Cellular/Molecular

EphA4 Signaling Regulates Phospholipase Cγ1 Activation, Cofilin Membrane Association, and Dendritic Spine Morphology

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Specialized postsynaptic structures known as dendritic spines are the primary sites of glutamatergic innervation at synapses of the CNS. Previous studies have shown that spines rapidly remodel their actin cytoskeleton to modify their shape and this has been associated with changes in synaptic physiology. However, the receptors and signaling intermediates that restructure the actin network in spines are only beginning to be identified. We reported previously that the EphA4 receptor tyrosine kinase regulates spine morphology. However, the signaling pathways downstream of EphA4 that induce spine retraction on ephrin ligand binding remain poorly understood. Here, we demonstrate that ephrin stimulation of EphA4 leads to the recruitment and activation of phospholipase Cγ1 (PLCγ1) in heterologous cells and in hippocampal slices. This interaction occurs through an Src homology 2 domain of PLCγ1 and requires the EphA4 juxtamembrane tyrosines. In the brain, PLCγ1 is found in multiple compartments of synaptosomes and is readily found in postsynaptic density fractions. Consistent with this, PLC activity is required for the maintenance of spine morphology and ephrin-induced spine retraction. Remarkably, EphA4 and PLC activity modulate the association of the actin depolymerizing/severing factor cofilin with the plasma membrane. Because cofilin has been implicated previously in the structural plasticity of spines, this signaling may enable cofilin to depolymerize actin filaments and restructure spines at sites of ephrin–EphA4 contact.

Key words: **synapse; plasticity; actin; hippocampus; neuron glia; phosphorylation**

Introduction

Dendritic spines are specialized protrusions from the dendritic shaft where excitatory synapses are formed in the brain. The stereotypic spine has an enlarged head that is connected to the dendritic shaft by a constricted neck. This morphology creates a biochemical compartment that accommodates the postsynaptic density (PSD), a dense region of ion channels and receptors that are complexed with scaffolding and other signaling proteins (Kim and Sheng, 2004). Remarkably, spines change their morphology within minutes (Dailey and Smith, 1996; Fischer et al., 1998; Dunaevsky et al., 1999) and this may adjust the physiology of synapses during processes such as learning and memory formation (Segal, 2005). Previous studies indicate that this structural plasticity relies on the dynamics of actin filaments, which are concentrated in spines and serve as their primary structural scaffold (Matus, 2000).

Among molecules that may control actin rearrangements in spines are proteins of the cofilin/actin depolymerization factor (ADF) family (Bamburg, 1999; Racz and Weinberg, 2006). Cofilin/ADF proteins bind, depolymerize, and sever actin filaments (Bamburg, 1999). Cofilin activity is negatively regulated by kinases [Lin-11, Isl-1, Mec-3 (LIM) kinase 1/2 and testis-specific (Tes) kinase] (Arber et al., 1998) and positively regulated by phosphatases (Slingshot and chronophin) through phosphocycling on a serine residue (Huang et al., 2006). Cofilin is also modulated by phosphoinositides, especially phosphatidylinositol 4,5-bisphosphate (PIP_2). Cofilin has multiple binding sites for PIP_2 (Yonezawa et al., 1990, 1991b) and cofilin–PIP_2 interactions tether cofilin to the cell membrane (Nagaoka et al., 1996; DesMarais et al., 2005) and modulate cofilin activity (Nagaoka et al., 1995). The cofilin–PIP_2 interaction also inhibits the enzyme phospholipase Cγ1 (PLCγ1) from cleaving PIP_2 when PLCγ1 is not tyrosine phosphorylated (Yonezawa et al., 1991b). PLCγ1 is

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activated by receptor tyrosine kinases (RTKs), enabling it to hydrolyze PIP_2 into IP_3 and DAG (Rebecchi and Pentyala, 2000). Cofilin and PLCγ1 are important regulators of cell morphology and implicated in synaptic plasticity (Reyes-Harde and Stanton, 1998; Micheva et al., 2001; Meng et al., 2002; Zhou et al., 2004). However, the receptors that control these proteins at synapses remain to be fully described.

Several classes of RTKs may regulate PLCγ1 and cofilin, including those of the Eph family. Eph receptors are divided into EphA and EphB subtypes (Kullander and Klein, 2002). In general, EphA receptors bind glycosylphosphatidylinositol-anchored ephrin-As, whereas EphB receptors respond to transmembrane ephrin-Bs. Eph receptors are important for spine morphogenesis and maintenance *in vitro* and *in vivo* (Ethell and Pasquale, 2005). Several signaling mechanisms have been elucidated for EphB receptors in spines, however, those downstream of EphA receptors remain to be described.

Here, we identify an EphA4 signaling pathway that regulates spine morphology. We show that EphA4 triggers PLCγ1 activation through an Src homology 2 (SH2) domain interaction with the juxtamembrane tyrosines of EphA4. PLC signaling is necessary for maintaining spine morphology and for ephrin-induced spine retraction. Remarkably, EphA4 and PLC signaling alter the membrane association of cofilin. We propose that EphA4 promotes PLCγ1 signaling to allow cofilin translocation away from the cell membrane, enabling it to depolymerize actin filaments in spines at sites of ephrin-A/EphA4 contact.

Materials and Methods

DNA constructs. Full-length mouse PLCγ1 was cloned from an expressed sequence tag vector (IMAGE clone 6854923; Open Biosystems, Huntsville, AL) through PCR and ligated in-frame into pcDNA3 with a V5 epitope tag (Invitrogen, Carlsbad, CA). The lipase-inactive PLCγ1 mutant (H335Q) (Huang et al., 1995; Ronnstrand et al., 1999; Rong et al., 2003) and EphA4 juxtamembrane tyrosine mutants (Y596E and Y602E) were created using standard PCR-based mutagenesis (Zisch et al., 2000; Cowan et al., 2005). The EphA4 kinase-dead construct has been described previously (Murai et al., 2003a). The N- and C-terminal SH2 domains of PLCγ1 were cloned into pGEX-4T1 (Promega, San Luis Obispo, CA) and used for glutathione *S*-transferase (GST) fusion protein production using standard procedures.

For RNAi constructs, a short hairpin sequence directed against mouse PLCγ1 (Patterson et al., 2002) was cloned into pSUPER (OligoEngine, Seattle, WA) containing the H1 promoter for driving the expression of a short-hairpin RNA and a phosphoglycerate kinase promoter for expressing green fluorescent protein (GFP). To more thoroughly delineate dendritic spines, the GFP sequence was replaced with membrane-targeted farnesylated enhanced GFP (EGFPf; BD Biosciences, San Jose, CA). To generate RNAi plasmids, two complementary oligos containing the PLCγ1 shRNA were annealed (sequences 5′GATCCCCAAACAACCGGCTCTTCGTCTTCAAGAGAGACGAAGAGCCGGTTGTTTTTTTTA3′ and 5′AGCTTAAAAAAAACAACCGGCTCTTCGTCTCTCTTGAAGACGAAGAGCCGGTTGTTTGGG3′) and cloned into the *Bgl*II and *Hin*dIII sites of pSUPER. A shRNA vector containing a scrambled sequence of the PLCγ1 shRNA sequence, which did not show homology to the mouse genome, was cloned in a similar manner and used in control experiments (sequences 5′GATCCCCTAGACCTATATCCCTGCGCTTCAAGAGAGCGCAGGGATATAGGTCTATTTTTA3′ and 5′AGCTTAAAAATAGACCTATATCCCTGCGCTCTCTTGAAGCGCAGGGATATAGGTCTAGGG3′).

Antibodies and recombinant proteins. The following antibodies were used in this study: mouse PLCγ1 (mouse monoclonal; Millipore, Upstate Division, Billerica, MA); PLCγ1 (rabbit polyclonal; Cell Signaling Technologies, Danvers, MA); pY783 PLCγ1 (Cell Signaling Technologies); pY771 PLCγ1 (Cell Signaling Technologies); pS1248 PLCγ1 (Millipore); EphA4 (Soans et al., 1994; Murai et al., 2003a); pY20 (BD Biosciences); synaptosomal-associated protein 25 (SNAP25) (a generous gift from Dr. P. McPherson, Montreal Neurological Institute), synaptophysin (Sigma, St. Louis, MO); PSD95 (BD Biosciences); NR1 (BD Biosciences); cofilin (Cell Signaling Technologies; Cytoskeleton, Denver, CO); V5 (Sigma); transferrin receptor (Invitrogen); GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Abcam, Cambridge, MA); anti-rabbit and mouse HRP (GE Healthcare, Fairfield, CT); and control mouse IgGs (Jackson Immunochemicals, West Grove, PA). The following recombinant proteins were used: human IgG Fc (Jackson Immunochemicals), EphA4 kinase domain (Cell Signaling Technologies), and ephrin-A3 and -A5 Fc (R & D Systems, Minneapolis, MN).

