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Change in the Shape and Density of Dendritic Spines Caused by Overexpression of Acidic Calponin in Cultured Hippocampal Neurons

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ABSTRACT: Dendritic spines are morphing structures believed to provide a cellular substrate for synaptic plasticity. It has been suggested that the actin cytoskeleton is the target of molecular mechanisms regulating spine morphology. Here we hypothesized that acidic calponin, an actin-binding protein, is one of the key regulators of actin filaments during spine plasticity. Our data showed that the overexpression of acidic calponin-GFP (green fluorescent protein) in primary cultures of rat hippocampal neurons causes an elongation of spines and an increase of their density as compared with those of GFP-expressing neurons. These effects required the actin-binding domains of acidic calponin. The close apposition of the presynatic marker synaptophysin to these long spines and the presence of specific postsynaptic markers actin, PSD-95, NR1, and GluR1 suggested the existence of functional excitatory synaptic contacts. Indeed, electrophysiological data showed that the postsynaptic overexpression of acidic calponin enhanced the frequency of miniature excitatory postsynaptic currents as compared with that of GFP-expressing neurons, but did not affect their properties such as amplitude, rise time, and half width. Studies in heterologous cells revealed that acidic calponin reorganized the actin filaments and stabilized them. Taken together, these findings show that acidic calponin regulates dendritic spine morphology and density, likely via regulation of the actin cytoskeleton reorganization and dynamic. Furthermore, the acidic calponin-induced spines are able to establish functional glutamatergic synapses. Such data suggest that acidic calponin is a key factor in the regulation of spine plasticity and synaptic activity. © 2005 Wiley-Liss, Inc.

KEY WORDS: actin cytoskeleton; acidic calponin; dendritic spine; spine plasticity; hippocampal neurons

INTRODUCTION

The vast majority of excitatory glutamatergic synapses are made on dendritic spines (Gray, 1959; Peters et al., 1976; Parnavelas et al., 1977; Harris and Kater, 1994). These small protrusions emerging from dendritic shafts have raised widespread interest because it is believed that spines may constitute a cellular substrate for synaptic plasticity in the brain (Andersen, 1999). Several evidences indicate that dendritic spines undergo changes in shape and number during development and adulthood (Papa et al., 1995; Dailey and Smith, 1996; Ziv and Smith, 1996; Boyer et al., 1998; Fiala et al., 1998; Ethell and Yamaguchi, 1999; Matus, 2000; Hering and Sheng, 2001). In adults, these changes are influenced by several factors, including age, hormones, neurotrophins, learning, sensory deprivation, disease (Bailey and Kandel, 1993; Horner, 1993; Harris and Kater, 1994; Moser et al., 1994; Comery et al., 1997; Lendvai et al., 2000; Irwin et al., 2000; 2001; Nimchinsky et al., 2001; Trachtenberg et al., 2002), synaptic activity, and plasticity (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999).

Little is known about the molecular mechanisms that modulate the shape and number of dendritic spines, particularly with respect to the regulation mechanisms of actin filaments. However, it is well known that dendritic spines are highly enriched with actin filaments (Fifkova and Delay, 1982; Matus et al., 1982). These actin filaments are the key target of molecular mechanisms regulating spine plasticity because drugs that inhibit actin dynamic provoked a loss of spines (Allison et al., 1998) and blockage of their motility (Fischer et al., 1998; Dunaevsky et al., 1999; Matus, 2000). It is essential to elucidate the molecular basis involved in the spine plasticity to understand the mechanisms of synaptic plasticity as well as some neurological disorders such as mental retardation (Rudelli et al., 1985; Hinton et al., 1991; Comery et al., 1997; Irwin et al., 2000; 2001; Meng et al., 2002).

We hypothesized that acidic calponin, an actin-binding protein, is one of the regulators of actin filaments during spine plasticity based on the following observations: (1) Calponin family is known to stimulate actin polymerization, bundling, and stabilization of actin filaments (Kake et al., 1995; Kolakowski et al., 1995; Danninger and Gimona, 2000; Ferhat et al., 2001); (2) Transfection of acidic calponin into HEK 293 cells induced reorganization of actin filaments, leading to a change in cell morphology (Ferhat et al., 2001); (3) Electron-microscopy studies showed

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that this actin-binding protein is found in dendritic spines of adult hippocampal neurons (Agassandian et al., 2000); and (4) Immunohistochemical studies revealed that acidic calponin is upregulated during dendritic spines plasticity following pilocarpine-induced seizures (Ferhat et al., 2003).

To confirm our hypothesis, we overexpressed acidic calponin in cultured hippocampal neurons and analyzed its effects on the regulation of dendritic spine morphology. In addition, we studied the electrophysiological consequences of this regulation. Here, we report that the overexpression of acidic calponin-GFP (green fluorescent protein) causes elongation of dendritic spines and an increase in their density when compared with those of GFP-expressing neurons and that these effects require the actin-binding domains of acidic calponin. Immunocytochemical studies and electrophysiological data suggested that spines induced by overexpression of acidic calponin-GFP formed functional excitatory synaptic contacts. Furthermore, in heterologous cells, acidic calponin induced a reorganization of actin filaments. The latter were more stable against a drug that inhibits actin dynamic than those without acidic calponin. Altogether, these results suggest that acidic calponin regulates spine morphology and density presumably via regulation of the actin cytoskeleton reorganization and dynamic.

MATERIALS AND METHODS

cDNA Constructs

The full-length acidic calponin (ac.CaP, NCBI accession number U06755) fragment was inserted into mammalian expression vectors pDsRed1-N1 and pEGFP-N1 (BD Bioscience Clontech, Palo Alto, CA). We have also used the mutant ac.CaP without actin-binding sites (ac.CaPΔABS, deletion of amino acid residues from 142 to 272), which was inserted in pEGFP-N1. The vectors pDsRed1-N1 and pEGFP-N1 encode for red fluorescent protein (RFP) and GFP, respectively. The constructs ac.CaP-GFP/-RFP and ac.CaPΔABS-GFP have been previously described (Ferhat et al., 2001). The mutant containing the actin-binding sites (ABS, amino acid residues from 142 to 187, Mezgueldi et al., 1992, 1995; Mino et al., 1998) was cloned by PCR, with full length DNA as template, using a sense primer containing a EcoRI site (5′TATATAGAATTCCAGCCATGGTTAAGTACGCAGAAAAA3′) and an antisense primer (5′ATATATGGATCCCCATGCCTCCGAGTCCCATAGGCTGTC3′) containing a BamHI site. The ABS insert was then inserted into the EcoRI and BamHI sites of pEGFP-N1. The obtained construct ABS-GFP was subsequently fully sequenced to verify the integrity of the fusion protein.

Cell Line, Transfection, and Immunofluorescence

The Chinese Ovary (CHO-K1) cells were obtained from the American Type Tissue Culture Collection (ATCC, Molsheim, France). They were grown in F12 (Invitrogen, Cergy Pontoise, France), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Invitrogen), 100 i.u./ml penicillin, and 100 mg/ml streptomycin (Sigma, Lyon, France). Cells were rinsed once with a serum-free medium (Opti-MEM, Invitrogen) and transfections were performed according to the manufacturer's protocol (Invitrogen). Briefly, cells were incubated in a solution containing 500 μl of Opti-MEM, 4 μl of plus reagent, 6 μl of lipofectamine reagent (Invitrogen), and 1 μg of either the GFP, the ac.CaP-GFP, the ac.CaPΔABS-GFP, or the ABS-GFP construct. After 4 h of incubation at 37°C, the transfection mixture was replaced by a fresh complete growth medium containing 10% FBS. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffer (PB, pH 7.2) for 20 min at room temperature (RT).

Quantitative analyses of the number of transfected cells were performed using a fluorescence microscope with a 20× objective. Twenty fields per cover slip per experiment ($n = 3$) were analyzed. Data were expressed as mean % of total cells per experiment ± SEM.

For the actin staining, the cells were incubated with 0.5% Triton X-100 and 5% normal goat serum (NGS) for 30 min and exposed for 2 h at RT to 0.5 unit per cover slip of Texas Red-X phalloidin (Molecular Probes, Leiden, Netherlands). Cells were then mounted with Fluoromount G (Electron Microscopy, Fort Washington, PA) and analyzed using Olympus laser scanning microscope.

In some experimental sets ($n = 4$), the microfilament-depolymerizing drug latrunculin B (Calbiochem, San Diego, CA; used at 1 μg/ml final concentration in 0.1% dimethylsulfoxide) was added into the medium to assess the stability of microfilaments of transfected cells. We used this drug because it is 10- to 100-fold more potent than that of cytochalasins (Spector et al., 1989; Saito et al., 1994). After incubation with latrunculin B for 8 min, cells were fixed with PFA for 20 min, washed three times with PB, and stained for F-actin as described above.

Primary Cultures of Rat Hippocampal Neurons and Transfection

To localize the endogenous ac.CaP in neuron, low-density hippocampal cultures were prepared according to Goslin and Banker (1991) with slight modifications. Briefly, embryonic 18-day-old (E18) Wistar rat hippocampi were dissected and dissociated by trypsin (Invitrogen), plated on cover slips coated with poly-L-lysine (0.1 mg/ml) at a density of 2,400 cells/cm^2, in minimal essential medium (MEM, Invitrogen) containing 10% NuSerum (BD Biosciences, Bedford, MA). After the cells were attached, the cover slips were transferred into a dish with glial cells and maintained in MEM with B27 supplement (Invitrogen) above the glial feeder layer (Brewer, 1995). Cytosine arabinoside (5 μM) was added to the cultures after 3 days to inhibit glial proliferation.

