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Calcium-Calmodulin-Dependent Protein Kinase II Isoforms Differentially Impact the Dynamics and Structure of the Actin Cytoskeleton

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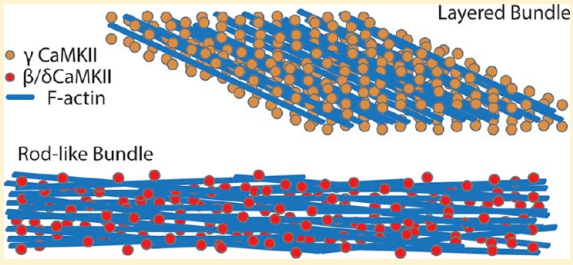
Supporting Information

ABSTRACT: Calcium-calmodulin-dependent protein kinase II (CaMKII) has been implicated in a wide variety of cellular processes, which include a critical regulatory role in actin cytoskeletal assembly. CaMKII is ubiquitous in cells, expressed as one of four isoforms termed α, β, γ, and δ. Characterization of the CaMKII−actin interaction has mainly focused on the β isoform, which has been shown to bundle actin filaments and sequester actin monomers in an activity-dependent manner. Much less is known about the interactions of other CaMKII isoforms with actin. In this work, isoform specific interactions of CaMKII with actin are described and reveal that the δ isoform of CaMKII bundles F-actin filaments like the β isoform while the γ isoform induces a novel layered structure in filaments. Using electron tomography, CaMKII holoenzymes are clearly identified in the complexes bridging the actin filaments, allowing direct visualization of the interactions between CaMKII isoforms and actin. In addition, we determined the isoform specificity of CaMKII-mediated inhibition of actin polymerization and discovered that all isoforms inhibit polymerization to varying degrees: $\beta > \gamma \approx \delta > \alpha$ (from most to least effective). Ca^{2+}/CaM activation of all kinase isoforms produced a robust increase in actin polymerization that surpassed the rates of polymerization in the absence of kinase inhibition. These results indicate that diversity exists between the types of CaMKII−actin interactions mediated by the different isoforms and that the CaMKII isoform composition differentially impacts the formation and maintenance of the actin cytoskeleton.



Properly regulated assembly and disassembly of actin filaments coupled with stabilization of the actin cytoskeleton are fundamental processes necessary for the proper functioning of eukaryotic cells.[1−3] Actin filaments serve a central role in molecular transport, providing tracks for motor protein-based cargo delivery, and participate in establishing the cellular cytoskeleton.[1] Polymerization of actin from the monomeric, G-actin, form to the filamentous, F-actin, form is asymmetric, where the growth is more rapid at the barbed end than at the pointed end. This asymmetric growth is responsible for mechanical force generation in cells.[4,5] Filament stability is controlled by capping proteins, such as gelsolin and tropomodulin, that interact with the filament ends to regulate polymerization rates.[1,2,6] There are also G-actin binding proteins, for example, thymosin and profilin, which offer additional regulation of filament growth by sequestering the monomeric form of actin. New filament formation depends on interactions between monomers that are initially unstable, and consequently, any factors that reduce actin availability can have a major impact on whether polymerization of filaments is initiated.[1] As a result, the nucleation of new filaments typically starts slowly, but once initiated, polymerization proceeds rapidly to allow for efficient response to cellular signaling.[2] Once polymerized, filaments can be stabilized by interactions

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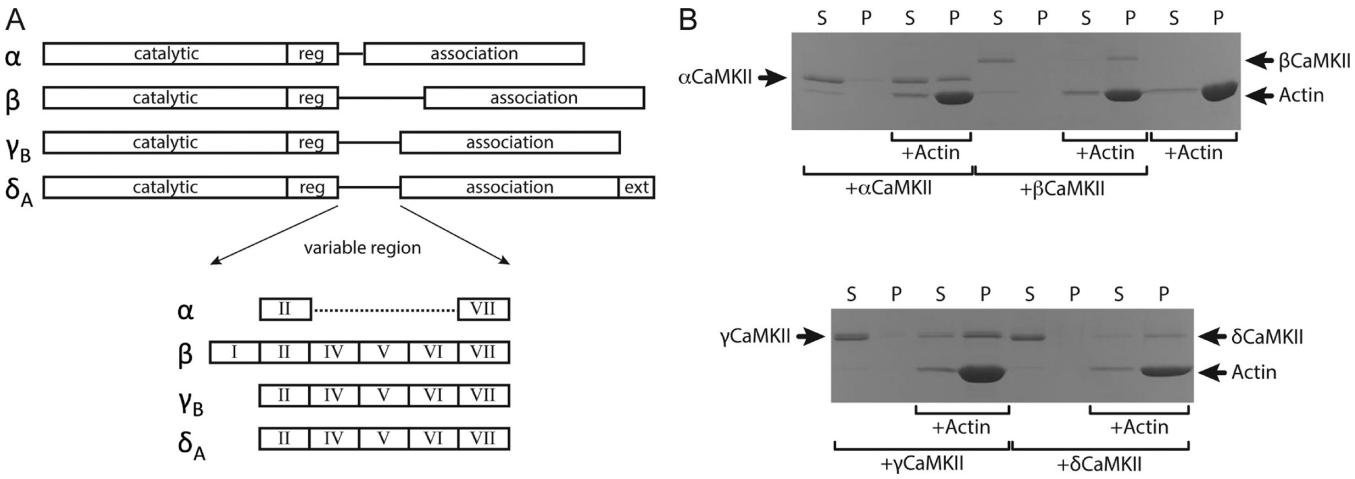

Figure 1. Domain map of CaMKII isoforms and their cosedimentation with F-actin. (A) The domain structures of all CaMKII isoforms used in these studies are highly conserved in sequence in the catalytic, regulatory, and association domains. The only noteworthy differences are found in the variable linker region where alternative splicing results in inclusion or omission of various exons denoted by Roman numerals. Additionally, the δ_A isoform contains a 21-amino acid C-terminal extension unique to this isoform. (B) F-Actin was incubated with CaMKII isoforms for 1 h and sedimented at 50000*g* at room temperature for 1 h. The supernatant was collected and made 1× in SDS sample buffer (total volume of 125 μL), and the pellet was resuspended in 125 μL of 1× SDS sample buffer. Equivalent volumes of supernatant (S) and pellet (P) fractions from each reaction mixture were then analyzed by SDS−PAGE, and the gel was Coomassie-stained. Control samples (reaction mixtures without actin) show that each kinase isoform remains in the supernatant (S) under these conditions. While all four kinase isoforms can be found in the pellet (P) with actin, there is a β, δ, γ, and α rank order of binding from strongest to weakest.

with multivalent actin binding proteins such as α-actinin, filamin, and spectrin, forming the structural network of the cellular cytoskeleton.[1]

