Regulation and Function of the Calcium/Calmodulin-dependent Protein Kinase IV/Protein Serine/Threonine Phosphatase 2A Signaling Complex*

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Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a member of the broad substrate specificity class of Ca2+/calmodulin (CaM)-dependent protein kinases and functions as a potent stimulator of Ca2+-dependent protein kinases that also includes CaMKI and CaMKII (1, 2). Whereas CaMKI and CaMKII are ubiquitously expressed, CaMKIV is present in a limited number of cells and tissues (3–5). Analysis of the phenotypic consequences caused by the absence of CaMKIV in genetically manipulated mice led to the hypothesis that CaMKIV is involved in the differentiation of cells in which it is expressed (6–11). At the cellular level, CaMKIV is a potent activator of Ca2+-dependent transcription mediated by a number of factors, including CREB, the CREB-related factor ATF-1, the MADS-box family members SRF and MEF2D, and the orphan receptors RORα, RORγ, and COUP-TF1 (12–15). CaMKIV-dependent activation of CREB, the most extensively studied cellular activity of the kinase, can occur through the direct phosphorylation of CREB on Ser-133 (16). However, CaMKIV can also stimulate CREB-mediated transcription by activating the CREB-binding protein through a mechanism that remains to be elucidated (17) but may also involve direct phosphorylation (18).

The regulation of CaMKIV has been the subject of a number of studies. As is the case for other CaM kinases, the active site of CaMKIV is sterically blocked by an autoinhibitory domain that prevents substrate binding to the enzyme. Binding of Ca2+/CaM to this region relieves autoinhibition by repositioning the autoinhibitory domain and exposes a critical residue in the activation loop, T200, to CaMKK-mediated phosphorylation (18, 20). T200 phosphorylation increases CaMKIV activity and leads to the generation of Ca2+/CaM-independent, or autonomous, activity. It has been proposed that phosphorylation of T200 is required for CaMKIV-mediated transcription (21, 22).

A unique aspect of CaMKIV activation in the cellular context is its transient nature. In lymphocyte-derived cell lines, both the Ca2+-dependent and autonomous activities of CaMKIV peak by 1 min following application of stimuli that increase intracellular Ca2+ and return to baseline levels by 5 min, indicating that activation/inactivation are brief and tightly regulated events (21, 23). A protein-protein interaction that may influence the kinetics of inactivation in a cellular context is the stable complex between CaMKIV and the broad substrate specificity protein serine/threonine phosphatase 2A (PP2A) (24). It is generally accepted that the formation of multiprotein complexes is one way to regulate specificity and timing of signaling events (25, 26). In the case of protein phosphorylation, macromolecular protein complexes may restrict access of kinases and phosphatases to specific microenvironments and position them to respond rapidly to appropriate signals. In recent years several kinases in addition to CaMKIV, including CK2, p70 S6 kinase, p21-activated kinase, Jnk2, IkB kinase, and protein kinase C, have also been shown to interact with PP2A (27–33).

However, in most of these cases details describing a precise functional link between the kinase and PP2A are lacking.

In the present study, we have further examined the...
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MATERIALS AND METHODS

Cells and Cell Culture—A 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 manufacturer.

Constructs—FLAG-CaMKIV WT, FLAG-CaMKIV Trunc 1–317, FLAG-CaMKIV Trunc 1–340, and FLAG-CaMKIV K75M, all cloned into the mammalian expression vector, pSG5 (Stratagene), were generated as described previously (21). The other CaMKIV mutants were generated by site-directed mutagenesis using single-stranded CaMKIV/pSG5 (without the FLAG tag) DNA as template and the protocol and reagents provided with the Mutma-Gene M13 kit (Bio-Rad). The single-stranded DNA was synthesized in C236 Escherichia coli that had been transformed with CaMKIV/pSG5 and grown with M13K07 helper phage (Invitrogen). The sense oligonucleotides used were as follows: CaMKIV Trunc 1–308, 5′-GCAATTTATGATAGACCTACGGC-TAAGAAAAGAAGCTCAAAAAAAACTTTGCCAAGAGTAAGTGGAAGCA-3′; antisense, 5′-GGATATCCGCGCTGGTGGCC-3′.