Immunoprecipitation and Western blot analysis. For COS7 cell experiments, EphA4 and juxtamembrane mutants were transfected with Lipofectamine 2000 (Invitrogen) and stimulated 24 h later for 45 min with dimeric control Fc, ephrin-A3 Fc, or ephrin-A5 Fc. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 20 mM Tris; 150 mM NaCl; 1 mM EDTA) containing protease inhibitors and orthovanadate. Lysates were subjected to immunoprecipitation using 2 μg of mouse anti-PLCγ1 or mouse IgG control and coupled to protein-G Sepharose (GE Healthcare). The degree of phosphorylation was determined using an antibody against pY783 and a protein-A HRP secondary antibody. The amount of immunoprecipitated protein was detected by stripping and reprobing membranes with rabbit anti-PLCγ1. Alternatively, transfected cell lysates were directly probed with anti-pY783, -pY771, or -pS1248 and stripped and reprobed for PLCγ1. For densitometry, the amount of phosphorylation was quantified using ImageJ and was normalized against the total PLCγ1 levels. Data was collected over three independent experiments.

For biochemistry involving hippocampal tissue, slices (300 μm thick) were prepared as described previously (Murai et al., 2003a) and kept *in vitro* for ~2–5 min before stimulation with Fc fusion proteins (9.5 μg/ml). After stimulation for 45 min, slices were lysed in RIPA buffer. Lysates were immunoprecipitated with either anti-EphA4 or anti-PLCγ1 antibodies coupled to protein-A or protein-G Sepharose, respectively. Phosphorylation of EphA4 was detected using an anti-phosphotyrosine antibody (PY20) coupled to HRP. PLCγ1 phosphorylation was detected with an anti-pY783 and protein-A HRP. Blots were stripped and reprobed by immunoblotting with anti-EphA4 or rabbit anti-PLCγ1 antibodies and anti-rabbit HRP to confirm that equal amounts of protein were immunoprecipitated from each condition.

The following procedure was used to coimmunoprecipitate EphA4 and PLCγ1 from mouse hippocampus. Hippocampi were dissected from the mouse brain at postnatal day 21 and homogenized in Triton lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1% Triton X-100, 10% glycerol, supplemented with protease inhibitors and sodium orthovanadate) using a Dounce homogenizer and lysed on ice for 15 min. Lysates were centrifuged at 13,000 rpm for 10 min at 4°C to pellet cell debris. Lysates were precleared for 1 h with protein-G Sepharose, then subjected to immunoprecipitation using 5 μg of mouse IgG control or mouse anti-PLCγ1 coupled to protein-G Sepharose for 5 h at 4°C. Immune complexes were washed three times with lysis buffer, boiled in 40 μl of 3× SDS sample buffer and resolved by SDS-PAGE. The association of EphA4 and PLCγ1 was detected by immunoblotting with a rabbit anti-EphA4 antibody.

GST pull-down assays. GST fusion proteins were prepared as described by Zisch et al. (1998). GST fusion proteins (GST, N-terminal SH2 PLCγ1, or C-terminal SH2 PLCγ1) coupled to agarose beads were incubated with transfected COS7 cell lysates [EphA4, EphA4 Y596E, or EphA4 Y602E solubilized in 1% Brij97 (Sigma) in PBS with protease inhibitors and orthovanadate] overnight at 4°C. The beads were subsequently washed with 1% Brij97/PBS (with protease inhibitors and orthovanadate), diluted with sample buffer, and boiled for 5 min. Supernatants were subjected to SDS-PAGE and blotted using anti-EphA4 antibodies and anti-rabbit HRP as described previously.

In vitro *kinase assay.* For *in vitro* kinase assays, endogenous PLCγ1 from COS7 cells was immunoprecipitated as described previously. PLCγ1 immunoprecipitates were resuspended in kinase buffer containing the following (in mM): 25 HEPES, pH 7.4, 25 β-glycerophosphate, 25 $MgCl_2$, 0.1 Na_3VO_4, 0.5 DTT, and 1 ATP. In conditions that included the activated kinase, 0.5 μg of recombinant EphA4 kinase domain was used.

The reaction mixture was incubated at 30°C for 30 min with gentle agitation every 10 min. The reaction was stopped by adding 25 μl of 3× SDS sample buffer and boiled for 5 min. Phosphorylation of PLCγ1 at Y783 was detected by Western blot analysis using pY783 and protein A-HRP. The total amount of PLCγ1 immunoprecipitated was determined by stripping and reprobing membranes with rabbit anti-PLCγ1. Data were collected from three independent experiments and quantified as described previously.

Synaptosome preparations. Synaptic proteins were purified as described in the supplemental information (available at www.jneurosci.org as supplemental material).

Cell-membrane preparations. Cell membrane fractionation experiments were performed as described previously (Cote et al., 2005). Briefly, cells were collected in PBS, centrifuged at 800 g for 5 min, and then resuspended in 300 μl of buffer A (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 1 μg/ml protease inhibitors). The cells were then subjected to a single freeze/thaw cycle in liquid nitrogen and a 37°C water bath and the membranes were pelleted using centrifugation at 16,000 g for 10 min. The pellet was then washed with 500 μl of buffer A and extracted with buffer B (buffer A plus 1% Triton X-100). Equal amounts of proteins were resolved by SDS-PAGE and the levels of membrane-associated cofilin analyzed by immunoblotting with cofilin antibodies. Equal loading between samples was confirmed by blotting for the transferrin receptor. For PLC inhibitor experiments, COS7 cells were seeded at a density of 1×10^6 cells/ml and the next day were treated with either the PLC inhibitor 1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122; EMD Biosciences, La Jolla, CA) or control compound 1-[6-([17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-2,5-pyrrolidine-dione (U73343; EMD Biosciences) at a final concentration of 10 μM for 3 h before collecting the cells. For ephrin-stimulation experiments, cells were seeded at a density of 1×10^5 cells/ml and the next day transfected with the cDNA for EphA4 using Lipofectamine 2000 (Invitrogen). Twenty-four hours after the transfection, the cells were treated with 8 μg/ml recombinant control Fc or ephrinA5-Fc fusion proteins for 40 min at 37°C. Membranes were then fractionated as described above. For densitometry, the amount of cofilin in each condition was quantified using ImageJ and normalized against the amount of transferrin receptor. Data was collected over three independent experiments.

For membrane fractionation in hippocampal slices after ephrin stimulation, 300 μm slices were made from postnatal day 10 mice and placed into stimulation media containing Fc or ephrin-A5 Fc proteins (9.5 μg/ml). After 45 min of stimulation, slices were coarsely homogenized, and then subjected to a single freeze/thaw cycle followed by the procedure described above. Data were collected from three independent experiments and quantified as described above.

Immunofluorescence in heterologous cells. For immunofluorescence studies in heterologous cells, COS7 cells were plated onto chambered slides (Nunc, Roskilde, Denmark) and transfected as described previously. Cells were fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 30 min, rinsed with TBS, and incubated for 1 h in blocking solution (5% goat serum/0.1% Triton X-100 in TBS). Cells were then incubated overnight (at 4°C) with anti-pY783 in blocking solution. The next day, the cells were washed with 0.1% Triton X-100/TBS and incubated with a goat anti-rabbit Alexa 568 secondary antibody (Invitrogen) for 1 h at room temperature. After washing cells with 0.1% Triton X-100/TBS, cells were coverslipped for microscopy.

Semliki Forest virus plasmid construction and virus preparation. For expressing PLCγ1 constructs and fluorescent proteins in hippocampal slices, Semliki Forest virus (SFV) constructs were created (Ehrengruber et al., 1999). PLCγ1 and membrane-targeted EGFPf (Clontech, Cambridge, UK) genes were each cloned 3′ to a viral subgenomic promoter in modified SFV vectors (Lundstrom et al., 2003). Viral particles were produced as described previously (Haber et al., 2006). SFV particles were injected into hippocampal slices with a Picospritzer (General Valve, Fairfield, NJ).

Hippocampal slice preparation. Organotypic hippocampal slices were prepared as described previously (Murai et al., 2003a). Briefly, 300 μm slices from postnatal day 6 (P6)–P7 mouse pups were made using a McIllwain tissue chopper (Stoelting, Kiel, WI) and transferred onto semiporous tissue culture inserts (0.4 μm pore size; Millipore) containing media (50% Minimum Essential Medium/25% Horse Serum/25% HBSS/6.5 mg/ml D-glucose/0.5% penicillin/streptomycin, pH ~7.2). Media was replaced every 2 d and slices were cultured for 1 week before viral gene delivery. Sixteen to 20 h postinfection, slices were fixed and mounted for confocal imaging.

For imaging hippocampal slices after stimulation, 300 μm slices were made from mice ~3 months of age and placed into stimulation media containing dimeric Fc or ephrin-A5 Fc and U73122 (PLC-inhibitor) or U73343 for 45 min. The slices were then fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 30 min. CA1 pyramidal cells were labeled using diolistics. Briefly, 1.3 μm tungsten particles (Bio-Rad, Hercules, CA) carrying the lipophilic dye DiI (Invitrogen) were coated onto the inner portion of plastic tubing, cut into cartridges, loaded into a Helios Gene Gun (Bio-Rad), and propelled into hippocampal slices at 120 psi using helium gas. The DiI was allowed to transport for 16 h in fixative before imaging by confocal microscopy.