Among actin regulatory proteins, βCaMKII has been shown to play multiple unique roles in actin assembly. It organizes and stabilizes F-actin into higher-order assemblies[7−11] and also controls the rate and extent of actin polymerization through binding to and sequestering G-actin.[11] Through such interactions, CaMKII can play the dual role of both an F-actin cross-linking protein and a sequestering protein by its ability to bind G-actin. These multivalent interactions with actin are made possible by the architecture of CaMKII holoenzymes which are composed of 12 subunits that oligomerize into a dodecameric complex. Each subunit is the product of one of four genes, α, β, γ, and δ,[12] that are assembled in a random stoichiometry, forming pure or mixed holoenzymes depending on the level of expression of each subunit.[13−15] The oligomeric complex formed by each isoform is similar in three-dimensional (3D) structure,[15] each with the potential to form multivalent interactions with multiple proteins. In addition to actin, the list of CaMKII binding partners is constantly expanding [e.g., α-actinin, the NMDA receptor, voltage-activated Ca^{2+} channels, to name a few (for reviews, see refs 12 and 16)], leading to the proposal that the kinase plays a role as a structural scaffold protein in addition to its role as a protein kinase. Expression of CaMKII isoforms is tissue specific with α and β isoforms highly enriched in nervous tissue and γ and δ isoforms found more broadly throughout the body.[12] The four genes are alternatively spliced to form almost 30 protein products, the roles of which are poorly defined.[12,17] From deletion and comparative studies of CaMKII isoforms (largely focused on the β isoform),[9,10,18,19] it has been suggested that the variable domain residing just C-terminal to the autoregulatory and CaM-binding domain of CaMKII (Figure 1A) is responsible for targeting the enzyme to the actin cytoskeleton and therefore contains the site for actin binding. However, one study examining deletion mutants and naturally occurring splice variants of the δ isoform of CaMKII concluded that the actin binding site was not in the variable domain but resides in a sequence further C-terminal to the variable domain in an area largely conserved among all the CaMKII isoforms.[20] Interestingly, interactions with actin were dependent on formation of the oligomeric state of the kinase; however, activity was not required.[8,9,18] It was shown that βCaMKII and not αCaMKII bundles actin filaments,[9−11] implying that structural differences exist between isoforms to mediate actin cross-linking. Furthermore, differences in inhibition of actin polymerization are observed between β and α isoforms,[11] suggesting potential isoform differences in the affinity for G-actin. Despite some ambiguity in the literature concerning the exact domain of CaMKII responsible for actin binding, converging data indicate that binding of Ca^{2+}/CaM to the enzyme causes the enzyme to dissociate from actin.[7−9,11] These studies indicate that CaMKII serves to sequester G-actin and to cross-link actin filaments, processes that occur in a Ca^{2+}-regulated manner and are mediated by the reversible binding of Ca^{2+}/CaM.

Much less is known about the influence of the γ and δ isoforms of CaMKII on actin cross-linking or polymerization, prompting us to investigate their potential roles in the maintenance of actin structure. In addition, we examined whether activation by Ca^{2+}/CaM binding would reverse association of G-actin with CaMKII, potentially leading to a burst in actin polymerization as has been proposed.[11] In this report, we present novel structural and kinetic data providing insight into the isoform specificity of the CaMKII−actin interaction. We show for the first time that the δ isoform of CaMKII bundles F-actin into rodlike assemblies like the β isoform and that the interaction of the γ isoform with F-actin results in a unique layered structural assembly. We describe the isoform dependence on CaMKII-mediated inhibition of actin polymerization and rank their capacity for inhibition ($\beta > \gamma \approx \delta > \alpha$). We also provide experimental evidence that Ca^{2+}/CaM activation of each CaMKII isoform, precomplexed with actin,

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does result in a rapid burst of actin polymerization. Finally, contrary to previous observations, we show that the α isoform of CaMKII interacts with actin, although weakly, and reveal its potential role in the regulation of the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Protein Preparation. Purification of actin was conducted as previously described.[11] The final purified protein was dialyzed into 5 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM dithiothreitol (DTT) (pH 8.0) and stored at −80 °C until it was needed. Preparations were shown to be more than 90% pure by SDS−PAGE analysis, and the protein concentration was determined by the BCA assay (Pierce Chemical Co.). The actin depolymerization reaction mixture consisted of 1 mg/mL G-actin, 5 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM Na_2ATP, 0.5 mM DTT, and 1 mM NaN_3 (pH 8.0). The reaction mixture was incubated on ice for 1 h and then allowed to undergo nearly complete depolymerization for ∼16 h at 4 °C. From this pool of G-actin, F-actin was produced by adding 10× polymerization buffer for 3 h at room temperature (RT) with final concentrations of 1 mg/mL G-actin, 5 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 1 mM Na_2ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 1 mM NaN_3 (pH 8.0). Expression of all CaMKII isoforms was completed using a baculovirus system exactly as previously described,[11,21] and purification was conducted using CaM-Sepharose affinity chromatography (for complete sequence comparison of isoforms used in this study, see Figure S1 of the Supporting Information). The purified protein was dialyzed into 20 mM HEPES, 0.5 M NaCl, and 10% glycerol (pH 7.4), and aliquots were frozen at −80 °C. The CaMKII protein was quantified using theoretical extinction coefficients of 1.226, 1.032, 1.068, and 1.104 for the α, β, γ, and δ isoforms, respectively. A codon-optimized cDNA of CaM was cloned into a pET3a vector (Novagen) containing a T7 promoter. Protein expression was conducted in *Escherichia coli* BL21(DE3) cells with a 0.4 mM IPTG induction at 32 °C for 4 h. Harvested cell pellets were lysed using an initial freeze−thaw cycle and then by sonication in 50 mM MOPS, 100 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (pH 7.5). $CaCl_2$ (5 mM) was added to lysis supernatant and applied to a High Trap Phenyl HP column (GE Healthcare) equilibrated with 50 mM Tris-HCl and 1 mM $CaCl_2$ (pH 7.5). Protein bound on the column was washed with 50 mM Tris-HCl, 1 mM $CaCl_2$, and 0.5 M NaCl (pH 7.5), and bound CaM was eluted with 10 mM Tris-HCl and 10 mM EDTA (pH 7.5). Purified CaM was then desalted into 50 mM HEPES, 100 mM NaCl, and 2.5 mM EDTA (pH 7.5) to remove Ca^{2+} and then finally desalted into 50 mM MOPS (pH 7.2).