For electrophysiology and transfections, mixed hippocampal cell cultures were prepared from E18 according to Brewer (1995) with slight changes. Briefly, hippocampal cells, isolated by trypsin treatment, were plated at densities of 4×10^4 cells/cm^2

on poly-L-lysine coated cover slips in MEM supplemented with 10% NuSerum. Ten days later, half of the medium was replaced by N2.1 medium containing MEM, Hepes (15 mM, Invitrogen), glucose (0.6%, Invitrogen), L-glutamine (2 mM, Invitrogen), pyruvic acid (1 mM, Sigma), albumin (0.1%, Sigma), and supplemented with insulin (5 μg/ml, Sigma), transferrin (100 μg/ml, Sigma), progesterone (6 ng/ml, Sigma), putrescine (15 μg/ml, Sigma), and selenium (3 ng/ml, Sigma).

At 13 days in vitro, the cultures were completely switched prior transfection to N2.1 culture medium. At 14 days in vitro, hippocampal neurons were transfected with GFP or ac.CaP fusion constructs using lipofectamine2000 reagent according to the manufacturer's protocol (Invitrogen). Briefly, 1 μg of plasmid DNA and 7 μl of lipofectamine2000 were resuspended in 300 μl of Opti-MEM and incubated at RT for 30 min. The transfection mixture was added to the cells and left at 37°C for 3 h. The cells were rinsed gently once with a fresh N2.1 culture medium and maintained at 37°C for 24–48 h. Following posttransfection, the cells were either used for electrophysiology recordings or fixed with 4% PFA in PB for 20 min at RT for immunofluorescence. The transfection efficiency was less than 1%.

Primary Antibodies

Antibodies were obtained from various sources. The ac.CaP protein was detected with a polyclonal antiserum raised in rabbits against the C-terminal of the rat protein. Using Western blot, the specificity of this antiserum has been previously assessed on the total homogenate of rat hippocampus (Plantier et al., 1999). The mouse monoclonal antibody against synaptophysin (Chemicon, Tenecula, CA), mouse monoclonal antibody against postsynaptic density (PSD-95, Upstate, Charlottesville, VA), polyclonal antibody against NR1 (Chemicon), and polyclonal antibody against GluR1 (Chemicon) were purchased as indicated.

Immunofluorescence

For double immunofluorescence labeling of ac.CaP/synaptophysin and ac.CaP/PSD-95, hippocampal neurons cultured for 3 weeks in vitro were rinsed for 30 min in potassium phosphate-buffered saline (KPBS) and preincubated for 1 h at RT in KPBS containing 0.3% Triton X-100 and 1% blocking reagent (Roche, Meylan, France). Cover slips were then incubated overnight at RT in a mixture of ac.CaP antiserum (1:300) and either synaptophysin (1:200), or PSD-95 (1/500) monoclonal antibodies diluted in KPBS containing 1% blocking reagent. After several rinses in KPBS, cover slips were incubated for 1 h at RT in biotinylated goat antirabbit IgG (1:200), rinsed in KPBS, and incubated in a mixture of Alexa 488-conjugated goat antimouse IgG (1:200, Jackson Immunoresearch, West Grove, PA) and Cy3-conjugated streptavidin (1:200, Jackson Immunoresearch) diluted in KPBS. The cover slips were then dipped in water and mounted with Fluoromount G.

For double staining of ac.CaP/actin filaments in primary hippocampal cultures, neurons at 3 weeks in vitro were incubated with 0.5% Triton X-100 and 5% NGS in 0.1M PB for 30 min and exposed for 2 h at RT to 0.5 unit per cover slip of Texas Red-X phalloidin in PB containing 5% NGS. After washing with PB, neurons were dipped in water and then mounted with Fluoromount G. Specimens were analyzed with Olympus fluoview-500 laser scanning microscope.

For immunostaining of transfected hippocampal neurons with ac.CaP, cells were fixed in 4% PFA in PB, blocked and permeabilized and incubated as described above with the following primary antibodies: synaptophysin, PSD-95, NR1 (1/100), or GluR1 (1/50). Bound antibodies were detected with appropriate secondary antibodies.

For double staining of synaptophysin/actin filaments in neurons transfected with ac.CaP-RFP, cells were fixed and immunostained with Oregon Green 488 phalloidin (Molecular Probes) using the same procedure described above.

In all cases, no labeling was detected when specific antibodies were replaced with normal rabbit or mouse serum or when primary antibodies were omitted.

Image Acquisition and Quantification

Images were acquired with an Olympus fluoview-500 confocal microscope using an oil immersion 60 × 1.4 lens. For observation of dendritic spines, 7–13 serial images of 0.5 μm thickness were projected onto one plane.

To analyze the effects of ac.CaP on spine morphology and number, hippocampal neurons transiently cotransfected with RFP and ac.CaP-GFP or RFP and mutants constructs (ac.CaP-ΔABS-GFP or ABS-GFP) were compared with RFP- and GFP-expressing neurons considered as control neurons. RFP was used to outline the morphology of the dendrites to analyze the length and dendritic spine number. Although the extent of RFP fluorescence does not provide measurements in an absolute sense, it allowed us to compare relative changes in the length and spine number between groups of neurons. After transfections, neurons were immunostained with antisynaptophysin monoclonal antibodies. Only protrusions morphologically characterized and that were associated with synaptophysin-stained presynaptic terminals were considered as spines and taken into account for quantification.

To make different experiments comparable, all pictures were taken with the same parameters. Ten transfected neurons were chosen randomly from three independent experiments for each construct and their entire dendritic segments were reconstructed with Image analysis system (Neurolucida, Microbrightfield, Inc., Cholchester, VT). For morphometric analysis, spine length and density were measured from project images using Neurolucida software. The numbers of spines were collected from at least four dendritic segments. The density of spines corresponds to the total number of spines per 100 μm. The length of spine was determined by measuring the distance between its tip and its base. In this study, we selected only spines ranged from 0.2 to 10 μm. For each construct, at least 779 spines were counted. The mean of the density and the

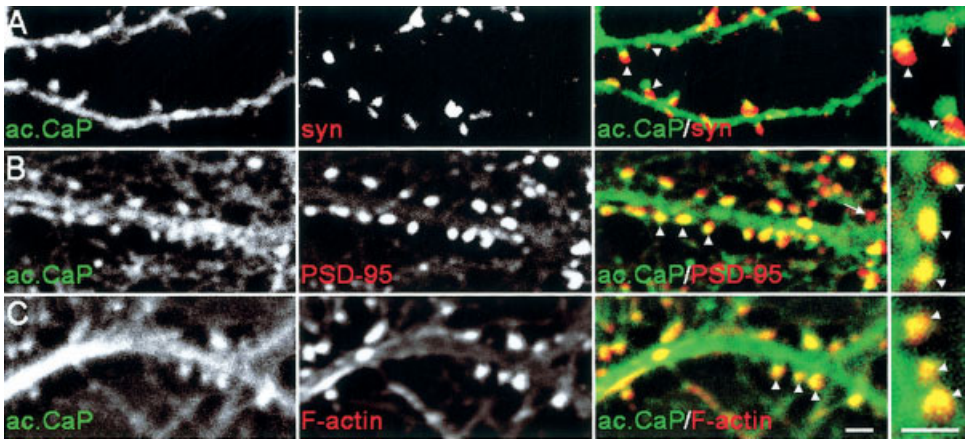

FIGURE 1. Acidic calponin colocalizes with PSD-95 and F-actin in dendritic spines of cultured hippocampal neurons. Twenty-one-day-old cultured hippocampal neurons were immunostained simultaneously with ac.CaP antiserum and either synaptophysin monoclonal antibody (A), PSD-95 monoclonal antibody (B), or Texas red-phalloidin (C). Arrowheads in A indicate that the immunostaining with acidic calponin antiserum (green) was closely adjacent to the staining with synaptophysin antibody (red). Examples of sites of colocalization (yellow) of ac.CaP (green) with either PSD-95 (red) or F-actin (red) are indicated by arrowheads in B and C respectively. Arrow in B indicates a PSD-95-positive dot that was not positive for ac.CaP. Similar results were observed in five independent experiments. Scale bars: 3 μm.

