Inositol 1,4,5-Trisphosphate 3-Kinase A Associates with F-actin and Dendritic Spines via Its N Terminus*

Michael J. Schell†§, Christophe Erneux¶, and Robin F. Irvine¶

From the †Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, United Kingdom and the ¶Free University of Brussels, Campus Erasme, Building C, 808 Route de Lennik, B-1070 Brussels, Belgium

The consequences of the rapid 3-phosphorylation of inositol 1,4,5-trisphosphate (IP3) to produce inositol 1,3,4,5-tetrakisphosphate (IP4) via the action of IP3 3-kinases involve the control of calcium signals. Using green fluorescent protein constructs of full-length and truncated IP3 3-kinase isoform A expressed in HeLa cells, COS-7 cells, and primary neuronal cultures, we have defined a novel N-terminal 66-amino acid F-actin-binding region that localizes the kinase to dendritic spines. The region is necessary and sufficient for binding F-actin and consists of a proline-rich stretch followed by a predicted α-helix. We also localized endogenous IP3 3-kinase A to the dendritic spines of pyramidal neurons in primary hippocampal cultures, where it is co-localized postsynaptically with calcium/calmodulin-dependent protein kinase II. Our experiments suggest a link between inositol phosphate metabolism, calcium signaling, and the actin cytoskeleton in dendritic spines. The phosphorylation of IP4 in dendritic spines to produce IP5 is likely to be important for modulating the compartmentalization of calcium at synapses.

Inositol 1,4,5-trisphosphate (IP3) signals in neurons are terminated predominantly via the action of calcium-stimulated IP3 3-kinases (IP3kinases) (1). These enzymes produce 1,3,4,5-tetrakisphosphate (IP4), which does not gate IP3 receptor calcium channels and may have signaling properties of its own (2–4). Brain has more IP3kin activity than other tissues (5), and this is due to the concentration of the A isoform in the dendrites of principal neurons, such as cerebellar Purkinje cells, hippocampal CA1 pyramidal cells, and dentate gyrus granule cells (6–8). Targeted disruption of IP3kin A isoform (IP3kinA) produces mice with enhanced long term potentiation (LTP) (9). By contrast, injecting IP4 into neurons specific function of modulating highly localized intracellular calcium signals.

EXPERIMENTAL PROCEDURES

Molecular Biology—Clone CP16 (21), containing the full-length rat cDNA for IP3kinA, was used as a polymerase chain reaction template, and various primer sets were designed to amplify parts of the cDNA that were cloned into pEGFPC3 and -N1 vectors (CLONTECH) for expression as green fluorescent fusion proteins. All polymerase chain reaction was carried out as described previously (13), using Pfu Turbo polymerase (Stratagene) in the presence of 10% Me2SO. The full open reading frame (aa 1–489) was amplified and cloned into pEGFPC3 with the following primer set: full sense (GAGAAGAGGCTATCGAGCTCGGATCCGGGCAACCGGC) and full antisense (GAGGAGCGCTCCCTGCTGTTCTGAGGCGCGCGCGC). The underlined regions depict HindIII and BamHI sites inserted into the primers. All other primers contained the same 5′ GAGA sequence. HindIII and BamHI restriction sites, and as required contained added or deleted start or stop codons or extra nucleotides to preserve reading frame, according to the manufacturer's instructions. These primer sequences are as follows: (restriction sites and added start or stop codons not shown); aa 66–489, 66.sense (GTTCCTAATGGGCTCCCG), and full antisense, aa 1–33, full sense and 33.antisense (TCATTCGGCACGCTTCGGGCGG), and full antisense, aa 34–46, 34.sense (CTGCGCCCCTGCTGGACGAGGCGGC) and 66.antisense (GACTTGTCCTCCCC-AGCGCGCTT); aa 1–66, full sense and 66.antisense; aa 34–459, 34.sense and full antisense; aa 109–489, 109.sense (TCGCACTCGCA-
The open reading frame for rat α-actin was amplified by polymerase chain reaction from a CDNA library made from hippocampal cultures (a gift of Dr. Hilmar Bading, Laboratory of Molecular Biology, Cambridge) and cloned into pEGFP-C3 at the HindIII/BamHI sites. All constructs were tested by restriction enzyme digest before use, and some (those used in these figures) were sequenced across the multicloning site to confirm identity and reading frame.

