Ca\(^{2+}\)/calmodulin-dependent protein kinase IV (CaM-KIV) is phosphorylated at Thr\(^{196}\) by Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaM-KK), resulting in induction of both autonomous activity and a high level of Ca\(^{2+}\)/CaM-dependent activity. We have shown that the kinetics of Thr\(^{196}\) phosphorylation of CaM-KIV by CaM-KK is well correlated with the generation of its autonomous activity, although Thr\(^{177}\) phosphorylation of CaM-KI does not induce its autonomous activity. The activities of CaM-KI chimera mutants fused with C-terminal regions (residues 296–469 and 296–350) of CaM-KIV are completely dependent on Ca\(^{2+}\)/CaM, which is also the case for CaM-KI. Unlike wild-type CaM-KI, however, phosphorylation of Thr\(^{177}\) in the chimera mutants by CaM-KK resulted in generation of significant autonomous activities, indicating that the phosphorylation of Thr in the activation loop is sufficient to partially release the autoinhibitory region of CaM-KIV from the catalytic core. Indeed, the CaM-KIV peptide (residues 304–325) containing minimum autoinhibitory sequences (residues 314–321) suppressed the activity of non-phosphorylated CaM-KIV with an IC\(_{50}\) of \(\sim 50\) \(\mu\)M, and this suppression was competitive with respect to the peptide substrate; however, the CaM-KIV peptide was not capable of inhibiting Thr\(^{196}\)-phosphorylated CaM-KIV. Taken together, these results indicated that the Thr\(^{196}\) phosphorylation of CaM-KIV by CaM-KK reduced the interaction of the catalytic core with the autoinhibitory region, resulting in generation of the autonomous activity.

Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaM-Ks)\(^{1}\) constitute a diverse group of enzymes, which are involved in many cellular responses mediated by an increase in the concentration of intracellular calcium (1–3). CaM-KIV, also known as CaM-kinase Gr, is one of the multifunctional CaM-Ks, and is predominantly localized in cell nuclei (4–6). Consistent with the nuclear localization of CaM-KIV, an important role has been demonstrated for CaM-KIV in the regulation of Ca\(^{2+}\)/CaM-dependent activity. We have shown that the induction of the total activity of CaM-KIV by CaM-KK phosphorylation was because of mainly Ca\(^{2+}\)/CaM-independent activity (autonomous activity) is generated by activation with CaM-KK. This important regulatory mechanism would allow a transient elevation in intracellular Ca\(^{2+}\) levels to produce a prolonged CaM-KIV activation to regulate gene transcription via the phosphorylation of transcription factor(s). Autonomous activity of CaM-KIV has been observed with purified enzyme from rat brain (20), immunoprecipitated enzyme from anti-TCR/CD3 monoclonal antibody-stimulated Jurkat cells (21, 22), and ionomycin-treated, transfected COS-7 and HeLa cells (23, 24). A recent study suggested that the autonomous activity appears to be required for CaM-KIV to regulate CREB-mediated transcription, indicating that it might be physiologically relevant (25).

We have shown that the induction of the total activity of CaM-KIV by CaM-KK phosphorylation was because of mainly decreasing \(K_{m}\) for its substrate (17). However, this does not account for generation of the autonomous activity. At a minimum, Thr\(^{196}\) phosphorylation by CaM-KK is required for generation of Ca\(^{2+}\)/CaM-independent activity of CaM-KIV, because a mutation of Thr\(^{196}\) by Ala has been shown to abolish the generation of autonomous activity (19, 23). Therefore, it has not been resolved whether the Ca\(^{2+}\)/CaM-independent activity is a direct consequence of Thr\(^{196}\) phosphorylation or whether it is the result of subsequent autophosphorylation.

In contrast, it has been well characterized that CaM-KII, another member of multifunctional CaM-Ks, is converted to the Ca\(^{2+}\)/CaM-independent form by autophosphorylation at Thr\(^{286}\) in its autoinhibitory region, which suppresses the autoinhibitory function of the enzyme (26–31). Thr\(^{286}\) autophosphorylation has been shown to be sensitive to the duration, magnitude, and frequency of the imposed calcium transient (32, 33) and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^{TM}\)/EBI Data Bank with accession number(s) L42810.

