The Autonomous Activity of Calcium/Calmodulin-dependent Protein Kinase IV Is Required for Its Role in Transcription*$$

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Calcium/calmodulin-dependent kinase IV (CaMKIV) is a multifunctional serine/threonine kinase that is positively regulated by two main events. The first is the binding of calcium/calmodulin (Ca$^{2+}$/CaM), which relieves intramolecular autoinhibition of the enzyme and leads to basal kinase activity. The second is activation by the upstream kinase, Ca$^{2+}$/calmodulin-dependent kinase kinase (CaMKK). Phosphorylation of Ca$^{2+}$/CaM-bound CaMKIV on its activation loop threonine (residue Thr$^{200}$ in human CaMKIV) by Ca$^{2+}$/calmodulin-dependent kinase kinase leads to increased CaMKIV kinase activity. It has also been repeatedly noted that activation of CaMKIV is accompanied by the generation of Ca$^{2+}$/CaM-independent or autonomous activity, although the significance of this event has been unclear. Here we demonstrate the importance of autonomous activity to CaMKIV biological function. We show that phosphorylation of CaMKIV on Thr$^{200}$ leads to the generation of a fully Ca$^{2+}$/CaM-independent enzyme. By analyzing the behavior of wild-type and mutant CaMKIV proteins in biochemical experiments and cellular transcriptional assays, we demonstrate that CaMKIV autonomous activity is necessary and sufficient for CaMKIV-mediated transcription. The ability of wild-type CaMKIV to drive cAMP response element-binding protein-mediated transcription is strictly dependent upon an initiating Ca$^{2+}$ stimulus, which leads to kinase activation and development of autonomous activity in cells. Mutant CaMKIV proteins that are incapable of developing autonomous activity within a cellular context fail to drive transcription, whereas certain CaMKIV mutants that possess constitutive autonomous activity drive transcription in the absence of a Ca$^{2+}$ stimulus and independent of Ca$^{2+}$/CaM binding or Thr$^{200}$ phosphorylation.

Calcium/calmodulin-dependent kinase IV (CaMKIV)$^1$ belongs to the family of broad specificity calcium/calmodulin-dependent kinases that includes CaMKI and CaMKII (1–4). It is expressed in distinct regions of the brain and in thymus, testis, bone marrow, and spleen (5–7) and generally exhibits a prominent nuclear localization (8). CaMKIV has been shown to activate transcription mediated by a number of transcription factors, including serum response factor, retinoid-related orphan receptor a/γ, and chicken ovalbumin upstream promoter-transcription factor 1 (9, 10). One mechanism of CaMKIV function in transcription is phosphorylation and regulation of specific transcription-related components such as histone deacetylase 4, cAMP-response element-binding protein (CREB), and CREB-binding protein (11–16).

Similar to other calcium/calmodulin-dependent kinases, CaMKIV is inhibited by autoinhibitory interactions, which are relieved upon calcium/calmodulin (Ca$^{2+}$/CaM) binding, leading to basal CaMKIV activity (17). Ca$^{2+}$/CaM binding also exposes the CaMKIV activation loop, which can then be phosphorylated on a specific threonine residue (Thr$^{200}$ in human CaMKIV; Thr$^{196}$ in mouse CaMKIV) by Ca$^{2+}$/calmodulin-dependent kinase kinase (CaMKK) (18, 19). This phosphorylation event is associated with a marked increase in the total activity of CaMKIV and the development of some Ca$^{2+}$/CaM-independent activity (20, 21). Mutation of the activation loop threonine of CaMKIV to a nonphosphorylatable alanine does not affect the basal activity of the kinase but precludes its activation by CaMKK as well as abrogating the ability of the protein to drive transcription in cellular assays (18, 20). This suggests that Thr$^{200}$ phosphorylation is necessary for CaMKIV transcriptional function. Recent work (22) further suggests that the most important role of Thr$^{200}$ phosphorylation is the generation of autonomous activity and implies that autonomous activity is necessary for CaMKIV-mediated transcriptional activity (22). CaMKIV transcriptional function may be negatively regulated by the protein phosphatase PP2A. PP2A can exist in a stable complex with CaMKIV and dephosphorylate CaMKIV on residue Thr$^{200}$; loss of the CaMKIV/PP2A interaction in cells leads to enhanced CaMKIV transcriptional activity (22, 23).

In this study, we further examined the relationship between CaMKIV activation, autonomous activity, and transcriptional function. Here we report that CaMKIV is transformed from a Ca$^{2+}$/CaM-dependent enzyme to a fully Ca$^{2+}$/CaM-independent or autonomous enzyme after phosphorylation of its activation loop threonine, residue Thr$^{200}$, by CaMKK. By analyzing the behavior of wild-type and mutant CaMKIV proteins in biochemical experiments and cellular transcriptional assays, we demonstrate that CaMKIV autonomous activity is necessary and sufficient for CaMKIV-mediated transcription, even in the absence of Ca$^{2+}$/CaM binding or Thr$^{200}$ phosphorylation.

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The abbreviations used are: CaMKI, -II, and -IV, calcium/calmodulin-dependent protein kinase I, II, and IV, respectively; CaM, calmodulin; Ca$^{2+}$/CaM, calcium-bound calmodulin; PP2A, protein phosphatase 2A; CaMKK, calcium/calmodulin-dependent protein kinase kinase; CREB, cAMP-response element-binding protein; WT, wild type; TBS, Tris-buffered saline.
**Experimental Procedures**

**Cells and Cell Culture**—The human embryonic kidney cell line, QBI-293A (293A), from Quantum Biotechnologies was used for all cell culture experiments and was cultured as recommended by the supplier.