Secondary Protein Structure Prediction—Three Internet-based programs (nnPredict, SOPM, and PSA) were used to predict the secondary structure of the actin-association region of IP3kinA. These can be found at the BCM search launch web site: searchlauncher.bcm.tmc.edu.

F-actin Co-sedimentation—The 1–66 actin association region (N66) of IP3kinA was fused to the bacterial protein NusA was produced by subcloning into the pET43A vector (Novagen) at the BamHI/Sall sites. This vector also incorporates His6, before and after the multicloning site. A 1:50 dilution of Escherichia coli strain Rosetta BL21(DE3) (Novagen) harboring the construct was grown for 3 h at 37 °C followed by cooling to 18 °C and overnight induction in the presence of 1 mm isopropyl-β-D-thiogalactopyranoside. The following day cells were harvested by centrifugation and disrupted in BugBuster reagent (Novagen) in the presence of a protease inhibitor mixture, according to the manufacturer’s instructions. The cleared, soluble extract was mixed batchwise with Talon beads (CLONTECH), loaded into a column, and eluted with the construct's instructions. The purified fusion protein fragments was negligible. Gels were dried and increases at high F-actin concentrations; however, even at the highest. Because the effective F-actin concentration changes in this way, at each concentration of F-actin, we multiplied the concentration of actin added to the assay by the portion appearing in the pellet as the effective F-actin concentration.

The F-actin binding curve was constructed by varying the concentration of skeletal muscle actin between 1.0 and 20 μM in the presence of a constant amount of NusA-N66 fusion protein (2.65 μM). This results in a range of actin concentrations, the portion appearing in the pellet as F-actin varied between 39% at the lowest concentration and 79% at the highest. Because the effective F-actin concentration changes in this way, at each concentration of F-actin, we multiplied the concentration of skeletal muscle actin, then mixed with BugBuster reagent (Novagen) in the presence of a protease inhibitor mixture, according to the manufacturer’s instructions. The cleared, soluble extract was mixed batchwise with Talon beads (CLONTECH) and eluted with the construct's instructions. The purified fusion protein fragments was negligible. Gels were dried and increases at high F-actin concentrations; however, even at the highest F-actin concentrations used, the trapping of the truncated (non-F-actin binding) fusion protein fragments was negligible. Gels were dried and scanned, and then the density of the gel bands was determined using the gel-pelleting macro of NIH image software (version 1.62). Only the largest (full-length) gel band for NusA-N66 was used for quantification. Curve fitting was done with KaleidaGraph software using the general form: y = m1x + m2.

Cell Culture and Transfection—Hippocampal cultures were prepared from neonatal rats (age 12–24 h), as described previously (22). Hippocampal were dissociated with papain (Worthington), grown for 2 days in Neurobasal media with B27 supplement (Life Technologies, Inc.) and 10% horse serum, and then maintained thereafter in serum-free media for up to 5 weeks. Cerebellar granule cells were prepared by trypsin digestion of postnatal day 6–8 rat cerebellum and then maintained as described for hippocampal cells, except that the KCl concentration in the media was increased to 25 mM and cytosine arabinoside (Sigma) was removed 48 and 72 h after plating. Transfection was achieved with a modified calcium-phosphate technique (23) that used a 1-h exposure of the cells to the DNA precipitate. Neurons were examined between 1 and 14 days after transfection. HeLa cells were maintained in DMEM plus 10% fetal calf serum and transfected by the same calcium-phosphate technique as for neurons, except that the exposure to the precipitate was for 8 h, with a 2-min glycerol shock at the end. COS-7 cells were maintained in DMEM plus 10% fetal calf serum and transfected with the Fugene reagent (Roche Molecular Biochemicals). HeLa and COS-7 cells were examined 1 day after transfection.

**RESULTS**

**Determination of an Actin Association Region in IP3kinA-Transfected HeLa Cells**—Our initial experiments to examine the targeting of IP3kinA, a neural specific protein, utilized.
HeLa cells as a more convenient vehicle for localizing the multiple constructs generated. Full-length IP3kinA (amino acids 1–459) fused N-terminal to GFP appeared to associate with the plasma membrane and cytoskeleton of HeLa cells (Fig. 1A). This localization was not an artifact of the GFP tagging, since the GFP could also be placed C-terminal to GFP (not shown); furthermore, untagged, full-length IP3kinA, when expressed in HeLa cells and stained with a polyclonal antibody to IP3kinA, produced a similar staining pattern (not shown). Double staining with fluorescent phalloidin revealed a high degree of colocalization with F-actin (Fig. 1B).