Cells—293A cells grown to 95% confluency in a p150 dish were transfected with Lipo-fectAMINE 2000 (Invitrogen); 750 μl of Opti-MEM (Invitrogen) containing a mixture of 6 μg of 1-Ag/CEP (plasmid kindly provided by Shirish Shenolakar), 4 μg of FLAG-CaMKIV/pSG5, and 14 μl of LipofectAMINE 2000 was pipetted into the culture media that was covering the cells. Other details of the transfection procedure followed were those recommended by the manufacturer. The next day, cell lysis and immunoprecipitation were carried out as described above.

CaMKIV protein by immunoblotting, samples were run on a 10% acrylamide gel and electrophoretically transferred to an Immobilon-P membrane (Millipore). The membrane was blocked for 1–2 h, incubated with PP2Ac monoclonal antibody (BD Biosciences) at a 1/1000 dilution, washed 3× (5 min each), incubated with horseradish peroxidase-conjugated anti-mouse IgG (Sigma) for 1 h, washed 4× (10 min each), and developed using the ECL kit from Amersham Biosciences. All incubations and washes were at room temperature. The membrane blocking and incubations with antibodies were in TBS (25 mM Tris base, pH 7.4, and 0.14 M NaCl) containing 5% milk, and the washes were with TBS containing 0.5% Tween 20. To detect CaMKIV P-T200 by immunoblotting, the procedure for detecting PP2Ac was followed except that the incubation with P-T200 antibody (Exalpha Biologicals Inc., Waterson, MA), at 1/1000, was in TBS containing 5% bovine serum albumin. To detect CaM by immunoblotting the following protocol was followed. Protein samples were run on a 15% acrylamide gel. After equilibrating the gel for 15 min in Buffer A (25 mM KH2PO4, pH 7.0), proteins were transferred to Immobilon-P membrane for 1 h at 4°C and 20 V in Buffer A. The membrane was then fixed in Buffer A containing 0.2% glutaraldehyde, washed 3× (10 min each) in Buffer B (TBS containing 5% milk), and then incubated 2 h with calmodulin monoclonal antibody (Upstate #05-173) diluted 1/1000 in TBS containing 2% bovine serum albumin. Next, the membrane was washed 3× (5 min each) in Buffer C (TBS containing 0.05% Tween 20 in 1 h in horseradish peroxidase-conjugated anti-mouse antibody diluted 1/5000 in Buffer B, washed 4× (10 min each) in Buffer C, and developed using the ECL kit from Amersham Biosciences.

CREB Assay—Six-well dishes were seeded with 0.2 × 106 293A cells per well. The next day, cells were transfected with 0.02 μg of β-galactosidase, 0.16 μg of Gal4 CREB, 0.4 μg of 5′-Gal4 luciferase reporter, and 0.4 μg of FLAG-CaMKIV/pSG5 DNA per well, using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s recommendations. The cells were stimulated with 2 μM ionomycin (calcium salt from Calbiochem, 407952) the following evening for 16 h and then lysed. Luciferase and β-galactosidase activities in the cell extracts were determined within 1–2 h in horseradish peroxidase-conjugated anti-mouse antibody dilution of 1/35. Transfection efficiency was normalized by β-galactosidase activity, and the control transfection was empty pSG5 vector (Stratagene).

In Vitro Kinase Assay—The kinase assay was initiated by diluting 100 μg of FLAG-CaMKIV in a final volume of 100 μl of kinase reaction buffer containing 25 mM Tris HCl, pH 7.4, 7.5 μM CaCl2, 50 mM MgCl2, 0.1% Tween 20, 100 μM MgOCC, 100 μM ATP, 2 μCi of [γ-32P]ATP, 100 mM NaCl, 200 mM GS-10 peptide (proline-leucine-arginine-arginine-threonine-leucine-serine-valine-alanine-alanine) substrate, and 1 μM Ca2+. In addition, either 1 μM CaCl2 or 1 mM EGTA was included in the reaction buffer, depending on whether Ca2+/CaM-dependent or Ca2+/CaM-independent activity was to be determined. Reaction mixtures were run for 10 min at 30°C at which point 20-μl aliquots of reaction mixture were spotted, in triplicate, onto phosphocellulose paper (p81 disks from Whatman) that was then rinsed in 75 mM phosphoric acid. The 32P-labeled peptide was quantitated by scintillation counting of the dried p81 papers.

Protein Determination—Protein concentrations were determined by the method of Bradford (36) using globulin as standard.