** Constructs**—FLAG-CaMKIV WT and FLAG-CaMKIV substitution 320-329 (FLAG-CaMKIV) plasmids (see legend of this paper) containing a mammalian expression vector pSG5 (Stratagene), were generated as previously described (22). The other CaMKIV mutants were generated by site-directed mutagenesis using single-stranded pSG5-FLAG CaMKIV as template and using the protocol and reagents provided with the Muta-Gen M13 kit (Bio-Rad). The single-stranded DNA was synthesized in C323 Escherichia coli that had been transformed with pBSK (+) digested with NotI and helper phage (Invitrogen). The sense oligonucleotides used were as follows: CaMKIV NAARIS 300–305, 5'-CTC CAG CAT CCG TGG AAT GCT GCT ATA CGA TCG AAT TTT GTA CAC ATG-3'; CaMKIV NAARIS 306–311, 5'-ACA GGT AAA GCA GCC AAT GCT GCT ATA CTA CGG GCC GCT GCT ATA CGA TCG ACC GCT CAA AAG AAG-3'; CaMKIV NAARIS 312–317, 5'-TTT GTA CTA CAT ATG GAT AAT GCT GCT ATA CGA TCG ACA TGA CCT AAG GC-3'; CaMKIV NAARIS 318–323, 5'-GCT CAA AAG AAG CTC AAT GCT GCT ATA CGA TCG ACC GCT CAA AAG AAG-3'; CaMKIV NAARIS 324–329, 5'-GAA TTC ACG GCG AAT GCT GCT ATA CGA TCG GTG ATG GCT GTG GTG-3'; CaMKIV NAARIS 330–335, 5'-AAG CTT ACG GCA GGC AAT GCT GCT ATA CGA TCG ACC GCT GCT ATA CGA TCG ACC GCT CAA AAG AAG-3'; CaMKIV NAARIS 336–341, 5'-AAG GCT GCT ATA CGA TCG ACC GCT CAA AAG AAG-3'; CaMKIV NAARIS 341–346, 5'-CC TCT TCC CGC CTG GGA AAT GCT GCT ATA CGA TCG ACC GCT CAA AAG AAG-3'; CaMKIV NAARIS 347–348, 5'-GCC AGC AGC AGC CAT AAT GCT GCT ATA CGA TCG ACC GCT CAA AAG AAG-3'; CaMKIV NAARIS 348–353, 5'-AGA GAC ACA TTA TAA GCT ACC AGC GTG GTG AAG CGA TCG ACC GCC CC-3'. The double-stranded linker sequence containing FLAG and Nhel overhangs was created by annealing the following primers: sense primer, 5'-CTA GCA TCG ACT ACA AAG AGC ACG ATG ACA AAG-3'; antisense primer, 5'-GCT AGA GAC ACA TTA TAA GCT ACC AGC GTG GTG AAG CGA TCG ACC GCC CC-3'. Nucleotide sequences of cloning sites and entire cDNA of each mutant was confirmed by automated sequencing (Duke University DNA Sequencing Facility).

**Expression and Purification of Recombinant Kinases**—293A cells (95–99% confluence) growing in 150 plates were transfected with DNA constructs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 16–24 h later. For most CaMKIV preparations, the cells were harvested by washing twice with Hank's balanced salt solution without Ca2+—prior to being scraped from the dish into 0.5 ml of lysis buffer described above but containing phosphatase inhibitors, 1 mM NaN3, and 1 mM NaF. The CaMKIV proteins were isolated as above but eliminating the λ-phosphatase treatment steps. Protein concentrations were determined by running samples on SDS-polyacrylamide gels along with β-galactosidase standard and quantifying Coomassie Blue-stained protein bands. All kinases were stored at −80 °C in 27% glycerol.

**Calmodulin Purification**—Chicken calmodulin was produced by bacterial expression as described previously (25). The eluted CaMKIV was recovered and passed through filter disks containing either Me2SO vehicle control or ionomycin to a final concentration of 2.5 μM. Cells were harvested ~16 h later. Luciferase and β-galactosidase activity in cell extracts were determined as previously described (22).

**PP2A/FLAG-CaMKIV Co-immunoprecipitation**—Unstimulated transfected cells were harvested in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 25 mM NaH2PO4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 100 μg okactate acid, which has been reported to enhance the co-immunoprecipitation of PP2A and CaMKIV (22). CaMKIV proteins were isolated using FLAG resin as described above but eliminating the phosphatase treatment steps.

**Western Blotting**—Samples were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp.) for detection of PP2A and CaMKIV, all blocking and antibody incubation steps were performed in TBS containing 5% nonfat milk. Washes were in TBS with 0.5% Tween 20. Anti-CaMKIV (catalog number 610276; BD Biosciences) was used at a 1:2000 dilution, whereas Anti-PP2Ac (catalog number 610555; BD Biosciences) was used at a 1:1000 dilution. Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. and were used at a 1:5000 dilution. Immunoreactivity was detected with the ECL kit provided by Amersham Biosciences. Detection of CaMKIV T200P was performed as above except that the primary antibody incubations were in TBS containing 5% bovine serum albumin. The T200P antibody (Exalpa Bioscience Inc., Waterton, MA) was used at a 1:1000 dilution.

**In Vitro Activation of CaMKIV by CaMKK**—For Western Blotting—Previous recombinant CaMKK (1.7 μg/ml final concentration) was incubated with purified recombinant CaMKK (1.7 μg/ml final concentration) at 30 °C for the indicated periods in buffer containing 41 mM HEPES, pH 7.5, 0.8 mM dithiothreitol, 16.7 mM MgCl2, 0.3 mM ATP, and 1.7 mM CaCl2 and 1.7 μM calmodulin or 3.3 mM EGTA. The reactions were terminated by the addition of 10× SDS loading dye and subsequent heating.