A series of truncated IP3kinA GFP constructs were made and expressed in HeLa cells. We first tested a 66–459-amino acid construct, which corresponds to a clone called C5 that was previously shown to produce a fully Ca²⁺/calmodulin-sensitive enzyme in bacteria (21). This construct, when fused to GFP, appeared completely cytosolic when transfected into HeLa cells (not shown), suggesting that the first 66 amino acids are necessary for targeting to the cytoskeleton. Further studies established that the first 66 amino acids were sufficient to target GFP to F-actin (Fig. 1, C and D). The cytosolic localization of construct 34–459 (Fig. 1E) suggested that the first 66 amino acids were necessary for co-localization with F-actin. Indeed, when the stretch of 66 amino acids was cut in half, neither 1–33 (Fig. 1G) nor 34–66 (Fig. 1H) could efficiently target GFP to F-actin.

Although in all cases placing the GFP at either end of the construct produced similar results, some subtle differences were observed. First, the 1–33 construct (but never the 34–66 constructs) sometimes appeared to localize partially with F-actin in cells with very high expression levels, but this was only observed when the GFP was placed C-terminal to 1–33. Second, constructs with the GFP at the C terminus had easier access to the nucleus; construct 66–459 with GFP placed at the N terminus was excluded from the nucleus but entered the nucleus when GFP was placed at the C terminus. Constructs 109–459 and shorter entered the nucleus freely, regardless of the site of GFP tagging (not shown). Third, in a subset of cells with very high expression levels, the presence of the actin association region appeared to stabilize or promote the formation of F-actin, as judged by our ability to pick out some of the transfected cells on the basis of enhanced phalloidin staining. This phenomenon was explored further in the transfected hippocampal cultures (see below and Fig. 4). The amino acid sequence of the actin-targeting region was examined through the use of computer programs that predict secondary structure (Fig. 1, black bar). Three programs agreed that the stretch 31–49 has a high likelihood of forming an α-helix. This contrasts to the most N-terminal stretch of 27 amino acids, which contains 6 prolines.

**Detergent Extraction of Living Cells**—Another criterion for an association with the actin cytoskeleton is resistance to extraction with the detergent Triton X-100. We therefore expressed the GFP-(1–66) and GFP-(66–459) constructs in HeLa and COS-7 cells, and we then extracted them with 1% Triton X-100 for 5 min at room temperature before fixation and staining with fluorescent phalloidin. In some cases, cells were pretreated with the actin-disrupting drug LTA (5 μM) for 15 min before extraction. The results of some of these experiments are depicted in Fig. 2. In both HeLa and COS cells, the 1–66 construct appeared to be highly resistant to detergent extraction (Fig. 2C), whereas the 66–459 construct was washed away so efficiently that it was impossible (HeLa cells) or difficult (COS cells; Fig. 2G) to determine which cells had been transfected with 66–459. Moreover, when HeLa cells expressing the 1–66 construct were pretreated with LTA for 15 min prior to extraction, no green fluorescent cells remained (not shown). These experiments demonstrate that the 1–66 region is likely to associate with F-actin directly and that disruption of F-actin is sufficient to liberate a cytosolic (Triton X-100-extractable) protein.

**Co-sedimentation with F-actin**—We next determined if the first 66 amino acids of IP3kinA bound F-actin directly in a purified system. The actin association region was difficult to express in bacteria. Our initial attempts to produce His-tagged 1–459 or 1–66 using the pQE-30 vector in the XL-10 gold strain of *E. coli* produced no detectable fusion protein in either the soluble or inclusion body fractions. To overcome this problem, we fused the 1–66 region (N66) downstream of the solubility-enhancing NusA protein in the PET43A vector and

