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AKAP-Independent Localization of Type-II Protein Kinase A to Dynamic Actin Microspikes

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Abstract

Regulation of the cyclic AMP-dependent protein kinase (PKA) in subcellular space is required for cytoskeletal dynamics and chemotaxis. Currently, spatial regulation of PKA is thought to require the association of PKA regulatory (R) subunits with A-kinase anchoring proteins (AKAPs). Here, we show that the regulatory RII α subunit of PKA associates with dynamic actin microspikes in an AKAP-independent manner. Both endogenous RII α and a GFP-RII α fusion protein co-localize with F-actin in microspikes within hippocampal neuron growth cones and the leading edge lamellae of NG108-15 cells. Live-cell imaging demonstrates that RII α -associated microspikes are highly dynamic and that the coupling of RII α to actin is tight, as the movement of both actin and RII α are immediately and coincidentally stopped by low-dose cytochalasin D. Importantly, co-localization of RII α and actin in these structures is resistant to displacement by a cell-permeable disrupter of PKA-AKAP interactions. Biochemical fractionation confirms that a substantial pool of PKA RII α is associated with the detergent-insoluble cytoskeleton and is resistant to extraction by a peptide inhibitor of AKAP interactions. Finally, mutation of the AKAP-binding domain of RII α fails to disrupt its association with actin microspikes. These data provide the first demonstration of the physical association of a kinase with such dynamic actin structures, as well as the first demonstration of the ability of type-II PKA to localize to discrete subcellular structures independently of canonical AKAP function. This association is likely to be important for microfilament dynamics and cell migration and may prime the investigation of novel mechanisms for localizing PKA activity.

Keywords

Signal transduction; cytoskeletal dynamics; kinase anchoring; compartmentalization; growth cones; lamellae

INTRODUCTION

The cAMP-dependent protein kinase, PKA, is a heterotetrameric enzyme comprising two regulatory (R) and two catalytic (C) subunits. Four R subunit isoforms - RI α , RI β , RII α , RII β - maintain the holoenzyme in an inactive state and release active C subunit upon cooperative binding of two molecules of cAMP per molecule of R. The N-terminus of each R subunit mediates homodimerization and also houses the region(s) responsible for subcellular localization of PKA (see below). The known and putative substrates for PKA are myriad and distributed widely throughout the cellular landscape (Howe 2004; Shabb 2001), *e.g.* the plasma membrane, cytoplasm, mitochondria, nucleus, and nearly every family of the cytoskeletal network, including microtubules, intermediate filaments, and actin

microfilaments (Howe 2004). A significant portion of the specificity of PKA signaling, therefore, is achieved through differential regulation of the enzyme in subcellular space. This regulation occurs largely through the functionally-related but structurally diverse A-kinase anchoring protein (AKAP) family of proteins (Michel and Scott 2002). AKAPs compartmentalize PKA – typically, but not always, via RII isoforms – to various subcellular regions or structures, and thus provide an invaluable measure of specificity to cAMP/PKA signaling. In addition to localization, many AKAPs direct the assembly of multi-enzyme complexes that serve as pre-assembled circuits capable of integrating multiple, diverse input signals to control the phosphorylation of a given target(s) with exquisite spatial and temporal specificity (Michel and Scott 2002).

A critical role for PKA in neuronal development and function is evidenced by the numerous and profound neurological defects and deficits observed in knockout mice lacking various subunits (Brandon et al. 1998; Brandon et al. 1995; Fischer et al. 2004; Hensch et al. 1998; Howe et al. 2006; Huang et al. 2002; Huang et al. 1995; Malmberg et al. 1997; Qi et al. 1996; Rao et al. 2004; Watson et al. 2006). While the role of PKA as a regulator of transcription likely contributes to some if not all of these cases, a significant body of literature has demonstrated that neuritogenesis and axon guidance are also specifically modulated by signaling via cAMP and PKA (Song and Poo 1999). For example, chemoattraction of the growth cones of *Xenopus* spinal neurons to netrin-1 is converted into chemorepulsion upon inhibition of PKA activity (Ming et al. 1997; Nishiyama et al. 2003; Song et al. 1998). Furthermore, the netrin-1-induced repulsion of growth cones from UNC5-expressing neurons is nullified by PKA inhibition (Nishiyama et al. 2003). Also, the collapse of chick growth cones induced by semaphorin 3A can be prevented by nerve growth factor (NGF), in a PKA-dependent manner (Dontchev and Letourneau 2003). Similarly, PKA is required for protection from nitric oxide-induced growth cone collapse by brain-derived neurotrophic factor (BDNF) (Gallo et al. 2002). During the migration of growth cones, PKA activity may be physiologically modulated by the binding of diffusible factors to their cognate receptors (*e.g.* BDNF (Gallo et al. 2002)) and also by the interaction of growth cone integrins with extracellular matrix (ECM) proteins (*e.g.* laminin (Hopker et al. 1999)). These and other studies (reviewed in (Gallo and Letourneau 2004; Song and Poo 1999)) convincingly demonstrate that PKA is an important facet of growth cone migration in response to neuronal guidance cues.

The principal mechanism that drives axon guidance is modulation of actin cytoskeletal dynamics (Dent and Gertler 2003). Polymerization of filamentous actin (F-actin) occurs in the periphery of neurons during neuritogenesis and of exploratory axonal growth cones to give rise to the various cellular structures involved in process formation. Most often, a broad, fan-shaped lamellae formed by branched actin arrays is populated by radially-arranged bundles of parallel actin filaments known as filopodia (if they extend past the margin of the lamellae) or microspikes (if they remain flush with the cell periphery). Filopodia are thought to probe the area beyond the lamellae to find suitable purchase for the advancing cellular structure and, upon doing so, relay signals that extend the lamellae in that direction. In turn, the lamellae serves, at least in part, to provide the raw materials required to recycle and regenerate more microspikes and filopodia to begin the process anew. A complex mixture of soluble and architectural extracellular guidance cues control the balance of filopodial and lamellar activities in order to properly regulate the timing, location, and trajectory of neurite outgrowth. Thus, these protrusive structures are also principally responsible for sensing differences in the extracellular environment - gradients of guidance cues and extracellular matrix molecules – in order to maintain the fidelity of neuronal process extension. While the importance of actin cytoskeletal regulation during neuritogenesis and axon guidance is firmly established, the signaling events responsible for

controlling cytoskeletal dynamics are still under intense investigation. Thus, delineation of the signaling pathways underlying neuronal cytoskeletal regulation is of critical importance.