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Autonomous Activity of CaM-KIV

also to be important in the regulation of synaptic plasticity and behavior in vitro (34). Although there is an equivalent Thr (Thr196) in the putative autoinhibitory region of CaM-KIV, mutation of Thr196 to Ala did not impair the generation of autonomous activity, indicating that the Thr196 is not involved in the generation of autonomous activity (23, 35). In addition, multiple autophosphorylation sites have been identified in either the N-terminal region of the catalytic domain or the C-terminal region of CaM-KIV (36–38), but none of them has been shown to be involved in its autonomous activity (35). Thus the precise mechanism of the autonomous activity of CaM-KIV generated by CaM-KK phosphorylation remains uncertain.

In this report, we have examined the mechanism of generation of the autonomous activity of CaM-KIV by CaM-KK phosphorylation using Escherichia coli-expressed recombinant enzymes, including CaM-KI/CaM-KIV chimeras and the autoinhibitory peptide of CaM-KII, and demonstrated that Thr196 phosphorylation reduced the affinity of its autoinhibitory region to the catalytic core and is likely sufficient to generate the autonomous activity of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—CaM-KIV cDNA (GenBank™ accession number L42810) was obtained from a rat brain cDNA library (18). Recombinant CaM-KK was expressed in E. coli and purified as described previously (39). Recombinant rat CaM was expressed in the E. coli strain BL21(DE3) using pET-CaM (kindly provided by Dr. Nobuhiro Hayashi, Fujita Health University, Toyoake, Japan) and purified by phenyl-Sepharose column chromatography (40). Anti-CaM-KV antibody and anti-CaM-KI antibody were obtained from Signal Transduction Laboratories (St. Louis, MO) and Santa Cruz Biotechnology, Inc., respectively. Anti-phospho-CaM-KIV at Thr196 and anti-phospho-CaM-KI at Thr177 monoclonal antibodies were obtained from Dako (Glostrup, Denmark) and Santa Cruz Biotechnology, Inc., respectively. Anti-phospho-CaM-KK antibody were obtained from Signal Transduction Laboratories (St. Louis, MO), and purified by phenyl-Sepharose column chromatography as described in the manufacturer’s protocol.

In Vitro Activation of CaM-KI and CaM-KIV by CaM-KK—Purified recombinant CaM-KK (0.1 mg/ml) were incubated with CaM-KKα (3 μg/ml) at 30 °C for the indicated periods in a solution containing 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM DTT, 2 mM CaCl2, 10 μM CaM, and 200 μM ATP. The reaction was initiated by addition of ATP and terminated by 10–20-fold dilution with ice-cold 50 mM HEPES (pH 7.5), 2 mM MgCl2, bovine serum albumin, 1% ethylene glycol, and 2 mM EDTA. Five μl of the diluted sample was then subjected to the protein kinase assay or Western blot analysis.

In Vitro Assay for CaM-KI and CaM-KIV Activity—CaM-KI and CaM-KIV were assayed at 30 °C for 5–10 min in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 40 μM syntide-2, 1 mM DTT, and 200 μM [γ-32P]ATP (1000 cpm/pmol) in the presence of either 1 mM EGTA (autonomous activity) or 1 mM CaCl2, 4 μg CaM. The reaction was initiated by the addition of the enzyme and terminated by spotting aliquots (15 μl) onto phosphocellulose paper (Whatman P-81) followed by several washes with 75 mM phosphoric acid (42). Phosphate incorporation into syntide-2 was determined by liquid scintillation counting of the filters.

