPH–YFP 30 min after 50 μM NMDA or cLTD treatment (control, 1.40 ± 0.12, $n = 12$; NMDA, 0.79 ± 0.06, $^{***}p < 0.001$, $n = 6$; cLTD, 0.86 ± 0.12, $^{**}p < 0.01$, $n = 5$), (see also Fig. 3) but no significant change in the spine/shaft ratio for CFP (control, 0.60 ± 0.05; $n = 12$; NMDA, 0.81 ± 0.05; $n = 6$; cLTD, 0.87 ± 0.06; $n = 5$; $p > 0.05$). Data were analyzed by one-way ANOVA with Tukey's *post hoc* test. Scale bars, 10 μm.

developmental age (Beattie et al., 2000; Carroll et al., 2001; Huber et al., 2001; Snyder et al., 2001; Malenka and Bear, 2004; Lee et al., 2005). Interestingly, agonist-induced mGluR-dependent LTD in hippocampal neurons does not require PLC signaling, despite the fact that these receptors are coupled to PLC activation through G_q proteins (Huang and Hsu, 2006). However, previous brain-slice electrophysiological studies have shown that induction of NMDAR-dependent LTD by LFS in both rat visual cortex and the CA1 region of the hippocampus is blocked by the PLC inhibitor U73122 (Reyes-Harde and Stanton, 1998; Choi et al., 2005; Lee et al., 2005). In visual cortex, the involvement of PLC in LFS-LTD actually depends on coactivation of a variety of G_q-coupled metabotropic receptors, including group I mGluRs (Choi et al., 2005); however, in the hippocampus of young rats when LFS-LTD is NMDAR dependent, it is almost entirely blocked by the PLC inhibitor U73122 (Reyes-Harde and Stanton, 1998; Lee et al., 2005) but only partially blocked by antagonism of mGluRs and other G_q-coupled receptors, suggesting a more direct role for NMDAR activation of PLC in LTD (Choi et al., 2005). In agreement with these findings, recent imaging studies of PKC activation in hippocampal neurons have shown that both mGluR and NMDAR activation can independently stimulate PLC, leading to PKC activation in dendrites (Codazzi et al., 2006).

Although NMDA receptors themselves are known to be both positively and negatively regulated by signaling pathways downstream of PLC, such as PKC, inhibition of PLC with U73122 on its own has no effect on NMDA receptor currents (Lei et al., 1999; Holohean and Hackman, 2004). Thus, U73122 inhibition of PLC is more likely preventing LTD induction by blocking signaling pathways downstream of NMDA receptors. One involvement of PLC is thought to be the mobilization of IP_3 receptor Ca^{2+} stores to generate retrograde messengers that lead to LTD of presynaptic glutamate release (Reyes-Harde et al., 1999a,b; Stanton et al., 2003). However, NMDAR-LTD is also expressed through postsynaptic mechanisms involving spine actin reorganization and AMPAR endocytosis (Carroll et al., 2001; Tada and Sheng, 2006). Although PIP_2 is also known to regulate membrane trafficking and actin cytoskeletal dynamics (Yin and Janmey, 2003; Downes et al., 2005), it has not been examined whether PLC is an important upstream regulator of postsynaptic NMDAR-LTD mechanisms that involve spine actin reorganization and PSD scaffolding interactions.

To further characterize the role of PIP_2 in dendritic spine regulation, cultured hippocampal neurons were transfected with C/YFP-tagged PLCδPH, a probe that binds specifically to PIP_2 (Tall et al., 2000), and either actin–YFP to mark dendritic spine heads (Fig. 1*A*) or PSD95–CFP to mark PSDs within spines (Fig. 1*B*), revealing prominent colocalization of these proteins in spine heads. Line-scan fluorescence intensity profiles for sections through dendritic spines and adjacent regions of dendrite shaft revealed the greatest enrichment of the PLCδPH–YFP probe in spine heads followed by the dendrite shaft plasma membrane with much lower levels present in the interior/cytoplasm of dendrite shafts (Fig. 1*B*,*D*). In contrast, after bath application of 50 μM NMDA for 30 min, PLCδPH–YFP spine localization was dramatically reduced, dendrite shaft plasma membrane localization was not detectable, and dendrite shaft cytoplasm localization was most prominent (Fig. 1*C*,*E*). Quantification of fluorescence mean intensity ratios for

spine heads versus dendrite shaft cytoplasm for multiple images confirmed enrichment of PLCδPH–YFP in spine heads in untreated neurons (spine/shaft ratio, 1.4 ± 0.12) and translocation of the probe into the shaft cytoplasm in response to NMDA treatment (spine/shaft ratio, 0.79 ± 0.06; $^{***}p < 0.001$) (Fig. 1*F*). As a control for any effects of NMDA-induced spine shrinkage on the amount of fluorescence present in spines, quantification of spine/shaft intensity ratios for cytoplasm-localized CFP showed slightly more distribution to the shaft cytoplasm than spine heads in untreated neurons (spine/shaft ratio, 0.60 ± 0.05) and no significant change in NMDA-treated neurons (spine/shaft ratio, 0.80 ± 0.05; $p > 0.05$) (Fig. 1*F*). This lack of change in CFP localization was in stark contrast to the large shift in spine/shaft distribution observed for the membrane-localized PLCδPH–YFP probe. Overall, these results indicate that PIP_2 is concentrated in the membrane of dendritic spines and that NMDAR activation strongly reduces this enrichment.

Consistent with AKAP79 direct binding to F-actin and PIP_2 *in vitro* (Dell'Acqua et al., 1998; Gomez et al., 2002), AKAP79–CFP showed prominent colocalization with actin–YFP (Fig. 2*A*) and PLCδPH–YFP in dendritic spines (Fig. 2*B*). In time-lapse imaging, bath application of 50 μM NMDA led to a rapid translocation of AKAP79–CFP from spines and dendrite shaft plasma membranes into the dendrite shaft cytosol (Fig. 2*C*), as shown in our previous studies (Gorski et al., 2005; Smith et al., 2006). In these AKAP79–CFP-expressing neurons, translocation of PLCδPH–YFP from membranes and spines was seen at $t = 5$ min (within 1 min of NMDA addition) and was complete at $t = 15$ min. In contrast, AKAP79–CFP translocation was not seen until $t = 10$ min (6 min after NMDA addition) and was not complete until $t = 20$ min, as quantified by normalized measurements of spine/shaft fluorescence ratios, which allow comparison to PLCδPH–YFP (Fig. 1*C*,*D*). These observations suggest that changes in spine PIP_2 levels in response to NMDAR activation precede loss of AKAP79 from spines and that these processes may mechanistically be related.