It is well established that PKA can regulate cytoskeletal dynamics and cell migration (Howe 2004). Furthermore, recent work has demonstrated that PKA is specifically up-regulated at the leading edge of migrating cells and that subcellular anchoring of PKA is important for this effect (Howe et al. 2005; Lim et al. 2007; Lim et al. 2008; Paulucci-Holthauzen et al. 2008). Indeed, both inhibition of PKA activity and inhibition of PKA interaction with AKAPs inhibit the formation of migration-associated morphological features as well as overall chemotactic cell movement (Howe et al. 2005; Lim et al. 2007; Lim et al. 2008; Paulucci-Holthauzen et al. 2008). In an ongoing effort to investigate the role of localized PKA activity in regulating neuronal cytoskeletal dynamics, we endeavored to test the hypothesis that AKAP-mediated localization of PKA was important for growth cone dynamics. During our initial investigations, Han *et al* (Han et al. 2007) confirmed this hypothesis by demonstrating that type-II PKA is enriched along growth cone filopodia, and that inhibition of PKA-AKAP interaction disrupts growth cone guidance (Han et al. 2007). In the current work, we also demonstrate that PKA localizes to actin-rich structures in neuronal growth cones and neuronal cell lamellae. However, unlike previous reports, we find that this pool of PKA is resistant to disruption or extraction by canonical inhibitors of AKAP function. Thus, we describe here a fraction of PKA that exhibits a heretofore unreported association with highly dynamic actin structures in the lamellae of neuronal cells in a manner that, surprisingly, is independent of traditional AKAP function.

MATERIALS AND METHODS

Cells and Reagents

NG108-15 mouse neuroblastoma x rat glioma somatic hybrid cells were obtained from the American Type Culture Collection (ATCC) and rat embryonic day 18 hippocampal tissue was obtained from BrainBits, LLC. and dissociated neurons were prepared and cultured according to the supplier's instructions. DMEM, Neurobasal, and Opti-MEM media, B27 supplement, HAT, Glutamax, and other routine cell culture supplies were from Invitrogen, while animal sera were from HyClone. Eugene 6 was from Roche Applied Sciences. Poly-D-lysine, cytochalasin D, protease inhibitors and other sundry chemicals and biochemicals were from Sigma. StHt31, StHt31P, and Ht31 peptides were from Promega, while the cell-penetrating Arg₁₁-Ht31 was synthesized by GenScript. The following antibodies and secondary reagents were used: rabbit polyclonal anti-PKA RII α (sc-909; Santa Cruz Biotechnology); monoclonal mouse anti-PKA RII α (612243; BD Biosciences); rabbit polyclonal anti-GAP43 (Ab16053; Abcam), mouse monoclonal anti-vimentin (V9; Sigma); mouse monoclonal anti-actin (AC-74; Sigma); rabbit polyclonal and mouse monoclonal anti-GFP (11120 and 11122, Invitrogen); mouse monoclonal anti-AKAP 79/150 (A31320, BD Biosciences); rabbit polyclonal anti-WAVE1 (A2142, Sigma); Alexafluor 488-, 594-, and 647-conjugated donkey anti-mouse and anti-rabbit secondary antibodies and phalloidins (Invitrogen); horseradish peroxidase-conjugated sheep anti-mouse and anti-rabbit secondary antibodies (Calbiochem). Glass coverslips (22 mm diameter; Fisher Scientific) were cleaned by immersion in 1N HCl at 55° C for 4 h, and then sonication for 30 min each in water, 50% ethanol (EtOH), 70% EtOH, and 95% EtOH. Coverslips were air dried in a sterile tissue culture hood and then coated overnight at 4° C with poly-D-lysine at 30 μ g/ml before cell seeding.

Plasmids

Standard PCR and cloning techniques were used to remove the stop codon and introduce a 5' EcoRI and an in-frame 3' AgeI site into the coding sequence of the murine PKA RII α

subunit, which was then fused to the 5' end of EGFP in pEGFP-N1 (Clontech). The I3,5S double point mutant (in which the isoleucines at positions 3 and 5 are changed to serines) and the $\Delta 2-5$ deletion mutant (which removes the indicated residues) that disrupt the interaction between RII α and AKAPs were generated using the Quikchange Mutagenesis Kit (Stratagene) using the following primers (all listed 5' to 3'):

I3,5Sfwd = GCCACCATGGGCCACAGCCAGAGCCCCGCCGGGGCTC;

I3,5Srev = CGGTGGTACCCGGTGTCGGTCTCGGGCGGCCCGAG;

$\Delta 2-5$ fwd = GAATTCGCCACCATGCCGCCGGGGCTC;

$\Delta 2-5$ rev = CTTAAGCGGTGGTACGGCGGCCCGAG.

Positive clones were verified by sequencing. The plasmid encoding mCherry-actin was generously provided by Dr. Frank Gertler (M.I.T.) with kind permission of Dr. Roger Tsien (UCSD) as described previously (Deming et al. 2008).

Cell Culture and Transfection

NG108-15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate, supplemented with 10% fetal bovine serum and hypoxanthine, aminopterin, and thymidine (HAT) at 100 μ M, 400 nM, and 16 μ M, respectively. For neuronal differentiation, cells at ~60% confluence were trypsinized and re-plated onto polylysine-coated surfaces in culture medium containing 5% fetal bovine serum. NG108-15 cells in 6 cm dishes were transfected with indicated plasmids (200 – 500 ng DNA) using Eugene 6 (at a 4:1 vol:wt ratio). Hippocampal neurons were dissociated from intact tissues and cultured according to the supplier's instructions. Briefly, intact hippocampi were incubated in 0.25% trypsin for 15 min at 37° C then triturated 15 times through a barely fire-polished Pasteur pipette. The cell suspension was then passed through a cell strainer (BD Biosciences), collected by centrifugation at 200 $\times$ g for 2 min, then resuspended in Neurobasal medium containing B27 and Glutamax supplements. Primary neurons were reverse-transfected using Eugene 6 and a slight modifications of the manufacturer's protocol for the SureFECT reagent (SA Biosciences). All cells were re-plated and analyzed 24 h post-transfection.