**In Vitro Kinase Assays**—For CaMKIV kinase assays performed in the absence of activation by CaMKK, CaMKIV was incubated at 30 °C, either alone or with CaMKK, for 10 min in the buffer described above containing Ca2+ and calmodulin. The incubation period was ended by the addition of either EGTA or water, and the kinase assays were initiated by the addition of GS-10 substrate (PLRRLTSLVA) and [γ-32P]ATP. Final concentrations were 25 mM HEPES, pH 7.5, 0.8 mM dithiothreitol, 0.1% Tween 20, 10 mM MgCl2, 0.2 mM ATP, 0.2 mM GS-10, 1 μCi of [γ-32P]ATP, 1 mM CaCl2, and 1 μM calmodulin and with or without 4 mM EGTA. The kinase reactions were terminated after 1 min by spotting aliquots of the reaction onto phosphocellulose paper (P-81; Whatman) and washing the filters extensively with 75 mM phosphoric acid. Phosphatase incorporation was determined by liquid scintillation counting of the filters. For CaMKIV kinase assays performed in the absence of activation by CaMKK, the incubation period prior to the kinase assays was eliminated, and the final kinase conditions were 25 mM HEPES, pH 7.5, 0.5 mM dithiothreitol, 0.1% Tween 20, 10 mM MgCl2, 0.2 mM ATP, 0.2 mM GS-10, 1 μCi of [γ-32P]ATP, and either 1 mM CaCl2 and 1 μM calmodulin or 2 mM EGTA.

**Calmodulin Overlays**—Calmodulin was labeled with 125I using Bolton and Hunter reagent from Amersham Biosciences (IM 5861).
following the provided instructions. CaMKIV proteins (100 ng each) were separated by SDS-PAGE, and the gels were then equilibrated for 30 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1 mM NaCl, pH 8.2) prior to transfer of proteins to Immobilon-P membranes. The membranes were then incubated in 0.1 M imidazole, pH 7.0, for 10 min and then in Solution G (20 mM imidazole, pH 7.0, 0.2 mM KCl, 0.1% bovine serum albumin, 0.05% Tween 20) containing either 1 mM CaCl₂ or 1 mM EGTA for 40 min. The membranes were then incubated for 2 h with Solution G containing either CaCl₂ or EGTA along with [³²P]CaM (1 × 10⁶ cpm/ml). The membranes were then washed with Solution G before being dried and subjected to autoradiography.

**Determination of K_CaM**—The calmodulin concentrations required for half-maximal Ca²⁺/CaM-dependent kinase activity (K_CaM) were determined for each CaMKIV protein by performing kinase assays under standard assay conditions in the presence of either 2 mM EGTA or 1 mM Ca²⁺ and with varying calmodulin concentrations. The activity of each kinase in EGTA was subtracted from its activity in Ca²⁺/CaM to determine its Ca²⁺/CaM-dependent activity. The K_CaM for each kinase was determined by analyzing the resulting substrate-velocity curve with GraphPad Prism software.

**RESULTS**

**Phosphorylation of Its Activation Loop Threonine Transforms CaMKIV from a Ca²⁺/CaM-dependent Kinase to a Fully Ca²⁺/CaM-independent or Autonomous Kinase**—It has been previously observed that activation of CaMKIV by CaMKK requires for half-maximal Ca²⁺/CaM but has no effect on the activity of CaMKIV T200A (Fig. A). The specificity of the anti-T200P antibody is demonstrated in vitro. WT CaMKIV is phosphorylated by CaMKKβ on residue Thr²⁰⁰ only in the presence of Ca²⁺/CaM (Fig. 1A). Although CaMKKβ is a Ca²⁺/CaM-responsive enzyme, it possesses significant Ca²⁺/CaM-independent activity (unlike CaMKKα) (24, 26). However, WT CaMKIV is not phosphorylated by CaMKKβ in the absence of Ca²⁺/CaM, indicating a requirement for Ca²⁺/CaM binding to WT CaMKIV for Thr²⁰⁰ phosphorylation, as has been previously reported (19). We next examined the effect of CaMKK on the kinase activity of CaMKIV. CaMKIV was incubated in buffer with Ca²⁺/CaM, either alone or with CaMKKβ, for 10 min at 30 °C. The activity of CaMKIV in the presence of EGTA or Ca²⁺/CaM was then assessed in kinase assays. Unactivated CaMKIV is completely Ca²⁺/CaM-dependent and has no kinase activity in the presence of EGTA (Fig. 1B). CaMKKβ induces an ∼2-fold increase in the activity of WT CaMKIV over its basal activity in Ca²⁺/CaM but has no effect on the activity of CaMKIV T200A (Fig. 1B). Surprisingly, the activation of WT CaMKIV by CaMKKβ was accompanied by the development of Ca²⁺/CaM-independent activity; activated WT CaMKIV was as active in EGTA as in Ca²⁺/CaM (Fig. 1B). Phosphorylation of Thr²⁰⁰ is critical for the generation of autonomy as the CaMKIV T200A mutant fails to develop autonomous activity after incubation with CaMKKβ (Fig. 1B), and WT CaMKIV also fails to develop autonomous activity if incubated with CaMKKβ in the absence of ATP (data not shown).