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**Fig. 1.** Association of IP3kin-GFP with F-actin in HeLa cells. **Upper panels** depict HeLa cells transfected with GFP constructs, and **lower panels** depict corresponding (and adjacent untransfected) cells stained for F-actin with fluorescent phalloidin. In all GFP constructs depicted, GFP was placed C-terminal to the stretch of IP3kinA. A and B, full-length IP3kinA (amino acids 1–459) fused to GFP. C and D, amino acids 1–66 fused to GFP. E and F, amino acids 34–459 fused to GFP. G and H, amino acids 1–33 fused to GFP. I and J, amino acids 34–66 fused to GFP. **Bottom,** schematic diagram depicting the actin association region of IP3kinA, which consists of an N-terminal proline-rich region (lightface numbers indicate position of prolines), followed by a predicted α-helix (black bar).
expressed the construct in the BL21(DE3) strain carrying a plasmid coding for rare tRNAs. This vector also incorporates one His$_6$ sequence between NusA and N66 and another His$_6$ after N66 to facilitate purification. Although this strategy resulted in substantial full-length NusA-N66 fusion protein purified from the bacterial extracts (about 47% of the total purified in our best preparations), the remainder of the purified protein was truncated (Fig. 3A, lanes 5 and 7). Our co-sedimentation experiments (Fig. 3A) confirmed that only the highest molecular weight band (containing the full N66 stretch) was able to bind F-actin (Fig. 3A, lanes 6 and 8) and also that NusA itself was not an F-actin-binding protein (lanes 2 and 4). Because skeletal muscle actin is known to have different properties than non-muscle actin when expressed in neurons (26), we tested both skeletal muscle and non-muscle (platelet) actin; the results with either type of F-actin appeared identical, at least qualitatively (lanes 6 and 8).

In order to estimate the stoichiometry and stoichiometry of our fusion protein for F-actin, we attempted to carry out binding assays whereby we varied the concentration of NusA-N66 against a fixed concentration of skeletal muscle F-actin, used at just above the critical concentration (~1 $\mu$M F-actin). However, because so much of the NusA-N66 protein was truncated (and unable to bind F-actin), we were unable to raise the concentration high enough in the assay to saturate the F-actin-binding sites (2.6 $\mu$M was the maximal concentration of apparently full-length NusA-N66 achieved in our assays). Then we tried titrating various F-actin concentrations against a maximal concentration of NusA-N66 (2.6 $\mu$M). F-actin binding assays done this way are theoretically possible, but there are a number of caveats that complicate the interpretation of the data (see below). Nevertheless, it was possible to generate a curve that resembled saturable binding and allowed us to estimate a rough stoichiometry of between 2 and 4 mol of actin per mol of fusion protein and to estimate the $K_{D}$ to be 2.7 $\mu$M (Fig. 3B). At least two caveats apply. First, the lowest point on the curve was obtained near or below the critical concentration (1 $\mu$M actin was added to the assay, and only 39% appeared in the pellet as F-actin). Second, even at the highest F-actin concentration tested (20 $\mu$M), some of the full-length NusA-N66 fusion protein (0.98 $\mu$M) remained in the unbound fraction when theoretically all of the fusion protein should pellet with the actin if the actin concentration is raised high enough. Perhaps some of the apparently full-length fusion protein was subtly modified so that it could no longer bind F-actin. The most reliable data on our curve lie in the points between 1 and 5 $\mu$M F-actin, where the average stoichiometry was 2.3 mol of F-actin per mol of fusion protein. These data serve as a rough guide for future studies using full-length, untruncated fusion protein in more conventional binding assays.

Expression of GFP-IP3kinA in Cultured Hippocampal Cells—Primary cultures of hippocampal cells (6–16 days in vitro) were transfected using a calcium phosphate procedure, fixed, and examined between 18 h and 14 days later. GFP-IP3kinA assumed the morphology of the F-actin in each of the various cell types in which it was expressed. The highest levels of expression were found in astrocytes, where fluorescence occurred mainly on spikey structures, stress fibers, and also cortical actin (Fig. 4). Transfected astrocytes also produced the clearest confirmation of the observations made in HeLa and COS-7 cells that the 1–66 region of IP3kinA sometimes stabilizes or promotes actin polymers. In Fig. 4C, phalloidin staining for F-actin is clearly increased compared with neighboring untransfected astrocytes. In the same experiment, the cultures were also transfected with GFP fused to $\gamma$-actin. Surprisingly, cells overexpressing actin did not show increased phalloidin staining compared with their neighbors (Fig. 4, B and D), thus serving as controls for the specificity of the IP3kinA F-actin-promoting effect. It is important to note that most of the transfected non-astrocytic cells did not exhibit enhanced staining for F-actin and that there was a clear correlation between a cell having very high GFP-IP3kinA fluorescence and its having enhanced levels of F-actin.