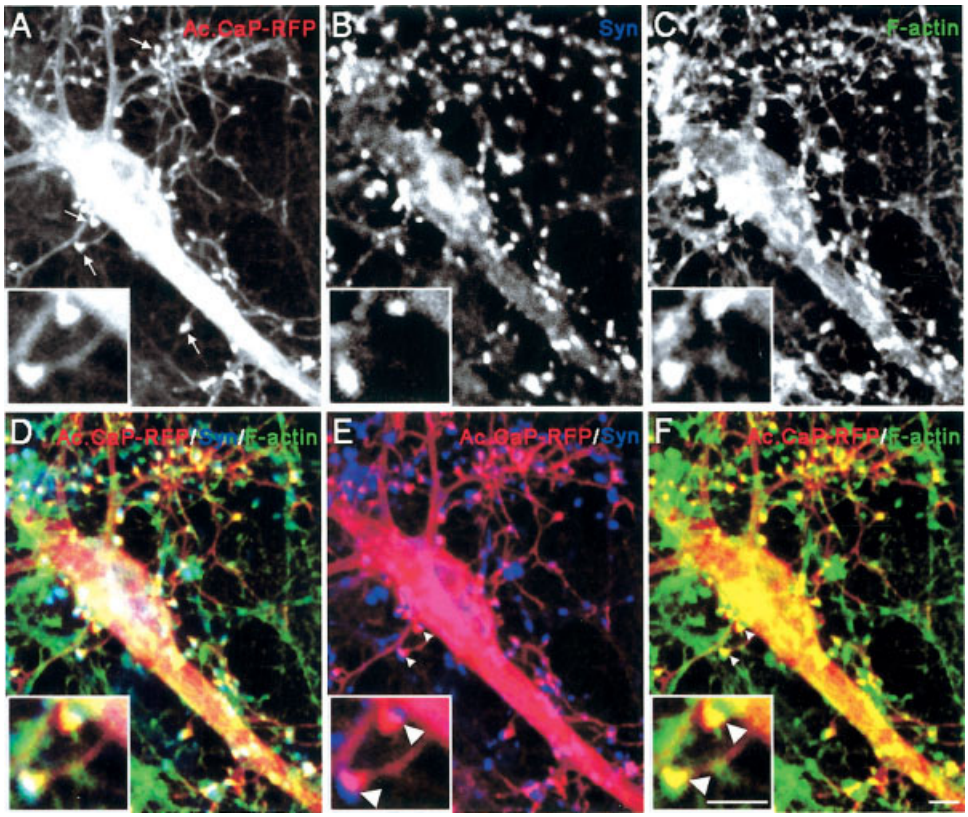

FIGURE 2. Postsynaptic localization of acidic calponin-RFP in cultured hippocampal neurons. Neuron transfected with ac.CaP-RFP at 2 weeks in vitro (A, red). Ac.CaP-RFP was seen in the cell body and dendritic shafts. Also, ac.CaP-RFP was seen in the protrusions (see arrows) that were associated with dendrites. Inset in A indicates an example of dendritic protrusions at high magnification. Neuron transfected with ac.CaP-RFP double-immunostained for a presynaptic marker, synaptophysin (B, blue), and a spine marker, F-actin (C, green). Insets in B and C indicate the same region of the dendritic protrusions shown in A, double immunostained for synaptophysin (B, blue), and F-actin (C, green) at high magnification. D indicates superimposition of color images (A, red), (B, blue), and (C, green). Inset in D indicates superimposition of color images of inset in (A) and (B). (C) and (E) indicates superimposition of color images (A, red) and (B, blue). Ac.CaP-RFP (E, red) and synaptophysin proteins (E, blue) did not colocalize (see small arrowheads); instead the two proteins were present in adjacent compartments (E, see large arrowheads), suggesting a postsynaptic localization of ac.CaP-RFP. F indicates superimposition of color image (A, red) and (C, green). Ac.CaP-RFP (F, red) and F-actin (F, green) did colocalize (see small arrowheads, yellow), confirming that ac.CaP-RFP is enriched in dendritic spines (inset, see large arrowheads). Scale bars: 5 μm.

length of spines were calculated for each construct (measurements are given as mean ± SEM).

Statistical analysis of differences in the means of the density and the length of spines between GFP neurons used as controls and ac.CaP-GFP, ac.CaPΔABS-GFP, and ABS-GFP-expressing neurons were performed with a Mann–Witney test. All measurements were performed on pyramidal neurons that were visually identified based on morphology (Benson et al., 1994).

Whole-Cell Recording

In an other set of experiments, miniature AMPA-mediated excitatory postsynaptic currents (mEPSCs) were recorded from visually identified pyramidal neurons overexpressing either GFP or ac.CaP-GFP. For this purpose, whole-cell recordings from 15 to 16-day-old cultured hippocampal neurons expressing either GFP or ac.CaP-GFP were performed at 35°C with 3- to 5-MΩ patch pipettes. Pipette solutions contained 135 mM K-gluconate, 10 mM Hepes, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM EGTA, 2 mM Na_2ATP and Rhodamine. Cultures were continuously superfused with a solution containing 112 mM NaCl, 3 mM KCl, 16 mM glucose, 8 mM Hepes, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05 mM D-2-amino-5-phosphovaleric acid (D-AP5, Tocris, Bristol, UK), 0.01 mM bicuculline (Tocris), and 0.001 mM tetrodotoxin (TTX, Sigma) at 1 ml/min. Fourteen GFP and sixteen ac.CaP-GFP-expressing neurons were recorded with EPC-9 amplifier (HEKA, Lambrecht, Germany) under visual guidance, with a 40× water-immersion objective, using Leica DM-LSF microscope. Transfected cells were identified before recording by fluorescence. Images from recorded cells were digitized with a CCD camera (C7500-Hamamatsu, Hamamatsu City, Japan). Current records were low-pass filtered at 5 kHz and stored on hard disk at 10 kHz via a Digidata 1,200 interface (axon instrument, Foster City, CA). Cells were held at −60 mV, and recording stability was monitored in real time with 5 mV steps every 30 s. Series resistances ranged between 8 and 23 MΩ. Recordings were made for 4–10 min from each cell. The mEPSCs were analyzed with MiniAnalysis (Synaptosoft, Decatur, GA) and Igor Pro (Wavemetrics, Lake Oswego, OR) softwares. The amplitude threshold for detection of synaptic events was set at 5 pA. Statistical analyses for frequency, amplitude, rise time, and half-amplitude duration were performed with the nonparametric Mann–Whitney test.

RESULTS

Localization of Endogenous Acidic Calponin in Cultured Hippocampal Neurons

It has been shown previously that ac.CaP is expressed both by glial cells and neurons (Ferhat et al., 1996; Plantier et al., 1999; Ferhat et al., 2003). Because of the high expression levels of ac.CaP in glial cells, it was difficult to define the neuronal compartment localization of endogenous ac.CaP in mixed cultures. To determine precisely its localization in neurons, we used glial-free monolayer cultures of E18 hippocampal neurons as described by Goslin and Banker (1991). These neuronal cultures form synapses and establish neuronal networks in the course of 3–4 weeks in vitro (Rao et al., 1998; Lee and Sheng, 2000).

To localize ac.CaP, we performed a series of double immunolabeling experiments on 3 weeks-old cultured primary hippocampal neurons ($n = 5$ independent experiments). First, we examined the distribution of ac.CaP (green) and synaptophysin (red), a specific marker of presynaptic terminals. As illustrated in Figure 1, ac.CaP was diffusely distributed along the dendritic shafts and also in small protrusions that emerged from dendrites. As indicated by the high magnification image, double immunostaining with an antisynaptophysin antibody revealed that ac.CaP and synaptophysin did not colocalize; instead the two proteins were present in adjacent compartments (Fig. 1A, see arrowheads), suggesting a postsynaptic localization of ac.CaP.

To confirm the postsynaptic localization of ac.CaP, we next performed double labeling for ac.CaP and two specific markers for spines, PSD-95 and actin filaments. Double immunostaining for ac.CaP (green) and PSD-95 (red) showed significant overlap along dendrites (Fig. 1B, see arrowheads), although occasionally there were some PSD-95-positive dots that were not positive for ac.CaP (Fig. 1B, see arrow). Double immunostaining for ac.CaP and actin filaments (red) further confirmed postsynaptic localization since ac.CaP colocalized with actin filaments, revealed by Texas Red-X phalloidin, in most spines (Fig. 1C, see arrowheads).

Localization of Exogenous Acidic Calponin in Cultured Hippocampal Neurons

To determine the neuronal localization of exogenous ac.CaP, we tranfected mixed cultures from rat hippocampus with ac.CaP-RFP construct (Fig. 2A, red). The transfection was performed on day 14 in vitro, and on day 15, transfected cells were fixed and double immunostained (Fig. 2D) for synaptophysin (Fig. 2B, blue) and F-actin (Fig. 2C, green). In ac.CaP-RFP transfected cells, a diffuse red fluorescence was observed within the cell body and along the dendritic shafts. In addition, we observed the presence of the red fluorescence within the protrusions reminiscent of dendritic spines (Fig. 2A, see arrows). As indicated in Figure 2E, immunostaining with an antisynaptophysin antibody revealed that ac.CaP-RFP (Fig. 2A) and synaptophysin (Fig. 2B) did not colocalize; instead the two proteins were present in adjacent compartments (Fig. 2E, see arrowheads), suggesting a postsynaptic localization of ac.CaP-RFP. We further confirmed this postsynaptic localization since ac.CaP-RFP colocalized with actin filaments in most dendritic spines (Fig. 1F, yellow, see arrowheads). Similar observations were obtained using ac.CaP-GFP construct (see Fig. 3). Taken together, these results demonstrate that RFP or GFP did not alter the neuronal localization of exogenous ac.CaP in mixed cultured hippocampal neurons.

Effects of Acidic Calponin on Spine Morphology in Cultured Hippocampal Neurons

We hypothesized that if ac.CaP, which is an actin-binding protein, is one of the regulators of actin filaments during spine plasticity, then the overexpression of ac.CaP in neurons would affect the morphology of dendritic spines. To test this hypothesis, mixed cultured hippocampal neurons transiently cotransfected with RFP and ac.CaP-GFP were compared with RFP and GFP-expressing neurons used as control neurons. RFP (Fig. 3A–D) was used to outline dendrites morphology. As visualized by cotransfected RFP (Fig. 3A,B), both GFP (Fig. 3A′) and ac.CaP-GFP (Fig. 3B′) were localized within dendritic shafts as well as in spines. In addition, remarkable morphological changes were observed between dendritic spines of ac.CaP-GFP-expressing neurons (Fig. 3B′) and those of GFP-expressing neurons (Fig. 3A′). Indeed, the dendrites of ac.CaP-GFP-expressing neurons showed longer spines (Fig. 3B,B′, see arrows) as compared with the ones observed in GFP-expressing neurons (Fig. 3A,A′). Some of these long spines could reach over 9 μm (see spines with a star in Fig. 3B,B′). Thus, the length of these long spines was reminiscent of the one of dendritic filopodia, which can reach about 10 μm (Ziv and Smith, 1996). However, the morphology of these long spines differed from that of dendritic filopodia. Indeed, while dendritic filopodia are long protrusions without heads, spines induced by ac.CaP-GFP were characterized as long protrusions with heads. Moreover, we showed that these long spines were actually associated with axon terminals (see Fig. 2E). Altogether, these observations suggest that these long spines are more mature than that of dendritic filopodia since the latter are considered to be the precursors of dendritic spines (Papa et al., 1995; Dailey and Smith, 1996; Ziv and Smith, 1996).