RESULTS

The Ca2+/CaM Binding-Autoinhibitory Domain of CaMKIV Is Required for Association of the Kinase with PP2A—PP2A has been shown to copurify with CaMKIV from brain, coimmunoprecipitate with CaMKIV from multiple cell types, and associate with GST-CaMKIV (24, 37). We find that PP2A also coimmunoprecipitates with a FLAG-tagged version of CaMKIV expressed in 293A cells (Fig. 1A). The absence of detectable...
PP2A coimmunoprecipitating with FLAG-CaMKI demonstrates the selectivity of the interaction (Fig. 1A). Because FLAG-CaMKIV expressed in 293A cells behaves similarly to its untagged counterpart in vitro and in cell-based transcription assays (data not shown), we have used this system throughout this study. To determine which region of CaMKIV is important for interaction of the kinase with PP2A, a series of FLAG-CaMKIV truncation mutants was tested for ability to coimmunoprecipitate PP2A from 293A cells. The most informative of these mutants are Trunc 1–308, Trunc 1–317, and Trunc 1–340, comprising the N-terminal 308, 317, and 340 residues of CaMKIV, respectively (see schematic in Fig. 1C). Trunc 1–308 fails to coimmunoprecipitate detectable PP2A, Trunc 1–317 coimmunoprecipitates a small amount, and Trunc 1–340 coimmunoprecipitates levels of PP2A similar to that coimmunoprecipitated by WT CaMKIV (Fig. 1B). All three mutants contain the entire kinase homology domain (residues 50–300) but differ from one another by including different portions of the autoregulatory domain of the kinase (residues 301–340, Fig. 1C). The dramatic difference in the amount of PP2A coimmunoprecipitated by these mutants implicates the autoregulatory domain as important for the kinase/phosphatase interaction. To test this idea further, mutants were generated in the context of full-length CaMKIV, by swapping 12 amino acid regions from the autoregulatory domain for corresponding residues from CaMKI. CaMKI was chosen, because it is the kinase that shares greatest homology with CaMKIV but does not associate with PP2A. CaMKIV Subst 312–323 and Subst 318–329, in which residues 312–323 and 318–329 have been replaced, respectively, were found to coimmunoprecipitate PP2A poorly (Fig. 1B). A third full-length CaMKIV autoregulatory domain mutant, Subst 320–321, was generated by replacing residues 320FN321 with DD, a change previously shown to disrupt autoinhibition of the enzyme (38). CaMKIV Subst 320–321 also failed to associate with PP2A (Fig. 1B). The behavior of the substitution mutants thus complements that of the truncation mutants. As previously reported (24), the catalytically inactive CaMKIV mutant, CaMKIV K75M, and the activation loop mutant, CaMKIV T200A, interact with PP2A similarly to CaMKIV WT (Fig. 1B). Collectively, these data indicate a re-
Fig. 2. Ca\(^{2+}\)/CaM inhibits the interaction between CaMKIV and PP2A. A, overexpressed FLAG-CaMKIV was immunoprecipitated from 293A cells in the presence or absence of EGTA and the PP2A and CaM present in the immunoprecipitates determined by immunoblotting (right-hand, upper and middle panels, respectively). Also shown is the Coomassie-stained membrane (right-hand, lower panel). B, FLAG-CaMKIV/PP2A complex isolated in the presence of EGTA was further incubated in EGTA, Ca\(^{2+}\)/CaM, Ca\(^{2+}\)/CaM, or with Ca\(^{2+}\)/CaM that had been preincubated with W13. The PP2A and CaM remaining in the complex after this incubation was determined by immunoblotting (right-hand, upper and middle panels, respectively). Also shown is the Coomassie-stained membrane (right-hand, lower panel). The average PP2A signal from the blots, quantitated by densitometry and normalized for CaMKIV protein, was plotted ± S.D. (n = 9 in A and n = 6 in B).

requirement for the autoregulatory domain of CaMKIV for association of the kinase with PP2A.