For RNAi experiments, shRNA plasmids (see above, DNA constructs) were delivered using biolistic gene transfer with a Helios Gene Gun (Bio-Rad). Briefly, PLCγ1 shRNA and control shRNA plasmids were precipitated onto 1.6 μm gold microcarriers and deposited on the inner lining of Tefzel tubing (Bio-Rad) to generate bullets. Hippocampal slices were prepared as described above from postnatal day 5 mice and cultured for 9–10 d before propelling gold microcarriers onto hippocampal slices at 100 psi using helium gas. To improve the efficiency of CA1 pyramidal cell transfection, a 3.0 μm membrane filter (Millipore) was placed between the gene gun nozzle and the hippocampal slices. The shRNA constructs were allowed to express for 72 h before fixation and imaging by confocal microscopy.

Confocal imaging. Confocal imaging was performed with a Yokogawa spinning disk confocal system (Perkin-Elmer, Wellesley, MA) connected to a Nikon (Tokyo, Japan) Eclipse TE2000. *Z*-stacks were collected using MetaMorph imaging software (Molecular Devices, Palo Alto, CA).

Image analysis. For analysis of dendritic spine morphology, images of dendrites were taken for each condition [control, PLCγ1 wild-type (wt), or PLCγ1 LIM, or PLCγ1 shRNA or control shRNA] from three independent experiments. Each image contained a *Z*-stack maximum projection of a primary apical dendrite from a CA1 pyramidal cell taken ~100 μm from the cell body (control, 16 dendritic segments with 568 spines total; PLCγ1 wt, 26 dendritic segments with 886 spines total; PLCγ1 LIM, 22 dendritic segments with 603 spines total). For RNAi experiments, 33 dendritic segments (with 889 spines) were used in the control shRNA condition and 41 dendritic segments (with 918 spines) in the PLCγ1 shRNA condition. All images were normalized for EGFPf signal intensity and thresholded in Photoshop (Adobe Systems, Seattle, WA). Geometric measurements of spine parameters (length, head width, area, and density) were acquired using the Reconstruct computer program (http://synapses.bu.edu/tools/index.htm). Spines were defined as any protrusion from the dendritic shaft that is not a dendrite branch. The dividing line of the spine head and neck was determined subjectively using the contours of the dendritic spine head. For spines without clearly defined head portions (i.e., stubby or elongated spines), the spine head area was considered equal to the total spine area and spine head width equal to the neck width. For each dendritic segment, the spine parameters were combined to generate an average value that was then used for comparisons. All quantifications were performed by an investigator blind to the experimental conditions. Using a computer to sort spines according to the measured parameters, spines were classified into four types (i.e., "mushroom," "stubby," "elongated," or "other"). The mushroom category corresponds to spines that have enlarged head regions with a constricted neck. Stubby spines lack neck regions. The elongated category includes filopodia-like spines and long spines with small, but well formed head regions. The other category includes spines with abnormal dimensions, such as those with large heads and highly branched spines that do not fit into the three other categories. Differences between samples were performed using a *t* test or ANOVA with a Student–Newman–Keuls *post hoc* comparison.

For stimulation experiments, images of dendrites were acquired sim-

ilar to as described above over three independent experiments. Thirty-five dendritic segments (with 752 spines) were used for the Fc plus U73343 condition, 33 segments (with 752 spines) were used for the Fc plus U73122 condition, 33 segments (with 686 spines) were used for the ephrin-A plus U73343 condition, and 31 segments (with 704 spines) were used for the ephrin-A plus U73122 condition. Quantifications were performed by an investigator blind to the experimental conditions and differences between samples were performed using a Kolmogorov–Smirnov test or ANOVA with a Student–Newman–Keuls *post hoc* comparison.

Results

EphA4 activates PLCγ1 in heterologous cells and hippocampal slices

We reported previously that EphA4 activation with ephrin-A ligands induces dendritic spine retraction (Murai et al., 2003a). However, the downstream signaling cascades of this receptor remain to be fully described. Using GST pull-down assays from hippocampal lysates, we initially found that EphA4 bound well to an SH2 domain of PLCγ1, a protein known to be important for phosphoinositide metabolism, cell morphology, and synaptic function (Rebecchi and Pentyala, 2000). To follow up on this observation, we transfected EphA4 into COS7 cells (which have little endogenous EphA4) while overexpressing PLCγ1 (Fig. 1*A*). EphA4 caused an increase in the phosphorylation of tyrosine 783 of PLCγ1, which is critical for PLCγ1 activity and is an indicator of its level of activation (Kim et al., 1991; Poulin et al., 2005). This phosphorylation event, however, was not detected after transfection of a kinase-dead form of EphA4, suggesting that kinase-dependent signaling is required for PLCγ1 activation downstream of EphA4.

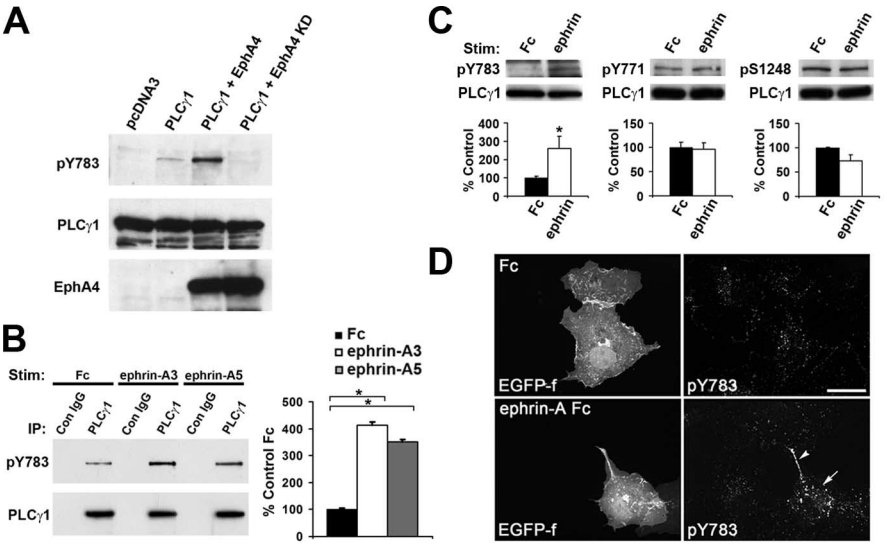

Figure 1. EphA4 activates PLCγ1. ***A***, Transfection of EphA4 increased the phosphorylation of overexpressed PLCγ1 on tyrosine 783, a site known to correlate with PLC activity. Expression of a kinase-dead form of EphA4, however, did not elevate PLCγ1 phosphorylation. Note that transfection of PLCγ1 alone led to a slight increase in phosphorylation over the control transfected condition (pcDNA3). Shown are blots of lysates derived from cells transfected with the indicated constructs. ***B***, Stimulation (Stim) of EphA4 transfected COS7 cells for 45 min with ephrin-A3 Fc- or ephrin-A5 Fc-induced phosphorylation of endogenous PLCγ1. PLCγ1 was immunoprecipitated after stimulation of cells with control Fc, ephrin-A3 Fc, or ephrin-A5 Fc and probed for phosphorylation on tyrosine 783. The membrane was subsequently stripped and reprobed for PLCγ1. Control IgGs were used to confirm the specificity of the immunoprecipitation. Quantification of these changes showed that both ephrin-A3 Fc and ephrin-A5 Fc significantly increased PLCγ1 phosphorylation (*$p < 0.05$, ANOVA). ***C***, Immunoblots of lysates of cells stimulated with control Fc or ephrin-A Fc. Ephrin-A stimulation caused a significant increase in phosphorylation of Y783, but not Y771 or S1248 of PLCγ1 (*$p < 0.02$, *t* test). ***D***, Immunofluorescence labeling showing increased labeling of EphA4 transfected COS7 cells with the anti-pY783 antibody. Note that the labeling was seen as clusters on the cell body (arrow) and processes (arrowhead) of EphA4, but not control transfected cells (EGFPf). KD, Kinase-dead. Scale bar, 30 μm. Error bars indicate SEM.

We were next interested in determining whether stimulation of EphA4 transfected COS7 cells with recombinant ephrin-A (vs control Fc) proteins would lead to an elevation in the phosphorylation of endogenous PLCγ1. In these experiments, we used endogenous PLCγ1 because we found that there is high basal level of PLCγ1 in COS7 cells. As shown in Figure 1*B*, ephrin-A stimulation caused a significant activation of PLCγ1 as indicated by its phosphorylation on tyrosine 783. This effect was observed with both recombinant ephrin-A3 Fc and ephrin-A5 Fc fusion proteins, but not control Fc (Fc vs ephrin-A3 Fc, $p < 0.05$; Fc vs ephrin-A5 Fc, $p < 0.01$, ANOVA). We further determined the specificity of this effect using lysates from EphA4-transfected cells stimulated with Fc or ephrin-A5 Fc and found that ephrin treatment caused a significant increase in PLCγ1 activation (pY783, 260% of control Fc stimulation; $p = 0.016$, two-tail *t* test) (Fig. 1*C*). Phosphorylation on tyrosine 771, a residue whose phosphorylation is not correlated with PLCγ1 activity (Kim et al., 1991), or serine 1248, which is potentially an inhibitory phosphorylation site for protein kinases C and A (Park et al., 1992) were not significantly changed (pY771, $p = 0.78$, two-tail *t* test, pS1248; $p = 0.091$, two-tail *t* test) (Kim et al., 1990, 1991). Consistent with these biochemical studies, we detected an increase in pY783 labeling of EphA4 transfected COS7 cells stimulated with ephrin-A Fc using immunofluorescence microscopy (Fig. 1*D*). Increased labeling was especially apparent as clusters on the cell body and processes of stimulated cells. These combined results show that EphA4 stimulation with ephrin ligands induces PLCγ1 activation by specifically enhancing its phosphorylation on tyrosine 783.