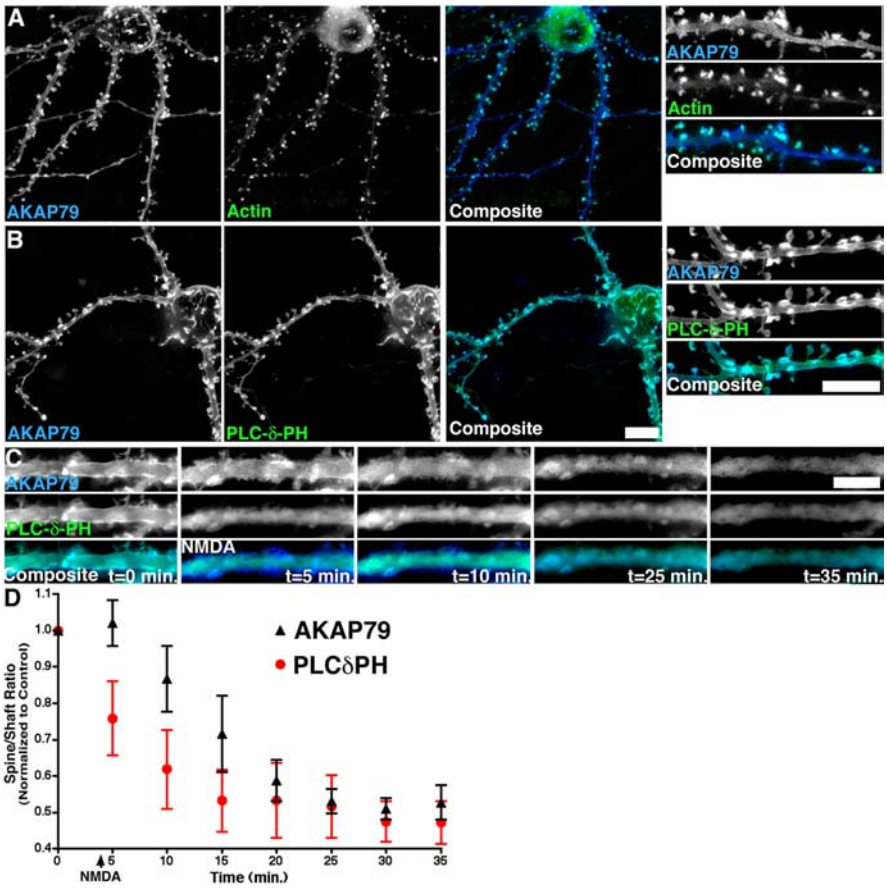

Figure 2. NMDA receptor activation leads to a rapid loss of PIP_2 preceding loss of AKAP79 from dendritic spines. ***A***, ***B***, AKAP79–CFP colocalizes with actin–YFP (***A***) and the PIP_2 probe PLCδPH–YFP (***B***) in dendritic spines of living hippocampal neurons. The small panels on the right show enlargements of dendrites. ***C***, Time-lapse images of dendrites from neurons transfected with PLCδPH–YFP and AKAP79–YFP taken from 0 to 35 min, as indicated, with NMDA (50 μM) addition 1 min before the $t = 5$ min image. ***D***, Quantification of PLCδPH and AKAP79 spine/shaft fluorescence ratios for the indicated times normalized to $t = 0$ min (before NMDA). Addition of NMDA (arrow; at $t = 4$ min) leads to a rapid PLCδPH spine/shaft ratio decrease seen at $t = 5$ min that reaches its maximum at $t = 15$ min ($n = 3$). AKAP79 spine/shaft ratio begins decreasing at $t = 10$ min and reaches its maximum by $t = 20$ min ($n = 3$). Decreases in spine/shaft ratios from $t = 0$–35 min are significant ($p < 0.0001$) for both PLCδPH and AKAP79 by two-way ANOVA with repeated measures. Scale bars, 10 μm.

NMDA chemically induced LTD leads to long-lasting decreases in actin, PIP_2, and AKAP79 localization to dendritic spines

To test whether brief applications of NMDA that have been shown to chemically induce LTD (cLTD; 30 μM NMDA for 3 min) in cultured neurons and hippocampal slices (Lee et al., 1998; Beattie et al., 2000; Lu et al., 2001) cause persistent decreases in AKAP79 and PIP_2 spine localization, as well as decreases in actin indicative of spine shrinkage and actin depolymerization, neurons were transfected with AKAP79–YFP, PLCδPH–YFP, or actin–YFP and PSD95–CFP (Fig. 3). cLTD-treated neurons imaged 30 min after stimulation revealed translocation of AKAP79 and PLCδPH–YFP from dendritic spines and membranes into the shaft cytosol when compared with control neurons (Fig. 3*A*,*B*). Likewise, actin–YFP fluorescence was found to be decreased in spines of cLTD-treated neurons relative to controls (Fig. 3*C*). In contrast, PSD95–CFP remained clustered in spines, thus allowing quantification of cLTD-induced changes in AKAP79–YFP, PLCδPH–YFP, and actin–YFP spine localization, as measured by significantly decreased fractional colocalization with PSD95–CFP for all three proteins (Fig. 3*D*). Similar to neurons treated with a stronger NMDA stimulus above, the translocation of PLCδPH–YFP from spines during cLTD resulted in a significant decrease in spine/shaft fluorescence ratio that was not observed for CFP alone (Fig. 1*F*).

Inhibition of PLC prevents decreases in PIP_2, actin, and AKAP79 localization to dendritic spines after NMDA receptor activation

The changes in PLCδPH spine localization that we observed with NMDAR activation are most likely caused by the known function of this probe as a reporter of PLC activity; PIP_2 hydrolysis by PLC leads to increases in cytosolic IP_3, which binds to the probe, fa-

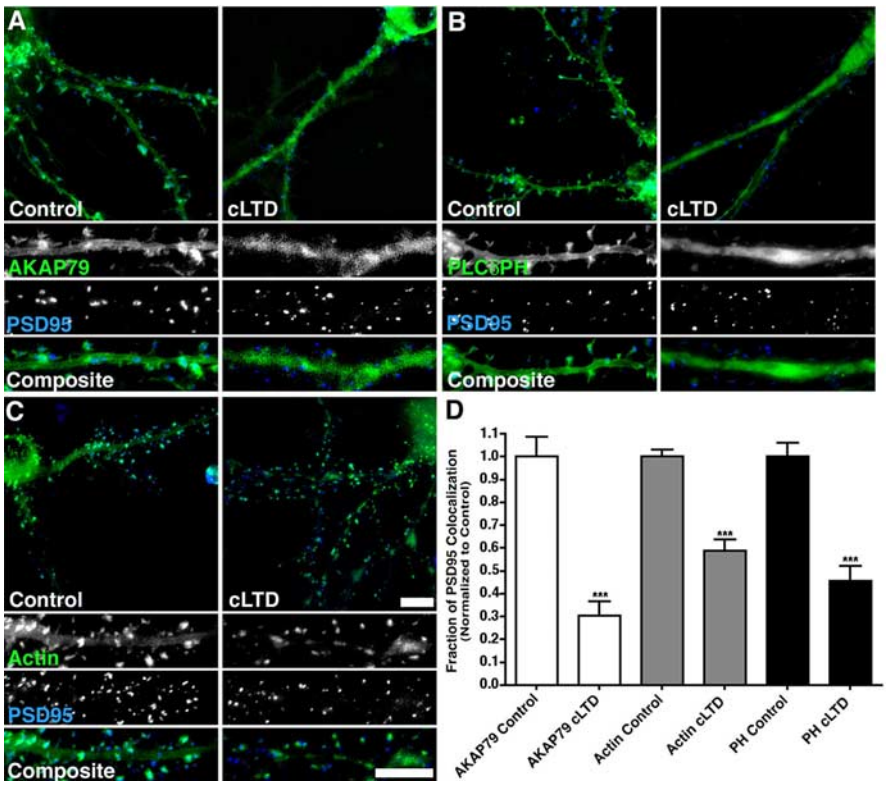

Figure 3. cLTD stimulation produces a long-lasting decrease in PIP_2, AKAP79–YFP, and actin–YFP localization to dendritic spines. ***A–C***, Dendritic spine localization of AKAP79–YFP (***A***), PLCδPH–YFP (***B***), and actin–YFP (***C***) with PSD95–CFP are all persistently decreased 30–45 min after cLTD stimulation (30 μM NMDA for 3 min) compared with untreated controls. The small panels show magnifications of dendrites. ***D***, Quantification of PSD95–CFP colocalization 30–45 min after cLTD normalized to untreated controls revealed a decrease in PSD95 colocalization of AKAP79–YFP (0.30 ± 0.06; $n = 8$), actin–YFP (0.59 ± 0.05; $n = 10$), and PLCδPH–YFP (0.46 ± 0.07; $n = 10$). ***$p < 0.0001$ by two-tailed Student's t test. Scale bars, 10 μm.