Binding of Ca\(^{2+}\)/CaM and PP2A to CaMKIV Is Mutually Exclusive—Whether by directly binding PP2A or through some other mechanism, our results indicate a newly identified role for the autoregulatory domain of CaMKIV. This small region of the kinase already has well defined functions. It is comprised of overlapping autoinhibitory and CaM binding regions that in the absence of Ca\(^{2+}\)/CaM sterically inhibit enzymatic activity. Because Ca\(^{2+}\)/CaM binding relieves inhibition by repositioning the autoinhibitory domain, we questioned whether Ca\(^{2+}\)/CaM might affect association of CaMKIV with PP2A. The standard cell lysis/immunoprecipitation buffer used in our experiments contains the Ca\(^{2+}\)-chelator, EGTA, for the purpose of inhibiting Ca\(^{2+}\)-dependent proteases. As a first step toward testing the effect of Ca\(^{2+}\)/CaM on the CaMKIV/PP2A interaction, EGTA was omitted from the standard protocol. This resulted in a dramatic reduction in the amount of PP2A coimmunoprecipitated by CaMKIV (Fig. 2A), and CaM was present only in the “without EGTA” samples (Fig. 2A). Next, the effect of Ca\(^{2+}\) on CaM on isolated CaMKIV/PP2A complex was examined. FLAG-CaMKIV was immunoprecipitated in buffer containing EGTA, which favors association between CaMKIV and PP2A. While still attached to FLAG resin, the CaMKIV/PP2A complex was further incubated in EGTA, Ca\(^{2+}\), CaM, Ca\(^{2+}\)/CaM, or in Ca\(^{2+}\)/CaM that had been preincubated with the CaM antagonist W13. Immunoblot analysis of the washed resin demonstrated that Ca\(^{2+}\)/CaM considerably decreased the amount of PP2A present, whereas the other conditions had no effect (Fig. 2B). We conclude that Ca\(^{2+}\)/CaM binding to CaMKIV inhibits its interaction with PP2A.

PP2A Negatively Regulates the Ability of CaMKIV to Regulate CREB-mediated Transcription in 293A Cells—We next examined the physiological role of the CaMKIV/PP2A interaction by comparing the cellular function of CaMKIV WT with that of the mutants that are unable to interact with PP2A. The position of the CaMKIV truncation and amino acid substitutions that render the kinase unable to interact with PP2A suggested that, in addition to compromised PP2A binding, autoinhibition and regulation of kinase activity by Ca\(^{2+}\)/CaM might also have been compromised. This prediction was tested before proceeding with the cell-based functional assays by characterizing the CaMKIV PP2A-binding mutant enzyme activities in vitro (Fig. 3A). Indeed, each mutant exhibited Ca\(^{2+}\)/CaM-independent activity, indicating that autoinhibition had been disrupted. The variation in levels of total and Ca\(^{2+}\)/CaM-independent kinase activity displayed by the different mutants probably reflects the different degrees to which autoinhibition has been disrupted. Least affected was CaMKIV Subst 318–329, because it exhibited a specific activity similar to WT CaMKIV and retained significant Ca\(^{2+}\)/CaM-dependent activity. Therefore, we chose the CaMKIV Subst 318–329 mutant to test the role of the CaMKIV/PP2A interaction by comparing it with WT kinase in a cell-based assay. The cell based assay used was the CREB transcription assay in which cells are cotransfected with GAL4-CREB, a GAL4-luciferase reporter, and CaMKIV (35). After stimulation of the cells with ionomycin to increase the intracellular Ca\(^{2+}\) concentration, cell extracts were made, from which luciferase activity was measured. Compared with CaMKIV WT, the binding mutant CaMKIV Subst 318–329 was found to stimulate CREB-mediated transcription with dramatically enhanced activity and to do so in a Ca\(^{2+}\)/CaM-independent manner (Fig. 3B). Fig. 3C illustrates that CaMKIV WT and mutant protein levels in cell extracts were similar. Thus, PP2A appears to negatively regulate CaMKIV in this cell-based system.

CaMKIV Becomes Transiently Phosphorylated on T200 in Response to Cell Stimuli That Increase Intracellular Ca\(^{2+}\); T200 Phosphorylation Correlates with Kinase Activation—Because CaMKIV is believed to require phosphorylation of T200...
immunoprecipitates, and again, there was no signal in the samples containing T200A.