**Activation of CaMKIV in Cells Is Accompanied by the Development of Autonomous Activity**—Having established that activation of CaMKIV in vitro leads to the generation of a fully autonomous enzyme, we examined whether this phenomenon occurred in cells. CaMKIV proteins, isolated from unstimulated 293A cells, or from 293A cells that had been stimulated with the calcium ionophore ionomycin, were assayed for Thr²⁰⁰ phosphorylation and for kinase activity. Ionomycin treatment leads to increases in intracellular Ca²⁺ and Ca²⁺/CaM levels, which influence both CaMKIV and CaMKK in cells (19, 21). Only a fraction of the CaMKIV in the cell became activated after the ionomycin stimulus, as demonstrated by our observations that only a fraction of CaMKIV was phosphorylated on residue Thr²⁰⁰ (Fig. 2A); correspondingly, autonomous activity generated was only a fraction of total CaMKIV activity (Fig. 2B). This partial activation of the cellular pool of CaMKIV led to an ∼5-fold increase in autonomous activity but had no significant effect on total CaMKIV activity (assayed with Ca²⁺/CaM). This result suggested that the physiologically relevant outcome of CaMKIV activation in cells might be the generation of autonomous activity rather than the increase in the total activity of CaMKIV. Another observation that supports the hypothesis that CaMKIV autonomous activity is biologically important is the fact that whereas WT CaMKIV drives transcription in a Ca²⁺ stimulus-dependent manner, the CaMKIV T200A mutant, which is incapable of developing autonomous activity, is transcriptionally incompetent (Fig. 2C) (24). Thus, we hypothesized that autonomous activity is important for CaMKIV function in transcription.

In order to pursue this hypothesis, we created a series of mutant CaMKIV proteins for use in biochemical experiments and cellular transcription assays. The regulatory domain was targeted because numerous published reports on CaMKIV and related Ca²⁺/CaM-dependent kinases have indicated that mutations in this region would lead to relief of CaMKIV autoinhi-
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bition and constitutive activity (27–30). A series of sequential 6-amino acid NAAIRS or NAAIRN substitution mutants were made spanning residues 300–352 and named CaMKIV N300, N306, N312, N318, N324, N330, N336, N341, and N347. (The NAAIRS and NAAIRN sequences are flexible substitutions that do not generally disrupt overall protein structure (31).) The numbering of mutants indicates the most N-terminal residue mutated. Mutants were made spanning residues 300–352 and named CaMKIV FNDD, which contains a double mutation that changes phenylalanine 320 and asparagine 321 to aspartic acid residues. Regions of CaMKIV important for autoinhibition, calmodulin binding, and PP2A binding were determined through analysis of these mutants.

CaMKIV Residues between 318 and 341 Are Important for Ca2+/CaM Binding. WT and mutant CaMKIV proteins and bovine serum albumin (BSA) used as a negative control in the leftmost lane. The blot was stripped and reprobed with CaMKIV as a loading control. B, WT and T200A CaMKIV proteins isolated from unstimulated (U) or from ionycin-stimulated (S) 293A cells were assayed for kinase activity in the presence of EGTA or Ca2+/CaM. C, 293A cells were transfected with either empty pSG5 vector (control), or with WT or T200A CaMKIV, together with Gal4-CREB and 5× Gal4 luciferase reporter. The cells were either unstimulated or were stimulated with 2.5 μM ionomycin, and after ~16 h, CREB transcription was assessed by assaying luciferase activity in cell lysates. Data in each panel are from one experiment that is representative of five independent experiments.

CaMKIV Residues 324–341 Are Important for Calmodulin Binding—CaMKIV mutants FNDD, N318, N324, N330, and N336 bound [125I]calmodulin poorly compared with WT CaMKIV (Fig. 4) in calmodulin overlay assays performed in the presence of Ca2+, implicating residues between 318 and 341 as important for calmodulin binding. (None of the CaMKIV protein bound [125I]calmodulin in the absence of Ca2+ (data not shown).) To further validate these results, KCaM values were determined for WT and mutant CaMKIV. The KCaM value was defined as the calmodulin concentration at which each kinase achieved half-maximal Ca2+/CaM-dependent activity, and was determined by analyzing the substrate-velocity curve with GraphPad Prism software (results summarized in Table I). Two of the mutants, N312 and FNDD, displayed very little or no Ca2+/CaM-dependent activity, and KCaM values could not be determined for these kinases using the above approach. WT CaMKIV was found to have a KCaM value of ~77 nM, which is consistent with values reported in the literature (17, 19). Of the kinases tested, three mutants had KCaM values much greater than WT CaMKIV. N324 and N330 had KCaM values over 100 μM each (more than a 1000-fold greater than WT), and N336 had an average KCaM of 329 nM, an ~4-fold increase over WT CaMKIV. These data suggest that residues between 324 and 341 are important for Ca2+/CaM binding, with residues between 324 and 335 being most important. There was an excellent correlation between the calmodulin overlay data and the KCaM data with the exception of one mutant, N318. This mutant had a KCaM value of 66 nM, close to the KCaM of 77 nM for WT CaMKIV (Table I), yet it bound [125I]calmodulin poorly compared with WT CaMKIV in the overlay assay (Fig. 4). Therefore, we could not make a definitive judgment on the calmodulin binding properties of N318, although we suspected that it might respond normally to Ca2+/CaM in cells based on its KCaM value.

CaMKIV Residues between 306 and 323 Are Important for PP2A Binding—PP2A and CaMKIV have been shown to exist in a stable complex, with PP2A apparently acting as a negative
Regulator of CaMKIV activity (23). We recently demonstrated that residues within the CaMKIV regulatory region are important for binding of CaMKIV to PP2A and that inhibition of the WT CaMKIV/PP2A interaction in cells could result in increased CaMKIV transcriptional activity, which correlated with increased levels of Thr200 phosphorylation (22). Using the additional CaMKIV mutants generated for this study, we expanded upon these findings by examining the ability of each CaMKIV protein to co-immunoprecipitate PP2A. Most of the CaMKIV mutants co-immunoprecipitated similar amounts of PP2A as compared with WT CaMKIV (Fig. 5). However, N306, N312, FNDD, and N318 co-immunoprecipitated very low amounts of PP2A, suggesting that CaMKIV residues between 306 and 323 are important for PP2A interaction. Based on these results, we anticipated that the activity of N306, N312, FNDD, and N318 might be misregulated in the cell, since PP2A normally acts to negatively regulate WT CaMKIV activity.