Microscopy of Fixed and Live Cells

Cells were plated at a density of ~2,000 cells per cm² onto acid-washed, polylysine-coated coverslips for fixed cell microscopy or onto polylysine-coated Δ T4 dishes (Bioprotechs) for live-cell microscopy. Where indicated, cells were fixed with warm 4% PBS-buffered formaldehyde (freshly prepared from the its para- polymer) for 20 min at RT, permeabilized with 0.1% Triton-X100 in PBS for 20 min at RT, and blocked overnight at 4° C in 3% BSA in PBS. Cells were then incubated with the indicated primary and secondary antibodies and phalloidin, all in 3% BSA in PBS, for 1 hr at RT, with 5 $\times$ 5 min washes in PBS after each incubation. For live-cell imaging, polylysine-coated Δ T4 dishes were rinsed with PBS and blocked with DMEM containing 5% FBS for 1 hour at 37° C before adding transfected cells. NG108-15 cells were plated and incubated at 37° C for 2 h, after which the plating media was exchanged for Hanks' buffered salt solution + 20 mM HEPES pH 7.4 for imaging purposes. Microscopy was performed as previously described (Deming et al. 2008) on a Nikon Eclipse TE2000-PFS inverted microscope equipped with 40 $\times$ dry and 60 $\times$ oil immersion objectives, Ludl shutters and filter wheels, Chroma optical filters, and an Exfo XCite 120 light source, all controlled by Nikon Elements software. During live-cell imaging, cells were maintained at 35°C using a Δ T4 dish heating system (Bioprotechs) with a heated lid to prevent media evaporation and condensation. Exposure times for RII-EGFP and mCherry-actin fusions were routinely 3 sec and 500 msec, respectively, and images were

typically captured every 5–7 sec for 10–30 min. Where indicated, some images were deconvolved using AutoQuant X software (Media Cybernetics), using 2D convolution algorithms and fluorophore-optimized settings. Time-lapse sequences were cropped, time-stamped and made into montages using ImageJ software (Wayne Rasband, NIH).

Kymography, Velocity Measurements, and Co-localization Analyses

Single pixel-wide, linear regions of interest (ROI) were used to generate kymographs from time-lapse image stacks using the Multiple Kymograph plugin of ImageJ. The region beneath the linear ROI is extracted from each image in the stack, and the resultant series of lines are juxtaposed, giving a representation of the dynamics of the selected region over time. ROIs were always drawn from the periphery of the cell inward, so centripetal motion of a given fluorescent speckle is depicted as a downward-sloping line (see Fig. 5B'). The x axis in these images is equal to the length of the selected ROI, in other words the scaled distance (in microns) of the linescan. The y axis represents time, with a scale equal to the image acquisition rate. Given this relationship, the velocity of a given particle is simply the slope of the line (distance over time) described by the particle over time, which is most easily calculated as the tangent of the angle described by the line relative to the horizontal (see Fig. 5B''). Distance and angular measurements were obtained from kymographs using ImageJ and GraphPad Prism was used to tabulate and analyze results to determine average values, standard deviations, and P values using an unpaired, two-tailed t-test.

For analysis of co-localization, two methods were used. First, linear regions of interest (ROI) were drawn in RGB color overlay images and pixel intensities within the region were plotted using the *Color Profiler* tool in ImageJ. In all cases of putative co-localization, the distance between the channel peaks was smaller than the resolution of the optical configuration for the corresponding image, thereby indicating co-localization. Second, square subcellular ROIs were specified in various images and the degree of co-localization within them was quantified using JACoP (Just Another Co-localization Plugin; written by S. Bolte & F. P. Cordelières) for ImageJ to determine Pearson's correlation coefficients. Because of the considerable differences in acquisition parameters and signal intensities between RII α and actin in some cases, we also determined and reported Manders' overlap coefficients (M1 & M2, or M_{RII α} & M_{actin}), which are based on Pearson's correlation coefficients but with the average intensity values taken out of the mathematical expression. For both Pearson's and Manders' coefficients, higher values indicate a higher degree of co-localization (with a maximum value of 1).

Immunoprecipitation and Metabolic Radiolabeling

Cells were harvested in modified RIPA lysis buffer and immunoprecipitations were performed essentially as described elsewhere (Howe et al. 2002). Briefly, lysates were pre-cleared by centrifugation, cleared by incubation with washed protein A/G-agarose beads (Santa Cruz Biotechnology), then incubated with the indicated antibodies for 2 h at 4°C. Immune complexes were collected on protein A/G-agarose, and the immunoprecipitates were washed four times with lysis buffer before boiling in Laemmli sample buffer and separation by SDS-PAGE. In some cases, the immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. In other cases, immunoprecipitates were prepared from cells that had been incubated in Met-free DMEM for 2 h, then metabolically labeled for 6 h with 100 μ Ci/ml Trans³⁵S-Label, containing both ³⁵S-methionine and ³⁵S-cysteine, (MP Biomedicals) in a 10:1 mixture of Met-free DMEM:regular DMEM. It was during this incubation that cells were re-plated onto polylysine-coated dishes (using their original labeling medium) and cultured as further described in the figure legends. Immunoprecipitates from radiolabeled cell extracts were separated by SDS-PAGE and visualized by autoradiography to film (Denville Scientific).

Growth Cone Particle Purification and Serial Extraction

The protocol is based on previously described methods for growth cone purification through differential centrifugation (Lohse et al. 1996; Pfenninger et al. 1983). Here, thirty 15 cm plates were seeded with NG108-15 cells at a density of 15,000 cells/cm² in DMEM + 5 % FBS + 1× HAT and incubated for 20 h before harvest. Cells were then scraped into EDTA buffer (0.54 mM EDTA in 1× PBS, containing the protease inhibitors aprotinin, AEBSF, leupeptin, benzamidine HCl, and pepstatin A). Cells were collected by centrifugation for 30 min at 400 × g at 4°C, resuspended in 6 ml of EDTA buffer, and homogenized by hand in a ground-glass homogenizer (Kontes) on ice for 45 min. Subsequently, 1.5 ml of homogenized cell material was loaded gently on top of four 500 µl cushions of 0.83 M sucrose in 2ml, thick-walled ultracentrifuge tubes (Beckman). Samples were then centrifuged at 240,675 × g for 40 minutes at 4°C in a Beckman tabletop ultracentrifuge, after which the growth cone fraction is located at the interface between the lysate and the 0.83 M sucrose solution. This material was then taken and mixed with 1.5 ml of a 0.32 M sucrose solution, triturated, and spun at 39,800 × g for 30 min at 4°, to generate a pellet containing purified growth cone particles. For differential, serial extraction, the growth cones particle pellet was resuspended in 40 µl cytoskeletal buffer (CB; containing 0.5% Triton X100, 10 mM PIPES pH 6.8, 140 mM KCl, 2 mM EGTA, 2 mM MgCl₂), briefly vortexed, triturated twenty times through a 28G Hamilton syringe, and incubated on ice for 20 min. The sample was then spun at 30,000 × g for 30 min at 4°C. The supernatant, containing Triton X-soluble proteins, was collected, and the pellet was resuspended in 40 µl CB containing Ht31 (at 0.4 µM, a concentration routinely used to inhibit RII-AKAP interactions *in vitro* (Alto et al. 2003; Gold et al. 2006)). The resuspended pellet was briefly vortexed, sonicated for two minutes at 4°C (Branson Sonifier), triturated as above, and incubated on ice for 20 min. The sample was then centrifuged at 16,800 × g for 10 minutes at 4°C. The supernatant, containing Ht31-extractable material, was collected and the pellet was extracted a second time with 40 µl CB + Ht31, and the entire sequence was repeated once again with 40 µl CB containing 2.5 µM cytoD. The minute pellet remaining after the sequential extraction was resuspended in 1× Laemmli sample buffer. The entire volume of each extraction was then analyzed by SDS-PAGE and immunoblotting with antibody against PKA RIIα (BD Biosciences) or actin (Sigma).