In our initial attempts to transfect neurons, we used short transfection times (20–48 h) and young neurons (3–9 days in culture). In these experiments, we found that (as was the case in HeLa cells, COS-7 cells, and astrocytes) the GFP-IP3kinA fluorescence was co-localized with F-actin, as evidenced by virtually complete co-localization with fluorescent phalloidin. Fluorescence was observed in a wide variety of F-actin-rich structures: some spikey, some string-like, and some punctate. The great variety of cell types and structures associated with GFP-IP3kinA in young cultures made it difficult to generalize about which structures were labeled, but they no doubt in-

**Fig. 2** Amino acids 1–66 of IP3kinA are associated with the Triton X-100-resistant cytoskeleton in COS-7 cells. Cells were transfected with GFP fused either to IP3kinA amino acids 1–66 (A–D) or 66–459 (E–H). 20 h later, cells were extracted (or not) with 1% Triton X-100 at room temperature for 5 min before fixation and staining with Alexa 568-phalloidin to visualize the F-actin cytoskeletons (B, D, F, and H).
and N66 (Coomassie Blue. Co-sedimentation is observed with full-length NusA-
and pellets were analyzed by SDS-PAGE followed by staining with
in the starting assay volume, and equal volumes of supernatants (lanes 1–4,
8). Amino acids 1–66 of IP3kinA (N66) were fused downstream of NusA, a
55-kDa bacterial protein. NusA alone or NusA-N66 was expressed in
bacteria, purified, and then used in co-sedimentation assays with F-
actin. A, F-actin (7.5 μm) purified from rabbit skeletal muscle (lanes 1,
2, 5, and 6) or non-muscle actin from platelets (lanes 3, 4, 7, and 8) was added to the fusion proteins, incubated in F-actin stabilization
buffer, and then pelleted at 200,000 g. Pellets (P) were resuspended in the starting assay volume, and equal volumes of supernatants (S) and pellets were analyzed by SDS-PAGE followed by staining with
Coomassie Blue. Co-sedimentation is observed with full-length NusA-
N66 (lanes 5 and 7) or with NusA protein alone (lanes 1–4), and no difference in co-sedimentation properties is observed between the two
kinds of actin. B, binding curve obtained by varying the concentration of F-actin in the presence of a constant concentration of 2.65 μm full-
length NusA-N66.

Fig. 3. Co-sedimentation of IP3kinA 1–66 region with F-actin.

While our “acute” transfection strategy worked well when the cultures were young and had not yet established synapses, we encountered great difficulty transfecting the neurons in the cultures once they had matured. If we transfected cells older than about 14 days in culture, the main cells transfected were glia and aspiny neurons. This presented a considerable prob-
elm, since IP3kinA is expressed very late in development, dur-
ning postnatal weeks 2 and 3 and is concentrated mainly in the
astrocytes and aspiny neurons. This presented a considerable prob-
elm, since IP3kinA is expressed very late in development, dur-
ning postnatal weeks 2 and 3 and is concentrated mainly in the
dentritic tree; additionally, some cells exhibited a lesser cyto-
solic staining that usually included the nucleus.

The punctate staining was associated with F-actin, as judged by double-staining with phalloidin (Fig. 6A, yellow). When the cultures were treated for 5 min with the actin-disrupting drug
LTA (5 μm), the phalloidin staining was greatly reduced relative
to controls, and the punctate F-actin staining pattern was
largely abolished (Fig. 6B, green). Likewise, following a 5-min
LTA treatment, IP3kinA staining had lost its punctate pattern and appeared more cytosolic (Fig. 6B, red). Some LTA-resistant
patches, usually located on distal dendrites, remained after the
LTA treatment, and they corresponded to patches of IP3kinA
more resistant to disruption. These experiments suggested that the punctate pattern of IP3kinA staining is due chiefly to its
ability to bind F-actin directly.