Cosedimentation Assay. Binding reactions (100 μL) were completed in 1 h at RT with 1 mg/mL F-actin (polymerized as described above) and 0.1 mg/mL CaMKII α, β, γ, or δ in the following buffer: 5 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 1 mM Na_2ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 1 mM NaN_3 (pH 8.0). Control samples containing either no actin or no kinase were prepared similarly. Reaction mixtures were centrifuged at 50000*g* and 24 °C for 1 h in a Beckman tabletop TL-1 ultracentrifuge. Supernatants were collected and were made 1× in SDS sample buffer; pellets were suspended in an equivalent volume of 1× SDS sample buffer, and the same volume of each sample was subsequently analyzed with 12% SDS−PAGE. The distributions of proteins between supernatant and pellet fractions were visualized with Coomassie Blue.

Polymerization Assay. Actin polymerization was assessed using fluorescently labeled pyrene-actin (pyr-actin) purchased as part of a kit from Cytoskeleton Inc. Depolymerization of pyr-actin was conducted for 1 h on ice and then for >16 h at 4 °C in general actin buffer with ATP [0.4 mg/mL pyr-actin, 5 mM Tris, 0.2 mM $CaCl_2$, and 0.2 mM Na_2ATP (pH 8.0)]. Polymerization of 0.25 μM actin was then assessed in the presence or absence of a 2-fold excess CaMKII subunit concentration (0.5 μM) or BSA (to control for nonspecific protein effects) at RT for 0.75−1.5 h in 5 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 1 mM Na_2ATP, and 0.2 mM $CaCl_2$ (pH 8.0). To observe the effects of CaMKII activation, a 1:1 CaM:CaMKII molar ratio (subunits) was added to experiments at the time of adding polymerization buffer, or 30 min after the start of polymerization. Fluorescence was monitored during the polymerization reaction in a PTI fluorimeter with 350 nm excitation and 405 nm emission. Excitation and emission slit widths were 1 and 10 nm, respectively. To avoid photobleaching from constant exposure over the time course of the experiments, data points were collected for a 1 s duration every 30 s and then rotated out of the path of the excitation beam.

Electron Microscopy. F-Actin−CaMKII reactions for electron microscopy were prepared as described in Cosedimentation Assay. After binding for 1 h at RT, 5 μL of each reaction mixture was placed on a glow-discharged Formvar carbon-coated copper grid (Ted Pella) and incubated for 1 min. Excess sample was wicked away, and the grids were washed once with methylamine tungstate (Nanoprobes Nano-W), stained for 30 s in methylamine tungstate, blotted, and air-dried. Electron micrographs were collected on a JEOL 1400 transmission electron microscope running at 120 kV with a 2000 × 1300 pixel Gatan Orius SC1000 camera. The same grids were used to collect tomographic tilt series on a 300 kV FEI Polara F30 electron microscope equipped with a 4000 × 4000 pixel Tietz CCD camera. Series were collected at 39000× magnification with a −5 to −10 μm defocus under a dose of ∼400 electrons/Å^2. Images were collected at 2° tilt increments from −60° to 60° with 2× binning that generated a final pixel size of 4.6 Å. Tilt series were aligned and tomographic reconstructions produced using Etomo, part of the IMOD software package.[22]

RESULTS

Isoform Dependence on CaMKII−F-Actin Interaction. For preliminary insight into interaction of CaMKII with actin filaments, we examined the cosedimentation of F-actin with α, β, γ_B, and δ_A isoforms of CaMKII (Figure 1). These conventional assays were designed such that the centrifugation parameters resulted in sedimentation of F-action while noninteracting CaMKII and monomeric G-actin remained in the supernatant (Figure 1B). Any CaMKII found in pellet fractions was assumed to interact with F-actin. Consistent with previously reported results, SDS−PAGE analysis illustrates that αCaMKII is found in both the supernatant and pellet fractions, indicating partial association with F-actin, while the vast majority of βCaMKII is found in the pellet, suggesting robust binding to actin filaments.[11] The majority of γCaMKII and δCaMKII were in association with F-actin, but the γ isoform also showed a minor band in the supernatant as well, indicating incomplete association under these conditions. These results suggest that interaction of CaMKII with F-actin is isoform-dependent with β binding actin most avidly, followed by δ, γ, and α.

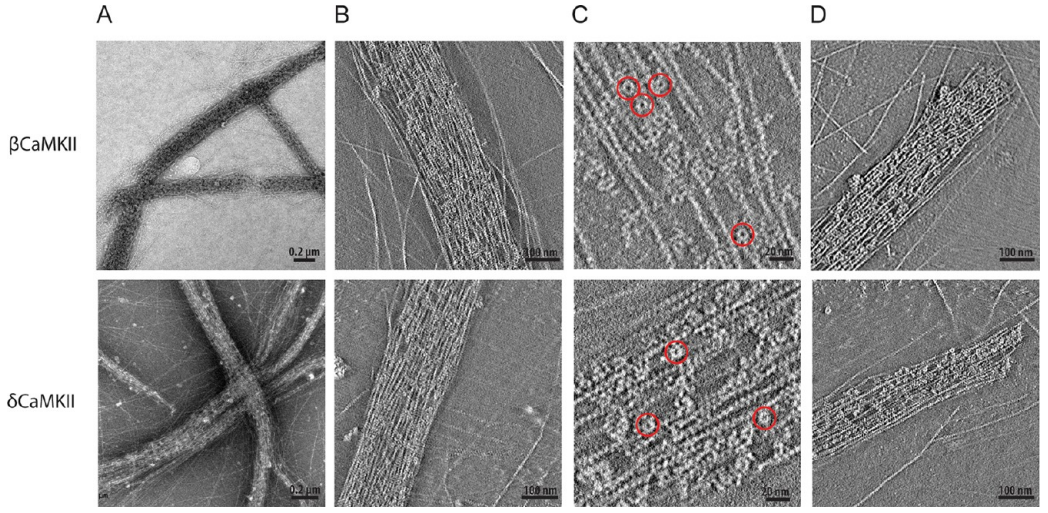

Figure 2. Structural analysis of actin bundles formed in the presence of the β and δ isoforms of CaMKII. (A) Left panels illustrate representative two-dimensional projections of low-power electron micrographs of negatively stained F-actin in the presence of β (top) and δ (bottom) isoforms ($n > 5$). (B) Panels show ~10 nm thick slices of two tomographic reconstructions to illustrate packing of β- and δCaMKII holoenzyme molecules within bundles. (C) Several CaMKII holoenzyme molecules are highlighted by red circles in a magnified region of a representative slice from the same reconstructions shown in panel B. (D) The right panels show ~10 nm slices through two different tomographic reconstructions that illustrate examples of blunt-ended bundles formed with β- or δCaMKII.