RESULTS

Activation and Phosphorylation of CaM-KIV and CaM-KI by CaM-KK—It has been shown that CaM-KIV is phosphorylated at Thr196 by CaM-KK in induction of a high level of Ca2+/CaM-dependent activity as well as generation of Ca2+/CaM-independent activity (17–19). Because CaM-KIV undergoes autophosphorylation of multiple residues subsequent to activation (36–38), the relationship between the kinase activity and Thr196 phosphorylation has not been precisely determined in vitro. Therefore, we have attempted to perform time course experiments on CaM-KIV activation and phosphorylation of Thr196 using anti-phospho-Thr196 monoclonal antibody (Fig. 1, A and B). We have expressed GST-fused enzymes and then GST was cleaved by PreScission protease treatment followed by removing GST and GST-fused protease to avoid the effect of GST on the enzyme activities and regulation. Thus the recombinant enzymes used in this study contain five residues (Gly-Pro-Ile-Leu-Glu) fused with the first Met. As shown in Fig. 1A, both Ca2+/CaM-dependent and -independent activities (−30% of total activity) of CaM-KIV were induced by CaM-KK treatment in the presence of Ca2+/CaM and Mg-ATP and were saturated for 20 min. This activation kinetics was well correlated with Thr196 phosphorylation under this condition (t50 = ~5 min, Fig. 1B). It is noteworthy that we did not observe a significant time lag between generation of the autonomous activity and phosphorylation of Thr196. These characteristic features of the recombinant CaM-KIV used in this study,
including generation of autonomous activity by activation and kinetic parameters of the enzyme ($K_m$ for ATP = 27 $\mu$M and $K_m$ for syntide-2 = 264 $\mu$M, Fig. 5C), were similar to the previous results obtained using enzymes expressed in Sf9 cells (16, 17, 19). We also performed similar experiments using CaM-KI and the same concentration (3 $\mu$g/ml) of CaM-KK as used for CaM-KIV activation (Fig. 1C). Protein kinase activity assay and Western blot analysis using anti-phospho-Thr$^{177}$ monoclonal antibody revealed that, in comparison to CaM-KI/CaM-KIV, CaM-KI was more rapidly activated and phosphorylated at Thr$^{177}$ by CaM-KK ($t_{50} = <1$ min) than CaM-KIV. However, significant autonomous activity of CaM-KI was not observed during the activation. These results indicated that the autoinhibitory mechanisms might differ between CaM-KIV and CaM-KI, at least for the phosphorylated form of enzymes, which in turn might affect the generation of the autonomous activity of CaM-KIV.

**Characterization of CaM-KI/CaM-KIV Chimera Mutants**—To examine the role of the autoinhibitory mechanism of CaM-KIV in the generation of autonomous activity, we have constructed, expressed, and purified chimera mutants in which the CaM-KI catalytic domain was fused with various C-terminal portions of CaM-KIV (residues 296–469 and residues 296–350; Fig. 2, A and B, upper panel), because it has been shown that CaM-KI does not undergo autoprophosphorylation in the catalytic domain subsequent to the activation (44) and also does not generate autonomous activity in association with Thr$^{177}$ phosphorylation (Fig. 1C). CaM-overlay analysis revealed that the chimera mutants possess the same degree of functional Ca$^{2+}$/CaM binding ability as the wild-type CaM-KI (Fig. 2B, lower panel). Without activation by CaM-KK, CaM-KI/CaM-KIV469 and CaM-KI/CaM-KIV350 were inactive in the absence of Ca$^{2+}$/CaM and exhibited kinase activity only in the presence of Ca$^{2+}$/CaM, which results are indistinguishable from those for the wild-type CaM-KI (Fig. 2C). This indicates that the autoinhibitory region in the C-terminal of CaM-KIV (within residues 296–350) functionally suppresses the catalytic activity of CaM-KI, which is conformationally neutralized by Ca$^{2+}$/CaM binding. When we treated these CaM-KI chimera mutants with CaM-KK in the presence of Ca$^{2+}$/CaM and Mg-ATP for 5 min as described in Fig. 1C, both mutants including wild-type CaM-KI were phosphorylated at Thr$^{177}$ (Fig. 2D, inset). However, unlike wild-type CaM-KI, which does not generate significant autonomous activity (Figs. 1C and 2D), CaM-KI/CaM-KIV469 and CaM-KI/CaM-KIV350 showed a high level of autonomous activity after their activation. This result strongly supports the idea that the autoinhibitory mechanism of CaM-KIV was partially disrupted by phosphorylation of the activation loop Thr residue, thereby resulting in generation of autonomous activity after their activation. These results strongly support the idea that the autoinhibitory mechanism of CaM-KIV was partially disrupted by phosphorylation of the activation loop Thr residue, thereby resulting in generation of autonomous activity, because it has been shown that CaM-KI is phosphorylated at Thr$^{177}$ by CaM-KK, but no other autophosphorylation in the catalytic domain has been observed (44).