Residues between 306 and 329 Are Involved in Autoinhibition of CaMKIV—In order to assess whether any of the mutations resulted in constitutive autonomous activity, we analyzed the kinase activities of unactivated wild-type and mutant CaMKIV proteins (either in 2 mM EGTA or in 1 mM Ca2+ and 1 μM CaM). WT CaMKIV has no significant autonomous activity in its unactivated state and is fully dependent on Ca2+/CaM for its activity (Fig. 6, A and B). In contrast, unactivated CaMKIV mutants N306, N312, FNDD, N318, and N324 each had activity in EGTA in the absence of activation (Thr200 phosphorylation) with specific activities of 17, 22, 4, and 16 nmol/min/mg, respectively, compared with less than 1 nmol/min/mg for WT CaMKIV (data not shown). These data indicate that residues between 306 and 329 are important for maintaining CaMKIV in an inactive state in the absence of bound Ca2+/CaM or Thr200 phosphorylation. Conversely, some of the mutants had significantly lower specific activities than WT CaMKIV in the presence of 1 mM Ca2+ and 1 μM CaM (Fig. 6A). For N324, N330, and N336, this was due to their poor Ca2+/CaM binding properties; in the presence of higher concentrations of calmodulin, these kinases achieved specific activities approaching that of WT CaMKIV (data not shown). The low activities of N300, N312, FNDD, and N318 were not improved by increased concentrations of Ca2+/CaM (data not shown). It is likely that the low activity of N300 is due to impairment of the catalytic function of the enzyme, since the mutated residue Val300 is a hydrophobic residue conserved among kinase catalytic domains. However, for N312, it seems probable that the same mutations that partially relieve autoinhibition also prevent the full relief of autoinhibition upon Ca2+/CaM binding, whereas FNDD and N318 may exhibit both this defect and impaired Ca2+/CaM binding.

CaMKIV Autonomous Activity in Cells Correlates with CaMKIV Transcriptional Activity—Having evaluated some of the regulatory properties of wild-type and mutant CaMKIV proteins in vitro, we next examined the function of each kinase in cells. We first looked at indicators of activation for each CaMKIV protein isolated from unstimulated cells or from ionomycin-stimulated cells. Wild-type and mutant CaMKIV proteins were immunoprecipitated from unstimulated 293A cells or from 293A cells that had been stimulated for 5 min with 2.5 μM ionomycin. The CaMKIV proteins were then analyzed for Thr200 phosphorylation status or assayed for kinase activity in the presence of EGTA to assess autonomous activity. We also examined the ability of each CaMKIV protein to drive transcription in CREB-luciferase assays in the absence of or in response to a Ca2+ stimulus (2.5 μM ionomycin). As observed previously, WT CaMKIV becomes phosphorylated on Thr200

### Table 1

Relationship between CaM concentration and kinase activity of WT and mutant CaMKIV proteins

| CaMKIV &nbsp; | K_{CaM} |
|--------------|---------|
| WT          | 76.9 ± 4.7 nM |
| N200A       | 80.9 ± 10.5 nM |
| N300        | 80.1 ± 12.9 nM |
| N306        | 51.5 ± 8.7 nM |
| N318        | 66.0 ± 8.4 nM |
| N324        | >100 μM |
| N336        | 328.5 ± 52.7 nM |
| N341        | 38.4 ± 5.5 nM |
| N347        | 50.4 ± 5.2 nM |

*a* N312 and FNDD display very little or no Ca2+/CaM-dependent activity, and K_{CaM} values could not be determined for these kinases using the above approach.

### Fig. 5

CaMKIV/PP2A co-immunoprecipitation experiments implicate CaMKIV residues between 306 and 323 as important for PP2A binding. FLAG-resin was used to immunoprecipitate (IP) FLAG-CaMKIV proteins from transfected 293A cells or used with untransfected cells (UNTF) as a negative control. PP2A and CaMKIV protein levels in the immunocomplex were detected by immunoblotting (IB) and Coomassie Blue protein staining, respectively. Data shown are from one experiment that is representative of three independent experiments.

### Fig. 6

Ca2+/CaM-dependent and -independent kinase activities of unactivated WT and mutant CaMKIV proteins. WT and mutant CaMKIV proteins (that had been treated with α-phosphatase to remove any activating Thr200 phosphorylation) were assayed for kinase activity in the presence of 1 mM Ca2+ and 1 μM calmodulin (A) or 2 mM EGTA (B). Data shown are means ± S.E. from six independent experiments.
after an ionomycin stimulus (Fig. 7A), and this is accompanied by the development of autonomous activity (Fig. 7B). Correspondingly, WT CaMKIV drives transcription only after an ionomycin stimulus (Fig. 7C), consistent with the hypothesis that CaMKIV autonomous activity is required for CaMKIV transcriptional activity. The behaviors of the CaMKIV mutants are also consistent with this hypothesis. Several of the mutants (N300, N341, and N347) behave similarly to WT CaMKIV, becoming phosphorylated on residue Thr200 (Fig. 7A), developing greater levels of autonomous activity (Fig. 7B), and driving transcription only after an ionomycin stimulus (Fig. 7C). The behavior of N300 in unstimulated cells is accompanied by only low levels of autonomous activity. It is tempting to speculate that the relatively low levels of autonomous activity of WT and N300 CaMKIV proteins isolated from unstimulated cells (which are not associated with transcriptional activity) may represent a snapshot of rapidly fluctuating levels of activity in the cell rather than sustained levels of autonomous activity.