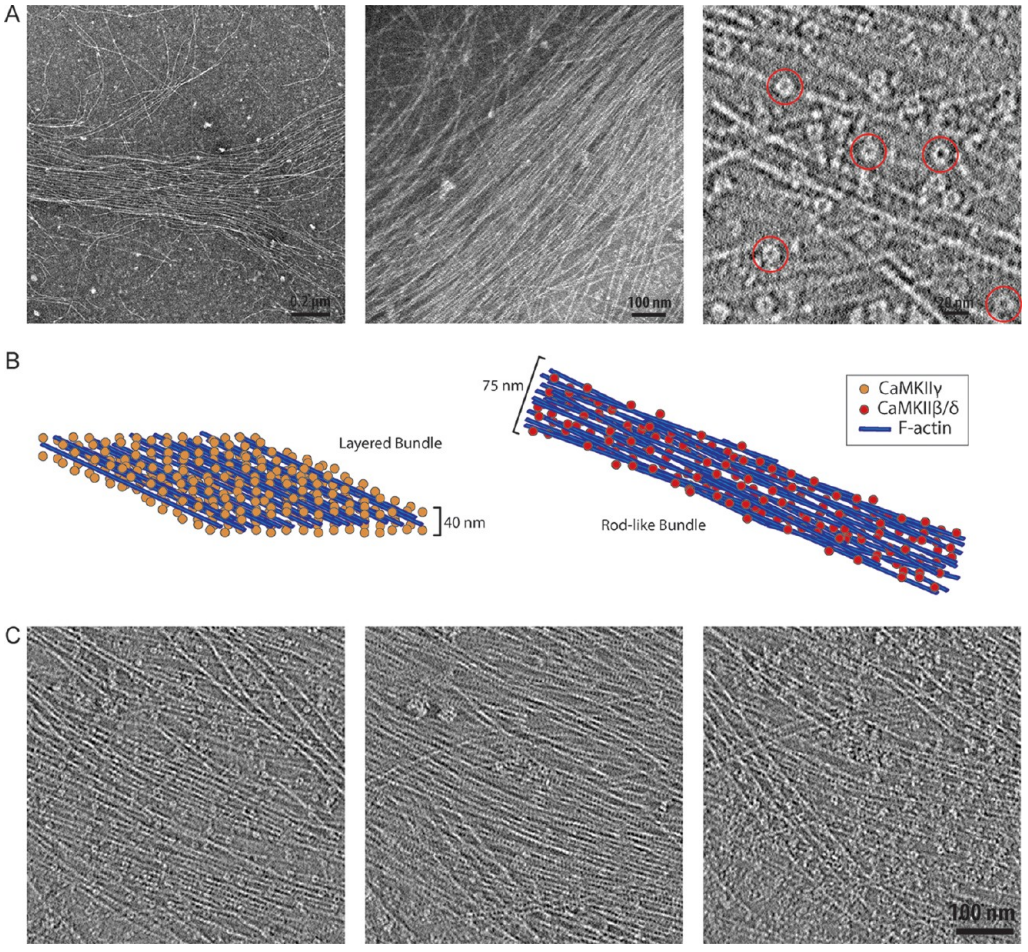

Figure 3. Structure of a layered γCaMKII bundle. (A) Left and center panels show representative electron micrographs of F-actin bundles in the presence of γCaMKII ($n = 6$). The right panel illustrates an ~10 nm slice from a tomographic reconstruction in which several CaMKII holoenzyme molecules are highlighted in red circles. (B) A cartoon illustrates structural differences between the layered bundles observed in the presence of the γ isoform (orange spheres) and the rodlike bundles observed in the presence of the β or δ isoform (red spheres). (C) Slices (~10 nm) from tomograms illustrate the top, center, and bottom z sections of the layered bundle in the left, center, and right panels, respectively.

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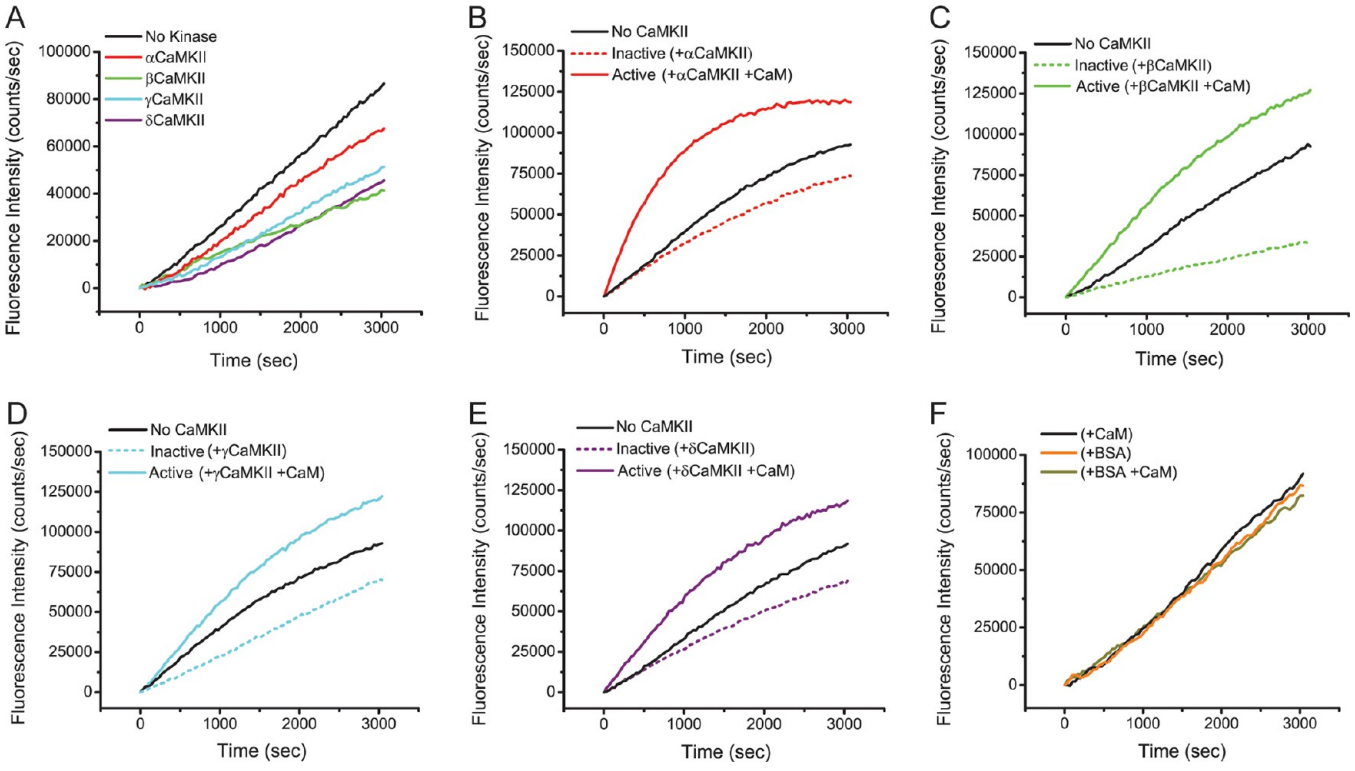