To determine whether ephrin stimulation could activate PLCγ1 signaling in the mouse hippocampus, we stimulated hippocampal slices with ephrin-A Fc and monitored the phosphorylation of EphA4 and PLCγ1. Similar to the experiments using COS7 cells, we found that ephrin-A Fc treatment caused an increase in phosphorylation of PLCγ1 on tyrosine 783 (Fig. 2*A*). To examine the time course for this activation, we stimulated slices for varying time periods and found that PLCγ1 activation was significantly increased after 45 min of ephrin stimulation (Fig. 2*B*,*C*). These results demonstrate that ephrin-A stimulation can activate endogenous EphA4 and PLCγ1 signaling in slices derived from the hippocampus. However, based on these results, we cannot conclude if PLCγ1 is activated earlier (i.e., between 15 and 45 min) after the onset of ephrin stimulation. Furthermore, we cannot exclude the possibility that EphA4 activates PLCγ1 through an intermediary protein or that other proteins are activated in parallel (i.e., a counteracting phosphatase) that cause the delayed kinetics of PLCγ1 activation.

EphA4 binds the C-terminal SH2 domain of PLCγ1 through juxtamembrane tyrosines

PLCγ1 is a multidomain signaling protein that contains, in addition to functional catalytic regions, two SH2 domains (N- and C-terminal) and an SH3 domain. The SH2 domains of PLCγ1, in particular, have been shown to play an important role in regulat-

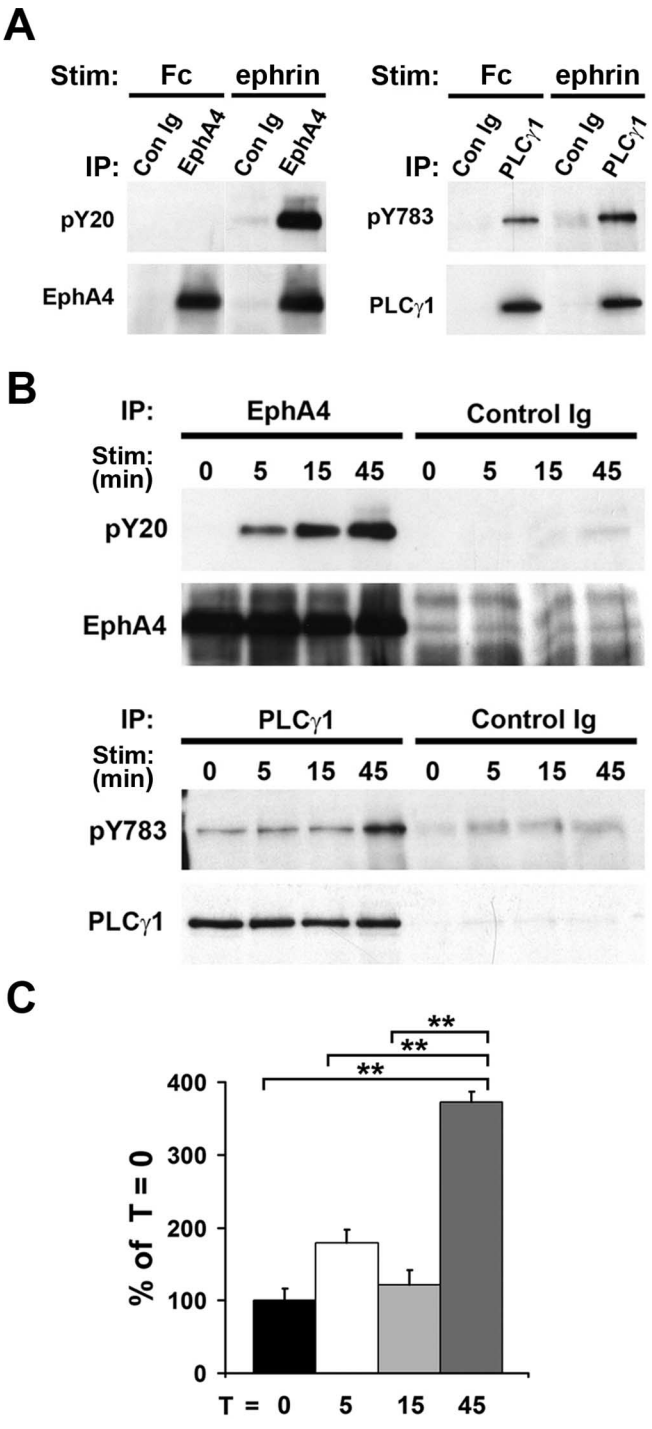

Figure 2. Ephrin stimulation induces the activation of PLCγ1 in hippocampal slices. ***A***, Stimulation (Stim) of hippocampal slices with ephrin-A Fc induced phosphorylation of endogenous EphA4 and PLCγ1. After 45 min of ephrin-A Fc or control Fc stimulation of slices, EphA4 or PLCγ1 were immunoprecipitated and blotted for phosphotyrosine (pY20) or pY783 of PLCγ1, respectively. Control IgGs were used to confirm the specificity of the immunoprecipitation (IP). Membranes were subsequently stripped and reprobed for either EphA4 or PLCγ1 to ensure that equal amounts of protein were initially immunoprecipitated. ***B***, ***C***, Time-course analysis showing PLCγ1 phosphorylation after 45 min of stimulation. Quantification of these changes showed a significant increase in phosphorylation at 45 min (**$p < 0.01$, ANOVA). Error bars indicate SEM.

ing its function through protein interactions with activated receptor tyrosine kinases (Rebecchi and Pentyala, 2000). We were interested in testing whether EphA4 may interact with PLCγ1 through binding its SH2 domains. Indeed, EphA4 has been reported to recruit SH2 domain containing proteins including Fyn, Src, and Vav2 through its highly conserved juxtamembrane tyrosine residues (Kalo and Pasquale, 1999; Cowan et al., 2005). To address whether EphA4 binds PLCγ1 through an SH2-domain interaction, we performed GST pull-down experiments using the SH2 domains of PLCγ1 fused to GST and EphA4-transfected COS7 cell lysates. We found that the C-terminal SH2 domain of PLCγ1 bound strongly to ephrin-A-stimulated EphA4 expressing COS7 cells (Fig. 3*A*). We could not detect binding of the N-terminal SH2 domain to EphA4. The binding to the C-terminal SH2 domain, however, was severely diminished after mutating either of the conserved juxtamembrane tyrosines of EphA4 to glutamic acid (Y596E or Y602E). Glutamic acid mutations have been shown to eliminate SH2-domain interactions of Eph receptors without interfering with kinase activity (Zisch et al., 2000; Cowan et al., 2005). We did observe very weak binding of the EphA4 Y596E mutant to the C-terminal SH2 domain of PLCγ1 after long film exposures (data not shown), but no binding to the Y602E mutant. Phosphorylation of these tyrosines may function cooperatively to recruit PLCγ1.

We further extended these findings by investigating the requirement of the EphA4 juxtamembrane tyrosines for PLCγ1 activation after ephrin stimulation. We found that both the Y596E and Y602E mutants were compromised in their ability to activate PLCγ1 above control Fc treatment levels in the presence of ephrin stimulation (Fig. 3*B*). However, the Y596E mutant appeared to have higher basal ability to cause PLCγ1 phosphorylation in the absence of ephrin, suggesting that this mutation may mimic phosphorylation on EphA4 and lead to a partial activation of PLCγ1 in the absence of ligand. The Y602E mutant, in contrast, only weakly phosphorylated PLCγ1 in the presence of ephrin, indicating that phosphorylation of tyrosine 602 of EphA4 is important for activating PLCγ1. Altogether, these results suggest that EphA4 uses juxtamembrane tyrosines to interact with the C-terminal SH2 domain of PLCγ1.

We also further tested the interaction between EphA4 and PLCγ1 by coimmunoprecipitation experiments, *in vitro* kinase assays, and double-label immunofluorescence in hippocampal slices. We found that EphA4 coimmunoprecipitated with PLCγ1 from lysates derived from COS7 cells (data not shown) and adult mouse hippocampus (Fig. 3*C*). The EphA4 kinase domain was also able to phosphorylate Y783 of PLCγ1 (Fig. 3*D*) in *in vitro* kinase assays. Furthermore, we found that EphA4 and PLCγ1 colocalized on at least a subset of spines (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). However, we could not rule out the possibility that we did not detect the full extent of these proteins because immunofluorescence is not a sensitive method to detect the subcellular location of proteins in tissues. Thus, these proteins may be more prevalent than what is observed in supplemental Figure 2 (available at www.jneurosci.org as supplemental material). Synaptosome fractionation, a more sensitive technique, was performed to further characterize the postsynaptic localization of PLCγ1.