voring its loss from membranes (Delmas et al., 2004). To confirm NMDAR activation of PLC, edelfosine, an inhibitor of PLC, was added to neurons transfected with PLCδPH–YFP and PSD95–CFP (Fig. 4*A*). Edelfosine was used in these imaging experiments because addition of U73122 caused some level of PLCδPH–YFP displacement from membranes and spines, making it difficult to quantify additional changes with NMDA (data not shown). Edelfosine alone had no effect on PLCδPH–YFP dendritic localization but did prevent PLCδPH–YFP translocation from spines with addition of NMDA (Fig. 4*A*), as quantified by the level of fractional colocalization with PSD95–CFP (Fig. 4*B*). Hence, translocation of PLCδPH–YFP is reporting hydrolysis of PIP_2 by PLC downstream of NMDAR activation.

Because PIP_2 controls proteins involved in regulating actin dynamics, we examined whether loss of actin from spines after NMDAR activation required PLC. Accordingly, addition of PLC inhibitor U73122 alone had no effect on the amount of actin–YFP localized with PSD95–CFP in spines but prevented loss of actin–YFP seen with NMDA (Fig. 4*C*,*D*). Our previous studies showed that AKAP79/150 binds both F-actin and PIP_2 and that its NMDAR-stimulated translocation from spines is linked to actin reorganization (Gomez et al., 2002). Thus, we tested whether inhibition of PLC would also prevent translocation of AKAP79. Addition of U73122 alone had no effect on AKAP79–YFP colocalization with PSD95–CFP in spines compared with control (Fig. 4*E*,*F*). However, U73122 prevented NMDA-stimulated translocation of AKAP79–YFP away from PSD95–CFP (Fig. 4*E*,*F*). Thus, PLC hydrolysis of PIP_2 regulates both the decrease in actin–YFP spine fluorescence and translocation of AKAP79 from dendritic spines in response to NMDAR activation.

Inhibition of PLC prevents decreases in endogenous F-actin and AKAP150 localization to dendritic spines during cLTD

Imaging actin–YFP fluorescence in living neurons above indicates that there is a decrease in the total amount of actin in spines that most likely relates to spine shrinkage associated with LTD; however, from these findings alone, we cannot say that this change reflects a decrease in F-actin caused by depolymerization. To address this issue and confirm that changes in actin and AKAP79/150 spine localization are also seen for endogenous proteins, cLTD-stimulated neurons were fixed and stained with antibodies to label rat AKAP150 and PSD95 to mark PSDs and Texas Red–phalloidin to visualize F-actin (Fig. 5). In control neurons, AKAP150 was clustered and colocalized with both PSD95 and F-actin in spines (Figs. 5*A*,*C*,*E*). In response to cLTD (Fig. 5*B*), levels of AKAP150 and F-actin colocalization with each other (Fig. 5*C*) and with PSD95 (Fig. 5*D*,*E*), which remained more clustered on spines, were significantly reduced 15 min after stimulation. Addition of U73122 alone had no effect on spine localization of AKAP150 or F-actin but prevented cLTD-induced changes in AKAP150 and F-actin localization (Fig. 5*A*,*C–E*). As a negative control, addition of U73343, a structural analog of U73122 that has similar chemical properties but does not inhibit PLC, did not prevent F-actin depolymerization and AKAP150 translocation after cLTD (Fig. 5*B–E*). However, addition of the unrelated PLC inhibitor edelfosine prevented F-actin depolymerization and AKAP150 translocation (Fig. 5*B–E*). Thus, NMDA receptor-dependent cLTD leads to activation of PLC, which is necessary for F-actin depolymerization and AKAP150 translocation from spines.

NMDA receptor activation of PLC is sufficient to decrease dendritic spine PIP_2, F-actin, and AKAP79/150 levels

G_q-coupled postsynaptic group I mGluRs activate PLCβ_1 and PKC but can use other downstream pathways, including tyrosine phosphatases, to induce forms of hippocampal LTD that are independent of PLC and NMDAR activation (Huang and Hsu, 2006); however, mGluRs can also cooperate with NMDARs in PLC activation (Codazzi et al., 2006) and NMDAR-LTD induction (Choi et al., 2005). To test whether NMDAR and mGluR coactivation is required for changes in dendritic PIP_2, neurons cotransfected with PLCδPH–YFP and PSD95–CFP were treated with NMDA in the presence of mGluR antagonist (S)-MCPG (Fig. 5*A*). Addition of MCPG did not prevent NMDA-induced PLCδPH–YFP translocation from spines, as quantified by measurements of spine/shaft fluorescence ratios (Fig. 6*C*), thus suggesting no requirement for mGluR coactivation. Accordingly, addition of NMDAR antagonist APV completely blocked

PLCδPH–YFP translocation (Fig. 6*C*). Likewise, addition of a group I mGluR agonist DHPG did not lead to any clear change in PLCδPH–YFP spine localization (Fig. 6*A*,*C*), suggesting that NMDAR activation under these conditions causes a much stronger PLC stimulation than DHPG–mGluR LTD induction (Huber et al., 2001; Snyder et al., 2001), which does not require PLC activity (Huang and Hsu, 2006). Importantly, similar experiments with DHPG or MCPG (Fig. 6*D*) confirmed that mGluR activation was not required for spine F-actin depolymerization or AKAP150 relocalization in NMDA cLTD (Fig. 6*F*,*G*), further emphasizing mechanistic difference between these two forms of hippocampal LTD.

Although all of the above results indicate that NMDAR activation of PLC is responsible for the observed changes in PIP_2, F-actin, and AKAP79/150 spine localization, conversion to PIP_3 by phosphatidylinositol 3-kinases (PI3-kinases) and/or changes in PIP_2 synthesis by phosphatidylinositol 4-phosphate 5-kinases (PI4P5-kinases) could also be involved. Importantly, edelfosine inhibits PI3-kinase in addition to PLC. To rule out involvement of PI3-kinases and PI4P5-kinases, neurons were incubated with a dose of wortmannin that inhibits both kinase families (Fig. 6*B*). Addition of wortmannin alone had no effect on PLCδPH–YFP localization and did not prevent PLCδPH–YFP translocation with NMDA (Fig. 6*B*,*C*). Similar wortmannin treatments (Fig. 6*E*) demonstrated that activation of PI-kinases are not required for F-actin depolymerization or AKAP150 relocalization from spines during cLTD (Fig. 6*F*,*G*).