In contrast to the behaviors of WT, N300, N341, and N347 CaMKIV proteins, the T200A CaMKIV mutant, which cannot be phosphorylated on the activation loop threonine (Fig. 7A), fails to develop autonomous activity in cells (Fig. 7B) and is transcriptionally inactive (Fig. 7C). Two other mutants, N330 and N336, also display poor transcriptional activity in these assays. N330 fails to become phosphorylated on residue Thr200 in response to an ionomycin stimulus (Fig. 7A), fails to develop autonomous activity (Fig. 7B), and correspondingly fails to drive transcription (Fig. 7C). N336, on the other hand, is poorly phosphorylated on Thr200 in response to an ionomycin stimulus (Fig. 7A), fails to develop autonomous activity (Fig. 7B), and correspondingly drives transcription to a low degree (Fig. 7C). The failure of N330 and N336 to develop autonomous activity in a cellular context can be attributed to their poor calmodulin binding properties, which preclude them from binding Ca2+/CaM and from being activated at physiological Ca2+/CaM concentrations. (Both N330 and N336 are phosphorylated on Thr200 and develop substantial levels of autonomous activity in vitro after incubation with CaMKK and high levels of Ca2+/CaM concentrations (supplemental Figs. S2 and S3). For the T200A, N330, and N336 mutants, their inability to drive transcription correlates with their failure to develop autonomous activity in cells, supporting the hypothesis that autonomous activity is necessary for CaMKIV-mediated transcription. Finally, several of the mutants, N306, N312, FNDD, N318, and N324, are capable of driving transcription in the absence of an ionomycin stimulus (Fig. 7C). Each of these kinases has significant levels of autonomous activity when isolated from unstimulated cells (Fig. 7B). This is not surprising, because each of these kinases has autonomous activity even in the absence of Thr200 phosphorylation (Fig. 6B). Additionally, N306, N312, FNDD, and N318 are phosphorylated on Thr200 to varying degrees when isolated from unstimulated cells. This is probably a consequence of their relatively poor ability to bind PP2A (Fig. 5), since these kinases would not be expected to be efficiently dephosphorylated by PP2A after activation resulting from normal cellular events such as transient Ca2+ fluxes. The transcriptional activities of N306, N318, and N324 are significantly increased after an ionomycin stimulus (Fig. 7C), correlating with increased levels of Thr200 phosphorylation (Fig. 7A) and autonomous activity in cells (Fig. 7B).2 These experiments illustrate a clear relationship between autonomous activity of CaMKIV and CaMKIV transcriptional function and suggest that CaMKIV autonomous activity is required for CaMKIV transcriptional activity. The behaviors of the CaMKIV mutants are also consistent with this hypothesis. Several of the mutants (N300, N341, and N347) behave similarly to WT CaMKIV, becoming phosphorylated on residue Thr200 (Fig. 7A), developing greater levels of autonomous activity (Fig. 7B), and driving transcription only after an ionomycin stimulus (Fig. 7C). The behavior of N300 in unstimulated cells is accompanied by only low levels of autonomous activity. It is tempting to speculate that the relatively low levels of autonomous activity of WT and N300 CaMKIV proteins isolated from unstimulated cells (which are not associated with transcriptional activity) may represent a snapshot of rapidly fluctuating levels of activity in the cell rather than sustained levels of autonomous activity.

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1. N347, which appears incapable of binding Ca2+/CaM (Fig. 4 and Table I), is able to be activated in cells upon a Ca2+ stimulus. This is because, unlike WT CaMKIV, N347 does not require Ca2+/CaM for Thr200 phosphorylation by CaMKK. N347 can be phosphorylated by CaMKKβ in the absence of Ca2+/CaM and in the presence of EGTA (supplemental data, Fig. S2). This is probably because the same mutations that partially relieve autoinhibition of the enzyme also expose residue Thr200 of N347, even in the absence of bound Ca2+/CaM.
that autonomous activity is the primary requirement for CaMKIV-mediated transcription.

**Autonomous Activity, in the Absence of Thr^{200} Phosphorylation, Is Sufficient to Drive Transcription—**For WT CaMKIV (and for some of the mutants), autonomous activity is tightly linked to Thr^{200} phosphorylation. In order to determine whether CaMKIV autonomous activity, in the absence of Thr^{200} phosphorylation, would be sufficient for CaMKIV transcriptional function, we made mutations of residue Thr^{200} to the nonphosphorylatable alanine in three of the mutants that have autonomous activity in vitro in the absence of Thr^{200} phosphorylation. We then looked at levels of Thr^{200} phosphorylation and autonomous activity of these three additional mutants, N312 T200A, FNDD T200A, and N318 T200A, when isolated from unstimulated or ionomycin-stimulated cells, and we also tested their ability to drive transcription in CREB-luciferase assays. N312 T200A, FNDD T200A, and N318 T200A were not phosphorylated on Thr^{200} when isolated from either unstimulated 293A cells or from 293A cells stimulated with ionomycin (Fig. 8A). Each of these three mutants had autonomous activity in cells that was not increased upon an ionomycin stimulus (Fig. 8B), and each drove transcription in a Ca^{2+}-stimulus-independent manner (Fig. 8C).

These results demonstrate that, at least in the context of some CaMKIV mutants, autonomous activity, even in the absence of Thr^{200} phosphorylation, is sufficient for CaMKIV transcriptional function.