RESULTS

To begin to test the hypothesis that AKAP-mediated localization of PKA was important for growth cone function, we examined the localization of the RIIα subunit of PKA in the growth cones of primary, dissociated rat hippocampal neurons by indirect immunofluorescence. Similar to the recently described localization of RIIα in *Xenopus* spinal neurons (Han et al. 2007), a significant portion of RIIα in hippocampal growth cones appeared to co-localize with F-actin-rich microspikes within the growth cone lamellae (Fig. 1A). Co-localization was confirmed by linescan analysis of fluorescence intensity across individual microspikes: the distance between the intensity peaks was smaller than the resolution of the optical configuration for the corresponding image, indicating co-localization of RIIα and actin (Fig. 1B). Determination of Pearson's correlation coefficients in selected regions of interest revealed that coefficients in regions containing microspikes were consistently and significantly higher than in regions devoid of microspikes, further confirming co-localization of RIIα and actin in these structures (Fig. 1C).

The subcellular localization of type-II (*e.g.* RII-containing) PKA is principally governed by the interaction between RII subunits and AKAPs (Michel and Scott 2002). This interaction occurs through a conserved region in the extreme N-terminus of RII subunits (Hausken et al. 1994) and a structurally conserved amphipathic alpha-helical region within all known AKAPs (Gold et al. 2006; Kinderman et al. 2006). The interaction of PKA with endogenous

AKAPs can be competitively inhibited by administration of exogenous peptides that contain such an amphipathic helical region (Gold et al. 2006; Michel and Scott 2002). Therefore, to assess the role of AKAPs in this localization, we treated the neurons with StHt31, a steared and thus cell-permeable peptide, derived from AKAP-Lbc (formerly known as Ht31), commonly used as an inhibitor of PKA anchoring (Michel and Scott 2002). As expected, treatment with StHt31P, a non-functional control peptide in which two critical isoleucine residues are substituted by prolines, had no effect on PKA localization (Fig. 1A–C). Surprisingly, however, StHt31 also had little discernable effect on the co-localization of RII α with actin microspikes (Fig. 1A–C), even under treatment conditions in excess of those known to disrupt canonical PKA anchoring (Stokka et al. 2006), PKA localization in spinal neuron growth cones (Han et al. 2007), and AKAP-dependent cell migration (Howe et al. 2005). The lack of displacement of RII α from actin microspikes was not due to a lack of effect or cell penetration of StHt31 under our particular experimental system, as similar treatment conditions were effective at disrupting the biochemical interaction of PKA with known AKAPs (data not shown and Fig. 6). These data suggest that the RII α subunit of PKA co-localizes with actin microspikes in an ostensibly AKAP-independent manner.

Because the actin cytoskeleton is highly dynamic, we wanted to investigate whether this subset of PKA exhibited similar dynamics. To this end, we transfected primary hippocampal neurons with a plasmid encoding PKA RII α fused to enhanced green fluorescent protein (EGFP), with the intent of imaging the dynamics of RII α -EGFP in live cells. Preliminary experiments revealed that high levels of expression of RII α -EGFP consistently altered the spreading and polarization behavior of primary rat hippocampal neurons. Specifically, while untransfected neurons or neurons transfected with EGFP alone exhibited morphologies classically associated with stage 1 through stage 4 neurons in culture ((Goslin and Banker 1989) and data not shown), neurons exhibiting bright, readily detectable levels of RII α -EGFP fluorescence consistently failed to initially spread with the same kinetics as control neurons, and frequently failed to develop distinct, mature neuritic processes (data not shown). While potentially quite interesting, the biological basis underlying this effect is completely unknown. Nonetheless, closer examination of transfected cultures revealed that neurons expressing very low levels of RII α -EGFP (assessed by eye and by microscopy and camera exposure settings) exhibited classical morphologies indistinguishable from those of untransfected cells (Supp. Info. Fig. 1). Importantly, as seen for endogenous PKA, there was also significant, specific co-localization of RII α -EGFP with actin microspikes in growth cone lamellae (Supp. Info. Fig. 1). However, these conditions did not allow assessment of RII α -EGFP dynamics by live-cell imaging, as the low level of EGFP fluorescence necessitated extremely long exposure times (see Supp. Info. Fig. 1 and below), which resulted in excessive phototoxicity and rapid cell death in the primary neurons (data not shown).

Therefore, to carry our investigation further, we employed NG108-15 cells, a neuroblastoma $\times$ glioma hybrid neuronal cell line, as a surrogate cell system with morphological features that are comparable to early stage hippocampal neurons (Smalheiser 1989), but are heartier in culture than primary neurons. To ensure that NG108-15 cells would faithfully recapitulate the distinct localization of PKA RII α subunits observed in hippocampal neurons, we plated the cells under differentiating conditions (*i.e.* on polylysine-coated coverslips in low serum; see Materials & Methods) and first examined the localization of endogenous RII α and F-actin by immunofluorescence. As seen in the hippocampal neurons, RII α exhibited striking, specific co-localization with actin microspikes within the growth cones of differentiated NG108-15 cells (Fig. 2). The degree of co-localization was confirmed by linescan analysis across microspikes as well as by determining Pearson's and Manders' coefficients in control- and microspike-rich regions (Fig. 2B). Furthermore, this localization was similarly AKAP-independent, as it was not displaced by treatment of cells with either StHt31 (data

not shown and Fig. 6) or with Ht31 fused to a cell-penetrating poly-Arg peptide (Arg₁₁-Ht31; Supp. Info. Fig. 2). As seen in the primary neurons, we found that high levels of expression of the RII α -EGFP fusion also tended to inhibit neuritic process formation in NG108-15 cells (data not shown). This necessitated the observation of only those cells with moderate to low levels of fluorescence (as an indicator of RII α -EGFP expression), which in turn required long (*e.g.* 3 sec) exposure times. However, NG108-15 cells proved far more robust than primary hippocampal neurons and survived such imaging regimens without any obvious signs of phototoxicity. Importantly, we found that exogenously expressed RII α -EGFP also exhibited strong co-localization with actin microspikes in differentiated NG108-15 cells (Fig. 2). Because of the long exposure times required to visualize RII α -EGFP compared to phalloidin-stained F-actin in fixed cells, we performed photobleaching experiments to confirm that the microspike-associated RII α -EGFP fluorescence was not due to bleedthrough or optical anomaly (Supp. Info. Fig. 3).