Dendritic spines are actin-rich structures in excitatory neu-
rons abutting presynaptic terminals. The punctate pattern we
observed for IP3kinA in our cultures suggested that, similar to
the situation in brain, endogenous IP3kinA in cultures was
concentrated in dendritic spines, which corresponded to the
punctate, actin-rich structures. Accordingly, we double-stained
with the presynaptic marker synapsin (Fig. 6C, green). A sub-
stantial portion of the punctate structures (Fig. 6C, red) indeed
localized to bona fide dendritic spines, i.e., they were adjacent to
but not co-localized with synapsin (Fig. 6C, arrow). When we
treated the cultures with LTA for 5 min, almost all of the
IP3kinA immunoreactivity lost its punctate pattern (Fig. 6D,
red) and had moved away from the presynaptic terminals. Pre-
vious studies have demonstrated that LTA treatment causes spine morphology to collapse and F-actin and its asso-
ciated binding proteins to disperse; however, LTA does not
disrupt the core postsynaptic protein complex consisting of,
among other things, PDZ-containing proteins and the NMDA

at least 2 weeks, even after synaptic connections had estab-
lished, and the levels of green fluorescence in transfected neu-
rons tended to be lower than for astrocytes. In cultures 2 weeks
old or older, scores of punctate-labeled transfected pyramidal
neurons were present on each coverslip. In these cells, GFP-
IP3kinA was observed in a pattern suggestive of dendritic
spines (Fig. 5, A and B). Likewise, in synaptically mature
cultures of cerebellar granule neurons (29 days in vitro), GFP-
IP3kinA appeared to be associated with synapses, as judged by
their apposition to axons of nearby green cells (Fig. 5C, arrow,
Ax). As in HeLa cells and COS-7 cells, truncation of the 66-
amino acid F-actin binding sequence produced a cytosolic pro-
tein (Fig. 5D). The GFP-(1–66) construct expressed in hip-
 PPCPcampal neurons also concentrated in actin-rich structures
such as putative spines (Fig. 5, E and F).

Co-localization of Endogenous IP3kinA with F-actin at the
Synapse—Although IP3kinA has been mapped in hippocampus
at both the light and ultrastructural levels (6–8), there are no
reports of its localization in primary hippocampal cultures. In
our initial attempts to visualize the IP3kinA in 1–2-week-old
cultures using an antibody, we observed no specific staining.
IP3kin activity in rat brain increases ~10-fold during the 1st 3
postnatal weeks (27), due to a massive increase in IP3kinA
expression between weeks 1 and 3. When we grew our cultures
for 2.5 weeks or longer, we could observe a subset of neurons
that stained intensely with the IP3kinA antibody; this sug-
gested that the developmental expression of the IP3kinA in
hippocampal cultures recapitulated expression in vivo. IP3kinA
immunoreactivity was confined mainly to very large
spiny neurons, most of which had a pyramidal cell soma. Many
other neurons and all glia were unlabeled, suggesting that the
antibody recognized isoform A selectively. The staining was
highly punctate (Fig. 7, A and C) and covered the whole soma-
dendritic tree; additionally, some cells exhibited a lesser cyto-
solic staining that usually included the nucleus.
FIG. 4. **GFP-IP3kinA overexpression can increase F-actin in cells.** Hippocampal cultures (6 days in vitro) were transfected with either GFP-IP3kinA (A and C) or GFP-γ-actin (B and D) and examined 10 days later. **Top panels** depict GFP fluorescence, and the **bottom panels** depict phalloidin staining. The arrows in the **lower left panel** point to two astrocytes transfected with GFP-IP3kinA that show enhanced phalloidin staining compared with nearby untransfected astrocytes. By contrast, cells transfected with the GFP-γ-actin construct had levels of phalloidin fluorescence indistinguishable from untransfected cells. **Bars,** 20 µm.

CaMKII is highly concentrated in putative dendritic spines, whereas end of a continuum of patterns observed. In this cell, IP3kinA—contrast, cells transfected with the GFP-IP3kinA that show enhanced phalloidin staining compared with nearby untransfected astrocytes. By contrast, cells transfected with the GFP-γ-actin construct had levels of phalloidin fluorescence indistinguishable from untransfected cells. **Bars,** 20 µm.