**DISCUSSION**

Numerous studies have reported that activation of CaMKIV by CaMKK in vitro, we found that high amounts of CaMKK (relative to CaMKIV) were required for maximal activation of CaMKIV (i.e. for maximal T200 phosphorylation and for generation of complete CaMKIV autonomy). In all of the experiments presented here examining activation of CaMKIV by CaMKK in vitro, we utilized approximately equal amounts of CaMKIV and CaMKK. In contrast, many of the previous studies examining CaMKK activation of CaMKIV have used catalytic amounts of CaMKK relative to CaMKIV (20, 32). Furthermore, studies performed prior to the identification of CaMKK as the activating kinase for CaMKIV (29, 33, 34) and prior to the cloning and characterization of CaMKKa/β (24, 35, 36) used brain extract (which probably contained relatively low levels of CaMKK) to activate CaMKIV in vitro. Under these conditions, only a fraction of CaMKIV appeared to be activated. This led us to suspect that in many previous studies looking at CaMKIV activation in vitro, levels of CaMKIV were the limiting factor for complete CaMKIV activation. This also raised the intriguing possibility that the relationship between CaMKIV and CaMKK could be stoichiometric rather than catalytic.

Numerous studies have suggested that activation of CaMKIV by CaMKK is important for CaMKIV transcriptional function. For example, co-transfection of CaMKK along with CaMKIV was found to greatly potentiate Ca^{2+}-dependent CaMKIV transcription in cells (24, 36). (This might indicate that low levels of endogenous CaMKK were limiting for activation of the exogenously expressed CaMKIV protein.) Other studies have shown that the T200A CaMKIV mutant, which is incapable of activation by CaMKK, is transcriptionally inactive (18, 24), suggesting that activation by CaMKK is actually critical for CaMKIV function in transcription. In this study, we observe that, in vitro, activation of CaMKIV by CaMKK converts CaMKIV to a fully Ca^{2+}/CaM-independent enzyme. This finding appears to be physiologically important, since our experiments indicate that the primary consequence of CaMKIV activation in cells is the generation of autonomous activity rather than any significant increase in total activity (Fig. 2B). In addition, the generation of autonomous activity of WT CaMKIV upon an ionomycin stimulus correlates with the ability of WT CaMKIV to drive transcription in cellular assays. Together, these observations led us to hypothesize that CaMKIV autonomous activity is required for transcription. In order to pursue this hypothesis, we created a series of mutants for use in comparative biochemical and cellular transcriptional assays.

We analyzed the regulatory properties of WT and mutant CaMKIV proteins in vitro, assessed their activation in cells, and examined their ability to drive CREB-mediated transcrip-
CaMKIV Autonomous Activity Required for Transcription

CaMKIV autonomous activity is required for transcription. In the case of each CaMKIV protein, transcriptional activity correlates with the generation of or levels of autonomous activity in cells. Additionally, it appears that Ca\(^{2+}\)/CaM binding and Thr\(^{200}\) phosphorylation are not strictly required for CaMKIV transcription.

The lack of a requirement for Ca\(^{2+}\)/CaM binding of CaMKIV for its role in transcription is exemplified by the N324 mutant. N324 fails to bind detectable levels of [\(^{125}\)I]calmodulin in overlay assays and has a \(K_{\text{CaM}}\) value in excess of 100 \(\mu\)M, indicating that this mutant is unlikely to bind Ca\(^{2+}\)/CaM under physiological conditions. Nevertheless, N324, which has constitutive autonomous activity, is capable of driving CREB-mediated transcription (Fig. 7C), suggesting that Ca\(^{2+}\)/CaM-binding is not absolutely required for CaMKIV transcription.\(^2\)

We also found that Thr\(^{200}\) phosphorylation was not strictly required for CaMKIV transcription. In order to determine whether autonomous activity, in the absence of Thr\(^{200}\) phosphorylation, would be sufficient for CaMKIV transcriptional activity, we made threonine 200 to alanine mutations in N312, Thr200 phosphorylation, is sufficient for CaMKIV transcriptional function through dephosphorylation of residue Thr\(^{200}\) and termination of the autonomous activity of CaMKIV.

Previous studies have shown that PP2A negatively regulates CaMKIV transcriptional function through dephosphorylation of CaMKIV residue Thr\(^{200}\) (22, 23). Our study corroborates the importance of PP2A in regulating CaMKIV function. For example, in unstimulated cells, CaMKIV mutants that are impaired in PP2A binding (Fig. 5) generally exhibit higher levels of Thr\(^{200}\) phosphorylation, autonomous activity, and transcriptional activity (Fig. 7) than CaMKIV proteins that bind PP2A normally. This is presumably because mutants that bind PP2A poorly are less efficiently dephosphorylated in cells after activation resulting even from ambient cellular Ca\(^{2+}\) transients. Thus, a fraction of the cellular pool of these mutants is somewhat activated even in unstimulated cells. The significance of this is exemplified by the behavior of N318 and N324. Although, in vitro, unactivated N318, which binds PP2A poorly (Fig. 5), has less autonomous activity than unactivated N324, which binds PP2A normally (Fig. 5), in cells, N318 exists in a more activated state even in the absence of an ionomycin stimulus (Fig. 7A), has greater autonomous activity (Fig. 7B), and drives transcription to a greater degree than N324 (Fig. 7C).