Figure 4. Impact of CaMKII binding on actin polymerization rates. (A) Representative plot of fluorescence intensity of pyr-actin measured in the presence or absence of each CaMKII isoform (n = 4). (B) Actin polymerization kinetics in the presence of αCaMKII where kinase (2-fold molar excess over actin) was preincubated with G-actin and then activated via the addition of equimolar CaM (to CaMKII subunits) at the same time polymerization was started. (C–E) Experiments identical to that described for panel B, but with βCaMKII, γCaMKII, and δCaMKII, respectively. (F) Control experiment showing a plot of the impact of BSA and Ca^{2+}/CaM, at the same concentration as in panel A added at the time of initiation of actin polymerization. Note that preincubation of kinase with G-actin followed by addition of Ca^{2+}/CaM concurrent with actin polymerization produces a robust burst in actin polymerization with all four CaMKII isoforms.

δCaMKII Bundles Actin Filaments Similarly to βCaMKII. Motivated by the apparent differences in F-actin association, we prepared EM grids from CaMKII binding reaction mixtures and negatively stained the grids for imaging. Electron micrographs of CaMKII isoforms with F-actin indeed illustrate isoform-dependent differences in F-actin–CaMKII complexes (Figures 2 and 3). As previously reported, βCaMKII binds to F-actin to produce actin bundles, parallel assemblies of filaments interspersed with bound CaMKII holoenzymes.[9,11] We observed that bundling by δCaMKII was very similar to that by βCaMKII in that bundles had comparable sizes and structures. Actin filaments were well organized in a mostly parallel fashion, with a degree of twist to the overall bundle (Figure 2A). The configuration of holoenzyme molecules within the bundle did not show any apparent pattern with regard to location or orientation. Bundle diameters on the order of 100 nm were consistently observed, and the width did not vary significantly throughout the length of the bundle, giving rise to relatively linear rodlike structures. Binding of either β or δ isoform resulted in bundles with considerable variability in length, ranging up to 15 μm. We observed that much of the F-actin on grids was incorporated into bundles and that bundles were discrete structures, seemingly unrelated to stray unbundled filaments in the same vicinity.

In addition to two-dimensional micrographs, we also compiled 3D tomographic reconstructions from series of tilt images of negatively stained samples to provide additional insight into the 3D structures of bundles. Slices (10 nm thick) from these series are shown in Figure 2B. In these images, the packing of the bundle can be appreciated, where the small ringlike structures of individual holoenzyme molecules can be clearly identified among actin filaments. An enlargement of representative slices for each isoform revealed several CaMKII holoenzymes identified among actin filaments (Figure 2C, highlighted in red circles). Actin bundles were tightly packed with a z axis thickness on the order of ~50–100 nm. One noteworthy observation was that bundle ends formed with β and γ often did not taper off but ended abruptly (Figure 2D). Rarely did individual filaments extend beyond the length of the bundle. We wondered if this squared end may suggest a role for β and δ isoforms of CaMKII in capping of filaments but could never discern holoenzyme molecules bound to the end of individual filaments. Overall, the bundle structures resulting from the β and δ isoforms were indistinguishable, suggesting that their mode of interaction with F-actin is similar and that they presumably play similar roles in the maintenance of intracellular actin structure.

Binding of γCaMKII to F-Actin Generates an Assembly with a Layered Structure. Remarkably, interaction of the γ isoform with F-actin resulted in a completely different organization of filaments. In contrast to the discrete structures produced by β and δ isoforms, binding of γCaMKII to F-actin resulted in wide and long filament bundles. These bundles contained a roughly parallel assembly of actin filaments, which appeared to be nonhomogeneously linked to a loose meshwork of filaments with multiple branching sites in the x–y plane (Figure 3A). Diameters were variable, and although their lack of boundary made it difficult to determine bundle boundaries,

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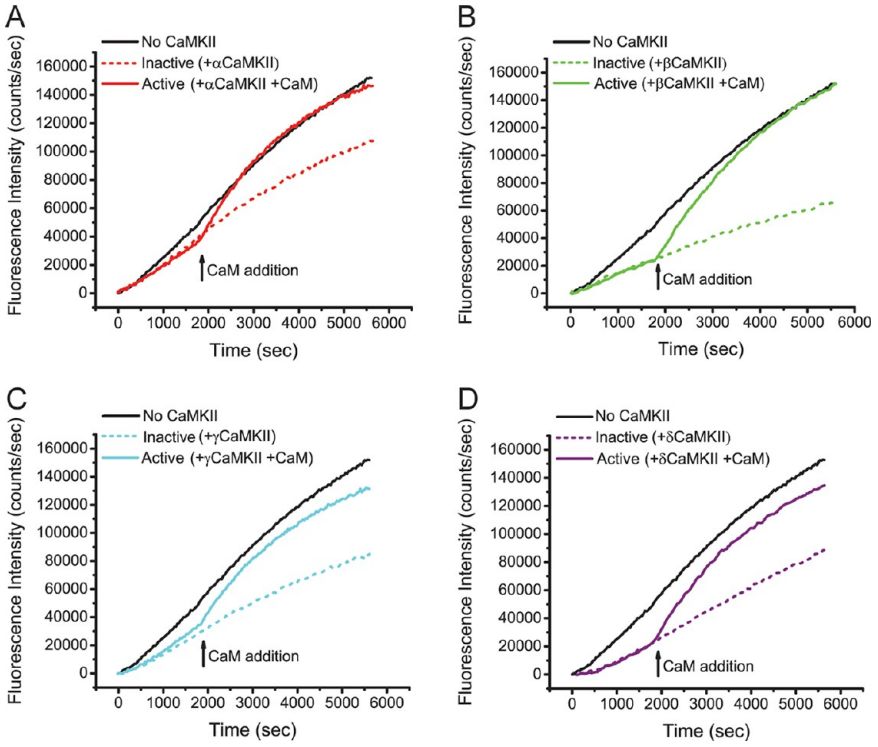

Figure 5. Impact of Ca^{2+}/CaM binding on CaMKII-inhibited actin polymerization rates. (A) Actin polymerization kinetics in the presence of αCaMKII where kinase was activated upon addition of CaM following actin polymerization for 30 min (saturating Ca^{2+} is already present in the reaction mixtures). CaMKII was added to the reaction mixture in 2-fold molar excess (subunit concentration) over actin, and CaM was added at a concentration equimolar to that of CaMKII subunits. (B–D) Experiments identical to that described in for panel A, but the panels display fluorescence traces showing the impact of addition of CaM (black arrows) to actin polymerization reaction mixtures with added βCaMKII (B), γCaMKII (C), and δCaMKII (D).