**Mapping of the Autoinhibitory Sequence in CaM-KIV**—To clarify the involvement of the autoinhibitory function in generation of the autonomous activity of CaM-KIV, we attempted to precisely map the autoinhibitory sequence in CaM-KIV. Previous studies have demonstrated that the truncation at Leu$^{313}$ (16) and a block mutation (Phe$^{316}$–Asn$^{317}$ to Asp–Asp) are converted to the Ca$^{2+}$/CaM-independent form of CaM-KIV (17), suggesting that the C-terminal region after Leu$^{313}$ contains the autoinhibitory sequence of CaM-KIV. Because the precise location of the autoinhibitory sequence has not been determined, we expressed and purified a series of C-terminal truncation mutants to map a minimum autoinhibitory sequence (Fig. 3A, B, and C, insert upper panel). As shown in Fig. 3B, measurement of the protein kinase activities of these mutants in either the absence or presence of Ca$^{2+}$/CaM revealed that truncation at Gly$^{336}$ did not alter either the Ca$^{2+}$/CaM dependence of the activity or the activity itself. The truncation mutants at Ala$^{331}$, Lys$^{327}$, and Lys$^{321}$ are completely inactive in either the absence or presence of Ca$^{2+}$/CaM, indicating that these mutants contain a functional autoinhibitory sequence. Semiquantitative analysis of the Ca$^{2+}$/CaM binding of these mutants using the CaM overlay method in the presence of Ca$^{2+}$ (Fig. 3B, insert lower panel) showed that the truncation mutant at Ala$^{331}$ fails to bind Ca$^{2+}$/CaM as well as other truncation mutants (1–327, 1–321, 1–313, and 1–309), suggesting that the residues between Ser$^{323}$ and Gly$^{336}$ are the C-terminal end of the Ca$^{2+}$/CaM binding sequence. Thus the 1–331, 1–327, and 1–321 mutants were not activated by Ca$^{2+}$/CaM. Further trun-
CaM-KI/KIV350 mutants (1 wild-type CaM-KI, CaM-KI/KIV469, and panel) or CaM-overlay analysis (panel). Massie Brilliant Blue staining (upper to 10% SDS-PAGE followed by either Coomassie Brilliant Blue staining (upper panel) or CaM-overlay analysis (lower panel). C, protein kinase activities of the wild-type CaM-KI, CaM-KI/KIV469, and CaM-KI/KIV350 mutants (1 μg/ml) were measured at 30 °C for 10 min in the presence of either 1 mM CaCl₂, 4 μM CaM (closed column) or 1 mM EGTA (open column) as described under “Experimental Procedures.” Results represent the mean ± S.E. of three experiments. D, wild-type CaM-KI and CaM-KI/KIV chimeric mutants were incubated with CaM-KK at 30 °C for 5 min as described for panel C in Fig. 1. After terminating the reaction, the samples were subjected to either Western blotting analysis using anti-phospho-Thr177 antibody (inset) or protein kinase assay at 30 °C for 5 min in the presence of 1 mM EGTA as described under “Experimental Procedures.” Results represent the mean ± S.E. of three experiments. Inset: lane a, wild-type CaM-KI; lane b, CaM-KI/KIV469; lane c, CaM-KI/KIV350.

fig. 2. Characterization of CaM-KI/KIV chimera mutants. A, a schematic representation of the wild-type CaM-KI and CaM-KI/KIV chimera mutants. Numbers without and within parentheses indicate the residue numbers of CaM-KI in CaM-KIV, respectively. B, purified recombinant wild-type CaM-KI (lane a), CaM-KI/KIV469 (lane b), and CaM-KI/KIV350 mutants (lane c) were subjected to 10% SDS-PAGE followed by either Coomassie Brilliant Blue staining (upper panel) or CaM-overlay analysis (lower panel). C, protein kinase activities of the wild-type CaM-KI, CaM-KI/KIV469, and CaM-KI/KIV350 mutants (1 μg/ml) were measured at 30 °C for 10 min in the presence of either 1 mM CaCl₂, 4 μM CaM (closed column) or 1 mM EGTA (open column) as described under “Experimental Procedures.” Results represent the mean ± S.E. of three experiments. D, wild-type CaM-KI and CaM-KI/KIV chimeric mutants were incubated with CaM-KK at 30 °C for 5 min as described for panel C in Fig. 1. After terminating the reaction, the samples were subjected to either Western blotting analysis using anti-phospho-Thr177 antibody (inset) or protein kinase assay at 30 °C for 5 min in the presence of 1 mM EGTA as described under “Experimental Procedures.” Results represent the mean ± S.E. of three experiments. Inset: lane a, wild-type CaM-KI; lane b, CaM-KI/KIV469; lane c, CaM-KI/KIV350.