Together, our data suggest a model for CaMKIV function in transcription where autonomous activity is the primary requirement for CaMKIV transcriptional activity. CaMKIV mutants with constitutive autonomous activity can bypass the requirements for Ca\(^{2+}\)/CaM and Thr\(^{200}\) phosphorylation, indicating that autonomous activity alone is sufficient for transcriptional activity. WT CaMKIV, however, achieves autonomous activity (and transcriptional competence) only after Ca\(^{2+}\)/CaM binding and subsequent Thr\(^{200}\) phosphorylation, whereas PP2A negatively regulates CaMKIV transcriptional function through dephosphorylation of residue Thr\(^{200}\) and termination of the autonomous activity of CaMKIV (Fig. 9).

The primary function of PP2A and CaMKK in the regulation of CaMKIV appears to be fulfilled through their opposing effects on CaMKIV Thr\(^{200}\) phosphorylation status. Our data (Fig. 5), along with previous studies (22, 23), indicate that PP2A and CaMKK may interact in a complex in cells. We explored the possibility that CaMKK might also share this ability to complex with CaMKIV, examining the ability of CaMKK to interact with CaMKIV in co-immunoprecipitation experiments. We found that WT CaMKIV can stably associate with CaMKK (supplemental Fig. S1), as has been previously reported (40, 41). However, the transcriptionally inactive T200A CaMKIV mutant also binds CaMKK, similarly to WT CaMKIV, when immunoprecipitated from either unstimulated or ionomycin-stimulated cells (supplemental Fig. S1). These data indicate that the interaction between CaMKK and CaMKIV is insufficient to confer CaMKIV transcriptional capability. This hypothesis, that the interaction between CaMKIV and CaMKK is not the primary determinant of CaMKIV transcriptional function, is supported by the behavior of other CaMKIV mutants. For example, N330 also binds CaMKK similarly to WT CaMKIV (supplemental Fig. S1) but is transcriptionally incompetent (Fig. 7C). Thus, CaMKK binding does not preclude the requirement for CaMKIV autonomous activity in CaMKIV-mediated transcription. It is possible that the main role of a tight interaction between CaMKIV and CaMKK is to facilitate efficient Thr\(^{200}\) phosphorylation upon a Ca\(^{2+}\) stimulus. Indeed, it has been reported that an RP-domain mutant of CaMKK, which fails to bind CaMKIV but is not catalytically impaired, is incapable of activating CaMKIV via phosphorylation of the Thr\(^{200}\) residue, although it phosphorylates other substrates (such as protein kinase B) similarly to wild-type CaMKK (40).

Previous work studying the relationship between PP2A and CaMKIV has indicated that a fraction of the cellular pool of CaMKIV interacts with PP2A in a complex, independent of CaMKIV activation status (23). We have also observed that only a small fraction of CaMKIV in cells appears to interact with CaMKK, based on the relative amounts of these two proteins that co-immunoprecipitate (data not shown), and this interaction occurs even in the absence of a Ca\(^{2+}\) stimulus.
Thus, it is fascinating to speculate that some portion of CaMKIV in cells ordinarily exists as a complex with one or both of its regulators. The autonomous activity of CaMKIV within such a complex could thus be very tightly regulated by the opposing activities of bound CaMKK and PP2A.

CaMKIV is an important effector of Ca\textsuperscript{2+} signaling events, and there are a number of reasons why autonomous activity might be important for its function in transcription. It has become increasingly clear that the amplitude, frequency, duration, source, and localization of Ca\textsuperscript{2+} signals are critical for determining how increases in intracellular Ca\textsuperscript{2+} are interpreted by the cell and which Ca\textsuperscript{2+}-sensitive pathways are activated (42–45). There has also been a tremendous amount of interest in understanding how short lasting Ca\textsuperscript{2+} signals lead to long term changes in cellular environment (46, 47). Many adaptive changes in response to Ca\textsuperscript{2+} signals involve gene transcription, and thus transcriptional transducers of Ca\textsuperscript{2+} signaling are key cellular regulators. Here we show that CaMKIV, once activated, is no longer dependent on Ca\textsuperscript{2+}/CaM for its activity. This has important implications, since it means that brief elevations in intracellular Ca\textsuperscript{2+} can lead to an activation of CaMKIV that outlasts the duration of the Ca\textsuperscript{2+} signal. In this way, CaMKIV may mediate transcription even after the fleeting Ca\textsuperscript{2+} signal has subsided. Under these circumstances, negative regulation by PP2A may be the primary mechanism for termination of Ca\textsuperscript{2+}-dependent CaMKIV transcription.

CaMKIV autonomy may have other implications as well. In cells, CaMKIV can be activated by CaMKK after intracellular Ca\textsuperscript{2+} elevations resulting from treatment with agents such as ionomycin or resulting from physiological stimuli such as CD3-mediated activation of T lymphocytes (21). However, it has been unclear where in the cell CaMKIV becomes activated (CaMKIV is primarily nuclear, and CaMKK is primarily cytoplasmic). One possibility is that CaMKIV (unaccompanied or within a complex), cycling in and out of the nucleus, becomes activated in the cytoplasm upon a Ca\textsuperscript{2+} signal. Activated CaMKIV could then translocate back into the nucleus to potentiate transcription. Because activated CaMKIV would no longer require elevated Ca\textsuperscript{2+} levels for its activity, the implication is that cytoplasmic Ca\textsuperscript{2+} signals, even if they never crossed into the nucleus, could lead to activation of CaMKIV and nuclear CaMKIV functions. These possibilities underscore the complexities of Ca\textsuperscript{2+} signaling and highlight the roles of multiple, converging regulatory mechanisms (Ca\textsuperscript{2+}/CaM binding, CaMKK activation, and PP2A dephosphorylation) in regulating the Ca\textsuperscript{2+}/CaM effector, CaMKIV.
The Autonomous Activity of Calcium/Calmodulin-dependent Protein Kinase IV Is Required for Its Role in Transcription

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