they were, in general, 2–3-fold larger than the bundles formed by β and δ isoforms. 3D tomographic reconstructions of these bundles revealed that while the organization of filaments in the x–y plane was somewhat nonuniform, a layered structure was detected along the z axis, with actin filaments sandwiched between layers of kinase holoenzymes. The heights of these complexes are approximately 40 nm compared to the 50–100 nm range for the rodlike bundles. Figure 3B illustrates a comparison of this layered bundle to rodlike bundles. In the left panel, a cartoon representing a segment of a layered bundle is depicted with γCaMKII holoenzymes represented as orange spheres and actin filaments as blue rods. The rodlike bundle is shown to the right with red spheres representing the β or δ isoform of CaMKII. The z axis height of the rodlike bundles allows for more stacking of filaments, while the layered bundle is dominated by one central sheet of roughly parallel filaments. Figure 3C shows three tomographic slices from the top, center, and bottom of a representative layered bundle (left, center, and right panels, respectively). In the center panel representing the center of the bundle, mainly actin filaments are observed with only a few holoenzyme molecules seen, indicating the center of the bundle is largely made of filaments. In contrast, the left and right panels of Figure 3C illustrate the outermost slices of the bundle where flat planes of holoenzyme molecules are positioned.

These results indicate that although all CaMKII isoform holoenzymes are structurally similar at low resolution,[15] their capacity to bundle F-actin and their organization of filaments are isoform-dependent. The nature of CaMKII-dependent bundling of F-actin seen in EM micrographs and 3D tomographic reconstructions is congruous with the findings in the cosedimentation assay suggesting there is a relationship between the isoform binding capacity and the type of bundling. β and δ isoforms showed nearly complete association with F-actin, which resulted in rodlike bundling, while the γ isoform was not as completely associated with F-actin, resulting in layered bundles. Despite extensive evaluation, under the assay conditions described here, αCaMKII did not show evidence of bundling filaments analyzed using EM perhaps because of a weak overall interaction with F-actin.

The Extent of CaMKII-Mediated Inhibition of Actin Filament Polymerization Is Isoform-Dependent. βCaMKII is able to bind both G- and F-actin[11] and decreases the actin polymerization rate, while the α isoform yields a modest reduction.[9,11] To examine how the γ and δ isoforms of CaMKII influence actin filament formation, actin polymerization was assessed in the presence of the various CaMKII isoforms. Polymerization was probed with fluorescent pyr-actin, an environmentally sensitive molecule that increases in fluorescence as monomers assemble into filaments. An initial depolymerization reaction was conducted in an effort to maximize the amount of starting material that was largely monomeric. Polymerization was then observed over a 50–100 min time course in the presence (or absence) of various CaMKII isoforms, where CaMKII subunits were in 2-fold excess of actin. Figure 4A is a representative plot of this experiment illustrating that all CaMKII isoforms decreased the rate of actin polymerization. As seen with previously reported data,[9,11] we observed a robust reduction in polymerization rate in the presence of the β isoform, while αCaMKII resulted in the

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least amount of inhibition. The rates of actin polymerization in the presence of the γ and δ isoforms (Figure 4A, blue and purple traces, respectively) lie between those of α and β (red and green traces, respectively), suggesting they inhibit polymerization with an intermediate capacity. We discovered that the amount of inhibition was highly dependent on the G-actin:CaMKII subunit ratio that varied somewhat depending on the effectiveness of the initial actin depolymerization reaction. However, the trend of inhibition by the various isoforms ($\beta > \gamma \approx \delta > \alpha$) was reproducible throughout multiple experiments ($n = 4$), clearly illustrating a robust isoform dependence on the CaMKII capacity to inhibit actin polymerization. The inhibition detected in these experiments was specific, as inclusion of BSA at the same concentration as CaMKII had no discernible effect on actin polymerization (Figure 4F).

CaMKII Activation Releases the Inhibition of Actin Polymerization and Promotes the Rapid Growth of Filaments. We previously demonstrated that βCaMKII binds G-actin and that binding was reduced upon Ca^{2+}/CaM activation of the kinase.[11] Others have reported that the F-actin−βCaMKII interaction was also weakened in the presence of Ca^{2+}/CaM.[7,9,10,19] These data led us to propose a dynamic mechanism of actin reorganization in which exploitation of the multivalent architecture of the 12-subunit holoenzyme provides a kinetic advantage for rapid filament growth.[11] In the basal state, our model predicts holoenzyme subunits serve to sequester monomeric G-actin and that Ca^{2+}/CaM activation of CaMKII−actin complexes would facilitate a burst of actin polymerization due to a local release of G-actin. To examine this possibility, we first assessed whether addition of Ca^{2+}/CaM to reaction mixtures in which CaMKII was preincubated with G-actin prior to the initiation of polymerization impacted the rate of actin polymerization. Figure 4B illustrates the time course of actin polymerization when G-actin was preincubated with αCaMKII (or not) and then the reaction was initiated with polymerization buffer with or without a 1:1 Ca^{2+}/CaM:CaMKII subunit molar ratio. While the level of inhibition is modest with αCaMKII (dotted red trace) compared to that without kinase (black trace), there is a significant increase in the rate of actin polymerization in reaction mixtures that also contained Ca^{2+}/CaM (solid red trace). Importantly, inclusion of BSA, to test for nonspecific protein effects, with or without the same concentration of added Ca^{2+}/CaM, produced no significant change in the polymerization rate of actin (Figure 4F). In an analogous design, preincubation of the β, γ, or δ isoform of CaMKII with actin similarly showed enhanced rates of actin polymerization when Ca^{2+}/CaM was added coincident with the actin polymerization buffer (panels C−E, respectively, of Figure 4). We conclude that the rate of actin polymerization associated with CaMKII when CaM is added is significantly increased compared to what is observed for the no CaMKII condition. Thus, the burst of polymerization we observe appears to be due to release of G-actin localized by CaMKII since otherwise, polymerization would have resembled that observed under the no CaMKII condition.

We next assessed whether addition of Ca^{2+}/CaM to reaction mixtures in which actin polymerization had progressed significantly in the presence of CaMKII would also have an impact on the rate of actin polymerization. In these reactions, we presume there is a mixture of polymerized F-actin and G-actin and that at least a portion of the CaMKII subunits in each reaction are interacting with the F-actin filaments. Figure 5A illustrates a time course in which actin polymerization was initially monitored in the absence of CaM to establish αCaMKII-mediated inhibition of polymerization (dotted red trace). After 30 min, αCaMKII was activated by addition of CaM (indicated by a black arrow; Ca^{2+} is already present in the polymerization reaction mixtures). As a control, the no kinase condition (black trace) reports polymerization in the absence of αCaMKII. We observed that activation of αCaMKII (solid red trace) resulted in a rapid increase in fluorescence compared to the inactive condition (red dotted line), indicating a dramatic increase in the rate of actin polymerization. We also analyzed the impact of CaM addition and binding of Ca^{2+}/CaM to complexes formed between β-, γ-, and δCaMKII and actin (panels B−D, respectively, of Figure 5) that were each permitted to polymerize before activation. Addition of CaM to each preformed complex led to a surge of actin polymerization similar to that observed with αCaMKII and actin (Figure 5A).