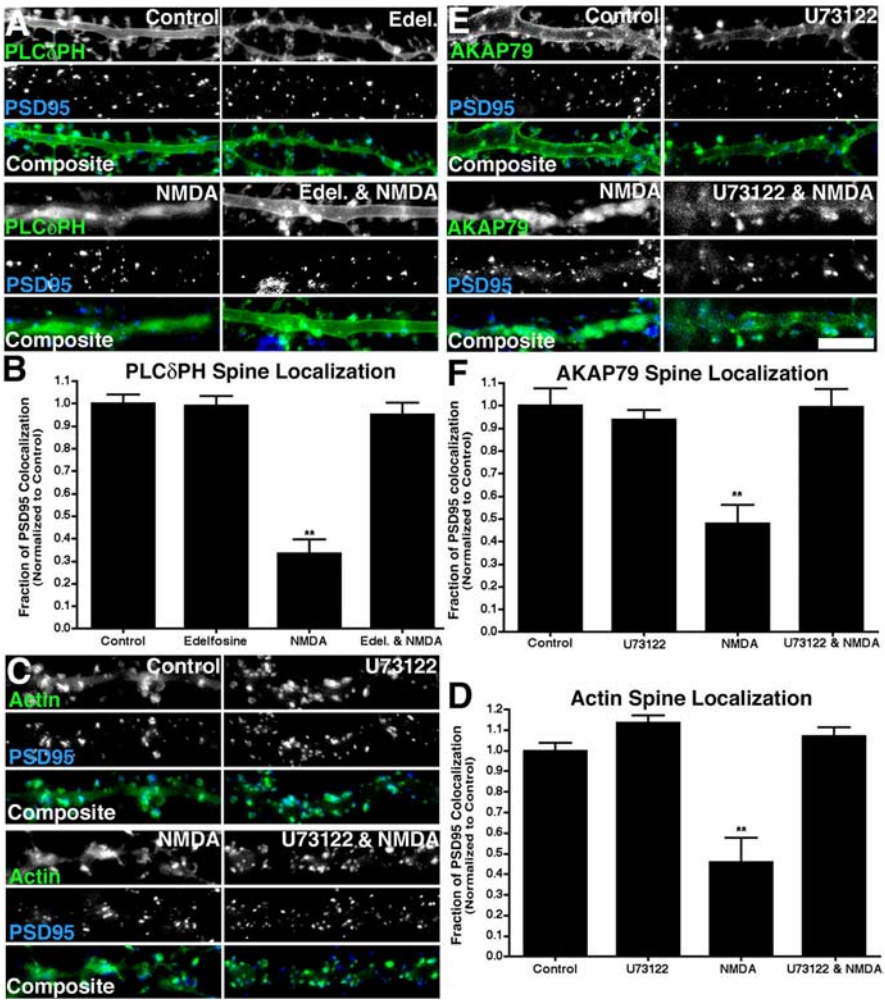

Figure 4. Inhibition of PLC prevents loss of PIP_2, actin–YFP, and AKAP79–YFP from dendritic spines after NMDA receptor activation. ***A***, Hippocampal neuron dendrites transfected with PLCδPH–YFP and PSD95–CFP treated with or without edelfosine (Edel; 50 μM) and NMDA (50 μM) as indicated. ***B***, Quantification normalized to control reveals a decrease in PLCδPH–YFP colocalization with PSD95–CFP 30–45 min after NMDA addition (0.34 ± 0.06; $^{**}p < 0.001$) that is blocked by edelfosine ($n = 6$ for all conditions). ***C***, Hippocampal neuron dendrites transfected with actin–YFP and PSD95–CFP treated with or without U73122 (50 μM) and NMDA (50 μM) as indicated. ***D***, Quantification normalized to control reveals a decrease in actin–YFP colocalization with PSD95–CFP 30–45 min after NMDA addition (0.46 ± 0.12; $n = 5$; $^{**}p < 0.001$) that is blocked by U73122 ($n = 6$ for control, U73122, and U73122 and NMDA). ***E***, Hippocampal neuron dendrites transfected with AKAP79–YFP and PSD95–CFP treated with or without U73122 (50 μM) and NMDA (50 μM) as indicated. ***F***, Quantification normalized to control reveals a decrease AKAP79–YFP colocalization with PSD95 30–45 min after NMDA addition (0.48 ± 0.08; $n = 6$; $^{**}p < 0.001$) that is blocked by U73122 ($n = 8$ for control; $n = 7$ for U73122; $n = 6$ for U73122 and NMDA). All data were analyzed by one-way ANOVA with Tukey's *post hoc* test. Scale bar, 10 μm.

NMDAR activation of PLC is not downstream of calcineurin activation

Previous work by ourselves and others have shown that NMDAR activation of CaN phosphatase activity is at least in part responsible for decreases in dendritic spine F-actin and AKAP79/150 localization associated with LTD (Halpain et al., 1998; Gomez et al., 2002; Zhou et al., 2004). Thus, PLC activation could be occurring in some manner downstream of CaN activation. However, inhibition of CaN activity with ascomycin did not block NMDA-induced translocation of PLCδPH–YFP from spines (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Hence, NMDAR activation of PLC is more likely to be parallel to CaN activation in LTD signaling.

Activation of PKC and release of intracellular Ca^{2+} through IP_3 receptors are not required for decreases in F-actin and AKAP150 localization to dendritic spines during cLTD

Hydrolysis of PIP_2 by PLC leads to the production of DAG, which activates PKC, and IP_3, which increases intracellular Ca^{2+} via IP_3 receptors. It has not been examined whether changes in spine F-actin associated with LTD involve PKC activation. Previous work showed that PKC phosphorylates the AKAP79 targeting domain to inhibit binding to PIP_2, F-actin, and cadherins *in vitro*, although previous studies found that PKC activity is not required for AKAP79/150 targeting regulation by NMDARs or induction of NMDAR-LTD (Dell'Acqua et al., 1998; Gomez et al., 2002; Malenka and Bear, 2004; Gorski et al., 2005). Accordingly, two different PKC inhibitors, a catalytic domain inhibitor, BIM, and a regulatory domain antagonist, CalC, failed to prevent AKAP150 translocation (supplemental Fig. 2*A*,*D*, available at www.jneurosci.org as supplemental material). Importantly, addition of either BIM or CalC (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material) also failed to prevent spine actin depolymerization seen as decreased F-actin colocalization with AKAP150 (supplemental Fig. 2*B*, available at www.