Inhibition of CaM-KIV Activity by CaM-KIV Autoinhibitory Peptide—Based on the mapping of the regulatory domain in CaM-KIV (Fig. 3), we were able to synthesize the CaM-KIV peptide (residues 304–325) containing the minimum autoinhibitory sequence (residues 314–321) to examine its inhibition of unphosphorylated and Thr196-phosphorylated CaM-KIV (Fig. 5A, upper and middle panels). We also confirmed by CaM overlay analysis that the Ca²⁺/CaM bindings of two enzymes were indistinguishable (Fig. 5A, lower panel). In the presence of Ca²⁺/CaM, the CaM-KIV peptide inhibited the total activity of unphosphorylated CaM-KIV with an IC₅₀ value of ~50 μM (Fig. 5B), which is ~3-fold less potent than the inhibition by the CaM-KI autoinhibitory peptide (residues 281–302, T286A) (17). Furthermore, inhibition of CaM-KIV by the CaM-KIV peptide (residues 304–325) was not competitive with ATP but was competitive with the peptide substrate syntide-2 (Fig. 5C). This kinetic profile of enzyme inhibition was similar to that observed for the inhibition of CaM-KIV by the CaM-KII autoinhibitory peptide (residues 281–302, T286A) (17). When we assayed the same concentration (2 μg/ml) of phosphorylated CaM-KIV by CaM-KK for 30 min (Fig. 1A), the CaM-KIV peptide at a concentration of up to 250 μM was unable to suppress the activity of activated CaM-KIV (Fig. 5B). We performed the same kinase assay as shown in Fig. 5B in the absence of the peptide substrate (syntide-2), but significant phosphate incorporation into the CaM-KIV peptide was not detected (data not shown). We also confirmed by mass spectrometry analysis that the autoinhibitory peptide was not phosphorylated at Thr196 by activated CaM-KIV (data not shown). This result indicated that the interaction between the catalytic core of CaM-KIV and the autoinhibitory peptide was abolished by Thr196 phosphorylation, resulting in a loss of the inhibitory potency of the peptide.
The enzymatic activities of Ca\(^{2+}\)/CaM-dependent protein kinases are thought to be strictly autoinhibited by intrasteric interaction between their own autoinhibitory segment and the catalytic core when the intracellular Ca\(^{2+}\) concentration is low enough to dissociate from CaM. Once the concentration rises upon stimulation of the cells, the Ca\(^{2+}\)/CaM complex binds to the Ca\(^{2+}\)/CaM binding segment of the kinases, which either overlaps or is adjacent to the autoinhibitory segment. Then Ca\(^{2+}\)/CaM binding conformationally neutralize the autoinhibitory function, resulting in the generation of protein kinase activity. Two members of the CaM-K family, CaM-KII and CaM-KIV, generate Ca\(^{2+}\)/CaM-independent activity in association with autophosphorylation at Thr\(^{286}\) and trans-phosphorylation at Thr\(^{196}\) by an upstream kinase (CaM-KK), respectively.