Co-localization of IP3kinA and CaMKII Isoforms—Previous biochemical results obtained in stimulated rat brain cortical slices established an important role for CaMKII in activating IP3kinA via its phosphorylation at threonine 317 in the sequence (12–14). We therefore co-localized endogenous IP3kinA and CaMKII isoforms in synaptically mature hippocampal cultures (Fig. 7). A monoclonal antibody specific for the α isoform stained most neurons in the cultures but not glia. A substantial portion of the immunoreactivity appeared to be cytosolic and stained axons as well as dendrites but, unlike IP3kinA, was excluded from the nucleus (Fig. 7, A versus C). Every cell that stained prominently for IP3kinA was also positive for CaMKIIα. However, we observed a great deal of cell-to-cell and culture-to-culture variation in the degree of punctate CaMKIIα staining. This is probably due to the fact that this protein is known to translocate from the cytosol to the synapse in response to synaptic activity (29, 30), and thus we were observing differences in the spontaneous synaptic activity of different neurons. The neuron depicted in Fig. 7, A and C, represents one end of a continuum of patterns observed. In this cell, IP3kinA is highly concentrated in putative dendritic spines, whereas CaMKIIα appears almost completely cytosolic. Nevertheless, high power images (Fig. 7, B and D) indicated that some IP3kinA spines also contained CaMKIIα. Overall, roughly half of the IP3kinA-positive punctate structures also contained punctate staining for CaMKIIα.

By contrast to the mainly cytosolic CaMKIIα, CaMKIIβ, like IP3kinA, is thought to be associated with F-actin (31). Indeed, when we double-stained for IP3kinA and CaMKIIβ, we observed many examples of neurons in which the two proteins appeared highly co-localized. One such example is depicted in Fig. 7, E and G. As was the case with the α isoform, the β isoform also exhibited heterogeneity in its degree of punctate staining. Despite its proposed ability to bind F-actin, some CaMKIIβ in some cells appeared cytosolic. This may be because this protein occurs in at least four splice forms (32), and some of the splicing occurs in the suggested actin-binding region (31). In summary, IP3kinA was usually found to be co-localized with CaMKII, its endogenous activator, and this co-localization occurred more often with the β than the α isoform.

Co-immunoprecipitation of IP3kinA with Actin and CaMKII—To determine if IP3kinA could interact directly with either of the CaMKII isoforms, we performed immunoprecipitation experiments using detergent extracts from rat forebrain. In our initial experiments, we immunoprecipitated IP3kinA from a 0.5% Triton X-100 extract that had been centrifuged at 48,000 × g at 4 °C. In these experiments, we indeed observed an association of IP3kinA with actin and both CaMKII isoforms (Fig. 8, left lanes 3). We wondered if this result could be explained by an incomplete removal of F-actin fragments from the extract. We therefore subjected the extract to further centrifugation at 200,000 × g before immunoprecipitation with the IP3kinA antiserum. It was important to establish that the 200,000 × g supernatant still contained IP3kinA, so we blotted this extract (and the starting 48,000 × g extract) with the IP3kin antibody (Fig. 8, top). Although removal of the remaining F-actin by centrifugation...
depleted IP3kinA levels roughly 50%, substantial IP3kinA protein remained soluble and thus available for immunoprecipitation. Substantial amounts of G-actin and both CaMKII isoforms were also present in the post-200,000 g supernatant (Fig. 8, right lanes 1). When we carried out a direct comparison of co-immunoprecipitation from the medium speed versus high speed extracts, the apparent association of IP3kinA with actin and CaMKII isoforms disappeared (Fig. 8, lanes 3). The most likely interpretation of these data is that IP3kinA does not directly associate with CaMKIIa, or CaMKIIβ, and that any association with these proteins occurs only as part of a larger F-actin-containing complex.

**DISCUSSION**

This is the first report establishing a link between the N terminus of IP3kinA and F-actin, which in brain is highly concentrated in dendritic spines. The data presented here stress the importance and novel function of an N-terminal
amino acid sequence in the localization of a major IP₃-metabolizing enzyme and contrast to the quite different multiple targeting mechanisms that have been reported (20) for the family of inositol polyphosphate-5-phosphatases. The targeting sequence of IP3kinA consists of an N-terminal proline-rich stretch followed by an 18-amino acid predicted α-helix that binds F-actin with high affinity. The N-terminal region of IP3kin isoform B has practically no sequence identity with the F-actin-binding region of IP3kinA. However, 5 of the first 25 amino acids in isoform B are prolines, followed by a 13-residue predicted α-helix. Recently, we expressed the first 66 amino acids of IP3kin isoform B as a GFP fusion protein and found it to be cytosolic and Triton X-100-extractable. The recently cloned isoform C, which is not expressed in brain or regulated by CaMKII (33), also shows no sequence similarity to isoform A in the N-terminal region. Thus we suggest that association with F-actin occurs selectively in isoform A, presumably to subserve a neuronal specific function of modulating highly localized intracellular calcium signals.