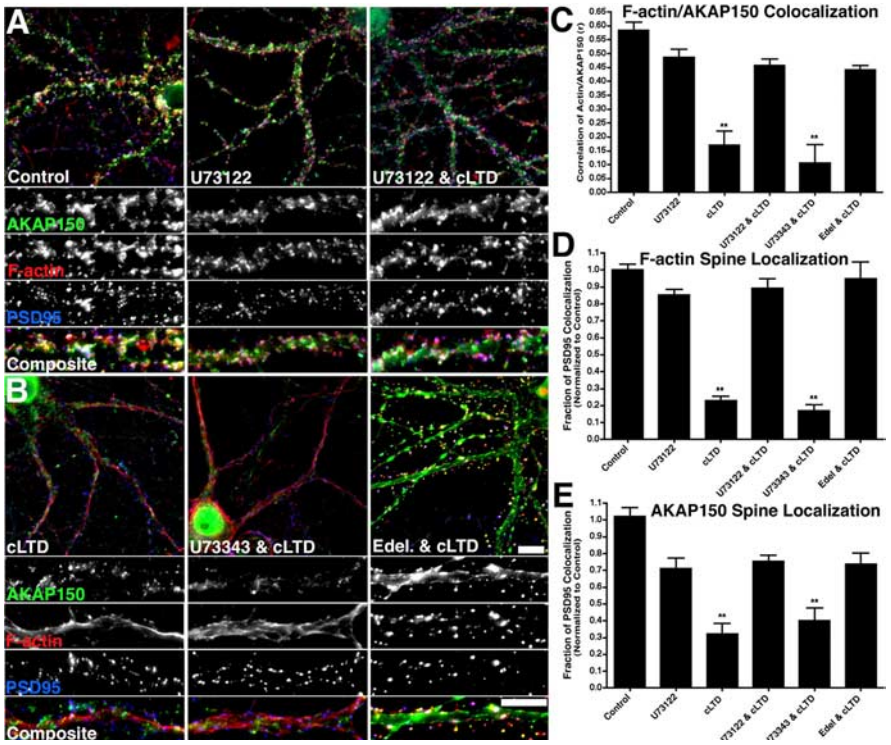

Figure 5. Decreases in endogenous F-actin and AKAP150 localization to dendritic spines during cLTD require PLC activation. ***A***, ***B***, Hippocampal neurons treated with U73122 (35 μM; ***A***), U73343 (35 μM; ***B***), or edelfosine (Edel.; 35 μM; ***B***) alone or during cLTD stimulation as indicated and stained with antibodies to label AKAP150 and PSD95 and phalloidin to visualize F-actin. The small panels show magnifications of dendrites. ***C***, Quantification of F-actin and AKAP150 colocalization in dendrites 15 min after cLTD stimulation as measured by a correlation value (*r*; see Materials and Methods). Induction of cLTD with or without U73343 (control, 0.58 $\pm$ 0.03; cLTD, 0.17 $\pm$ 0.05; cLTD and U73343, 0.11 $\pm$ 0.07) shows a reduction in the amount of F-actin colocalized with AKAP150, but when PLC is inhibited by either U73122 or edelfosine (U73122, 0.49 $\pm$ 0.03; U73122 and cLTD, 0.46 $\pm$ 0.02; Edel. and cLTD, 0.44 $\pm$ 0.01), F-actin colocalization with AKAP150 is maintained at levels similar to untreated controls ($n = 10$). ***D***, Quantification of F-actin colocalization with PSD95 in dendritic spines 15 min after cLTD stimulation normalized to untreated controls shows a loss of F-actin from dendritic spines in cLTD with and without U73343 treatment (cLTD, 0.23 $\pm$ 0.06; U73343 and cLTD, 0.17 $\pm$ 0.04). The decrease with cLTD is blocked by U73122 and edelfosine ($n = 10$). ***E***, Quantification of AKAP150 colocalization with PSD95 in dendritic spines 15 min after addition of drug normalized to untreated controls shows a loss of AKAP150 from dendritic spines in cLTD with or without U73343 treatment (cLTD, 0.32 $\pm$ 0.06; U73343 and cLTD, 0.40 $\pm$ 0.08). The cLTD decrease is blocked by U73122 and edelfosine ($n = 10$). $^{**}p < 0.001$. All data were analyzed using one-way ANOVA with Tukey's *post hoc* test. Scale bars, 10 μm.

jneurosci.org as supplemental material) and PSD95 in response to cLTD (supplemental Fig. 2*C*, available at www.jneurosci.org as supplemental material). As mentioned above, NMDAR activation of CaN is required for LTD induction (Mulkey et al., 1994) and is involved in both F-actin depolymerization and AKAP79/150 loss from spines (Halpain et al., 1998; Gomez et al., 2002; Zhou et al., 2004). Additional release of Ca^{2+} from IP_3 receptor stores could favor greater CaN activation; however, an IP_3 receptor antagonist, xestospongin D, did not prevent F-actin depolymerization or AKAP150 translocation after cLTD (supplemental Fig. 2*A–D*, available at www.jneurosci.org as supplemental material). Thus, neither PKC nor IP_3 receptors are required downstream of PLC–PIP_2 hydrolysis for F-actin depolymerization or AKAP150 translocation from spines during cLTD.

Inhibition of PLC prevents decreased spine PSD95 levels and decreased AMPA receptor surface expression in response to cLTD

Depression of AMPAR synaptic strength in LTD is thought to be mediated by dephosphorylation of GluR1 Ser845 (Lee et al., 1998, 2000, 2003; Banke et al., 2000; Ehlers, 2000) and removal of receptors from the PSD through GluR2-regulated endocytosis (Carroll et al., 2001; Lee et al., 2004). Importantly, this LTD regulation requires CaN activation, is opposed by PKA activation, and may involve anchoring of these enzymes to AKAP79/150 (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Tavalin et al., 2002; Hoshi et al., 2005; Snyder et al., 2005; Smith et al., 2006). Thus, to test whether PLC activation is also required for AMPAR removal in LTD, neurons were treated with U73122 for a prolonged period (5 min before, during, and 15 min after cLTD stimulation) or for a shorter period just during cLTD stimulation. Endocytosis was stopped 15 min after cLTD stimulation by cooling neurons to 4°C, and then surface AMPARs were labeled with antibodies that recognize the GluR2 N-terminal domain, followed by imaging in parallel with control neurons using identical settings (Fig. 7*A*). The cLTD-treated neurons showed decreases in mean surface GluR2 fluorescence in dendrites compared with controls, indicating a decrease in AMPAR surface expression, as expected (Fig. 7*A*,*B*). Importantly, prolonged treatment with U73122 alone led to increased surface expression of GluR2 on its own and prevented surface expression decreases in response to cLTD stimulation (Fig. 7*A*,*B*). Similar results were obtained with surface staining for GluR1 (data not shown). Because prolonged pretreatment with U73122 increased AMPAR surface labeling on its own and blocked cLTD-induced decreases in AMPAR surface labeling, we next evaluated whether addition of U73122 only during the cLTD stimulation would also block GluR2 internalization. Importantly, incubation with U73122 alone for only 3 min did not increase GluR2 surface labeling above basal levels; however, coapplication of U73122 with NMDA during the 3 min cLTD stimulation did prevent a decrease in GluR2 surface levels (Fig. 7*C*). Finally, short-term coapplication of the alternate PLC inhibitor edelfosine with NMDA also blocked cLTD-induced decreases in GluR2 surface labeling (Fig. 7*C*). Thus, PLC hydrolysis of PIP_2 regulates LTD-related AMPAR trafficking events.

In order for LTD to lead to long-lasting decreases in synaptic AMPARs, it is believed that the number of docking slots for receptors in the PSD must be decreased. In particular, PSD95 is thought to function as an AMPAR PSD slot protein (Schnell et al., 2002; Stein et al., 2003; Ehrlich and Malinow, 2004). Previous studies have indicated that cLTD leads to a decrease in the amount of PSD95 clustered in dendritic spines through a process that involves polyubiquitination and degradation by the proteasome. This PSD95 degradation requires CaN activation, is blocked by PKA activation, and is necessary for AMPAR internalization (Colledge et al., 2003). To test whether PLC activation is also required for PSD95 decrease in spines, neurons were treated with either U73122 or edelfosine during cLTD stimulus and then fixed and immunostained for PSD95, followed by imaging using identical settings in parallel with control neurons. In agreement

with previous observations (Colledge et al., 2003; Smith et al., 2006), PSD95 remained clustered in cLTD-treated neurons but showed decreased PSD95 cluster mean fluorescence intensity 15 min after stimulation (Fig. 7*D*,*E*). Interestingly, this decrease in PSD95 cluster intensity was substantially blocked by U73122 and completely blocked by edelfosine (Fig. 7*D*,*E*). However, the number/density of PSD95 dendritic clusters did not change with any of the above treatments, suggesting that the decrease in PSD95 cluster intensity was caused by a decrease in the amount of PSD95 in the PSD and not a loss of synapses/spines. These results show that activation of PLC is required for both PSD95 decreases and AMPAR internalization during LTD. Thus, overall, PLC appears to function, along with CaN, as a common upstream regulator coordinating structural and functional postsynaptic modifications underlying NMDAR-dependent LTD.