Here we have shown that the phosphorylation of Thr\(^{196}\) in the activation loop by CaM-KK was likely sufficient to generate the autonomous activity of CaM-KIV because of: 1) the in vitro kinetics of the activation and Thr\(^{196}\) phosphorylation of CaM-KIV were well correlated; 2) CaM-KI chimera mutants containing the regulatory domain of CaM-KIV generated autonomous activities that were associated with Thr\(^{177}\) phosphorylation by CaM-KK, whereas the CaM-KI wild-type did not exhibit Ca\(^{2+}\)/CaM-independent activity by Thr\(^{177}\) phosphorylation and also did not undergo autophosphorylation in the catalytic domain subsequent to the activation (44); 3) as shown in the experiment with a truncation mutant (CaM-KIV-(1–336)), the C-terminal of CaM-KIV (residues 337–469) is not directly involved in generation of autonomous activity; and 4) CaM-KIV autoinhibitory peptide (residues 304–325) failed to inhibit activated CaM-KIV without phosphorylation by CaM-KIV. Although multiple autophosphorylation sites in the N-terminal region of the catalytic domain of CaM-KIV have been identified (36–38), a previous study has shown that the deletion of six N-terminal Ser residues (Ser\(^{8}\), Ser\(^{10}\), Ser\(^{12}\), Ser\(^{13}\), Ser\(^{15}\), and Ser\(^{16}\) in the human enzyme) did not impair the generation of autonomous activity subsequent to the activation by CaM-KK (35). Collectively, these results suggest that the Thr\(^{196}\) phosphorylation is most likely sufficient to generate the autonomous activity of CaM-KIV. However, although it is unlikely, we cannot completely exclude the possibility that the autophosphorylation at unidentified residue(s) subsequent to phosphorylation at Thr\(^{196}\) by CaM-KK may be involved in the generation of autonomous activity.

**DISCUSSION**

**FIG. 3. Mapping of the autoinhibitory sequence of CaM-KIV.** A, schematic representation of CaM-KIV truncation mutants. Arrows indicate the residues of truncation. Underlined portions indicate the sequence of the synthetic peptide used in Fig. 5. B, protein kinase activities of the purified CaM-KIV truncation mutants, including the wild-type enzyme (10 \(\mu\)g/ml), were measured at 30 °C for 10 min in the presence of either 1 mM Ca\(_{\text{Cl}_2}\), 4 \(\mu\)M CaM (closed column) or 1 mM EGTA (open column) as described under "Experimental Procedures." Results represent the mean ± S.E. of three experiments. Recombinant enzymes were subjected to SDS-PAGE followed by either Coomassie Brilliant Blue staining (insert upper panel) or CaM overlay analysis (insert lower panel).
Interestingly, CaM-KI is also phosphorylated on Thr\(^{177}\) in the activation loop by CaM-KK, resulting in a large increase in Ca\(^{2+}\)/CaM-dependent activity without generation of autonomous activity (45, 46). This result, together with our present data, suggests that the autoinhibitory mechanisms of CaM-KI and CaM-KIV are distinct, which is supported by the fact that the CaM-KI chimera mutants fused with the regulatory domain of CaM-KIV, generating Ca\(^{2+}\)/CaM-independent activity by Thr\(^{177}\) phosphorylation, whereas the CaM-KIV autoinhibitory domain completely suppressed the catalytic activity of unphosphorylated chimera mutants in the absence of Ca\(^{2+}\)/CaM. Therefore, the autoinhibitory segment of CaM-KIV no longer tightly binds to the Thr\(^{177}\)-phosphorylated CaM-KI catalytic core in the absence of Ca\(^{2+}\)/CaM, but that of CaM-KI does. Consistent with these findings, we directly demonstrated that the inhibitory function of the autoinhibitory peptide of CaM-KIV (residues 304–325), by preventing the substrate binding, was completely lost toward phosphorylated CaM-KIV on Thr\(^{196}\).

This observation clearly indicates that the autonomous activity of CaM-KIV is generated by a reduction of the interaction between the catalytic core and the autoinhibitory region. Notably, the Ca\(^{2+}\)/CaM-independent activity of activated CaM-KIV accounts for \(~30\)% of the total activity suggesting that the autoinhibitory region is partially but not fully released from the catalytic core. This may indicate that the intramolecular interaction of the catalytic core with the autoinhibitory region is not completely destroyed by Thr\(^{196}\) phosphorylation, as distinct from our results with the intermolecular inhibition by the autoinhibitory peptide. The crystal structure of the autoinhibited form of CaM-KI revealed that the regulatory segment of CaM-KI (residues 286–316) interacted with the catalytic core at some distance from Thr\(^{177}\) (47).