To determine how phosphorylation of CaMKIV on T200 correlated with enzyme activation, aliquots of the immunoprecipitates shown in Fig. 4A were assayed in vitro for both Ca^{2+}/CaM-dependent and autonomous activity, using GS-10 peptide as substrate. CaMKIV WT developed autonomous activity that peaked at 5 min and had returned to baseline level by 15 min, similar to the time course of P-T200 (Fig. 4B). CaMKIV T200A, which cannot be phosphorylated on residue T200, did not develop autonomous activity. We conclude from these data that autonomous activity correlates well with P-T200. In contrast, Ca^{2+}/CaM-dependent activity of CaMKIV is significant, the small activated fraction does not appear above the relatively high background. In contrast, “unactivated CaMKIV” has no autonomous activity. Thus, against this low background, the small activated fraction can be detected. We draw two conclusions from these experiments: 1) the rapid activation of CaMKIV in 293A cells that occurs in response to cell stimulation is followed by inactivation of the kinase; 2) the kinetics of activation and inactivation of CaMKIV parallel the accumulation and subsequent disappearance of P-T200. These findings support a role for dephosphorylation in the negative regulation of CaMKIV and suggested to us an important role for PP2A.

Manipulations That Prevent Interaction between CaMKIV and PP2A Result in Increased Phosphorylation of CaMKIV on T200—To test the idea that PP2A may negatively regulate CaMKIV by dephosphorylating T200, we examined whether the failure of CaMKIV to bind PP2A would alter P-T200 levels. For this analysis we again used the CaMKIV-PP2A binding mutant, CaMKIV Subst 318–329, which was immunoprecipitated from 293A cells before and after stimulation of the cells with ionomycin, and examined for P-T200 by immunoblotting (Fig. 5A). The mutant was found to differ profoundly from wild type CaMKIV. It displayed levels of P-T200 that were higher than WT at all time points following stimulation with ionomycin, and this was accompanied by increased autonomous kinase activity (Fig. 5B). The mutant also displayed significant P-T200 activity (Fig. 5C). The PP2A plot showed no significant differences with ionomycin treatment, whereas the PP2A plot showed a significant increase in P-T200 activity. The PP2A plot is shown in Fig. 6A. Because the basal Ca^{2+}/CaM-dependent activity of CaMKIV is significant, the small activated fraction does not appear above the relatively high background. In contrast, “unactivated CaMKIV” has no autonomous activity. Thus, against this low background, the small activated fraction can be detected. We draw two conclusions from these experiments: 1) the rapid activation of CaMKIV in 293A cells that occurs in response to cell stimulation is followed by inactivation of the kinase; 2) the kinetics of activation and inactivation of CaMKIV parallel the accumulation and subsequent disappearance of P-T200. These findings support a role for dephosphorylation in the negative regulation of CaMKIV and suggested to us an important role for PP2A.

The coexpression of CaMKIV with SV40 small t antigen (t-Ag), a highly selective inhibitor of PP2A, has been previously shown to enhance CaMKIV-mediated transcription (24). We therefore questioned whether t-Ag coexpression might serve as an alternative means for disrupting the physical association between CaMKIV and PP2A. CaMKIV alone, or when coexpressed with t-Ag, was immunoprecipitated from 293A cells that had been either untreated or stimulated with ionomycin. PP2A and P-T200 present in the immunoprecipitates were determined by immunoblotting, and the total CaMKIV protein in the samples was visualized by Coomassie Blue staining (Fig. 6A). The changes in PP2A and P-T200 as a function of time after ionomycin treatment, normalized for total CaMKIV protein, are shown in Fig. 6B and C, respectively. The PP2A plot...
demonstrates that t-Ag partially inhibits the physical association between CaMKIV and PP2A. The partial inhibition suggested that the level of t-Ag in the cells was limiting, but our attempts to increase expression levels by increasing the amount of transfected DNA resulted in cell death (not shown). Nevertheless, as revealed in the P-T200 plot, the t-Ag-dependent reduction in associated PP2A correlates with increased levels of P-T200. Particularly noteworthy is the large effect of t-Ag on samples from unstimulated cells. The transient peak of P-T200 observed when t-Ag is present likely results from that fraction of CaMKIV that has remained associated with and negatively regulated by PP2A. Thus, disruption of the CaMKIV/PP2A interaction provided by two different approaches, CaMKIV mutagenesis or coexpression of t-Ag, results in the elevated phosphorylation of CaMKIV on T200. These data are consistent with a model in which the PP2A in complex with CaMKIV plays an important role in dephosphorylating T200.