Discussion

AMPARs are linked to F-actin by scaffold proteins, and actin depolymerization triggers loss of AMPARs and certain scaffolds from synapses (Allison et al., 1998; Allison et al., 2000; Shen et al., 2000; Zhou et al., 2001; Gomez et al., 2002). However, although actin depolymerization is necessary for spine shrinkage in NMDAR-LTD (Okamoto et al., 2004; Zhou et al., 2004), it is not required for AMPAR synaptic depression (Zhou et al., 2004; Morishita et al., 2005) or endocytosis (Beattie et al., 2000). Thus, we do not fully understand mechanisms coordinating regulation of spine structure and AMPAR function in LTD. Here, we found that PLC is a novel regulator of spine actin and AMPARs in processes underlying NMDAR-LTD. In particular, we show that NMDAR stimulation of PLC coordinately regulates spine actin, PSD scaffolding, and AMPAR internalization.

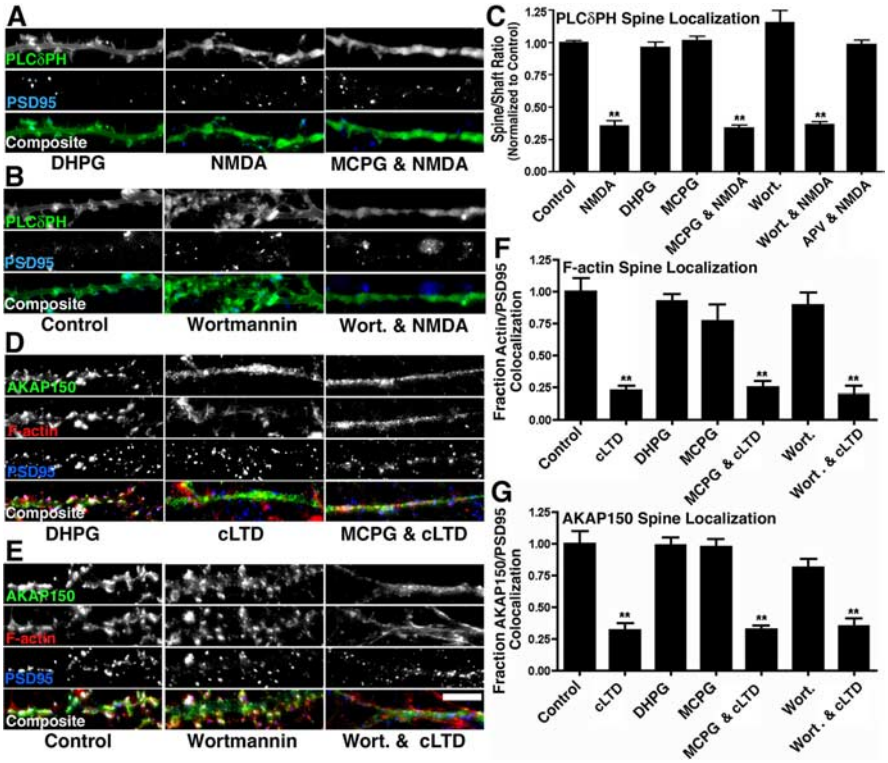

Figure 6. mGluRs, PI3-kinase, and PI4P5-kinase are not involved in the decreases of PIP_2, F-actin, or AKAP150 in dendritic spines after NMDA receptor activation. ***A***, Dendrites from neurons transfected with PLCδPH–YFP and PSD95–CFP treated with DHPG (100 μM), NMDA (50 μM) alone, or NMDA with MCPG (100 μM; added 30 min before NMDA). ***B***, Dendrites from neurons transfected with PLCδPH–YFP and PSD95–CFP untreated or treated with wortmannin (200 μM) or NMDA (50 μM) with wortmannin (Wort.; added 30 min before NMDA). ***C***, Quantification of PLCδPH–YFP spine/shaft fluorescence ratios from experiments in ***A*** and ***B*** normalized to untreated controls 30–45 min after NMDA show decreased PLCδPH–YFP localization to dendritic spines (0.35 ± 0.04). DHPG, MCPG, and wortmannin alone have no effect on PLCδPH–YFP spine localization. Addition of MCPG or wortmannin with NMDA do not prevent decreases in the spine/shaft ratios (0.34 ± 0.02 and 0.37 ± 0.02, respectively; $n = 6$; $^{**}p < 0.001$). Addition of D-APV (100 μM; 30 min before NMDA) prevents the effects of NMDA. ***D***, Dendrites of hippocampal neurons treated with DHPG (100 μM), cLTD alone, or cLTD with MCPG (100 μM; 30 min before cLTD) and stained with antibodies to label AKAP150 and PSD95 and phalloidin to visualize F-actin. ***E***, Dendrites of AKAP150-, PSD95-, and F-actin-stained neurons as in ***D*** untreated or treated with wortmannin or cLTD with wortmannin. ***F***, Quantification of F-actin colocalization with PSD95 in dendritic spines 15 min after cLTD stimulation normalized to controls for experiments in ***D*** and ***E*** shows a loss of F-actin from spines in neurons treated with cLTD alone, cLTD with wortmannin, or cLTD with MCPG (0.23 ± 0.04, 0.20 ± 0.07, and 0.25 ± 0.05, respectively; $^{**}p < 0.001$). These decreases are not replicated in neurons treated with DHPG, MCPG, or wortmannin alone ($n = 15$). ***G***, Quantification of AKAP150 colocalization with PSD95 in dendritic spines 15 min after cLTD stimulation for experiments in ***D*** and ***E*** normalized to controls shows a loss of AKAP150 from spines in neurons treated with cLTD alone, cLTD with wortmannin, or cLTD with MCPG (0.32 ± 0.06, 0.35 ± 0.06, and 0.33 ± 0.02, respectively; $^{**}p < 0.001$). These decreases are not replicated in neurons treated with DHPG, MCPG, or wortmannin alone ($n = 15$). All data were analyzed using one-way ANOVA with Tukey's *post hoc* test. Scale bar, 10 μm.

In hippocampal neurons, we observed PLC hydrolysis of PIP_2 during NMDAR stimulation, as reported by translocation of the membrane PLCδPH probe from spines into dendrite shaft cytoplasm. This NMDAR-PLC activation was independent of mGluR activation. Our imaging observations are in agreement with other recent studies in hippocampal neurons showing that mGluRs and NMDARs cooperate in PLC regulation, but NMDARs stimulate PLC independently (Codazzi et al., 2006). Although hippocampal G_q-coupled group I mGluRs stimulate PLCβ1, they can use other pathways independent of PLC to induce AMPAR internalization and LTD (Huang and Hsu, 2006). However, mGluRs and other G_q-coupled receptors can still contribute to LFS induction of NMDAR-LTD in visual cortex and CA1 hippocampus (Choi et al., 2005; Lee et al., 2005). Nonetheless, in CA1 of young rats when LFS-LTD is NMDAR-dependent, as shown by APV block, it is almost entirely blocked by the PLC inhibitor U73122 (Reyes-Harde and Stanton, 1998; Lee et al., 2005) but only partially blocked by antagonism of mGluRs and other G_q-coupled receptors (Choi et al., 2005), suggesting a more direct role for NMDAR activation of PLC in LTD. Thus, based on these electrophysiology studies and our imaging studies in hippocampal neuron cultures, we propose that NMDAR activation of PLC is required upstream of multiple pathways regulating dendritic spine structure and function in LTD (Fig. 8). The PLCδ and PLCη isoforms that, unlike PLCβ1, are not regulated by G_q but are strongly activated by Ca^{2+} are possible candidates for participating in NMDAR-LTD signaling (Delmas et al., 2004; Cockcroft, 2006; Codazzi et al., 2006).