To access if ac.CaP exerts its effect via interaction with actin filaments, we cotransfected hippocampal neurons with RFP (Fig. 3C) and ac.CaPΔABS-GFP (Fig. 3C′), a mutant of ac.CaP-GFP that lacks the actin-binding domains (Ferhat et al., 2001). As visualized by cotransfected RFP (Fig. 3C), ac.CaP-ΔABS-GFP was also detected in the dendritic shafts as well as in the spines (Fig. 3C′). In contrast to ac.CaP-GFP, ac.CaPΔABS-GFP did not induce morphological changes in dendritic spines. These results indicate that: (1) the effect of acidic calponin on spine morphology is a specific effect of the overexpression of ac.CaP-GFP and (2) the elongation of dendritic spines induced by ac.CaP-GFP requires its actin-binding domains.

We further investigated whether the overexpression of actin-binding domains of ac.CaP are sufficient to induce the elonga-

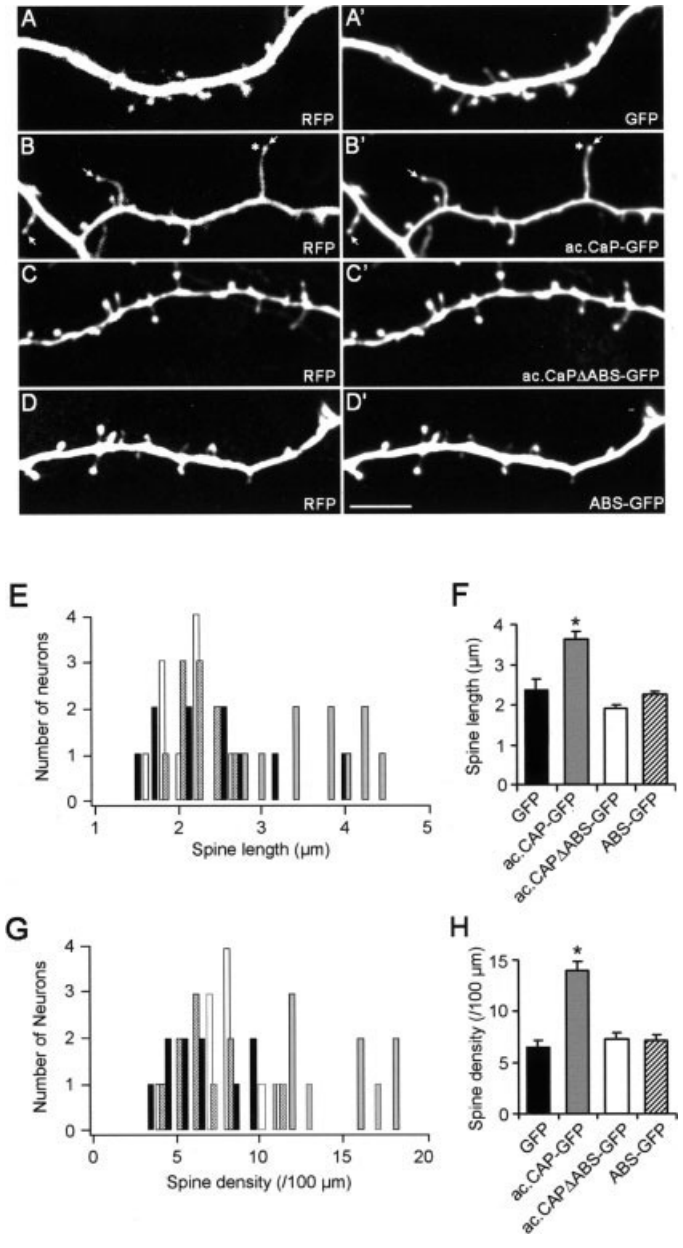

FIGURE 3. Acidic calponin overexpression in cultured hippocampal neurons affects the morphology of dendritic spines. Cultured hippocampal neurons were cotransfected at 14 days in vitro with RFP (A) and GFP (A′), RFP (B) and ac.CaP-GFP (B′), RFP (C) and ac.CaPΔABS-GFP (C′), or RFP (D) and ABS-GFP (D′). After 2 days of transfection (16 days in vitro), neurons were fixed and then examined. RFP channel is shown in all panels to outline dendrite morphology. In all cases, the fluorescence was seen within dendritic shafts as well as in spines. In the latter, the fluorescence was more concentrated in the head than that in the neck. Some spines labeled with ac.CaP-GFP (B′, see arrows) were markedly longer than that of the GFP (A′), ac.CaPΔABS-GFP (C′), or ABS-GFP-labeled spines (D′). Scale bars: 10 μm. (E–H) Quantification of acidic calponin-GFP effects on spine length and density. Histograms showing the distributions of the average length (E, bin size = 0.5 μm) and density (G, bin size = 1/100 μm) of the spines labeled with GFP (black), ac.CaP-GFP (gray), ac.CaPΔABS-GFP (white), or ABS-GFP (hatched). The length of 779 spines of GFP-expressing neurons, 1,729 spines of ac.CaP-GFP-expressing neurons, 902 spines of ac.CaPΔABS-GFP-expressing neurons, and 1,348 spines of ABS-GFP-expressing neurons was measured. The average length and density of spines were calculated for each type of neuron. Histograms, showing the average length (F) and density (H) of spines of 10 GFP-expressing neurons, 10 ac.CaP-GFP expressing neurons, 10 ac.CaPΔABS-GFP-expressing neurons, and 10 ABS-GFP-expressing neurons. *, $P < 0.001$.

tion of dendritic spines. For this purpose, we cotransfected hippocampal neurons with RFP (Fig. 3D) and ABS-GFP (Fig. 3D′), a construct that contains only the actin-binding domains of ac.CaP (Mezgueldi et al., 1992, 1995; Mino et al., 1998). As revealed by coexpressed RFP (Fig. 3D), ABS-GFP was detected in the dendritic shafts as well as in the spines (Fig. 3D′). In contrast to ac.CaP-GFP, ABS-GFP did not induce morphological changes in dendritic spines. We conclude from these observations that the actin-binding domains of ac.CaP are necessary but not sufficient to induce an effect on spine morphology. In addition to the actin interaction, ac.CaP needs other molecular interactions to exert its effects on spine morphology.

Quantitative Analysis of the Morphological Changes Induced by the Overexpression of Acidic Calponin

Because differences in the morphological changes were observed consistently for ac.CaP-GFP as compared with GFP, ac.CaPΔABS-GFP, or ABS-GFP, we conducted a quantitative analysis to determine the relative extent of these changes. Quantification of the changes was performed on mixed cultured hippocampal neurons that were cotransfected at 14 days in vitro with RFP and the different constructs described above. Two days after transfection (16 days in vitro), neurons were fixed, labeled with antisynaptophysin, and the length of spines (from the base of the neck to the furthest point on the spine head) as well as their density were measured. For this purpose, we measured from confocal microscopic pictures, the length of 779 spines of 10 GFP-expressing neurons, 1,729 spines of 10 ac.CaP-GFP-expressing neurons, 902 spines of 10 ac.CaP-ΔABS-GFP-expressing neurons, and 1,348 spines of 10 ABS-GFP-expressing neurons. Figure 3E shows the distribution of the average spine length in GFP, ac.CaP-GFP, ac.CaPΔABS-GFP, or ABS-GFP-expressing neurons. The distribution of the average spine length in ac.CaP-GFP-expressing neurons is clearly shifted to the right (upper values) as compared with those of GFP, ac.CaPΔABS-GFP, or ABS-GFP. Quantitative data showed that, in ac.CaP-GFP-expressing neurons, the average spine length was significantly longer (3.62 ± 0.19) than that in GFP (2.39 ± 0.24; $P < 0.005$), ac.CaPΔABS-GFP (1.91 ± 0.09; $P < 0.001$), and ABS-GFP-expressing neurons (2.26 ± 0.08; $P < 0.001$) (Fig. 3F). However, the average spine length of ac.CaPΔABS-GFP and ABS-GFP-expressing neurons were not significantly different from that of GFP-expressing neurons ($P = 0.19$ and $P = 1.00$, respectively) (Fig. 3F).

Figure 3G shows the distribution of density of the spines labeled with GFP, ac.CaP-GFP, ac.CaPΔABS-GFP, and ABS-GFP. Similar to the spine length, the distribution of the average spine density in ac.CaP-GFP-expressing neurons is clearly shifted to the right (upper values) as compared with that of GFP, ac.CaPΔABS-GFP, or ABS-GFP (Fig. 3G). In ac.CaP-GFP-expressing neurons the average spine density was significantly higher (13.94 ± 0.84) than that in GFP-expressing neurons (6.53 ± 0.65; $P < 0.001$), ac.CaPΔABS-GFP-expressing neurons (7.31 ± 0.56; $P < 0.001$), or ABS-GFP-expressing neurons (7.17 ± 0.60; $P < 0.001$) (Fig. 3H). However, the average spine density of ac.CaPΔABS-GFP and ABS-GFP-expressing neurons were not significantly different from that of GFP-expressing neurons ($P = 1.81$ and $P = 1.52$) (Fig. 3H).