During the evaluation of the localization of endogenous RII α and RII α -EGFP in NG108-15 cells, it became readily apparent that the co-localization between RII α and actin microspikes was not complete: some filaments and microspikes were highly decorated with RII α while other, equally prominent actin features were devoid of RII α signal (see Fig. 2). This heterogeneity suggested that the localization of RII α to these actin structures might be regulated. This in turn underscored the importance of evaluating the dynamics of RII α in live cells. Indeed, live cell imaging revealed that the filament-like structures described by RII α -EGFP in neuronal cell lamellae are extremely dynamic, with individual filaments exhibiting assembly and dissolution, and comet-like radial and lateral movements (Fig. 3 and Supp. Info. Movies 1 and 2). The dynamics of the RII α -filaments are reminiscent of the dynamics of previously described actin-based structures in neuronal lamellae such as microspikes (Bray and Chapman 1985), inductopodia (Thompson et al. 1996), and intrapodia (Rochlin et al. 1999), which further supported our prior observations of co-localization between RII α and F-actin microspikes. To confirm that these dynamic RII α filaments were indeed coincident with the actin cytoskeleton, we co-expressed RII α -EGFP and mCherry-actin and imaged both in live in NG108-15 cells. As expected, there was significant co-localization between the dynamic RII α -EGFP filaments and dynamic actin microspikes (Fig. 4 and Supp. Info. Movie 3): determination of regional Manders' coefficients over time showed that RII α /actin co-localization increased as microspikes formed and ebbed as the structures dissolved (Fig. 4C and Supp. Info. Fig. 4).

To confirm the putative coupling of the movement of PKA RII α subunits and actin in neuronal cell lamellae, we examined the dynamics of RII α -EGFP and mCherry-actin in live cells before and after the addition of a low concentration of cytochalasin D, a cyclic peptide well known for its ability to disrupt the actin cytoskeleton by binding the fast-growing barbed end of actin filaments. At very low concentrations (50–200 nM), actin filaments remain intact but monomer addition to the barbed end is blocked, resulting in inhibition of filament dynamics and retrograde flow (Bear et al. 2002). Empirically, we found 50 nM cytoD to be an optimal concentration for achieving these results under our experimental conditions. Importantly, addition of this concentration of cytoD rapidly halted not only actin cytoskeletal dynamics in the lamellae, but also the movements of RII-rich actin microspikes (Fig. 5A and Supp. Info. Movie 4), strongly supporting the notion that the dynamics of RII-rich microspikes within neuronal lamellae are closely coupled to actin microfilament dynamics. In addition to visualizing large-scale lateral movements of actin bundles and microspikes, the rapid rates of acquisition and relatively low levels of expression used here allowed us to routinely observe retrograde flow of actin along individual bundles/microspikes (see, for example, Supp. Info. Movie 4). Interestingly, lamellar RII α was almost always observed to flow in the same direction and ostensibly at the same rate. Therefore, to further confirm the coupling of RII α subunits and actin within the lamella, we used

kymography to determine the retrograde flow rate of both actin and RII α in individual microspikes, before and after the addition of cytoD (Fig 5B & C). The average velocity for actin, in the absence of drug, was found to be between $\sim 3.7 \mu\text{m}/\text{min}$, quite comparable to velocities reported by other groups for the retrograde actin flow observed in the similarly-shaped lamellae of *Aplysia* growth cones (Lin et al. 1996) and fish keratocytes (Jurado et al. 2005). Importantly, the velocities for actin and RII α were indistinguishable, both before ($P=0.6242$) and after ($P=0.9897$) addition of cytoD, providing additional strong evidence that RII α and actin are closely linked in neuronal lamellae.

The subcellular localization of type-II (*e.g.* RII-containing) PKA is mediated by the interaction of RII subunits with AKAPs (Michel and Scott 2002). However, as discussed above, treatment of primary hippocampal neurons with StHt31 failed to displace RII α from actin microspikes (Fig. 1). Also, endogenous RII α associated with microspikes in NG108-15 cells showed little or no overlap with ezrin (Supp. Info. Fig. 5), an AKAP known to be associated with the actin cytoskeleton (Michel and Scott 2002), and no overlap with AKAP79/150 (data not shown), a prominent AKAP in neurons whose loss of function is closely associated with several, diverse deficits in neuronal morphology and function (Michel and Scott 2002; Carnegie and Scott 2003; Dell'Acqua et al. 2006). Therefore, to begin to investigate the nature of the cytoskeletal localization of RII α in neuronal cell lamellae, we performed live-cell imaging of NG108-15 cells expressing RII α -EGFP and mCherry-actin in the absence and presence of a high concentration of the AKAP inhibitor, StHt31, as described in Fig. 1. The RII α -EGFP in StHt31-treated cells still exhibited a prominent co-localization with dynamic actin microspikes that was indistinguishable from control (StHt31P-treated) cells (Fig. 6A, Supp. Info. Fig. 6 and Movie 5). The treatment conditions (*i.e.* concentration and length of incubation) used here exceed those that have been reported to have significant effect on RII localization and activity in other cell systems (Han et al. 2007; Howe et al. 2005) and are, in fact, in the range in which the effects of the Ht31 peptide extend to inhibiting, *in vitro* or *in vivo*, the anchoring of RI-containing/type-I PKA (Alto et al. 2003; Ruppelt et al. 2007). Indeed, the lack of effect of StHt31 treatment on the RII α -EGFP microspikes was not due to a lack of overall efficacy of the inhibitor, as identical treatment conditions lead to a complete disruption of the interaction between RII α and two known AKAPs – WAVE1 and AKAP150 – as well as to the displacement of several other RII α -binding proteins (Fig. 6B). These data support our earlier observations in primary hippocampal neurons (Fig. 1), but are nonetheless surprising in that they suggest that the close coupling of RII α with peripheral actin microspikes occurs through a mechanism that is independent of traditional AKAP function.

To further investigate the possibility that the cytoskeletal association of PKA RII α in the periphery of neuronal cells is mediated by an AKAP-independent mechanism, we performed biochemical experiments in differentiated NG108-15 cells wherein growth cone particles were fractionated and purified from cell body components by gradient centrifugation (Lohse et al. 1996; Pfenninger et al. 1983), and then sequentially and differentially extracted with lysis buffers formulated to retain or liberate cytoskeletal elements and/or AKAP-bound proteins. The fidelity of our subcellular fractionation protocol was first confirmed by separation of extracts of isolated fractions by SDS-PAGE and analysis by immunoblotting for vimentin and GAP43 (Fig. 7A), markers for neuronal cell bodies and growth cones, respectively (Lohse et al. 1996). In subsequent experiments, purified growth cone pellets were extracted with a Triton X100-containing lysis buffer then centrifuged at high speed (see Materials and Methods), which preferentially solubilizes non-cytoskeletal elements into the supernatant buffer and thus leaves behind the detergent-insoluble cytoskeleton and associated complexes. Subsequently, the remaining, cytoskeleton-enriched pellet was further and sequentially extracted with Triton X100-buffer containing either Ht31 (twice) or cytoD to disrupt AKAP function or actin microfilament integrity, respectively (outlined in Fig.