Proline-rich stretches are present in many actin-binding proteins (34) and often promote or stabilize the formation of F-actin. Although our GFP fusion proteins required the full 66-amino acid N-terminal amino acids to produce complete co-localization with F-actin, we did observe that merely the first 33 amino acids (containing the proline-rich region) could sometimes confer a lesser degree of actin association when fused upstream (but not downstream) of GFP. This might be explained by an ability of GFP to partially substitute for the α-helix stretch. The 34–66 region alone fused to GFP showed no tendency to associate with F-actin. Proline-rich stretches are also the binding sites for Src-homology 3 (SH3) domains (35). The minimal core requirement for SH3 domain binding is the PXXP motif, two of which occur in the IP3kinA F-actin-binding region. Perhaps the targeting to F-actin localizes IP3kinA near some type of synaptic scaffolding, and it is feasible that SH3 domain-containing proteins and F-actin might compete for the same binding site on IP3kinA.

The ability of IP3kinA to bind F-actin may explain the discrepancy between biochemical studies, reporting IP3kin activity and protein to be cytosolic (18, 19), and electron microscopy studies, which demonstrate an enrichment in both the cytosolic matrix and membranes of dendritic spines (7, 8). Relevant here is the previous, extensive evidence that calpains cleave the 53-kDa full-length kinase to produce a ladder of catalytically active, calcium-regulated soluble fragments (18, 19). Our results show that truncation of merely the first 33 amino acids (which would be liberate a protein of predicted size of 49.6 kDa) destroys actin binding and produces a cytosolic protein that probably has access to the nucleus. Thus, most or all known calpain-generated fragments would be cytosolic. A recent proteomic cataloguing of proteins associated with the postsynaptic

2 M. J. Schell and R. F. Irvine, unpublished observations.
density did not report the presence of IP3kinA (36). Taken together, this suggests that IP3kinA, although highly enriched at synaptic sites in dendrites, is only dynamically or loosely associated with membranes and cytoskeleton. Mild detergent (such as the 0.1% Chaps used during its purification (19)), rapid actin disruption by the drug LTA, or limited proteolysis are sufficient to generate a cytosolic protein.

Although IP3kinA is known to be concentrated in dendritic spines in brain (7, 8), this is the first report of the localization of endogenous IP3kinA in cultured hippocampal neurons. To visualize endogenous IP3kinA in our cultures, it was important to grow the cells for longer than 2 weeks, a time when synaptic junctions have begun to stabilize. A previous study using hippocampal neurons made from IP3kinA knockout mice reported that the global calcium signals of these cells were indistinguishable from cultures made from control animals (9). Their cultures were grown for 7–10 days before use, and one reason for the lack of effect on the calcium signal in the knockouts may simply have been that the cultures weren’t grown for a time sufficient for IP3kinA to be expressed fully in the controls.

IP3kinAs have a variety of cellular functions (4), one of which is to remove IP3 by a calcium-regulated reaction which would be of particular significance in dendritic spines. A downstream target for the IP3 produced by IP3kinA, if any, is unclear; however, the activation of voltage-gated calcium channels by IP3 has been implicated in a number of studies (10, 37, 38). IP3 production in a spine would be expected to shift the effect of a receptor’s signal away from calcium stores and toward calcium entry. Recently, Penner and colleagues (39) have demonstrated that GAP1IP4BP is present in dendritic spines, but if it were near the spine apparatus it would have the capability to block Ca2+-entry. If GAP1IP4BP is present in dendritic spines, but if it were near the spine apparatus of Purkinje cells is sufficient to generate a cytosolic protein. Indeed, calpain inhibitors can block long term potentiation (50). In conclusion, the role of IP3kin in a dendritic spine is likely to involve the restriction of IP3-generated calcium signals to individual synapses. Moreover, the rapid, localized synthesis of IP3 may in turn have complex effects on cytosolic calcium and on the molecular mechanisms that control learning and memory.

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