Together, we interpret these results as evidence of rapid nucleation of localized actin monomers through Ca^{2+}/CaM-mediated release from CaMKII, supporting the model in which release of G-actin, whether the kinase is bound to filaments or not, promotes an increased probability of new filament formation and/or an increased rate of addition of actin to existing filaments. These data indicate that all four of the isoforms of CaMKII can bind to and slow the polymerization rate of actin, including αCaMKII, and that Ca^{2+}/CaM binding leads to an apparent dissociation of G-actin with subsequent increased rates of polymerization.

DISCUSSION

In this report, we have shown that the products of each of the four mammalian genes of CaMKII, α, β, γ, and δ, interact with, and influence, aspects of actin polymerization and actin filament organization. CaMKII has been termed a multifunctional protein kinase, largely based on the fact that many proteins serve as substrates for the enzyme. In addition to this aspect of multifunctionality, CaMKII also serves a role as a structural scaffold, interacting with a number of proteins distinct from those that interact with the substrate-binding pocket.[12,16] Importantly, one of these interacting proteins is actin, the assembly and bundling of which are impacted by binding of CaMKII to both G- and F-actin. This earlier work was dominated by analysis of the α and β isoforms of CaMKII, the consensus being that β interacts with the actin cytoskeleton while α does not[19,23] and that a domain unique to β is therefore responsible for actin-dependent interactions. The majority of this work also relied on colocalization experiments inside cells or photobleaching recovery and was not derived from direct in vitro tests. The interaction of the γ and δ isoforms with actin or their influence on the actin cytoskeleton was largely unknown. The γ and δ isoforms have been shown to differentially localize to cellular actin rich structures,[20] with δ showing stronger targeting to the actin cytoskeleton, but a direct interaction with G- or F-actin in vitro had not been analyzed.

We showed previously that compared to βCaMKII, αCaMKII weakly interacted with F-actin and could modestly impact the rate of G-actin polymerization.[11] We reproduced that experimental finding in this report and showed that αCaMKII has a modest but significant impact on actin polymerization and that it binds, albeit weakly, to F-actin in pull-down experiments. This latter result is actually quite similar to the result of the pull-down experiments described by Shen et al.,[23] who showed that αCaMKII bound to purified F-

actin, although less effectively than βCaMKII. These results suggest that while less efficient at binding to actin, αCaMKII does possess a site for interaction, complicating previous conclusions identifying the unique domain of βCaMKII as the site for actin-binding. Caran et al.[20] reached a similar conclusion from results evaluating distinct actin interactions of truncation mutants or naturally occurring splice variants of δCaMKII expressed inside cells. They showed that a domain beginning at amino acid 351, including the C-terminal oligomerization domain but lacking the variable domain, was targeted as efficiently to actin as full-length δCaMKII. Further, they showed that the δc splice variant lacking the variable domain sequences also targets the actin cytoskeleton.[20] We also evaluated the interactions of the δ and γ isoforms of CaMKII with actin in our panel of in vitro assays. Both bound to F-actin filaments, although γ appeared to bind with a lower apparent affinity than δ in pull-down assays. Both also inhibited actin polymerization to similar extents, similar to that of βCaMKII but more effectively than αCaMKII. These results indicate that all four isoforms of CaMKII can interact with G- and F-actin but do so to different extents. Our results suggest there is no clear correlation between CaMKII linker sequence and actin remodeling, and therefore, we predict that alternative splicing of the linker region is only one aspect of a multifaceted regulatory mechanism responsible for the differences in isoform association with actin. Although it has been suggested that the additional variable domains present in the linker region of the β isoform could recognize actin (refer to Figure 1A), the results presented here demand that a binding site must exist within the primary sequence of the α isoform. It is plausible to suggest then that while all isoforms contain the actin-binding site, it is the length of the linker region that is responsible for influencing access to actin binding, resulting in variable affinities for each isoform. Variations in the length of the linker have been proposed to govern the frequency of conformational exchange within the enzyme,[24,25] which may serve to influence the exposure of the actin binding site to alter affinity. This notion of altered affinities might explain previous results reported by O'leary et al.,[10] who showed that splice variants in the variable domain of βCaMKII exhibited altered actin binding. In addition, Fink et al.[19] showed that inserting the variable domain of β into αCaMKII strengthened the interaction of the chimeric construct with actin, but that it did not reconstitute actin interaction of the same magnitude as that found with βCaMKII.

We[11] and others[7,9,10] showed using EM approaches that βCaMKII interacts with F-actin to produce bundles of filaments. Using EM tomography, βCaMKII holoenzymes could be clearly identified as cross-linking the actin filaments.[11] In this report, we extended this analysis to include higher-order actin filament structures assembled with the γ and δ holoenzymes of CaMKII. We showed previously using single-particle EM reconstructions that the four CaMKII isoforms used in this study assemble into dodecameric complexes that appear to be largely similar in overall architecture.[15] Here we showed δCaMKII formed bundles of actin filaments that were indistinguishable from those formed by βCaMKII. These bundles were somewhat heterogeneous in length and could extend for many micrometers, sometimes crossing entire grid squares. Interestingly, the actin filaments within these complexes often terminated in a nearly identical fashion that produced distinct blunt ends to the bundles. Because we cannot distinguish CaMKII holoenzymes bound to the ends of filaments, we can only speculate about why we observe blunt ends in our experimental system. Perhaps the structure of the bundle provides the stability of individual actin filaments, where filaments that extend beyond the length of the bundle are subject to turnover or alternatively are susceptible to sheer force when samples are disturbed during application on the grid or staining. The CaMKII holoenzymes were clearly visible within the bundles and were relatively randomly distributed around the width and length of the bundle axes. However, the distance between filaments within the bundles was less variable, and it appeared that CaMKII holoenzymes were helping to maintain a relatively consistent (20−30 nm) spacing between individual filaments within the bundles. This spacing is best appreciated in the movies of the tomographic reconstructions (Movies 1 and 2 of the Supporting Information). A cartoon model summarizing observations of the relative orientation of CaMKII within the bundles is presented in Figure 3 (middle panel). In contrast to the tightly packed 3D actin bundles formed by β- and δCaMKII, γCaMKII produced distinct higher-order, loosely associated large sheetlike structures. In these complexes, the γCaMKII holoenzymes could be identified in layers sandwiching a layer of actin filaments between them. Note that we did not detect multiple layers of actin filaments with kinase molecules sandwiched between them. This would indicate that the growth of these complexes is less restricted in the *x* and *y* axes but is capped in the *z* axis. The 3D arrangement of these layers and the holoenzymes bracketing them were again best appreciated in tomographic reconstructions (Movie 3 of the Supporting Information). It is presently unclear why γCaMKII would produce different types of higher-order actin complexes than β- or δCaMKII. Perhaps the rigidity in the linker region distinguishes the type of interactions formed with actin filaments, giving a constraint preference or requirement for the binding interaction. Alternatively, other domains of CaMKII could interface with actin to modify the orientation of binding or serve to sterically restrict the types of interactions that can be formed with actin filaments. A thorough understanding of the isoform-dependent orientation of binding awaits definitive biochemical identification of the actin binding site(s) in CaMKII. Regardless of the underlying mechanism, to form the type of structures found with γCaMKII suggests an asymmetry in the orientation in which the kinase interacts with the filaments. While it is unclear whether such bundles or layers form inside cells with the different CaMKII isoforms, the data clearly indicate that the β, γ, and δ isoforms can form multivalent interactions with actin filaments, leading to the potential for organizing the actin cytoskeleton in unique ways.