Effects of Acidic Calponin-GFP Overexpression on Synaptic Function

Since dendritic spines are known to constitute a site of most excitatory glutamatergic synapses (Gray, 1959; Peters et al., 1976; Parnavelas et al., 1977; Harris and Kater, 1994), we investigated whether the long spines induced by ac.CaP-GFP formed functional excitatory glutamatergic synapses. For this purpose, we first studied the presence of specific markers of glutamatergic synapses, including anchoring proteins PSD-95 (red), glutamatergic receptors NMDA (NR1, red), and AMPA (GluR1, red) (see Sheng, 2001) in neurons overexpressing ac.CaP-GFP (green). Our confocal microscopy data showed that the long spines induced by ac.CaP-GFP (Fig. 4A–C) were labeled with the postsynaptic proteins PSD-95 (Fig. 4A′,A″, yellow, see arrowheads), NR1 (Fig 4B′,B″, yellow, see arrowheads) or GluR1(Fig. 4C′,C″, yellow, see arrowheads).

We next investigated whether ac.CaP-GFP overexpression was associated with changes in the electrophysiological properties of excitatory glutamatergic synapses. Whole-cell recordings were performed on cultured hippocampal neurons overexpressing either GFP used as a control (Fig. 5A, top left panels) or ac.CaP-GFP (Fig. 5A, top right panels). Miniature mEPSCs were isolated at −60 mV in the presence of TTX to block action potential triggered spontaneous events, bicuculline to block $GABA_A$ IPSCs, and D-AP5 to block NMDA-mediated currents in cultured neurons transfected with GFP or ac.CaP-GFP (Fig. 5B).

In the neurons overexpressing ac.CaP-GFP, the distribution of the average frequencies of mEPSCs (frequency range: 1.6–27.1 Hz) was shifted to higher values as compared with that of GFP-expressing neurons (frequency range: 0.3–9.5 Hz, Fig. 5C). The average frequency of mEPSCs was significantly increased in neurons overexpressing ac.CaP-GFP (7.4 ± 1.6 Hz, $n = 16$) when compared with that of GFP neurons (2.7 ± 0.6 Hz, $n = 14$, $P < 0.05$, Fig. 5C, inset). However, the distributions of mEPSC amplitudes were similar (Fig. 5D). The average amplitudes of mEPSCs were 13.7 ± 0.8 pA and 14.3 ± 1.3 pA respectively in GFP-expressing and ac.CaP-GFP-expressing neurons ($P = 0.16$, Fig. 5D, inset). Rise-times (GFP: 0.87 ± 0.07 μs; ac.CaP-GFP: 0.85 ± 0.09 μs, $P = 0.50$, Fig. 5E left) and half-width durations (GFP: 4.01 ± 0.52 μs; ac.CaP-GFP: 3.56 ± 0.42 μs, $P = 0.43$, Fig. 5E right) were comparable.

Altogether, these observations show that the overexpresssion of ac.CaP in neurons increases excitatory synaptic function, likely because of the increase in spine density.

Effects of Acidic Calponin-GFP on the Organization and Stability of Actin Microfilaments

We next investigated the mechanisms by which ac.CaP-GFP causes elongation of dendritic spines. Actin filament is the

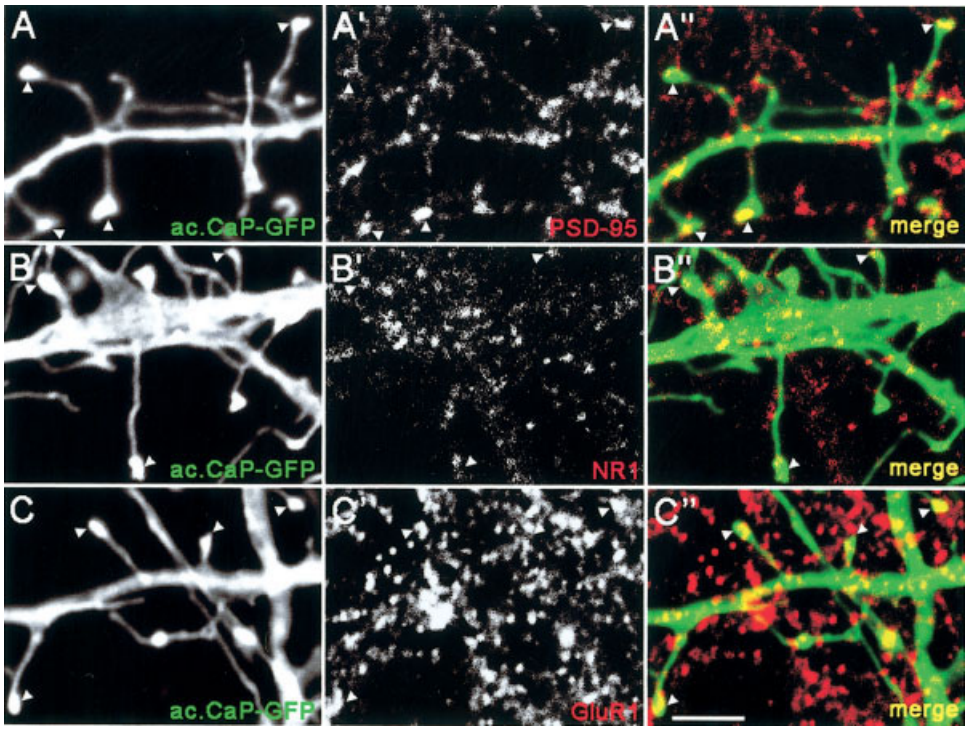

FIGURE 4. Spines labeled by acidic calponin-GFP expressed markers of excitatory synapses in cultured hippocampal neurons. Mixed hippocampal cultures were transfected at 14 days in vitro with ac.CaP-GFP (A–C), and 1 day after transfection (15 days in vitro), cells were fixed, immunostained for PSD-95 (A′), NR1 (B′), and GluR1(C′), and then the transfected neurons were examined. Confocal microscopy revealed that ac.CaP-GFP was localized at spines. The fluorescence at these spines was weak in their necks compared with that in their heads. The spines labeled with ac.CaP-GFP (A–C) contained markers of excitatory synapses, since ac.CaP-GFP colocalizes with the postsynaptic proteins such as PSD-95 (A″), NR1 (B″), and GluR1(C″). Note that, in addition to dendrites of transfected neurons, PSD-95, NR1, and GluR1 immunostainings revealed also dendrites of nontransfected neurons. Scale bars: 3 μm.

major cytoskeletal element of dendritic spines, which is thought to be important for determining spine morphology and dynamics (Fisher et al., 1998). So, it is possible that the overexpression of ac.CaP-GFP may affect the organization and dynamic of actin filaments in spines, leading to alterations in their shape. To test this hypothesis, we overexpressed ac.CaP-GFP in CHO-K1 cells and analyzed its effects on the organization and stabilization of actin filaments. CHO-K1 cells were chosen because, in contrast to neurons, they do not endogenously express ac.CaP (data not shown). Therefore CHO-K1 cells provide a model of choice for visualizing the cellular and stability of microfilaments, following ac.CaP expression.

Reorganization of F-actin by acidic calponin-GFP

Microfilament organization was studied in transfected cells using Texas Red-X phalloidin. Figure 6 shows the effects of overexpression of ac.CaP-GFP on the organization of actin filaments in CHO-K1 cells. Cells transfected with GFP (Fig. 6A–A″) used as a control, displayed the presence of actin stress fibers (see arrowheads) that cross over the cytoplasm, and bundles of actin filaments (see arrows) beneath the plasma membrane (Fig. 6A′,A″). Furthermore, these cells did not show any morphological changes compared with untransfected cells (data not shown). In contrast to GFP, ac.CaP-GFP overexpression in CHO-K1 cells does not only alter the organization of the microfilament network, but also changes the entire morphology of the cells from a more "loose" shape to a "hairy" phenotype with several extending cell processes (Fig. 6B–B″). Ac.CaP-GFP (Fig. 6B) mainly colocalized with microfilaments (Fig. 6B′,B″, yellow). In contrast to GFP transfected cells (Fig. 6A–A″), cells transfected with ac.CaP-GFP had lost both actin stress fibers and cortical actin bundles (Fig. 6B–B″). Instead, these ac.CaP-GFP transfected cells displayed a striking microfilament reorganization (Fig. 6B′,B″). Indeed, we observed thick and twisted bundles of microfilaments in the cytoplasm (see arrowheads). In addition, these actin filaments projected within the processes (see arrows). Over 39% of the cells expressing ac.CaP-GFP displayed remodeling of actin filaments, which was associated to a drastic change in cell morphology (observed in 39.6 ± 1.2% of the cells), as shown in Figure 6B–B″. The effects of ac.CaP-GFP on cell morphology and microfilament reorganization in CHO-K1 cells were similar to that described in HEK 293 cells (Ferhat et al., 2001).