PLCγ1 is localized in several compartments of brain synaptosomes and cofractionates with postsynaptic density proteins

Several reports have implicated PLCγ1 in synaptic transmission and plasticity (Reyes-Harde and Stanton, 1998; Micheva et al., 2001; Gartner et al., 2006). It also has been shown that PLCγ1 is detected in the adult brain and hippocampus (CA1–3 regions) through *in situ* hybridization and immunohistochemistry (Gerfen et al., 1988; Ross et al., 1989). One report suggested that PLC

isoforms function postsynaptically to block long-term synaptic plasticity (Reyes-Harde and Stanton, 1998). This was observed after infusion of the PLC inhibitor U73122 into CA1 pyramidal cells, which blocked the induction of hippocampal long-term depression (LTD). However, it remains unclear whether PLCγ1 is positioned to influence the postsynaptic terminal, including dendritic spines. To pursue this issue, we purified brain synaptosomes and further separated them into presynaptic and postsynaptic elements (Phillips et al., 2001). We found that PLCγ1 is found in multiple synaptic compartments, showing cofractionation with presynaptic active zones and extrajunctional regions. However, similar to PSD-95, PLCγ1 was readily apparent in postsynaptic density fractions, including the Triton-insoluble "core" PSD fraction (PSD III) (supplemental methods, available at www.jneurosci.org as supplemental material) (Fig. 4). These results indicate that PLCγ1 is localized to various regions of the synapse, including postsynaptic terminals, and could play a role in regulating postsynaptic properties including dendritic spine structure.

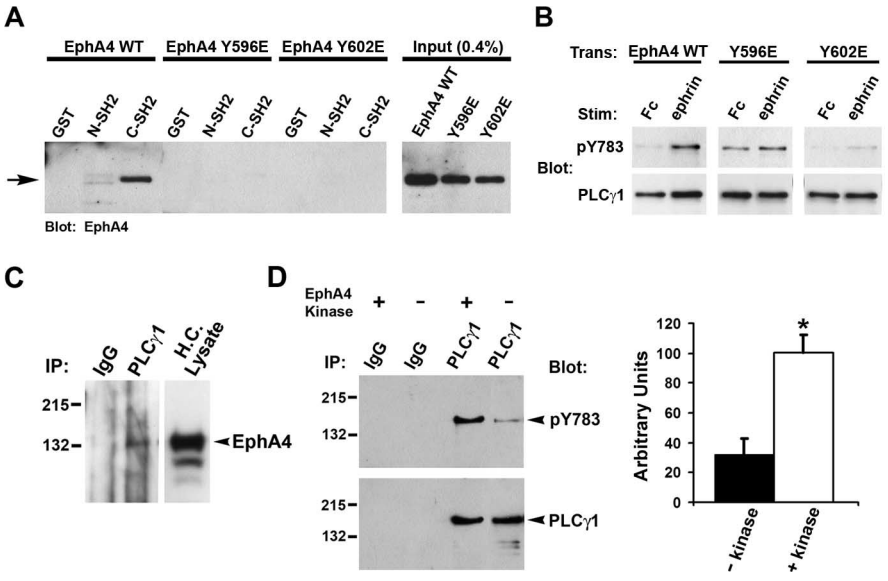

Figure 3. EphA4 interacts with PLCγ1. ***A***, GST pull-down assay using the N- (N-SH2) and C-terminal SH2 domains (C-SH2) of PLCγ1 and lysates from COS7 cells transfected with wild-type EphA4, Y596E EphA4 mutant, or Y602E EphA4 mutant and stimulated with ephrin-A Fc. EphA4 strongly bound the C-terminal SH2 domain of PLCγ1. We observed very weak binding of the C-terminal SH2 domain to the Y596E mutant after long film exposures. Lysates to the right (representing only 0.4% of total input) confirm the expression of the transfected receptors in each of the conditions used for the pull-down experiments. ***B***, Stimulation of transfected (trans) COS7 cells expressing wild-type, Y596E, or Y602E mutants of EphA4 with ephrin-A Fc showed that both Y596E and Y602E EphA4 mutants have compromised ability to activate PLCγ1 after ephrin-A stimulation (Stim). PLCγ1 was immunoprecipitated after stimulation of cells with control Fc or ephrin-A Fc and probed for phosphorylation on tyrosine 783. The membrane was subsequently stripped and reprobed for PLCγ1. The Y596E mutant appeared to have higher basal ability to activate PLCγ1. The Y602E mutant showed only low levels of PLCγ1 activation after ephrin-A stimulation. ***C***, EphA4 coimmunoprecipitated with PLCγ1 from postnatal day 21 mouse hippocampus. PLCγ1 or control IgG immunoprecipitates (IP) were blotted for EphA4. ***D***, PLCγ1 immunoprecipitates were subjected to *in vitro* kinase assays with or without a recombinant EphA4 kinase domain. The EphA4 kinase domain phosphorylated PLCγ1 on tyrosine 783. The blots were subsequently stripped and reprobed for PLCγ1 protein (*$p < 0.05$, *t* test). Error bars indicate SEM.

PLC signaling maintains dendritic spine morphology

Because PLCγ1 is found in postsynaptic fractions of synaptosomes and is known to be involved in synaptic function, we were interested in testing whether PLCγ1 activity may influence postsynaptic dendritic spine morphology. To test this possibility, we used Semliki Forest viruses to introduce wild-type PLCγ1 or a lipase-inactive mutant of PLCγ1 (PLCγ1 LIM) into CA1 pyramidal cells of organotypic hippocampal slices (Fig. 5*A*). The latter of which has been shown to function as a dominant-negative protein (Huang et al., 1995; Ronnstrand et al., 1999; Rong et al., 2003). We analyzed the morphology of CA1 pyramidal cell spines after 20 h of expression. No apparent changes in overall dendrite length or branching, or signs of dendritic pathology were observed in any of the conditions. Overexpression of wild-type PLCγ1 did not significantly affect the structural properties of dendritic spines when compared with EGFPf controls. Interestingly, the PLCγ1 LIM induced profound changes in spine morphology. In particular, spines showed a significant increase in length and area whereas the number of spines per unit length was reduced (Fig. 5). In some cases, the spines showed an abnormal phenotype, having multiple head portions with a very complex architecture. However, most spines in this condition retained a bulbous appearance with an enlarged head portion attached to the dendritic shaft through a narrow neck region. This was further corroborated by classifying spines into mushroom, stubby, elongated, or other-type morphologies. We found that expression of the PLCγ1 LIM caused a significant loss of stubby spines. Although expression of wild-type PLCγ1 did slightly reduce the amount of stubby spines, this effect was not significant when compared with

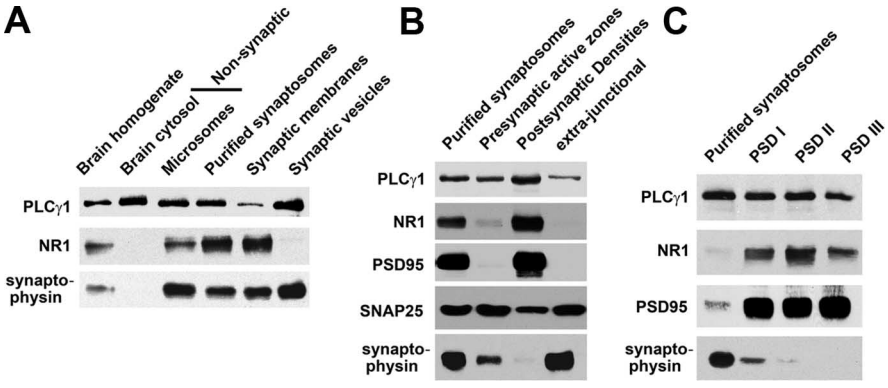

Figure 4. PLCγ1 is found in multiple compartments of synaptosomes, but is enriched in postsynaptic density fractions. ***A***, PLCγ1 was found in nonsynaptic fractions (cytosolic and microsomal) and in synaptic membranes and vesicles in the adult mouse brain as shown by immunoblotting with the anti-PLCγ1 polyclonal antisera. Antibodies against NR1 and synaptophysin were used as synaptic membrane and synaptic vesicle markers, respectively. ***B***, PLCγ1 was present on both sides of the synapse. PLCγ1 was detected in presynaptic active zones and extrajunctional membrane fractions and was readily apparent in the PSD fraction. SNAP25 and PSD95 were used as presynaptic and postsynaptic density markers, respectively. ***C***, PLCγ1 is strongly associated with PSDs. PLCγ1 was insoluble to 1% Triton X-100 (PSD I and II) and 3% sarcosyl (PSD III) and remained associated with the "core" PSD (PSD III) (supplemental methods, available at www.jneurosci.org as supplemental material).