Actin is the main spine cytoskeletal protein and is linked to PSD scaffolds, AMPARs, NMDARs, and transsynaptic adhesion molecules (Tada and Sheng, 2006). For example, AKAP79/150 is

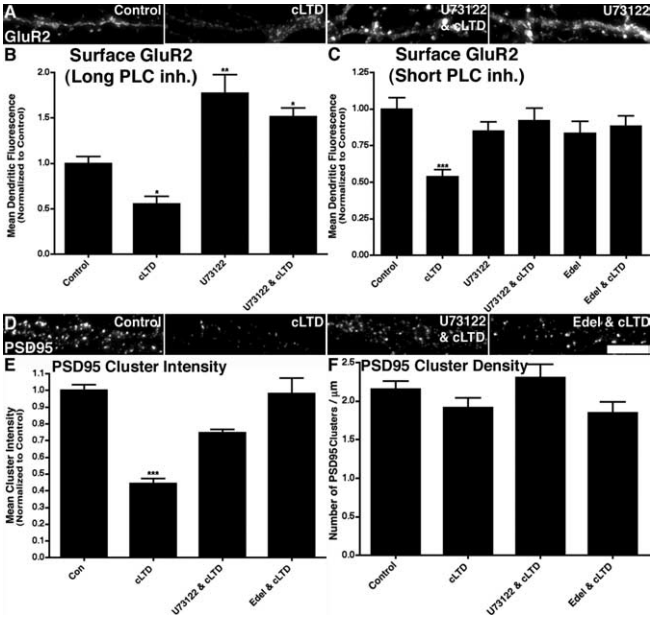

Figure 7. Decreased AMPA receptor surface expression and PSD95 degradation during cLTD requires PLC activation. ***A***, Dendrites from neurons treated with cLTD (30 μM NMDA for 3 min; 15 min recovery) with or without U73122 (35 μM; added 5 min before NMDA) or U73122 alone, stained with an antibody to the extracellular N-terminal domain of GluR2 to label surface AMPA receptors, and imaged using identical acquisition and normalization settings. ***B***, Quantification of dendritic surface GluR2 mean fluorescence intensity normalized to control shows that U73122 added 5 min before cLTD, during cLTD induction, and during the 15 min recovery period (long PLC inh.) increases AMPAR surface staining (1.77 ± 0.2; $n = 10$; $^{**}p < 0.01$) and blocks decreases in AMPAR surface staining seen with cLTD (cLTD, 0.55 ± 0.08, $n = 15$; U73122 and cLTD, 1.51 ± 0.1, $n = 10$; $^{*}p < 0.05$). ***C***, Quantification of dendritic surface GluR2 mean fluorescence intensity normalized to control shows that U73122 or edelfosine (Edel; 35 μM) added just during cLTD induction (short PLC inh.) do not increase AMPAR surface staining (U73122, 0.85 ± 0.06, $n = 9$; Edel, 0.84 ± 0.08, $n = 9$), but block decreases in AMPAR surface staining seen with cLTD (cLTD, 0.54 ± 0.05, $^{***}p < 0.001$, $n = 9$; U73122 and cLTD, 0.92 ± 0.09, $n = 10$; Edel and cLTD, 0.88 ± 0.07, $n = 10$). ***D***, Dendrites from neurons treated with cLTD, U73122 (35 μM for 5 min) alone, cLTD with U73122, or cLTD with edelfosine (35 μM; added 5 min before NMDA) stained for PSD95 and imaged using identical acquisition and normalization settings. ***E***, Quantitation of PSD95 mean cluster intensity in dendritic spines shows that U73122 and edelfosine block the decrease in PSD95 intensity seen after cLTD (cLTD, 0.44 ± 0.03; $n = 10$; $^{***}p < 0.001$). All data were analyzed using one-way ANOVA with Tukey's *post hoc* test. Con, Control. ***F***, Quantification of the number of PSD95 clusters per micrometer of dendrite, showing that cLTD treatment with or without PLC inhibitors present does not change the number/density of dendritic PSD95 clusters (control, 2.1 ± 0.1; cLTD, 1.9 ± 0.1; cLTD and U73122, 2.3 ± 0.2; cLTD and Edel, 1.8 ± 0.1; $p > 0.05$). All data were analyzed by one-way ANOVA with Tukey's *post hoc* test. Scale bar, 10 μm.

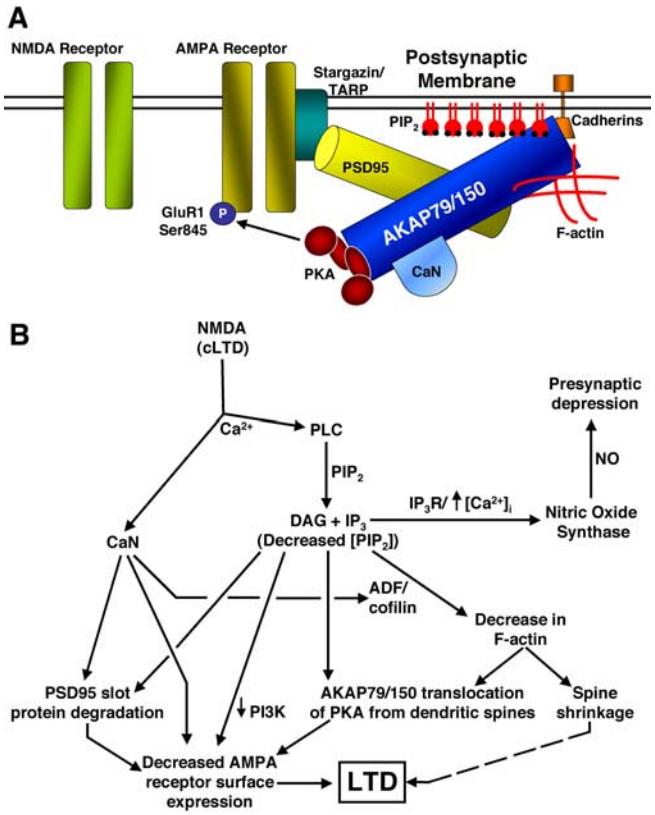

Figure 8. Model of signaling pathways downstream of NMDA receptor PLC activation regulating postsynaptic structure and function during LTD. ***A***, Postsynaptic organization of AKAP79/150 complexes with PSD95 showing targeting interactions, anchoring of PKA and CaN, and recruitment to AMPARs that are important for regulation of GluR1 Ser845 phosphorylation. Not shown are additional linkages of AKAP79/150 to AMPARs by SAP97 (synapse-associated protein 97) and to NMDARs by PSD95. ***B***, Proposed signaling pathways activated downstream of PLC and CaN during LTD. Shown are potential roles for PLC and CaN signaling in regulation of AKAP79/150 translocation from spines, depolymerization of spine F-actin, degradation of PSD95, and decreased AMPAR surface expression. For more details, see Discussion. TARP, Transmembrane AMPA receptor regulatory protein.

targeted to the PSD through N-terminal basic domains that bind PIP_2, F-actin, and cadherins (Dell'Acqua et al., 1998; Gomez et al., 2002; Gorski et al., 2005) (Fig. 8*A*). AKAP79/150 is further localized to NMDA and AMPARs through PSD95-family scaffolds (Colledge et al., 2000). Localization of AKAP79/150 to AMPARs allows for phosphorylation and dephosphorylation of GluR1 Ser845 by PKA and CaN anchored to AKAP79/150 (Colledge et al., 2000; Dell'Acqua et al., 2002; Tavalin et al., 2002; Hoshi et al., 2005). F-actin reorganization in cLTD is associated with disruption of AKAP79/150 binding to PSD95 and cadherins and its loss from spines (Gomez et al., 2002; Gorski et al., 2005). AKAP79/150 translocation removes PKA from the PSD to perhaps maintain dephosphorylation of GluR1 Ser845 during LTD (Smith et al., 2006).