The Enhanced Transcriptional Activity of CaMKIV Observed When Interaction with PP2A Is Prevented Is T200-dependent—The results shown in Figs. 5 and 6, suggested to us that the enhanced CaMKIV-mediated transcriptional activation ob-

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**Fig. 4.** A, the CaMKIV activation loop phospho-T200 antibody recognizes CaMKIV from cells stimulated with ionomycin and does not recognize the CaMKIV T200A mutant. 293A cells transfected with FLAG-CaMKIV WT or T200A mutant were either untreated or stimulated for 5, 15, and 30 min with 2 μM ionomycin. Cell extracts or FLAG-CaMKIV immunoprecipitates were then probed for P-T200 by immunoblotting using CaMKIV P-T200 antibody (upper panels). The Coomassie-stained membranes are shown in the lower panels. Shown is one representative experiment (n = 4). B, phosphorylation of CaMKIV on T200 correlates with autonomous kinase activity. Aliquots of immunoprecipitated FLAG-CaMKIV shown in A were assayed in vitro for kinase activity using GS-10 peptide as substrate in the presence of CaM and either EGTA (B) or Ca²⁺ (C). Shown are averages ± S.D. values from one representative experiment (n = 4).
served when interaction of the kinase with PP2A has been prevented (Fig. 3), is a consequence of the increased P-T200 that accumulates in this situation. To verify that the enhanced transcriptional activation exhibited by CaMKIV Subst 318–329 was T-200-dependent, we generated a new mutant, CaMKIV Subst 318–329 T200A. As shown in Fig. 7A, CaMKIV Subst 318–329 T200A expresses well in 293A cells and, like CaMKIV Subst 318–329, interacts poorly with PP2A. However, CaMKIV Subst 318–329 T200A showed no enhanced ability to stimulate CREB-mediated transcription, compared with CaMKIV WT (Fig. 7B). The fact that CaMKIV Subst 318–329 T200A stimulated transcription at all was quite surprising to us, because CaMKIV was previously believed to require T200 phosphorylation to catalyze this function. Nevertheless, we conclude that the increased cellular activity of CaMKIV, which occurs when interaction with PP2A is prevented, is likely a consequence of elevated P-T200.

**DISCUSSION**

Here we report the novel observation that the interaction of CaMKIV with PP2A is regulated by the autoregulatory domain of the kinase and that binding of PP2A and Ca
2+/CaM to CaMKIV appears to be mutually exclusive. The finding that the residues of CaMKIV critical for the interaction of the kinase with PP2A map to the autoregulatory domain of the kinase was unexpected, because this relatively small region of CaMKIV has other critical functions. Studies with prototypical CaM kinase family members have established that these enzymes contain an autoinhibitory domain that blocks the catalytic domain and inactivates the kinase (39). Binding of Ca
2+/CaM to a region overlapping the autoinhibitory domain removes the autoinhibitory sequence from the catalytic core, exposing the active site for binding and phosphorylation of protein substrate. Based on sequence homology, the autoinhibitory and overlapping CaM binding regions of CaMKIV, collectively referred to as the autoregulatory domain, are presumed to span residues 300–340. In support of this assumption, residues 329HMDT312 and 320FN321 of CaMKIV have been experimentally determined to have a role in autoinhibition of the kinase (38). The region of CaMKIV that we identified by mutagenesis to be critical for PP2A binding corresponds precisely to the autoregulatory domain of the kinase (residues 308–340, Fig. 1).

CaMKIV has been shown to mediate the CREB-dependent signaling responsible for inhibition of cell proliferation during mitochondrial dysfunction (37). In this study, the authors observed that the increased intracellular Ca
2+ concentration that accompanies mitochondrial dysfunction results in disruption of the protein-protein interaction between CaMKIV and PP2A and suggested this as a mechanism that might lead to increased CaMKIV enzyme activity. Our finding that Ca
2+/CaM inhibits formation of CaMKIV/PP2A complexes in cell extracts and displaces PP2A from isolated CaMKIV/PP2A complexes (Fig. 2) may explain this earlier observation. It is possible that Ca
2+/CaM produces these effects by competing with PP2A for binding to the same site within the autoregulatory domain of the kinase. However, because all identified mutations of CaMKIV that prevent PP2A binding exhibit at least partial autonomous activity, we cannot distinguish with certainty between the possibilities that PP2A binds directly to the autoregulatory domain of CaMKIV or to some other part of the autoinhibited conformation of the kinase. The MAPKs have recently been shown to have a common docking site to which either the upstream MAPKK or the inactivating phosphatase can directly bind (40). Thus, there is precedence for activators and inactivating phosphatases to share the same binding site on their target kinase. At any rate we find that the autoregulatory domain of CaMKIV plays a critical role in regulating PP2A binding to CaMKIV and have thus defined a novel function for the autoregulatory domain of CaMKIV. Furthermore, our data raise the possibility that the CaMKIV/PP2A interac-