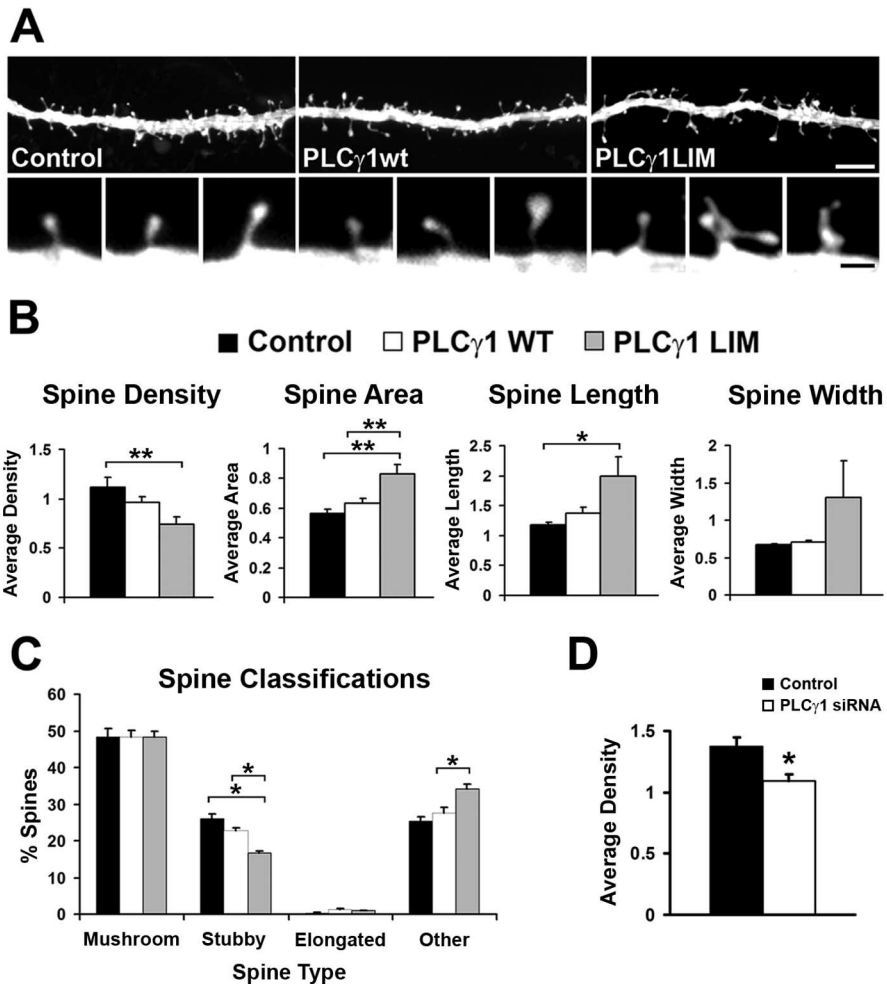

Figure 5. PLCγ1 activity is necessary for maintaining CA1 dendritic spine morphology. ***A***, Examples showing abnormal dendritic spine morphology after expression of the PLCγ1 LIM when compared with control CA1 cells expressing membrane-targeted EGFPf. ***B***, Quantification of spine parameters showed that spine density was significantly reduced after expression of the PLCγ1 LIM when compared with control. Significant increases in area and length of spines were also found. Spine width, although on average larger in the PLCγ1 LIM condition, was not significantly different among groups. No prominent changes were observed after overexpressing wild-type PLCγ1 ($*p < 0.05$, $**p < 0.01$, ANOVA with Student–Newman–Keuls *post hoc* comparisons). ***C***, Expression of the PLCγ1 LIM also induced a significant decrease in the number of stubby-type spines ($*p < 0.05$, $**p < 0.01$ ANOVA with Student–Newman–Keuls *post hoc* comparisons). ***D***, RNAi in organotypic slices after biolistic delivery of a vector expressing a PLCγ1 shRNA showed that PLCγ1 is necessary for maintaining spine density ($*p < 0.05$, *t* test). Scale bars: ***A***, 5 μm; high-magnification images of individual spines, 1 μm. Error bars indicate SEM.

the control. These results suggest that PLCγ1 signaling is required for spine stability and maintaining dendritic spine architecture, especially those with a stubby, retracted appearance.

In a separate set of experiments, we also tested the requirement of PLCγ1 in maintaining dendritic spine morphology. To investigate this, we performed RNA interference (RNAi) by biolistically delivering a plasmid to CA1 pyramidal cells that simultaneously induces the expression of a short-hairpin RNA directed against PLCγ1 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and drives the expression of EGFPf. The sequence used for RNAi has been characterized previously and shown to selectively knock down PLCγ1 expression, although leaving the expression of the closely related family member PLCγ2 intact (Patterson et al., 2002). A plasmid that drives the expression a scrambled sequence of the PLCγ1 shRNA was used as a control. Consistent with the experiments above using the PLCγ1 LIM, knockdown of PLCγ1 expression significantly reduced the density of dendritic spines. However, we could not detect significant changes in overall spine morphology or classification (data not shown). A potential explanation for this may be that the RNAi experiments were performed after 72 h of knockdown in CA1 cells (vs 20 h for the PLCγ1 LIM experiments). It is possible that spines with perturbed morphology collapsed during the 72 h period of PLCγ1 knockdown which, in turn, caused the reduction in spine density.

We attempted to rescue the spine phenotype using a mutated form of PLCγ1 (with the RNAi site mutated in four wobble positions), but this was not successful (data not shown). However, many technical issues could have complicated the rescue. One reason may be the timing of expression of the PLCγ1 shRNA and the rescue protein, which use different promoters (H1 RNA pol III and cytomegalovirus, respectively) and different cellular mechanisms for their production. Based on our experiments with PLCγ1 RNAi in NIH3T3 cells (which found that 72 h was needed for efficient PLCγ1 knockdown), we assessed spine morphology and density in CA1 neurons in hippocampal slices after 72 h of expression. However, it was unclear whether the lack of rescue was attributable to insufficient levels of the rescue protein present during PLCγ1 knockdown.

PLC signaling is necessary for ephrin-induced spine shrinkage

We next wanted to determine whether PLCγ1 signaling was required for transmitting signals downstream of EphA4 in dendritic spines. Previously, we showed that EphA4 is localized on dendritic spines and 45 min of ephrin treatment of hippocampal slices induced spine retraction in a kinase-dependent manner (Murai et al., 2003a). We further showed that EphA4 is the main EphA class receptor mediating these effects. Furthermore, stimulation of EphB receptors only induced small changes in dendritic spines in area CA1 of the hippocampus. To address whether PLC signaling was required for the acute effects of ephrin treatment on dendritic spine morphology, we incubated hippocampal slices with either ephrin-A or control Fc proteins in the presence of either the PLC inhibitor U73122 (see Materials and Methods) or control analog U73343 (Fig. 6). We found that incubation of slices with control Fc proteins along with the PLC inhibitor did not significantly affect the individual parameters of dendritic spines. The PLC inhibitor, however, caused a significant reduction in mushroom-type spines when compared with the control condition (Fc with control analog) (Fig. 6*E*), while not affecting the percentage of stubby, elongated, or other-type spines. This result differs from what we observed previously after longer-term expression of the PLCγ1 LIM and may reflect differential effects on spines under acute (PLC inhibitor) or prolonged (PLCγ1 LIM) blockade of PLC signaling. Similar to what was

reported previously (Murai et al., 2003a), acute ephrin-A treatment induced spine retraction (Kolmogorov–Smirnoff two-sample test, $p < 0.05$) and significantly promoted the generation of stubby spines (Fig. 6*E*). The generation of these spines was likely at the expense of mushroom-type spines because overall spine density was not significantly perturbed in any of the treatments. Remarkably, simultaneous treatment of slices with U73122 along with ephrin blocked the effects of ephrin on inducing the stubby dendritic spine phenotype (Fig. 6*D*,*E*). The percentage of both mushroom and stubby type spines was similar between conditions composed of the U73122 with either Fc or ephrin-A. These results suggest that PLC signaling is necessary for the effects of ephrin-A-induced generation of stubby and retracted dendritic spines. It should be noted that U73122, although specific for PLC enzymes, can inhibit multiple PLC isoforms (Smith et al., 1990). However, the only other PLC isoform that is known to signal downstream of RTKs other than PLCγ1 is PLCγ2, whose expression is restricted to the anterior lobe of the pituitary and the cerebellum (Tanaka and Kondo, 1994). However, we cannot rule out the possibility that Eph receptors can signal through other PLC proteins (i.e., PLC forms that are not commonly associated with RTKs) and that this signaling would be blocked by U73122.