F-actin depolymerization and AKAP79/150 loss from spines involves NMDAR activation of CaN (Halpain et al., 1998; Gomez et al., 2002). We now show that PLC hydrolysis of PIP_2 during cLTD is also upstream of changes in F-actin and AKAP79/150 localization (Fig. 8*B*). Inhibition of PLC by U73122 and edelfosine prevented loss of F-actin and AKAP79/150 from spines; two different inhibitors were used to control for nonspecific drug actions. Previous work by our group showed that AKAP79 is phosphorylated by PKC *in vitro* to regulate PIP_2, F-actin, and cadherin binding (Dell'Acqua et al., 1998; Gomez et al., 2002; Gorski et al., 2005). However, we found that PKC inhibition did not prevent AKAP79/150 relocalization or spine F-actin loss during cLTD. We also observed that changes in F-actin and AKAP79/150 were not attributable to IP_3 receptor Ca^{2+} release or PI3-kinase conversion of PIP_2 to PIP_3. Thus, PLC activation may be regulating AKAP79/150 localization in LTD more directly through loss of targeting domain PIP_2 binding and actin depolymerization (Fig. 8*B*).

PLC hydrolysis of PIP_2 could be altering spine actin through a number of mechanisms including: (1) actin cross-linking by α-actinin, (2) actin polymerization through Rho family GTPases, and/or (3) actin depolymerization by actin-depolymerizing factor (ADF)/cofilin (Yin and Janmey, 2003; Hilpela et al., 2004). PIP_2 binding to α-actinin promotes F-actin cross-linking (Stossel et al., 2006), and α-actinin provides linkages to PSD proteins, including NMDARs (Hering and Sheng, 2001). Thus, PLC hydrolysis of PIP_2 could inhibit α-actinin and destabilize actin link-

ages modulating NMDAR currents in LTD (Morishita et al., 2005). Activation of the Rho GTPase Rac1 promotes spine formation and enlargement through actin polymerization (Nakayama et al., 2000). Interestingly, knock-out of WAVE1 in mice, an effector of Rac regulating actin polymerization, produces dendritic spine loss, hippocampal plasticity alterations, and learning deficits (Soderling et al., 2003, 2007; Kim et al., 2006). In addition, Rac activates LIM kinase (LIMK), which phosphorylates and inactivates ADF/cofilin. (Sarmiere and Bamburg, 2004). Accordingly, LIMK1 knock-out mice display altered spine morphology and hippocampal plasticity (Meng et al., 2002; Sarmiere and Bamburg, 2002). During LTD, it is thought that CaN activates ADF/cofilin through the phosphatase Slingshot (Sarmiere and Bamburg, 2004; Zhou et al., 2004) (Fig. 8*B*). Importantly, PIP_2 can bind and inhibit Slingshot and ADF/cofilin and activate, either directly or through conversion to PIP_3 by PI3-kinase, guanine nucleotide exchange factors that activate Rac (Yin and Janmey, 2003; Hilpela et al., 2004; Sarmiere and Bamburg, 2004; Stossel et al., 2006). Thus, PLC hydrolysis of PIP_2 could, along with CaN, contribute to ADF/cofilin activation and actin depolymerization (Fig. 8*B*). Indeed, ADF/cofilin inhibitory peptides prevent spine shrinkage during LTD (Zhou et al., 2004). However, these same ADF/cofilin peptides, although they block depression of NMDAR responses, do not block depression of AMPAR responses, indicating involvement of additional PLC signals (Zhou et al., 2004; Morishita et al., 2005).

AMPAR depression in LTD is associated with GluR1 Ser845 dephosphorylation and GluR1/2 and GluR2/3 receptor removal from synapses by endocytosis through pathways involving CaN (for review, see Carroll et al., 2001; Malinow and Malenka, 2002; Song and Huganir, 2002; Henley, 2003; Malenka and Bear, 2004). We show here that brief PLC inhibition by U73122 or edelfosine blocks GluR2 internalization during cLTD. These results agree with electrophysiological studies discussed above showing that PLC inhibition blocks induction of NMDAR-LTD in hippocampus (Reyes-Harde and Stanton, 1998; Lee et al., 2005). However, mechanisms downstream of PLC responsible for AMPAR depression have not been established. One previous study suggested a role for postsynaptic PLC activation in producing retrograde second messengers regulating presynaptic LTD expression (Reyes-Harde et al., 1999b) (Fig. 8*B*). However, our findings demonstrate a requirement for PLC in AMPAR internalization, indicating that it is also important for postsynaptic LTD mechanisms.

As mentioned above, PLC-dependent movement of AKAP79/150 anchored PKA away from spines could promote prolonged GluR1 Ser845 dephosphorylation to prevent receptor recycling during LTD (Ehlers, 2000; Esteban et al., 2003; Snyder et al., 2005; Smith et al., 2006) (Fig. 8*B*). In addition, the GluR2-binding protein PICK1 regulates AMPAR endocytosis through mechanisms that are sensitive to Ca^{2+} and phosphoinositides (Jin et al., 2006; Sossa et al., 2006). Finally, because PI3-kinase activation is required for AMPAR membrane insertion during LTP, PLC activation may favor decreased AMPAR surface expression by lowering the amount of PIP_2 in spines available for conversion to PIP_3; in the absence of synthesis by PI3-kinase, lipid phosphatases such as PTEN would cause spine PIP_3 decreases (Man et al., 2003; Opazo et al., 2003). Thus, decreasing PI3-kinase activity could be another important step favoring receptor internalization over insertion in LTD (Fig. 8*B*). In contrast, PLC inhibition may favor membrane insertion over internalization, as shown by increased GluR2 surface expression, which we observed with longer U73122 treatments. Interestingly, coactivation of NMDARs and mGluRs with pharmacologic PI3-kinase inhibition induces spread of NMDAR-homosynaptic LTD to additional synapses leading to heterosynaptic LTD (Daw et al., 2002).

Another mechanism proposed to maintain decreased AMPAR surface expression in LTD is to remove PSD docking slots. PSD95 is considered to be a slot protein that recruits AMPARs to synapses through stargazin/transmembrane AMPA receptor regulatory proteins (Schnell et al., 2002; Stein et al., 2003; Ehrlich and Malinow, 2004; Tomita et al., 2005). During LTD, PSD95 levels in spines decrease through pathways involving polyubiquitination and degradation; this PSD95 spine decrease contributes to AMPAR internalization and may be under control of AKAP79/150-anchored CaN and PKA (Colledge et al., 2003). We show here that PLC inhibition by U73122 or edelfosine prevented decreases in PSD95 seen in cLTD. Thus, signaling events downstream of PLC and CaN may regulate AMPAR trafficking in part through PSD95 decreases (Fig. 8*B*). In summary, PLC functions as an upstream regulator of multiple downstream postsynaptic LTD pathways that coordinately control AMPAR surface expression, F-actin dynamics, and PSD scaffolding.

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