**Fig. 5.** A, CaMKIV Subst 318–329 exhibits elevated levels of P-T200. 293A cells transfected with FLAG-CaMKIV WT or FLAG-CaMKIV Subst 318–329 were either untreated or stimulated with 2 µM ionomycin for 5, 15, and 30 min. The kinase was then immunoprecipitated from the cells and probed for P-T200 by immunoblotting (upper panels). Coomassie-stained membranes are shown in the lower panels. Shown is one representative experiment (n = 4). B, FLAG-CaMKIV Subst 318–329 exhibits increased autonomous kinase activity compared with FLAG-CaMKIV WT. Aliquots of immunoprecipitated FLAG-CaMKIV shown in A were assayed in vitro for kinase activity using GS-10 peptide substrate in the presence of CaM and EGTA. Shown are averages ± S.D. from one representative experiment (n = 4).
tion may be a dynamic one regulated by changes in the intracellular Ca\(^{2+}\) concentration and/or by the active state of the kinase.

We show that when the interaction between CaMKIV and PP2A is prevented in the cell by mutagenesis of CaMKIV, there is dramatically increased CaMKIV-mediated gene transcription. This result is consistent with a model in which PP2A negatively regulates CaMKIV enzymatic activity, an idea originally proposed by Westphal et al. (24). One way that PP2A might negatively regulate CaMKIV is by dephosphorylating T200. This implies that, once phosphorylated on T200, CaMKIV is subsequently dephosphorylated as a means for returning the kinase to the inactivated state. The P-T200-specific antibody used in this study has allowed us to track the phosphorylation of CaMKIV on T200 in stimulated cells, and in so doing we have confirmed this idea. Thus, using mutagenesis of CaMKIV and coexpression of tAg, we have shown that the CaMKIV-associated PP2A negatively regulates CaMKIV by dephosphorylating the kinase on T200 and failure to dephosphorylate T200 leads to enhanced CaMKIV cellular activity.

We found it interesting that disruption of the CaMKIV-PP2A interaction resulted in P-T200 levels that were not only higher than normal in stimulated cells, but were also quite high in unstimulated cells. In contrast, CaMKIV from resting cells normally exhibits undetectable or very low levels of P-T200. This suggests that, in the absence of an applied stimulus to increase intracellular Ca\(^{2+}\), the resting Ca\(^{2+}\) concentration, or perhaps spontaneously occurring Ca\(^{2+}\) transients, is sufficient to result in the phosphorylation and activation of CaMKIV. When able to interact with PP2A, the P-T200 levels of CaMKIV are maintained at low levels. Disruption of the interaction leads to P-T200 accumulation, resulting in higher levels of activated kinase. Thus PP2A plays a critical role in regulating CaMKIV in resting cells. This may explain why the PP2A binding mutant, CaMKIV Subst 318–329, can stimulate CREB-mediated transcription in the absence of applied stimuli that increase intracellular Ca\(^{2+}\).

An unexpected issue arose from characterization of the CaMKIV Subst 318–329 T200A mutant. This mutant was designed to confirm that the enhanced cellular activity of CaMKIV Subst 318–329 was a consequence of the elevated P-T200 exhibited by this mutant. However, although the mutation of T200 to Ala, in the context of CaMKIV Subst 318–329, resulted in an enzyme with greatly reduced ability to stimulate transcription, the fact that the kinase could stimulate transcription at all in the absence of T200 was unexpected. This is
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is required for interaction of PP2A with the kinase. Functionally, the CaMKIV-associated PP2A dephosphorylates T200, which results in loss of autonomous kinase activity and correlates with termination of CREB-dependent transcription. Our data indicate that PP2A influences the activity of CaMKIV in both resting and stimulated cells, suggesting that the reciprocal interaction of Ca²⁺/CaM and PP2A with CaMKIV may be highly dynamic and potentially relevant to maintenance of cellular functions that are controlled by regulation of Ca²⁺ homeostasis.

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