To assess that ac.CaP exerts its effect via interaction with actin filaments, CHO-K1 cells were transfected with ac.CaP-ΔABS-GFP (Fig. 6C–C″), a mutant of ac.CaP-GFP that lacks the actin-binding domains (Ferhat et al., 2001). Then, we studied its effects on the organization of microfilaments. In contrast to ac.CaP-GFP, ac.CaPΔABS-GFP (Fig. 6C) was dif-

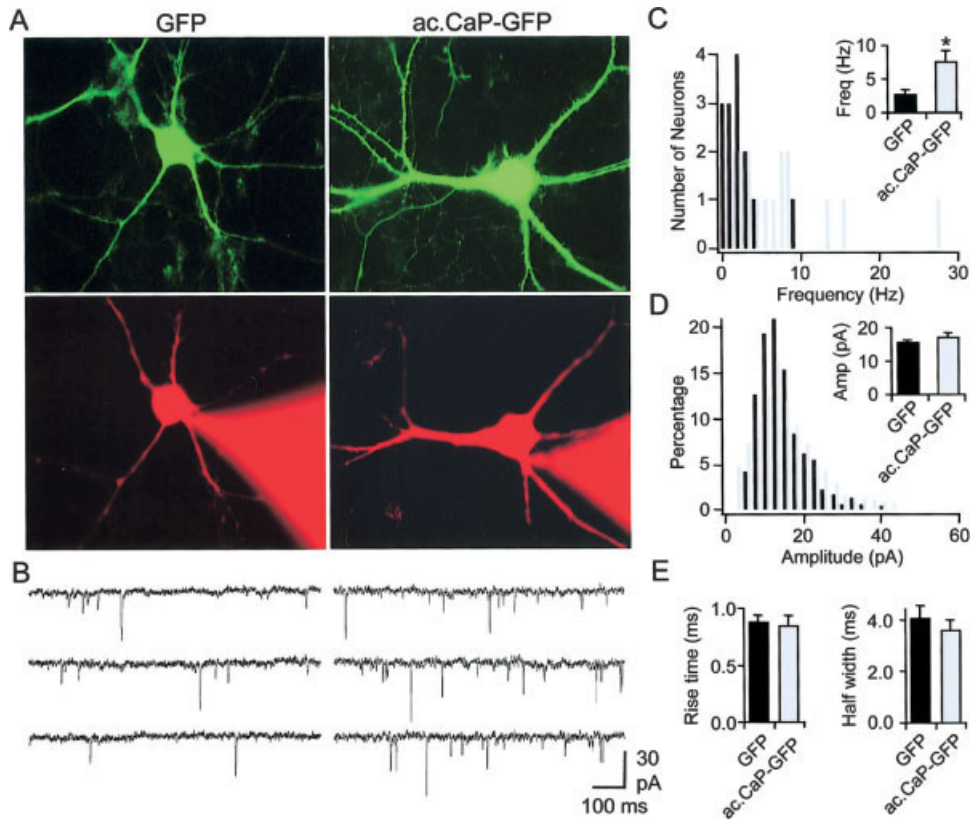

FIGURE 5. Effects of acidic calponin-GFP overexpression on synaptic function. Mixed hippocampal cultures were transfected at 14 days in vitro with GFP or ac.CaP-GFP, and 1–2 days later, the transfected neurons were recorded. Electrophysiological recordings of neurons transfected with GFP used as control were compared with those of ac.CaP-GFP. (A) The recordings of GFP (top, left panel, green) and ac.CaP-GFP-expressing neurons (top, right panel, green) were performed with a solution containing rhodamine (bottom, left and right panels, red). (B) Recordings of mEPSCs from GFP and ac.CaP-GFP-expressing neurons. (C) Distribution of the average frequencies of AMPA-mediated mEPSCs in GFP (black) and ac.CaP-GFP-expressing neurons (gray, bin size = 1 Hz). The average frequency for each construct is shown in inset. (*, $P < 0.05$). (D) Distribution of the average amplitudes of mEPSCs in GFP (black) and ac.CaP-GFP-expressing neurons (gray, bin size = 2.5 pA). The average amplitude for each construct is shown in inset. (E) Histograms showing the average rise-time and half-width of mEPSCs in GFP (black) and ac.CaP-GFP-expressing neurons (gray).

fused within cell body and nucleus and only a small proportion was codistributed with actin filaments (Fig. 6C′,C″, see arrows, yellow). Moreover, the ac.CaPΔABS-GFP transfected cells did not display any reorganization of actin filaments or change in cell morphology (Fig. 6C–C″). Indeed, the organization of actin filaments in ac.CaPΔABS-GFP transfected cells was similar to that observed in GFP transfected cells.

We next wanted to assess whether the actin-binding domains of ac.CaP were sufficient by themselves to induce actin remodeling and change in cell shape. To test the ability of these actin-binding domains, CHO-K1 cells were transfected with ABS-GFP, a mutant that contained the actin-binding domains (Fig. 6D–D″) and we examined their effects on the organization of microfilaments and cell shape. In ABS-GFP transfected cells (Fig. 6D–D″), some of ABS-GFP (Fig. 6D) was diffused within cell body and nucleus. However, it was clear that ABS-GFP bound to actin stress fibers (see arrowheads) and bundles of cortical actin (see arrows)(Fig. 6D′,D, yellow). In contrast to ac.CaP-GFP, ABS-GFP failed to induce remodeling of actin filaments or change in cell shape. Thus, the actin-binding domains are required but not sufficient for actin remodeling and change in cell shape.

Stability of F-actin by acidic calponin-GFP

To study the stabilizing effects of ac.CaP-GFP on microfilaments, GFP and ac.CaP-GFP transfected cells were treated with the microfilament-depolymerizing drug latrunculin-B for 8 min and microfilaments were stained using Texas Red-X phalloidin (Fig. 7). In all GFP-transfected cells (Fig. 7A–A″, four independent experiments), latrunculin-B treatment produced a typical fragmentation of actin stress fibers and cortical actin bundles (Spector et al., 1989), since Texas Red-X phalloidin revealed a patchy dot-like aggregation in the cytoplasm (Fig. 7A′,A″). However, in all ac.CaP-GFP transfected cells (Fig. 7B–B″), the twisted bundles of actin filaments in the cell body as well as the bundles within the processes persisted after latrunculin-B treatment (Fig. 7B′,B″, yellow, 4 independent experiments). Similar obser-

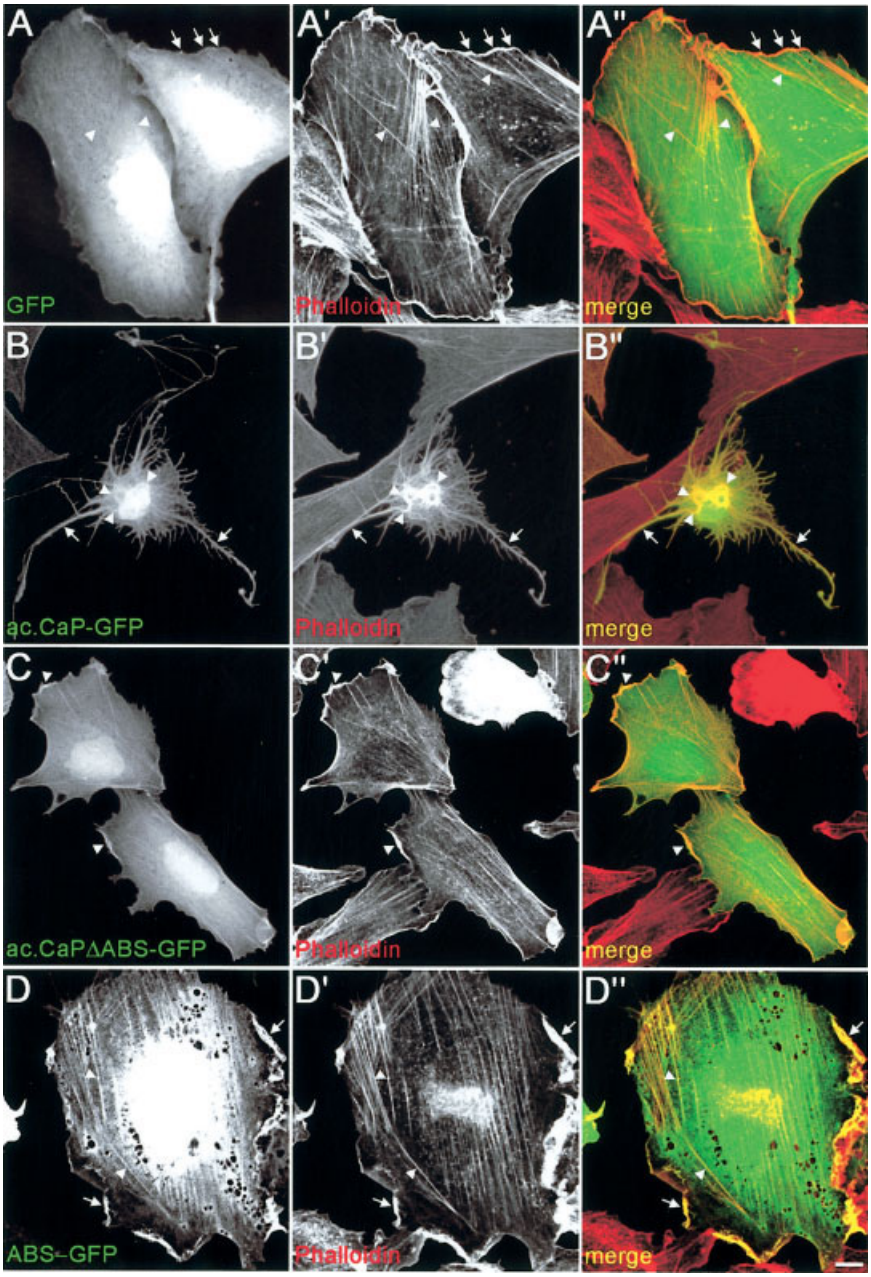

FIGURE 6. Acidic calponin induces a reorganization of microfilament arrays. Microfilament organization in cells transfected with GFP (A), ac.CaP-GFP (B), ac.CaPΔABS-GFP (C), and ABS-GFP (D) assessed with Texas Red-X phalloidin. (A′) In GFP transfected cells, Texas Red-X phalloidin detected the presence of actin stress fibers (see arrowheads) in the cytoplasm and bundles of cortical actin (see arrows) beneath the plasma membrane. A″ corresponds to the merge of panels A and A′. (B′) In contrast to GFP, ac.CaP-GFP caused a striking remodeling of actin filaments, which was associated to a drastic change in cell morphology. B″ corresponds to the merge of panels B and B′. The thick and curving bundles of actin filaments colocalized with ac.CaP-GFP in the cell body (see arrowheads, yellow) and extensions (see arrows, yellow). (C′) As with GFP, ac.CaP-ΔABS-GFP did not alter the organization of actin filaments. C″ corresponds to the merge of panels C and C′. Note that a small proportion of ac.CaPΔABS-GFP colocalized with actin filaments (see arrowheads, yellow). (D′) In contrast to ac.CaP-GFP, ABS-GFP did not induce any reorganization of actin filaments. D″ corresponds to the merge of panels D and D′. Note that ABS-GFP clearly bound to actin stress fibers (see arrowheads, yellow) and bundles of cortical actin (see arrows, yellow). Scale bars: 10 μm.

vations were obtained using the microfilament-depolymerizing drug cytochalasin B (data not shown).