7B). Proteins from each serial extraction, as well as the proteins remaining in the pellet after the final extraction (which were solubilized in SDS-containing RIPA buffer) were then analyzed by SDS-PAGE and immunoblotting for RII α . Some, but certainly not all of the RII α in the growth cones was solubilized in the initial extraction with Triton X-buffer alone (Fig. 7C, 'TX-S'). A far greater portion of the RII α was liberated during the first extraction of the remaining pellet with buffer containing Ht31 (Fig. 7C, 'Ht31 #1'), consistent with the idea that the residence of RII α in the cytoskeletal pellet was due at least in part to AKAP-mediated anchoring. Little detectable RII α was solubilized during the second Ht31 extraction (Fig. 7C, 'Ht31 #2'), supporting the efficiency of the first Ht31 extraction. Interestingly, however, a significant amount of RII α remained after these two rounds of Ht31 extraction: a considerable fraction of this Ht31-resistant pool of RII α was solubilized by extraction with buffer containing cytoD (Fig. 7C, 'cytoD'), while even more remained in the heavily extracted pellet, only to be liberated by solubilization in Laemmli sample buffer (Fig. 7C, 'P'). Of note, actin immunoblotting revealed that a considerable amount of actin also remained in the pellet, despite the cytoD extraction, and was ultimately solubilized in Laemmli buffer (Fig. 7C). Taken together, these biochemical data suggest that a minor, but quite significant portion of the RII α present in neuronal growth cones is in tight, AKAP-independent association with the actin cytoskeleton.

To this point, all of the data suggesting an AKAP-independent localization of RII α to the cytoskeleton depend on the ability of the Ht31 peptide to disrupt PKA-AKAP interactions. Given the somewhat heretical nature of our results, we wanted confirm our hypothesis of AKAP-independent localization with a complimentary approach that was independent of Ht31 and other such pharmacological inhibitors of AKAP function. Thus, we opted for a molecular genetic approach in which we introduced into the N-terminal region of RII α -EGFP two different mutations – I3,5S and Δ 2–5 – that have been previously described to preserve R subunit dimerization but disrupt the interaction of RII α with all known AKAPs (Hausken et al. 1994; Hausken et al. 1996). We confirmed that these mutations precluded AKAP interaction by immunoprecipitating the mutant RII α -EGFP expressed in NG108-15 cells and blotting for the known AKAPs WAVE1 and AKAP150. As expected, interaction between these AKAPs and the wild-type RII α -EGFP was readily apparent (Fig. 8A), while these interactions were completely absent in both the I3,5S mutant (Fig. 8A) and the deletion mutant (data not shown). Despite this, live cell imaging of NG108-15 growth cone lamellae showed that both mutants localized to highly dynamic filamentous structures (Fig. 8B and Supp. Info. Movie 6) that were indistinguishable from those seen with the wild-type RII α -EGFP. To confirm that these filamentous structures formed by the mutant RII α -EGFP isoforms were, indeed, indistinguishable from the wild-type RII α in their association with actin microspikes, we first examined the mutants' co-localization with phalloidin-stained F-actin structures in fixed cells and with mCherry-actin in live cells. In both cases, there was strong co-localization between the mutant RII α -EGFPs and peripheral actin microspikes (Fig. 9A and B). Also, the structures decorated with AKAP-deficient (I3,5S)-RII α -EGFP showed similar, coordinate sensitivity to cessation of actin filament dynamics with low-dose cytoD (Fig. 9C). Taken together, these data demonstrate that PKA RII α subunits are tightly associated, through an AKAP-independent mechanism, with dynamic actin cytoskeletal structures in neuronal cell lamellae.

DISCUSSION

Taken together, our observations demonstrate for the first time the close physical coupling of PKA to the dynamic actin cytoskeleton in a manner that defies the paradigm for PKA anchoring. Two significant questions arise from the current study. First, what is the molecular mechanism, if not traditional AKAP-mediated anchoring, for localization of PKA to the actin cytoskeleton? Second, what cellular and/or physiologic purpose does this

specific pool of PKA play in neuronal function? For answering both of these questions, it will be important and useful to determine whether this localization is specific to neuronal cells or whether similar associations can be found in non-neuronal cells, as well.

While seemingly heretical, there is precedent for the localization of PKA signaling through non-canonical anchoring mechanisms. A recent, prominent example is the direct interaction between type-I PKA and the integrin $\alpha 4$ subunit (Lim et al. 2007). This interaction seems to require interaction of the integrin with the intact, heterotetrameric PKA holoenzyme, instead of just the RI subunit, and like the interaction described in the current report, this binding cannot be disrupted by inhibitors of known AKAP function (*e.g.* Ht31). Although there are slightly different molecular determinants within the amphipathic helices of AKAPs specific for each form of R subunit (Alto et al. 2003; Gold et al. 2006; Kinderman et al. 2006), the overall mechanisms for traditional anchoring of type-I and type-II PKA are quite similar. Thus, in a manner parallel to the work of Lim *et al* (Lim et al. 2007), our data provide an important addition to the literature describing the compartmentalization of PKA signaling by demonstrating the existence of a non-canonical anchoring mechanism specific for type-II PKA, as well. The majority of known AKAPs have been discovered using purified R subunits in blot overlay or 'far-western' techniques (Carr and Scott 1992), and specific categorization of R subunit-binding proteins as AKAPs has been through competition with the Ht31 peptide. Clearly, new approaches must be taken in order to identify non-canonical PKA anchoring proteins. Our current efforts along these lines include using purified, AKAP-deficient mutant RII α subunits in overlay assays, affinity chromatography, and *in vivo* cross-linking experiments, to identify potential candidates. However, additional efforts that make use of the intact type-II tetrameric holoenzyme as 'bait' may ultimately be needed.