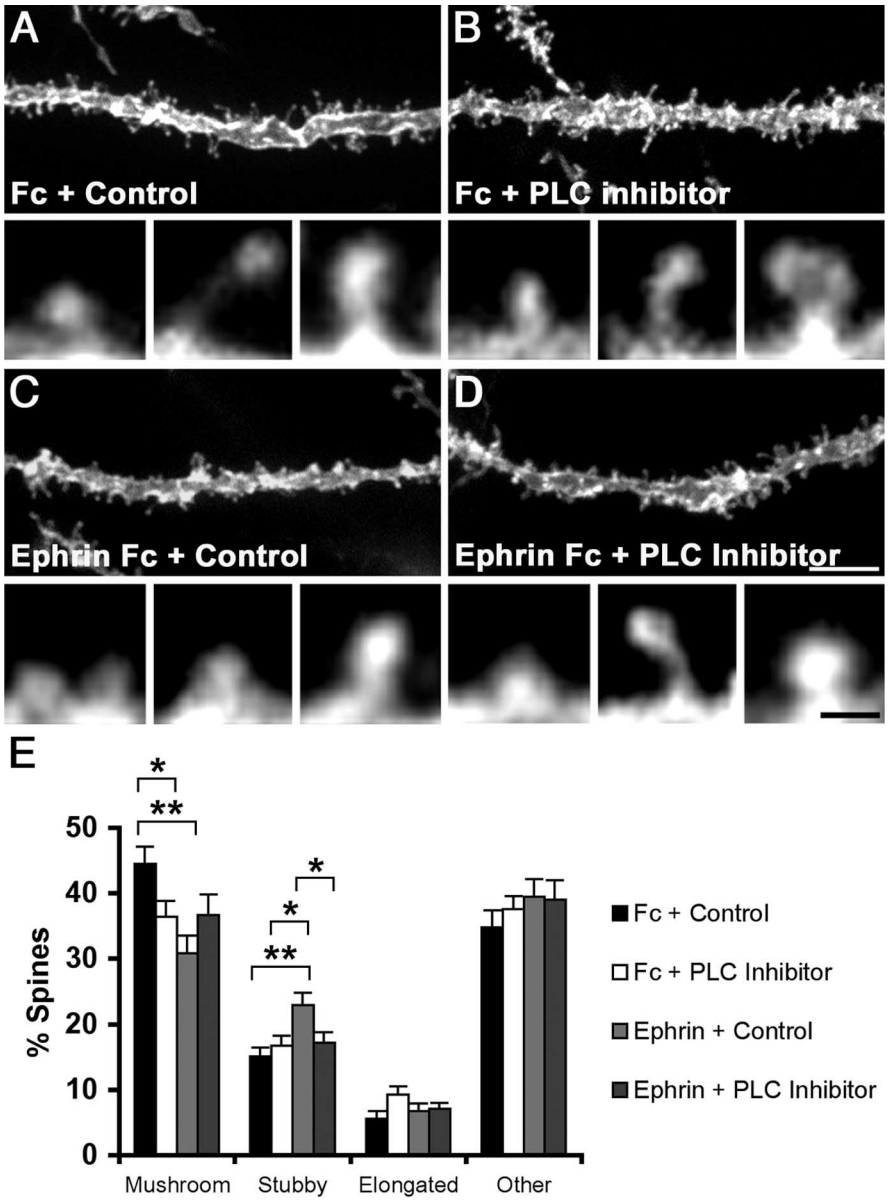

Figure 6. PLC activity is necessary for ephrin-A-induced dendritic spine retraction. ***A–D***, DiI-labeled CA1 dendritic spines in hippocampal slices stimulated with Fc and control analog (***A***), Fc and PLC inhibitor (***B***), ephrin Fc and control analog (***C***), or ephrin Fc and PLC inhibitor (***D***). Note the retracted appearance of dendritic spines in ***C***. Classification of spines showed that application of the PLC inhibitor blocked the ability of ephrin to induce a retracted, stubby spine phenotype. Treatment of slices with Fc or ephrin Fc with the PLC inhibitor significantly decreased the percentage of mushroom-shaped spines, suggesting that blocking PLC activity alone can destabilize mushroom-type spines (*$p < 0.05$, **$p < 0.01$ ANOVA with Student–Newman–Keuls *post hoc* comparisons). Scale bar: ***A***, 5 μm; high-magnification, 0.5 μm. Error bars indicate SEM.

PLC signaling regulates the localization of the actin-depolymerizing and -severing factor cofilin

Previous studies have proposed that PLCγ1, by generating IP_3 and DAG second messengers from cleavage of PIP_2, plays an important role in regulating cell membrane levels of PIP_2. PIP_2 itself can have dramatic effects on cell behavior and cytoskeletal-plasma membrane adhesion (Raucher et al., 2000). PIP_2 may act as a second messenger by binding and modifying the activity state of various proteins including ion channels and actin-binding proteins such as cofilin, a protein previously implicated in the structural plasticity of spines (Zhou et al., 2004). Cleavage of PIP_2 by PLCγ1 may thus serve to modulate PIP_2–protein interactions. It has been shown that PIP_2 tethers gelsolin to the cell surface and releases it after EGF stimulation and PLCγ signaling (Chou et al., 2002). We were interested in investigating the possibility that EphA4 and PLCγ1 signaling regulates cofilin association with the cell membrane because cofilin is highly expressed in dendritic spines, binds PIP_2, and has been implicated in mediating the effects of dendritic spine shrinkage after low-frequency, LTD-inducing stimuli (Zhou et al., 2004; Racz and Weinberg, 2006). To test this, we performed cellular fractionation experiments in which we isolated membrane components of COS7 cells after U73122 or control analog treatment. We found that blocking PLC activity with U73122 significantly enhanced the level of cofilin that was associated with the cell membrane (Fig. 7*A*). This is in contrast to the transferrin receptor, a nonraft localized receptor associated with the cell membrane, whose membrane association was not altered by U73122 treatment. These results are in accordance with other reports that indicate that a portion of cofilin is associated with the cell periphery (Suzuki et al., 1995; Heyworth et al., 1997). Interestingly, we also found that transfection of EphA4 alone could reduce the levels of cofilin found in the membrane fraction. We could also further decrease this amount with ephrin-A stimulation (Fig. 7*B*). Similarly, ephrin stimulation of hippocampal slices derived from postnatal day 10 mouse

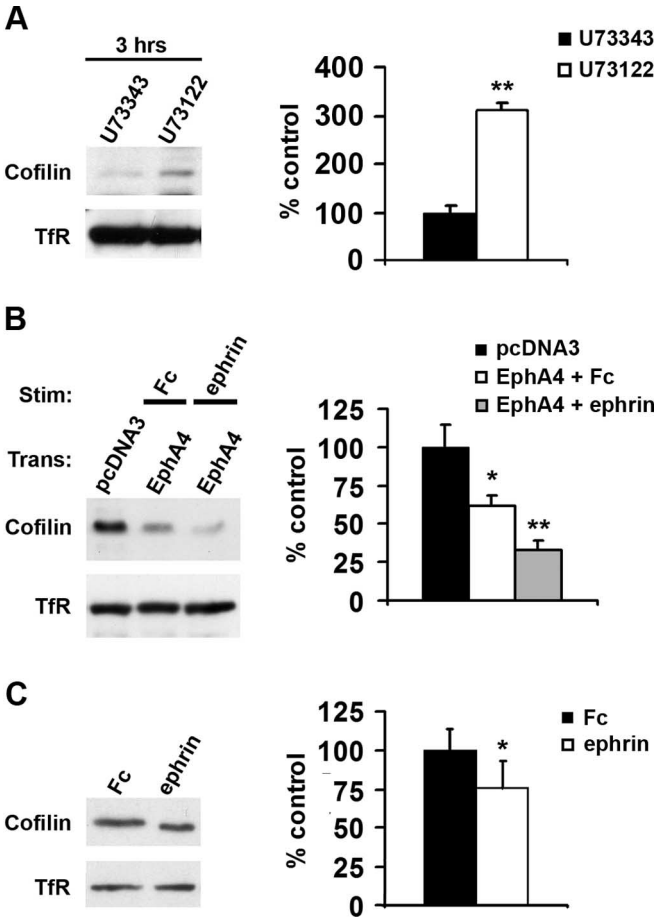

Figure 7. PLC activity and ephrin stimulation alter the membrane association of the actin-depolymerizing/severing protein cofilin. ***A***, Application of the PLC inhibitor (3 h) increased the levels of cofilin associated with the cell membrane of COS7 cells. The transferrin receptor (TfR) was used to ensure equal loading among lanes. ***B***, EphA4 expression in COS7 cells reduces the membrane-association of cofilin. This association was further reduced by ephrin stimulation ($*p < 0.05$, ANOVA with Student–Newman–Keuls *post hoc* comparisons; $**p < 0.01$, *t* test or ANOVA with Student–Newman–Keuls *post hoc* comparisons). ***C***, Ephrin treatment of postnatal day 10 hippocampal slices also reduced the level of cofilin associated with the cell membrane ($*p < 0.05$, *t* test). Error bars indicate SEM.

brain also significantly caused a reduction in the level of cofilin associated with the cell membrane (Fig. 7*C*). However, because we are using hippocampal tissue, the effects that we observed may be complicated by cofilin located in other cell types in addition to neurons that are found in slices such as oligdendrocytes and astrocytes.

These collective results suggest that EphA4 signaling and PLC activation contribute to the release of a pool of cofilin associated with the cell membrane. Removal of the membrane tethering of cofilin may release it from inhibition and allow it to bind and depolymerize actin filaments (DesMarais et al., 2005).