To determine that ac.CaP-GFP exerts a specific effect also on the stabilization of actin filaments, via its interaction with actin filaments, we transfected CHO-K1 cells with ac.CaPΔABS-GFP (Fig. 7C–C″) and then compared its effects on the stability of actin filaments with that of ac.CaP-GFP (Fig. B–B″). As with GFP transfected cells (Fig. 7A–A″), ac.CaPΔABS-GFP transfected cells (Fig. 7C) did not display any resistance to latrunculin-B treatment. Indeed, both the stress fibers and cortical bundles were disrupted and transformed into patchy dot-like aggregates in the cytoplasm (Fig. 7C′,C″).

To determine that the actin-binding domains of ac.CaP are able to exert an effect on the stabilization of actin filaments, we transfected CHO-K1 cells with ABS-GFP and then compared its effects on the stability of actin filaments with that of ac.CaP-GFP. As with GFP (Fig. 7A–A″) and ac.CaPΔABS-GFP transfected cells (Fig. 7C–C″), ABS-GFP (Fig. 7D) did not exhibit any resistance to latrunculin-B treatment (Fig. 7D′,D″). Thus, the actin-binding domains of ac.CaP are necessary but not sufficient to exert an effect on the stabilization of actin filaments. The isolated actin-binding domains overexpression had no effect on spines morphology and number, presumably because they cannot reorganize (see Fig. 6C–C″) and stabilize actin filaments (see Fig. 7D–D″) within dendritic spines. In addition, the overexpression of all the N- or C-terminal constructs with the actin-binding domains, failed also to reorganize and stabilize actin filaments and to induce change in cell shape

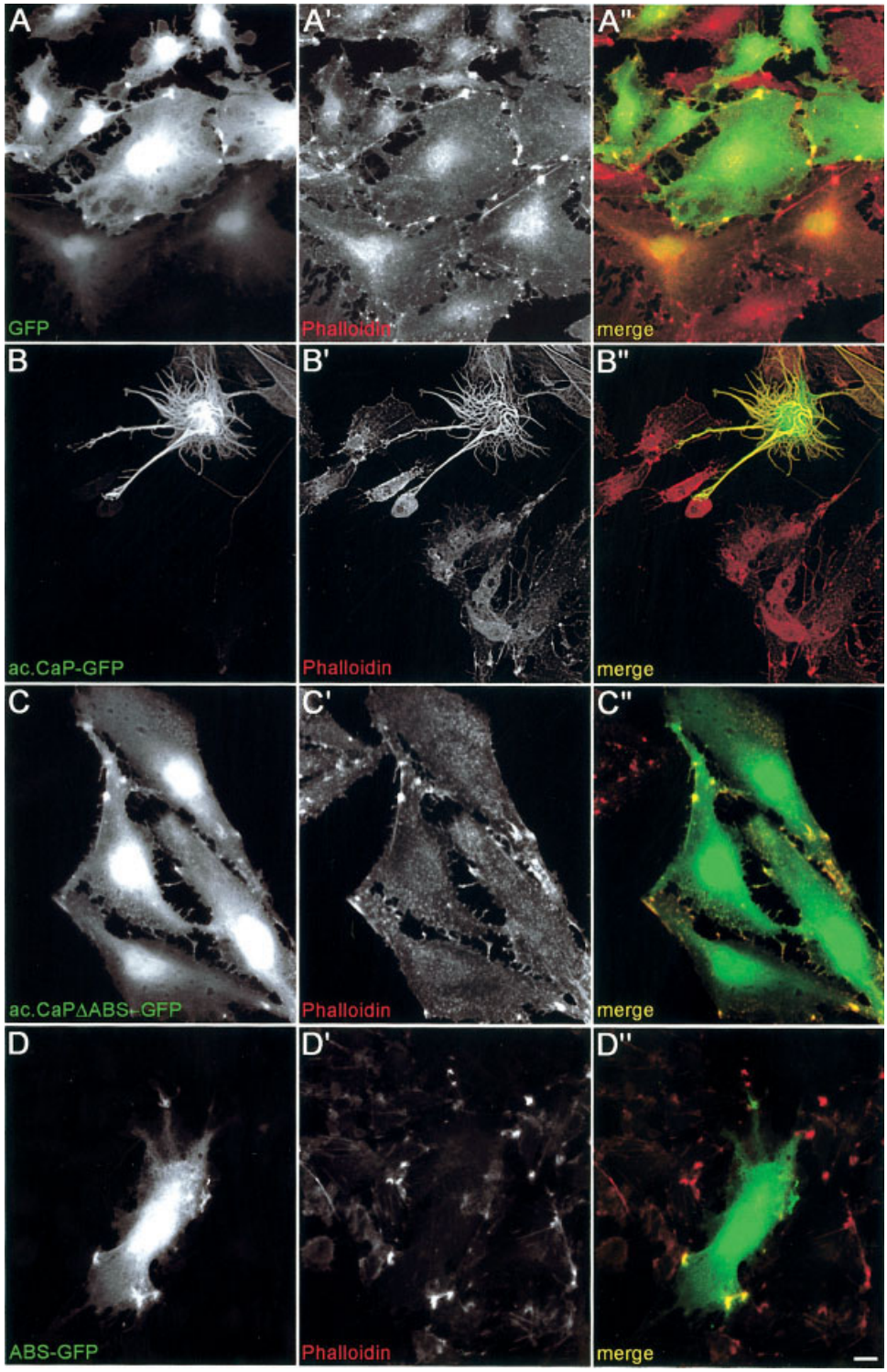

FIGURE 7. Effects of latrunculin-B on the organization of actin microfilaments in cells transfected with acidic calponin. Microfilament organization treated with latrunculin-B (8 min, 1 μg/ml) in cells transfected with GFP (A), ac.CaP-GFP (B), ac.CaP-ΔABS-GFP (C), and ABS-GFP (D) assessed with Texas Red-X phalloidin. (A′) GFP transfected cells treated with latrunculin-B and labeled with Texas Red-X phalloidin. Latrunculin-B affected microfilament organization since actin stress fibers and bundles of cortical actin were disrupted. A″ corresponds to the merge of panels A and A′. (B′) Ac.CaP-GFP transfected cells treated with latrunculin-B and labeled with Texas Red-X phalloidin. In contrast to GFP and ac.CaPΔABS-GFP transfected cells, latrunculin-B did not affect microfilament organization in the ac.CaP-GFP transfected cells. B″ corresponds to the merge of panels B and B′. (C′) Ac.CaPΔABS-GFP transfected cells treated with latrunculin-B and labeled with Texas Red-X phalloidin. Similar to GFP transfected cells, latrunculin-B affected microfilament organization of ac.CaPΔABS-GFP transfected cells. C″ corresponds to the merge of panels C and C′. (D′) ABS-GFP transfected cells treated with latrunculin-B and labeled with Texas Red-X phalloidin. Similar to GFP and ac.CaPΔABS-GFP transfected cells, latrunculin-B affected microfilament organization of ABS-GFP transfected cells. D″ corresponds to the merge of panels D and D′. Scale bars: 10 μm.

in CHO-K1 cells (data not shown). These data suggest that only the full length ac.CaP molecule can induce the reorganization, stabilization, and change in cell shape in CHO-K1 cells. Altogether, these observations in CHO-K1 cells show that the effects of ac.CaP-GFP on the organization and stabilization of microfilaments depend on its interaction with actin filaments.

DISCUSSION

In this study, we identified a new player, ac.CaP, that is able to regulate spine morphology, density, and synaptic activity.

Calponins were originally identified as molecules involved in the regulation of the contraction/relaxation cycle in smooth

muscle cells (Gimona and Small, 1996), in stabilizing the structural integrity of blood vessels (Taniguchi et al., 2001), in the organization of actin cytoskeleton in nonmuscle cells (Fukui et al., 1997; Danninger and Gimona, 2000; Ferhat et al., 2001), and in the formation of cell processes in HEK 293 cells (Ferhat et al., 2001). However, nothing is known about the function of ac.CaP in neurons and specifically in spines, where it is mainly present (Agassandian et al., 2000). The present study provides the first evidence for involvement of ac.CaP in dendritic spine plasticity, likely via regulation of the actin cytoskeleton reorganization and dynamic.

Our data with ac.CaP are in keeping with other studies showing that other actin-binding proteins are important for the regulation of spine morphology and/or density, e.g., drebrin (Hayashi and Shirao, 1999), cortactin (Hering and Sheng, 2003), and α-actinin2 (Nakagawa et al., 2004).

Acidic Calponin May Be Involved in Spine Formation

In this study, we show that ac.CaP is localized in dendritic spines of cultured hippocampal neurons. This result is consistent with previous immuno-electron microscopy studies showing the postsynaptic localization of ac.CaP in adult hippocampus in vivo (Agassandian et al., 2000). In addition to its presence in spines, ac.CaP is also detected in dendritic shaft (Agassandian et al., 2000; present study). This distribution is not surprising because most cytoskeletal proteins are distributed throughout neurons from dendrites to axons. Several postsynaptic proteins, Kalirin-7 (Penzes et al., 2001) and α-actinin2 (Nakagawa et al., 2004), are also localized in dendritic shafts, cells bodies, and axons. Thus, the ubiquitous localization of ac.CaP indicates that ac.CaP does not function only in spines. Indeed, a role for acidic calponin in dendrite trafficking is suggested by its interaction with microtubules (Fattoum et al., 2003) and phosphatydylserine vesicles (Bogatcheva and Gusev, 1995; Fujii et al., 2002). Such function has been also suggested for Kalirin-7 (Penzes et al., 2001).