Our observations that cytoskeletal association of PKA RII α subunits is AKAP-independent are seemingly at odds with those of Han *et al*, who recently reported a similar cytoskeletal and/or filopodial localization of RII α but a strong disruption of this localization by StHt31 (Han et al. 2007). The reason for this disparity is not known. Effects of differences in culture systems are quite likely – the current study focuses mostly on cells early on in the process of neurite extension while the previous study used cells with established, polarized axons (*e.g.* *Xenopus* spinal neurons ~10 h *in vitro* or rat hippocampal neurons 2–3 d *in vitro*). Indeed, the overall morphology of the cellular structures under observation in these two reports is quite distinct, and this may, in turn, suggest functional differences for the structures and proteins involved. The growth cones reported by Han *et al* bore long filopodia that extended well-beyond the concave margins of membrane between them. In contrast, the bundled actin structures to which PKA is localizing in the present study rarely protrude from their surrounding, convex lamellae, and thus appear to be more akin to microspikes (Bray and Chapman 1985), inductopodia (Thompson et al. 1996), or intrapodia (Rochlin et al. 1999) than to true filopodia. Finally, it should be noted that while treatment with StHt31 exhibited clear effects on the abundance of RII in growth cone filopodia and on the downstream effectors identified by Han *et al*, close inspection of the data in that report reveals that the residual RII immunofluorescence in StHt31-treated samples is largely restricted to streaks and puncta present along the filopodia (see Fig. 1F in (Han et al. 2007)), consistent with our observations. We have no doubts regarding the relevance of previous work establishing a functionally relevant pool of AKAP-associated PKA in neuronal growth cones. However, our current results suggest that another, presumably smaller pool of PKA also exists within neuronal cell processes – one that is associated with highly dynamic actin structures through an extremely tight and apparently novel mechanism. While the exact functional consequences of this pool of PKA remain to be identified, it is likely – given the importance and the variety of the effects of PKA on both cytoskeletal dynamics and neuronal function –

that this localization is a mechanism through which a pool of PKA is brought specifically to bare on neuronal cytoskeletal dynamics.

This brings to mind the second question posed, regarding the consequences of actin-associated PKA for neuronal cell function. As discussed in the Introduction, PKA has myriad functions in the developing and adult nervous system (Brandon et al. 1997). These range from regulation of neuron-specific programs of gene expression, ion channel function, membrane and receptor dynamics, and axon guidance. Not surprisingly, there is considerable and increasing evidence that underscores the importance of specific compartmentalization and/or localization of PKA signaling in neuronal function as well (reviewed in (Carnegie and Scott 2003; Dell'Acqua et al. 2006)). Given the close association with dynamic actin structures, a reasonable hypothesis is that this pool of PKA would preside over one or more actin-driven processes such as neurite outgrowth, neuronal polarity, or axon guidance. However, given the vast number of PKA substrates (Shabb 2001), even within the more focused sphere of actin dynamics (Howe 2004), the possibilities seem almost limitless. Indeed, potential targets exist within several, higher-order cytoskeletal regulatory pathways as well as cytoskeletal structural components all the way down to monomeric actin itself (Howe 2004). In their study, Han *et al* demonstrated that PKA anchored to filopodia contributes to axon guidance through phosphorylation of inhibitor-1 (I-1) and consequent regulation of protein phosphatase-1 activity (Han et al. 2007). It seems likely, however, that additional targets may also play a role. PKA can phosphorylate and/or regulate many cytoskeletal and/or cell adhesion regulatory proteins that are known to play critical roles in neurite outgrowth, polarity, and guidance. The list includes, but is certainly not limited to, the non-receptor tyrosine kinases FAK and Src, Mena/VASP proteins, the small GTPases RhoA and Cdc42, p21-activated kinase (PAK) and the p21-activated kinase-interacting exchange factor β PIX, the Nck adaptor protein, and on and on (see (Dent and Gertler 2003; Guan and Rao 2003; Howe 2004; Luo 2002; Shabb 2001) and references therein). We and others also recently described that PKA can regulate the dynamics and polarized accumulation of phosphatidylinositol-3,4,5-trisphosphate (Deming et al. 2008; Lim et al. 2008), a phospholipid that is intimately linked to cell polarization and is similarly linked to the development of neuronal polarity (Arimura and Kaibuchi 2005; Menager et al. 2004). Clearly, considerable effort will be required to identify the discrete targets for this highly specialized and discrete pool of PKA. This endeavor will be greatly facilitated, however, by elucidation of the mechanism(s) involved for anchoring PKA to the cytoskeleton, and both efforts are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PKA RII α localizes to F-actin microspikes in hippocampal growth cones in an AKAP-independent manner

(A) Neurons from E18 rat hippocampus were dissociated and plated onto polylysine-coated coverslips for 5.5 h, then left untreated or treated with 25 μ M StHt31 or the inactive StHt31P, as a control, for 30 min. Cells were then fixed and processed for immunofluorescence using an anti-RII α antibody, Alexa 488-conjugated secondary antibody, and Alexa 594-conjugated phalloidin to visualize F-actin. Bar = 5 μ m. (B) Assessment of RII α -actin co-localization in microspikes by linescan analysis of the regions specified by the white lines (*left*). Fluorescence intensities of RII α -GFP and mCherry-actin are depicted as green and red lines, respectively. (C) Pearson's correlation coefficients, as a measure of co-localization, were calculated for control regions devoid of microspikes (*a*) and regions containing obvious microspikes (*b*).

Figure 2. Endogenous PKA-RII α and exogenous GFP-tagged RII α localize to similar peripheral actin-rich structures

(A) NG108-15 cells were plated for 4 h on polylysine-coated coverslips. After 4 h, cells were fixed and stained with an antibody against PKA RII α (*anti-RII α*) and then with Alexa 594-conjugated secondary and Alexa 488-conjugated phalloidin to visualize F-actin. Alternatively, NG108-15 cells were transfected with a plasmid encoding RII α fused to EGFP (*RII α -GFP*). Thirty hours after transfection, cells were replated onto coated coverslips for 4 h, fixed and stained with Alexa 594-conjugated phalloidin, then imaged to visualize RII α -GFP and F-actin. Arrowheads in the overlay depict actin-rich microspikes to which RII α co-localizes, while arrows depict microspikes that show little to no RII α localization. Bar = 10 μ m. (B) Assessment of RII α -actin co-localization in microspikes by linescan

analysis and determination of Pearson's and Manders' coefficients ($M_{R\text{II}\alpha}$ refers to the proportion of $R\text{II}\alpha$ that is co-localized with actin, with a maximum value = 1) for regions devoid of microspikes (*blue*) and regions containing obvious microspikes (*white*).

Figure 3. PKA RII α localizes to dynamic filamentous structures in neuronal cell lamellae
 NG108-15 cells were transfected with a plasmid encoding PKA RII α fused to EGFP for 24 h, then replated on polylysine-coated glass-bottom dishes for 4 h and imaged by time-lapse fluorescence microscopy (one frame every 10 sec). (A) A portion of an RII α -EGFP-expressing cell, taken at an optimal exposure of 5 sec. (B) The same image as in (A), with brightness linearly adjusted to visualize the faint fluorescence signal in the peripheral lamella. (C) Montage of time-lapse images of RII α -GFP localization in the cell from (B) (See Movie 1 in Supplemental Material). Frames are 20 sec apart. (D) An enlargement of the boxed region in (C), with frames 10 sec apart. (E) A montage of frames (10 sec apart) from another, similarly transfected cell from a different experiment, further illustrating the dynamic appearance and disappearance of RII α filaments (*arrowheads*). Bars = 20 (B) or 5 (E) μ m.