This may account for the finding that the autoinhibitory mechanism of CaM-KI appeared not to be affected by Thr\(^{177}\) phosphorylation. We could therefore speculate that the autoinhibitory domain of CaM-KIV might be located closer to the activation loop in its autoinhibited form of unphosphorylated kinase to prevent the substrate binding. Subsequent to phosphorylation by CaM-KK, the interaction of the autoinhibitory segment with the catalytic core of CaM-KIV could be reduced by either Thr\(^{196}\) phosphorylation directly or by conformational changes of the catalytic domain mediated by Thr\(^{196}\) phosphorylation in the absence of Ca\(^{2+}\)/CaM, resulting in the partial release of the autoinhibitory domain from the catalytic core. In contrast to the findings for CaM-KIV, it has been extensively characterized that direct autophosphorylation of the autoinhibitory region at Thr\(^{286}\) in CaM-KII suppresses the autoinhibitory function that is necessary to generate CaM-KII autonomous activity (26–31). Therefore, closely related multifunctional CaM-Ks appear to show different modes of regulating the autoinhibitory mechanism in association with the activation. Because the three-dimensional structure of CaM-KIV has not been determined, future structural studies will be needed to clarify the mechanism of the enzymatic regulation of CaM-KIV in greater detail, and to reveal the diverse range of autoinhibitory mechanisms among the CaM-K family.

![Fig. 4. Generation of autonomous activity of the CaM-KIV-(1–336) mutant by CaM-KK treatment. Recombinant CaM-KIV-(1–336) mutant was incubated with CaM-KK for 0–30 min at 30 °C in a solution containing 50 mM HEPES (pH 7.5), 10 mM Mg\(_{2+}\)Ac\(_2\), 1 mM DTT, and 200 \(\mu\)M ATP in the presence of 2 mM CaCl\(_2\), 10 \(\mu\)M CaM. After terminating the reaction, autonomous activity was measured at 30 °C for 5 min in the presence of 1 mM EGTA as described under “Experimental Procedures.” Results represent duplicate experiments. The same samples after terminating the activation reaction (40 ng of CaM-KIV) were subjected to Western blotting analysis using either anti-CaM-KIV antibody (upper inset) or anti-phospho-Thr\(^{196}\) antibody (lower inset).](http://www.jbc.org/Downloaded_from)

![Fig. 5. Inhibition of CaM-KIV activity by CaM-KIV autoinhibitory peptide (residues 304–325). A, wild-type CaM-KIV was either treated with CaM-KK at 30 °C for 30 min as described in Fig. 1 A (+) or left untreated (−), and then the enzymes (40 ng) were subjected to Western blotting analysis using anti-CaM-KIV antibody (upper panel), anti-phospho-Thr\(^{196}\) antibody (middle panel), or CaM overlay analysis in the presence of Ca\(^{2+}\) (lower panel). B, protein kinase activities of either the CaM-KK-treated (closed circle, 2 \(\mu\)g/ml) or untreated CaM-KIV wild-type (open circle, 2 \(\mu\)g/ml) were measured with various concentrations of the synthetic peptide corresponding to residues 304–325 of CaM-KIV (0–250 \(\mu\)M) in the presence of 1 mM CaCl\(_2\), 4 \(\mu\)M CaM at 30 °C for 5 min (closed circle) or 10 min (open circle). Activities are expressed as a percentage of the value in the absence of the peptide. Results represent the duplicate experiments. C, kinetic analysis of CaM-KIV inhibition. CaM-KIV was assayed in either the presence (closed circle) or absence (open circle) of 100 \(\mu\)M CaM-KIV peptide (residues 304–325) as described in panel B except for concentrations of substrates. For titration of syntide-2 (left panel), 200 \(\mu\)M \([\gamma^{32}P]ATP and 50–400 \(\mu\)M syntide-2 were used. For titration of ATP (right panel), 400 \(\mu\)M syntide-2 and 25–200 \(\mu\)M \([\gamma^{32}P]ATP were used. The results were performed in duplicate for each point and are presented as double-reciprocal plots (Lineweaver-Burk).](http://www.jbc.org/Downloaded_from)
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