Primary cultures of rat hippocampal neurons provide an excellent in vitro system in which the process of spine formation can be investigated (Papa et al., 1995; Ziv and Smith, 1996; Boyer et al., 1998). In these cultures, spine density increases in the course of 3–4 weeks in vitro (Papa et al., 1995; Ethell and Yamaguchi, 1999; Takahashi et al., 2003). Using this system, we showed that spine density along dendrites was significantly increased in ac.CaP-GFP-expressing neurons. Since the increase in spine density was observed during the second week in vitro, we suggest that ac.CaP is involved in spine formation. This suggestion is strongly supported by our recent data showing that in the rat model of temporal lobe epilepsy (TLE), the increased levels of ac.CaP were observed during the period of spine density recovery (Ferhat et al., 2003). By contrast to ac.CaP, the overexpression of drebrin, another actin-binding protein, did not induce significant changes in spine density in cultured cortical neurons (Hayashi and Shirao, 1999). Thus, the increase in spine density is specific to ac.CaP and can be related to the biochemical properties of the calponin family, which is known to stimulate polymerization of actin filaments in vitro (Kake et al., 1995). Consistent with this idea, it has been reported that Purkinje cells of transgenic mice expressing a constitutively active small GTPase Rac1, known to regulate actin polymerization (Hall, 1994), displayed spines that were reduced in size but increased in number (Luo et al., 1996).

Several time-lapse observations have revealed a high level of structural dynamics of dendrites during development, including both outgrowth and resorption of dendritic spines (Dailey and Smith, 1996; Lendvai et al., 2000; Prange and Murphy, 2001; Trachtenberg et al., 2002). Another mechanism that could increase spine density may involve alteration of dynamic turnover of spines via stabilization of actin filaments. Our data show that ac.CaP protects actin filaments from latrunculin B destabilization. So, it is possible that when ac.CaP is expressed at high levels, it might stabilize actin filaments in spines and therefore alters their dynamic turnover (resorption). This inhibition of spine retraction, thus, may result in the increase of spine density in ac.CaP-expressing neurons. Altogether, these observations suggest that the increased spine density in ac.CaP-expressing neurons is the result of either one or two independent mechanisms: polymerization of actin or/and stabilization of actin filaments.

It has been reported that actin filaments in the spines displayed particular stability against the actin-depolymerizing reagents as compared with those of dendritic shafts or cell bodies (Allison et al., 1998). The high stability of actin filaments within the spines might result from the presence of several stabilizing actin proteins in them such as ac.CaP and drebrin, which this later was shown to stabilize actin filaments of neuroblastoma cells (Asada et al., 1994). Thus, ac.CaP, in addition to drebrin, may help to maintain the turnover of actin filaments within dendritic spines at a low rate.

Acidic Calponin May Be Involved in Spine Morphogenesis

In addition to remodeling of spine density, change in spine shape was observed in ac.CaP-GFP-expressing neurons. Indeed, the overexpression of ac.CaP-GFP in cultured hippocampal neurons caused elongation of dendritic spines, similar to the spines observed in GFP-drebrin (Hayashi and Shirao, 1999), cortactin (Hering and Sheng, 2003) or α-actinin2-expressing neurons (Nakagawa et al., 2004). This effect was clearly due to ac.CaP-GFP, as ac.CaPΔABS-GFP, which lacks actin-binding activity, did not show any morphological change. This observation shows also that the elongation of spines induced by ac.CaP-GFP requires its interaction with actin filaments. Altogether, these evidences suggest that ac.CaP directly binds to actin filaments in spines to induce morphological changes.

Changes in spine shape have been also associated with some forms of mental retardation, including fragile X syndrome (Irwin et al., 2000; 2001). This syndrome is caused by a mutation in the FMR1 gene, leading to lack of the fragile X mental retardation protein. Both patients (Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2000; 2001) and FMR1 knock-out mice (Comery et al., 1997; Irwin et al., 2000; Nimchinsky et al., 2001) exhibited long dendritic spines and increased density,

similar to spines observed in ac.CaP-expressing neurons. Interestingly, the patients and Knock out mice displayed a high susceptibility to develop seizures (Musumeci et al., 2000; Incorpora et al., 2002). Similar plastic changes in dendritic spines were also observed in the kainate (Represa et al., 1993) and pilocarpine (Isokawa, 1998, 2000) models of TLE. In the latter, ac.CaP levels were shown to be increased (Ferhat et al., 2003). Thus, it is tempting to speculate that actin misregulation through an increase of ac.CaP levels is one of the major problems occurring in neurons in pathological situations. This hypothesis is reinforced by the data showing that an increase in Profilin, another actin-binding protein, mimics the phenotype of *Drosophila* FMR1 mutants (Reeve et al., 2005).

Acidic Calponin Overexpression Induces Functional Excitatory Contacts

The close apposition of the presynaptic marker synaptophysin to these long protuberances and the presence within them of specific postsynaptic markers such as actin, PSD-95, NR1, and GluR1 suggest the existence of excitatory synaptic contacts.

To investigate whether the elongated spines were the sites for functional glutamatergic synapses, electrophysiological recordings of miniature glutamatergic activity were performed in GFP- and ac.CaP-GFP-expressing neurons. An increase in the frequency of mEPSCs has been reported in PSD-95-overexpressing neurons (El-Husseini et al., 2000; Beique and Andrade, 2003; Losi et al., 2003). Like PSD-95, ac.CaP can induce important morphofunctional changes following its overexpression. Indeed, the frequency of synaptic AMPA mediated currents was higher in ac.CaP-GFP-expressing neurons as compared with that of GFP-expressing neurons, while the amplitudes of the synaptic currents were comparable. Therefore, the increased frequency of mEPSCs in ac.CaP-GFP could be due to the increased number of functional synapses. Alternatively, an increase in the probability of release of glutamate at excitatory terminals could also explain such a result. Nevertheless, the first hypothesis is reinforced by the fact that ac.CaP displayed an increased number of spines as compared with that of GFP-expressing neurons. The fact that the amplitude and kinetics of AMPA-mediated synaptic currents were not changed suggests that at the postsynaptic level the number and biophysical properties of the AMPA receptor channels were not different in the newly formed or already established glutamatergic synapses in ac.CaP-GFP-expressing neurons.

How Can Acidic Calponin Induce Elongation of Spines?

Several mechanisms may be involved in the elongation of spines after overexpression of ac.CaP. Because ac.CaP has been shown to bind with F-actin (Applegate et al., 1994; Fujii et al., 2002; Abouzaglou et al., 2004), it is possible to assume that ac.CaP can compete with other actin-binding proteins. Thus, when ac.CaP is present at high levels within neurons, the actin-binding proteins would be dissociated from F-actin. Therefore, the organization and the dynamic of actin filaments would be altered, leading to the alteration in the shape of dendritic spines. In agreement with this scenario, our data show that overexpression of ac.CaP in nonneuronal cells resulted in the disappearance of stress fibers and the appearance of thick, curving bundles of actin filaments, and consequently the formation of several processes that often branched (Ferhat et al., 2001; present findings). Moreover, our data show that these actin filaments with ac.CaP were not disrupted by latrunculin B, but those without ac.CaP were disrupted. Therefore, we suggest that the overexpression of ac.CaP may affect the organization and dynamic stability of actin filaments in spines, leading to the elongation of dendritic spines.

Another possibility to induce elongation of dendritic spine is that ac.CaP stimulates by itself the actin polymerization, resulting in abnormal growth of F-actin in spines of ac.CaP-expressing neurons. Indeed, a biochemical study revealed that, in vitro, calponins are able to stimulate actin polymerization (Kake et al., 1995). However, ac.CaP could recruit another actin-binding protein, such as profilin, known to stimulate actin polymerization (Buss et al., 1992; Rothkegel et al., 1996; Carlsson et al., 1977), causing elongation of dendritic spines. Such mechanism has been suggested in the case of drebrin (Hayashi and Shirao, 1999).

Actin filaments are the major cytoskeletal components of dendritic spines (Fifkova and Delay, 1982; Matus et al., 1982). Because myosin is also detected at high levels in spines (Drenckhahn and Kaiser, 1983; Morales and Fifkova, 1989), actomyosin-based motility may be involved in the elongation of spines in ac.CaP-GFP-expressing neurons. It has been also shown that the calponin family inhibited actin-base ATPase of myosin and inhibited the movement of actin over a myosin-bound surface in the sliding actin motility assay (Shirinsky et al., 1992; Haeberle, 1994; Gimona and Small, 1996; Winder and Walsh, 1996; Winder et al., 1998). So, when ac.CaP predominates within spines, it may reduce the motility or the sliding of actin filaments on myosin and therefore spines do not retract. Consistent with this idea, overexpression of drebrin, which also affects the actomyosin machinery (Hayashi et al., 1996), has been shown to induce an elongation of dendritic spines in cortical neurons (Hayashi and Shirao, 1999). Lin et al. (1996) showed that inhibition of the motor activity of myosin at the growth cone induces attenuation of retrograde F-actin flow. This phenomenon is accompanied by filopodia elongation of growth cone due to the polymerization process (Lin et al., 1996). On the basis of all these observations, we propose that elongation of spines induced by ac.CaP results from superimposition of two additive mechanisms: actin polymerization and actomyosin activity inhibition.

In summary, we have shown that the overexpression of ac.CaP regulates spine plasticity and synaptic activity. It is compelling to suggest that this protein may be used not only in the regulation of muscle contraction but also in neuroplasticity.

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