Figure 4. PKA RII α co-localizes with dynamic actin microspikes in neuronal cell lamellae
 NG108-15 cells were co-transfected with plasmids encoding PKA RII α -EGFP and mCherry-actin for 24 h, then replated on polylysine-coated glass-bottom dishes for 4 h and imaged by time-lapse fluorescence microscopy at a rate of one frame every 5 sec for a duration of approximately 8 min (See Movie 3 in Supplemental Material). (A) A montage of time-lapse images, 15 frames (75 sec) apart, of a typical neuronal lamella under these conditions, illustrating co-localization of RII α (*top panels*) and actin (*lower panels*) in dynamic, peripheral microspikes. Bar = 20 μ m. (B, C) Enlarged time-lapse montages, with higher time resolution, of the boxed regions in (A). The graph in (C) shows the quantification of co-localization of RII α and actin over time, reported as the Manders' coefficients for the panels above the graph.

Figure 5. PKA RII α movement in lamellar microspikes is coupled to actin dynamics
 NG108-15 cells expressing PKA RII α -EGFP (RII α) and mCherry-actin (Actin) were replated onto polylysine-coated glass-bottom dishes for 4 h and imaged by time-lapse fluorescence microscopy (one frame every 8 sec) before (Control) and after the addition of 50 nM cytochalasin D (cytoD) (A) A montage of time-lapse images, 40 sec apart, of a typical neuronal lamella under these conditions (cytoD was added at 240 sec; see Movie 4 in Supplemental Material). Actin cytoskeletal dynamics and the lateral movement of an RII-rich actin microspike (arrows) are rapid without drug, while the addition of cytoD essentially freezes the movement of both actin filaments and RII-rich microspikes (arrowheads). (B) A representative kymograph used to calculate retrograde flow velocities of actin and RII α . A linear region of interest was drawn perpendicular to the margin of the lamella. Linescans under this region were extracted from each frame of the movie and placed side-by-side, giving a graphical representation of the fluorescence dynamics in this region over time (B'). The grey/white streaks in the kymograph depict the retrograde movement of fluorescent actin (B') or RII α (not shown), which dramatically decreases upon addition of cytoD (arrowhead in B'). The slope of these streaks represents the velocity of retrograde flow, which was determined from tangent of the angle θ (B''), as described in Materials and Methods. (C) Retrograde flow velocities of actin and RII α before and after addition of 50 nM cytoD. The graph shows average velocities, $\pm$ s.d., derived from 100 slope angles, derived from at least four separate kymographs from five cells from four separate experiments.

Figure 6. Actin-associated RIIα is not displaced by disruption of PKA anchoring by StHt31
 (A) NG108-15 cells expressing PKA RIIα-EGFP (*RIIα*) and mCherry-actin (*Actin*) were replated onto polylysine-coated glass-bottom dishes for 4 h, treated with 25 μ M StHt31 or the inactive control peptide StHt31P for 15 min, then imaged by time-lapse fluorescence microscopy (see Movie 5 in Supplemental Material). For each treatment, the panels depict the same cell 2 min after the start of image acquisition, with frames approximately 30 s apart. (B) Untransfected NG108-15 cells were cultured and treated as described in (A), but were harvested 20 min after treatment for immunoprecipitation (IP) of PKA RIIα. In one set of experiments, the cells were labeled with 35 S-methionine for a total of 6 h before harvest, and RIIα immunoprecipitates were separated by SDS-PAGE and autoradiography (Autorad). In another set, immunoprecipitates from non-radiolabeled cells were separated by SDS-PAGE and analyzed by western blotting (WB) with the indicated antibodies (lower three panels). The positions of molecular weight markers (in kDa) is indicated.

Figure 7. Ht31-independent association of PKA RII α with Triton-insoluble cytoskeletal fractions from purified growth cones

(A) Differentiated NG108-15 cells were fractionated by sucrose gradient centrifugation into growth cone particles (GCP) and cell bodies (CB) as described in *Experimental Methods*. Lysates from each fraction were analyzed by immunoblotting with antibodies against vimentin and GAP43. (B) Simplified experimental scheme for the results shown in (C), wherein purified growth cone particles are initially extracted in Triton X100-containing lysis buffer, and the Triton-insoluble cytoskeletal pellet is serially extracted twice with lysis buffer containing Ht31 (500 nM), then once with buffer containing cytoD (2.5 μ M). (C) The extracts, as well as the remaining pellet (P), the initial Triton-soluble supernatant (TX-S),

and an equal amount of whole cell extract (*wce*) were analyzed by immunoblotting with antibodies against RII α and actin. All results are representative of three independent experiments. In all blots, the position of a 50 kDa molecular weight marker is indicated.

Figure 8. Mutation of the AKAP-binding domain of PKA RIIα does not disrupt its localization to dynamic filamentous structures

(A) NG108-15 cells were transfected with a plasmid encoding either wild-type (WT) RIIα-EGFP or RIIα (I3,5S)-EGFP for 24 h, then replated on polylysine-coated dishes for 4 h. Cells were harvested for immunoprecipitation with anti-GFP antibody. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies against AKAP150, WAVE1, and RIIα as indicated. The positions of molecular weight markers at 98 and 50 kDa are indicated. (B) NG108-15 cells were transfected with plasmids encoding either RIIα (I3,5S)-EGFP or RIIα (Δ2-5)-EGFP for 24 h, then cultured and imaged as described in Figure 3. Images were acquired every 7–10 sec and a montage of time-lapse images for each mutant is shown (see Movie 6 in Supplemental Material).

Figure 9. AKAP-binding mutants of RIIα co-localize with actin and are coupled to actin dynamics

(A) NG108-15 cells were transfected with plasmids encoding either RIIα(I3,5S)-EGFP or RIIα(Δ2-5)-EGFP, then replated on polylysine-coated coverslips for 4 h. Cells were then fixed, stained with Alexa 594-phalloidin (to visualize actin) and imaged by fluorescence microscopy. (B) NG108-15 cells were transfected with plasmids encoding RIIα(I3,5S)-EGFP and mCherry-actin, then cultured and imaged as described in Figure 4. Images were acquired every 7 sec and a montage of time-lapse images is shown (see Movie 7 in Supplemental Material). The upper panels show a longer time span, with images ~ 90 sec apart (to show overall dynamics) while the lower panels show a higher time resolution series, with images every 14 sec apart (to track the dynamics of an individual microspike). (C) NG108-15 cells expressing RIIα(I3,5S)-EGFP and mCherry-actin were cultured and imaged (one frame every 9 sec) as described in Figure 5, before (*Control*) and after the addition of 50 nM cytochalasin D (*cytoD*). A montage of time-lapse images, 45 sec apart, is shown (see Movie 8 in Supplemental Material). Arrows depict lateral movement of an RIIα-rich actin microspike before drug addition, while arrowheads depict a stationary actin/RIIα microspike after cytoD addition.