Binding of βCaMKII to actin filaments has been documented in a number of reports to be reversed by Ca^{2+}/CaM binding.[7−10] We extended these observations by showing previously that Ca^{2+}/CaM binding also causes βCaMKII to release G-actin[11] and proposed that CaMKII might serve as a local reservoir for actin monomers that could be released following Ca^{2+} influx. In the study presented here, we tested this hypothesis by evaluating the impact of addition of Ca^{2+}/CaM to actin polymerization assays that had been incubated with CaMKII to inhibit the rate of polymerization. As predicted, Ca^{2+}/CaM addition produced an increased rate of actin polymerization that exceeded the rate of polymerization in the absence of CaMKII. The general observation was consistent for all four CaMKII isoforms, where addition of Ca^{2+}/CaM to the inhibited reaction mixtures led to a burst of actin

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polymerization. There are a number of possible explanations for the rate of polymerization to increase beyond that found in the absence of CaMKII. First, CaMKII is a dodecamer, and we previously showed that 10−12 actin molecules could bind to one holoenzyme.[11] This provides the opportunity that, upon addition of an excess of Ca^{2+}/CaM, the release of localized G-actin would increase the probability for nucleation events, initializing multiple new filaments. The localized pool of G-actin then can facilitate growth of these new filaments as well as already formed actin filaments. Incubation with CaMKII might additionally lead to a change in the conformation of actin because of either phosphorylation, which was reported previously,[10] or some stabilization of actin caused by its cycle through binding to the kinase that increases its probability for interactions with G- or F-actin. Finally, it is possible that actin remains bound to CaMKII, while in competition for Ca^{2+}/CaM binding, and this bound actin might have a greater probability of initiating new filament formation. While these possibilities remain to be investigated, it is clear that binding of CaMKII to actin slows its polymerization and that the addition of Ca^{2+}/CaM leads to a burst in new filament formation and/or existing filament growth, supporting the idea that CaMKII can link increases in Ca^{2+} concentration (through CaM activation) to alterations in polymerization of the actin cytoskeleton. We suggest that this model is applicable for all four isoforms, each able to bind both G- and F-actin, although we reiterate that the α isoform was not shown to assemble filaments into higher-order structures.

Numerous studies have reported an impact of CaMKII on the cellular actin cytoskeleton, and interestingly, many of them do not depend on the activity of the kinase. Perhaps the most studied example is that of the activity-dependent reorganization of the actin cytoskeleton in neurons. βCaMKII was shown to impact neurite extension and synapse formation in primary cultures, and this activity remained evident with catalytically inactive mutants but required oligomerization of the subunits.[9,19] Similar conclusions were reached for a role of βCaMKII in the formation of microspikes in primary cortical cultures.[18] In addition to βCaMKII, a role for binding of δCaMKII to the actin cytoskeleton and in neurite outgrowth has been proposed.[26,27] CaMKII has been shown to regulate the reorganization of the actin cytoskeleton at synapses, leading to activity-dependent increases in synaptic efficacy.[9,28] In this scenario, increases in Ca^{2+} concentration lead to CaM activation and binding to CaMKII. On the basis of previous and current results, we propose a model in which Ca^{2+}/CaM binding leads to dissociation of CaMKII from the actin cytoskeleton and also increases the size of the local pool of G-actin, which results in a local increase in the extent of actin filament formation and growth. In addition, the release of CaMKII from actin is permissive for its recruitment and binding to other molecules with the synapse, for example, in the postsynaptic density.[16,29] When the Ca^{2+} concentration returns to baseline levels, CaM dissociates from the kinase and CaMKII again binds to the actin cytoskeleton to form higher-order complexes and to sequester available free actin monomers. Sequestration of free actin would act to further stabilize the actin cytoskeleton by decreasing the probability of treadmilling. While this model was largely developed from results examining interactions of actin with βCaMKII, on the basis of the results presented here, we can extend this model to cells that express the γ and δ isoforms of CaMKII as well.

We have shown that each isoform has a unique ability to promote variation in structural assemblies and actin polymerization, which is undoubtedly significant for reorganization of the actin cytoskeleton. Our results contribute to the emerging picture in which CaMKII decodes Ca^{2+}-mediated reorganization of the actin cytoskeleton and the exploitation of its isoforms provides for a tunable response. We have shown this multifaceted regulation of actin is controlled at the structural level through isoform specific bundling and polymerization of F-action. The interaction of CaMKII with actin is also regulated by activation of the kinase,[7,9−11,19] where the Ca^{2+}/CaM affinity[15] and the response to Ca^{2+} oscillations[24] are isoform specific. Tuning is further accomplished through the hetero-oligomerization of CaMKII into holoenzymes that are composed of varying ratios of different isoforms.[12,13] Thus, these multifaceted levels of regulation are elegantly controlled by the selectivity and efficiency of isoforms as well as their composition within cells, offering the ability to tune Ca^{2+}-mediated actin reorganization through CaMKII.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete amino acid sequence alignment of CaMKII isozymes used in these studies, rat α, rat β, human γ_B, and rat δ_A (Figure S1) and three movies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; G-actin, globular or monomeric actin; F-actin, filamentous or polymerized actin; IPTG, isopropyl β-D-1-thiogalactopyranoside; pyr-actin, pyrene-labeled actin; BSA, bovine serum albumin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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