Discussion

The structural properties of dendritic spines are believed to be closely linked to the physiology of excitatory synapses (Segal, 2005). Spine anatomy is also disrupted in several diseases affecting the brain including Down, Fragile-X, and Williams syndromes, suggesting altered synapse morphology is related to cognitive impairments (Sawa and Snyder, 2002). Previous studies have elucidated the molecular composition of spines and mechanisms that govern their morphology (Tada and Sheng, 2006). However, few reports have revealed the signaling cascades that couple receptors to direct regulators of the actin-rich cytoskeleton in spines. Here, we report a novel signaling pathway downstream of the EphA4 receptor tyrosine kinase linking PLCγ1 to the actin- depolymerizing/severing protein cofilin. We found that ephrin stimulation promotes the interaction between the juxtamembrane tyrosines of EphA4 and the C-terminal SH2 domain of PLCγ1. PLCγ1 signaling is critical for maintaining spine morphology and PLC activity is required for ephrin-induced spine retraction. Remarkably, the amount of cofilin associated with the cell membrane is regulated by PLC and EphA4 activity. This signaling may be important for the local remodeling of the actin cytoskeleton in spines at sites of ephrin-A/EphA4 contact.

Spines rely on actin filaments for their dynamics and these filaments are in a constant state of equilibrium, cycling between filamentous actin (F-actin) and globular actin (G-actin) forms. Time-lapse imaging has shown that actin is rapidly reorganized in spines over minutes (Fischer et al., 1998). Long-term potentiation (LTP)-inducing stimuli increases F-actin in spines that occurs during spine enlargement (Okamoto et al., 2004). Low-frequency stimulation, in contrast, increases G-actin levels and causes spine shrinkage. Furthermore, pharmacological inhibition of actin polymerization blocks the induction and maintenance of LTP in hippocampal slices (Kim and Lisman, 1999). Thus, actin filament dynamics are related to bidirectional changes in the structural and physiological plasticity of excitatory synapses.

Previous studies indicate a critical role for Eph receptors in spine morphogenesis and maintenance (Murai and Pasquale, 2004). EphB2 induces spine development in hippocampal neurons by phosphorylating the proteoglycan syndecan-2 (Ethell et al., 2001) and assembling a complex that elicits RhoA signaling (Moeller et al., 2006). Other studies have shown that EphB2 activates the exchange factors intersectin and kalirin to promote spine development through Cdc42 and Rac, respectively (Irie and Yamaguchi, 2002; Penzes et al., 2003). There is some redundancy for EphB receptors because only neurons from double and triple knock-outs of EphB1, EphB2, and EphB3 exhibit changes in spine density and morphology (Henkemeyer et al., 2003; Hoogenraad et al., 2005). Interestingly, F-actin is accumulated in dendritic shafts rather than spines of EphB knock-out mice, suggesting that EphB receptors influence the distribution of actin filaments (Henkemeyer et al., 2003). To date, the only EphA receptor shown to modulate spine morphology is EphA4 (Murai et al., 2003a). EphA4 is enriched in the developing and adult mouse hippocampus and is localized on spines (Murai et al., 2003a,b). Activation of EphA4 by ephrin-A results in spine retraction (Murai et al., 2003a). This could be mediated by EphA4 signaling induced by contact with ephrins on neurons or glia. Interestingly, during the review of this paper, a study showed that EphA4 mediates spine development through the Rho-family GTPase exchange factor, ephexin-1, and the serine/threonine kinase Cdk5 (Fu et al., 2007). *In vivo*, EphA4 knock-out mice have disorganized and abnormally shaped spines (Murai et al., 2003a), and decreases in early-phase LTP and in LTD (Grunwald et al., 2004). However, mice with the cytoplasmic portion of EphA4 replaced by GFP appear to have normal LTP and LTD under standard induction paradigms. Thus, EphA4 may have kinase-dependent and independent functions that control the structural and physiological plasticity of excitatory synapses. These collective studies demonstrate that Eph receptors are important determinants of spine shape *in vitro* and *in vivo*.

Our findings suggest that EphA4 regulates spine morphology

through a pathway different from what has been described previously for Eph receptors. Many of the events downstream of Eph receptors require activation of small GTPases that remodel the spine's actin cytoskeleton. Eph receptors also interact with the PDZ (PSD-95/Discs large/zona occludens-1)-domain proteins GRIP (glutamate receptor-interacting protein) and AF6 (Hock et al., 1998; Torres et al., 1998). These interactions, however, may be more pertinent to receptor trafficking (Hoogenraad et al., 2005). Our results suggest that EphA4 likely uses tyrosine 602 in the juxtamembrane region to activate PLCγ1 through its C-terminal SH2 domain. Coimmunoprecipitation experiments further indicate that EphA4 and PLCγ1 can interact directly or within the same molecular complex in the hippocampus. EphA4 is known to recruit and bind several proteins including Src, Fyn, and Vav2 through SH2-domain interactions (Ellis et al., 1996; Zisch et al., 1998; Cowan et al., 2005). The significance of these interactions at synapses has not been reported. Interestingly, cortical neurons derived from Fyn-null mice show reduced spine density (Kalo and Pasquale, 1999; Morita et al., 2006). Thus, Fyn and PLCγ1 may similarly bind to the juxtamembrane tyrosines of EphA4 to modify synaptic structure. However, the delayed kinetics of PLCγ1 activation after ephrin treatment and the fact that EphA4 and PLCγ1 only partially colocalize in a subset of spines leaves open the possibility that an intermediary protein downstream of EphA4 induces the activation of PLCγ1. Additional experiments are necessary to fully develop the direct or indirect nature of the EphA4-PLCγ1 interaction.

After activation by RTKs, phosphorylated PLCγ1 plays diverse roles in cellular behavior (Rebecchi and Pentyala, 2000). Previous data suggests that PLCγ1 controls the level of membrane-bound PIP_2, which by itself acts as a potent second messenger that modifies actin–plasma membrane interactions and cell adhesion (Raucher et al., 2000; DesMarais et al., 2005). PIP_2 also modulates the function of many proteins including potassium and TRP (transient receptor potential) channels (Lopes et al., 2005; Rohacs et al., 2005) and actin-binding proteins (Sechi and Wehland, 2000). Furthermore, PIP_2 actively competes with actin for binding cofilin and inhibits its actin depolymerizing ability *in vitro* (Yonezawa et al., 1990, 1991b). Reciprocally, cofilin binding to PIP_2 blocks PLCγ1-mediated cleavage of PIP_2 (Yonezawa et al., 1991a). PLCγ-PIP_2 interactions are known to be important for the dynamic regulation of the actin cytoskeleton and for cell motility after EGF stimulation (Chou et al., 2002). Ephrin/EphA4 signaling may provide a trigger for PLCγ1-dependent regulation of cofilin at the cell surface in dynamic compartments of neurons such as spines and growth cones. At the same time, generation of the second messengers IP_3 and DAG by PIP_2 hydrolysis may influence synaptic function (Lynch et al., 1988; Nagase et al., 2003; Taufiq et al., 2005). Additional experiments are needed to determine whether EphA4-PLCγ1 interactions modify spine morphology and synaptic function through these second messenger systems.

Cofilin may exist in different transient states that are important for spine morphology. Reports have suggested that cofilin association near the cell membrane is enhanced when it is in the dephosphorylated, activated state (Mulholland et al., 1994; Suzuki et al., 1995; Nagaoka et al., 1996). Phosphorylation of cofilin/ADF proteins on serine-3 also blocks their ability to bind actin (Morgan et al., 1993; Moriyama et al., 1996). Thus, PIP_2 may tether cofilin to the cell surface and maintain it in a "primed" (dephosphorylated but inactive) state awaiting RTK autophosphorylation. After PLCγ1 activation by EphA4, cofilin may be released to depolymerize and sever actin filaments in the spine (Matus, 2000). Cofilin may need to be released from PIP_2 inhibition and from the outer membrane perimeter of the spine to play a role in the "core" of the spine to alter its structure (Racz and Weinberg, 2006).

After release, LIM and Tes kinases may decrease cofilin activity through phosphorylation on serine-3. Indeed, LIM kinase 1 (LK1) knock-out mice have reductions in cofilin phosphorylation and spine size (Meng et al., 2002). This is consistent with cofilin promoting actin filament disassembly and spine shrinkage (Zhou et al., 2004). Physiological recordings and behavioral analysis of LK1 knock-out mice have shown that basal synaptic transmission is normal, however, the mice have elevated LTP and impairments on memory tasks (Meng et al., 2002). Additionally, a microRNA that reduces the translation of LK1 is modulated by BDNF and blocks spine development (Schratt et al., 2006). Cofilin is reactivated by Slingshot proteins through dephosphorylation of serine-3 (Niwa et al., 2002) and the mRNAs for these proteins are found in the developing and adult mouse brain (Ohta et al., 2003). The role of Slingshot proteins in regulating spine morphology, however, remains unknown. Remarkably, a previous study suggests that cofilin phosphorylation/dephosphorylation events are not necessarily required for cofilin activity in dynamic leading edges of carcinoma cells in response to EGF treatment (Song et al., 2006). In this context, translocation of cofilin is likely critical for its function in cellular remodeling. This supports the hypothesis that cofilin exists in different states and is regulated at multiple levels to control its localization and activity.

In summary, our results provide new insight into how EphA receptors control spine morphology. The combinatorial control over multiple downstream targets of activated EphA and B receptors, including signaling through PLCγ1, ephexin-1, Rho-family GTPases, and cofilin, is likely required for proper spine development and maintenance.

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