Plant Chimeric Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase

ROLE OF THE NEURAL VISININ-LIKE DOMAIN IN REGULATING AUTOPHOSPHORYLATION AND CALMODULIN AFFINITY

Received for publication, January 28, 2000, and in revised form, June 1, 2000
Published, JBC Papers in Press, June 5, 2000, DOI 10.1074/jbc.M000771200

P. V. Sathyanarayanan‡, Christine R. Cremo§, and B. W. Poovaiah¶

From the ‡Laboratory of Plant Molecular Biology and Physiology, Department of Horticulture, Washington State University, Pullman, Washington 99164-6414 and the §Department of Biochemistry, School of Medicine and School of Agriculture, University of Nevada, Reno, Nevada 89557

Chimeric Ca\(^{2+}\)/calmodulin-dependent protein kinase (CCaMK) is characterized by a serine-threonine kinase domain, an autoinhibitory domain, a calmodulin-binding domain and a neural visinin-like domain with three EF-hands. The neural visinin-like Ca\(^{2+}\)-binding domain at the C-terminal end of the CaM-binding domain makes CCaMK unique among all the known calmodulin-dependent kinases. Biological functions of the plant visinin-like proteins or visinin-like domains in plant proteins are not well known. Using EF-hand deletions in the visinin-like domain, we found that the visinin-like domain regulated Ca\(^{2+}\)-stimulated autophosphorylation of CCaMK. To investigate the effects of Ca\(^{2+}\)-stimulated autophosphorylation on the interaction with calmodulin, the equilibrium binding constants of CCaMK were measured by fluorescence emission anisotropy using dansylated calmodulin. Binding was 8-fold tighter after Ca\(^{2+}\)-stimulated autophosphorylation. This shift in affinity did not occur in CCaMK deletion mutants lacking Ca\(^{2+}\)-stimulated autophosphorylation. A variable calmodulin affinity regulated by Ca\(^{2+}\)-stimulated autophosphorylation mediated through the visinin-like domain is a new regulatory mechanism for CCaMK activation and calmodulin-dependent protein kinases. Our experiments demonstrate the existence of two functional molecular switches in a protein kinase regulating the kinase activity, namely a visinin-like domain acting as a Ca\(^{2+}\)-triggered switch and a CaM-binding domain acting as an autophosphorylation-triggered molecular switch.

Ca\(^{2+}/\text{CaM}\)\(^{3+}\)-dependent protein phosphorylation is believed to play a pivotal role in amplifying and diversifying Ca\(^{2+}/\text{CaM}\) mediated signals and has been implicated in regulating a broad range of physiological and developmental processes in animals (1). A number of different kinds of Ca\(^{2+}/\text{CaM}\)-dependent protein kinases have been cloned and characterized from animal systems (2). Understanding of CaM-regulated protein kinase systems as mediators in Ca\(^{2+}\)-signal transduction in plants is very limited. The predominant forms of Ca\(^{2+}\)-regulated protein kinases in plants are Ca\(^{2+}\)-dependent but CaM-independent protein kinases, and they are widely expressed and abundant in plants (3, 4). Three Ca\(^{2+}\)/CaM-dependent protein kinases sharing some features of mammalian multifunctional protein kinase (CaMK II) have been reported to exist in plants (5–7), but only chimeric Ca\(^{2+}\)/CaM-dependent protein kinase (CCaMK) isolated from lily (6) has the neural visinin-like Ca\(^{2+}\)-binding domain. CCaMK is highly tissue-specific and developmentally regulated (8). Biochemical characterization has revealed that it requires both Ca\(^{2+}\)/CaM for activation (9).

CCaMK has a serine-threonine kinase domain, a regulatory domain consisting of an autoinhibitory domain and a CaM-binding domain (highly similar to mammalian CaMK II), and a neural visinin-like Ca\(^{2+}\)-binding domain (6). Neural visinin-like Ca\(^{2+}\)-binding proteins are members of a family of Ca\(^{2+}\)-sensitive regulators, which include frequenin, neurocalcin, and hippocalcin and have not been reported to exist in plants (6). These proteins are activated at nanomolar concentrations of Ca\(^{2+}\) (10), and they are not reported to exist as chimeric proteins or to be associated with any CaM kinases in animal systems. The C-terminal visinin-like Ca\(^{2+}\)-binding domain of CCaMK makes this kinase unique among all other Ca\(^{2+}\)/CaM-dependent kinases known in animal and plant systems and raises the question of its role in regulating the kinase activity. Here we demonstrate that Ca\(^{2+}\)-stimulated autophosphorylation, mediated by the C-terminal neural visinin-like domain, significantly tightens the binding of CaM to CCaMK. This increase in affinity is autophosphorylation-dependent and also Ca\(^{2+}\)-dependent. We also show that EF-hand deletions in the visinin-like domain abolish autophosphorylation and increased affinity for CaM. The neural visinin-like domain of CCaMK could function as a Ca\(^{2+}\)-sensitive switch regulating autophosphorylation and CaM affinity. Our results demonstrate for the first time the autophosphorylation-dependent changes in affinity for CaM by a plant protein kinase.

EXPERIMENTAL PROCEDURES

Expression and Purification of CCaMK and Deletion Mutants—CCaMK cDNA from lily (Lilium longiflorum Thunb cv. Nellie White) and deletion mutants were cloned in pET3b vector (Novagen, Inc.) and expressed in Escherichia coli. These proteins were purified as described previously (9).

Expression and Purification of \(^{35}\)S-Labeled CaM—Potato calmodulin (PCMI cDNA cloned in expression vector pET3b was labeled using \(^{35}\)S)methionine (NEN Life Science Products) as described by Fromm and Chua (11). \(^{35}\)S-Labeled CaM was purified from bacteria using phenyl-Sepharose CL4B (Amersham Pharmacia Biotech) chromatography (11).

Autophosphorylation Assays—The autophosphorylation assay of...
CCaMK and deletion mutants were carried out for 10 min at 30 °C in the presence of 50 mM HEPES, pH 7.5, containing 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM [32P]ATP, and either 2.5 mM EGTA or 0.2 mM CaCl2 (9). The autophosphorylated CCaMK and mutants were analyzed on a 15% SDS-polyacrylamide gel electrophoresis, and the [32P]PO4 incorporation were measured by isolating the protein bands from the gels and counting them using a scintillation counter. The data were analyzed using the parameter SAP (stimulated autophosphorylation) index, which is the ratio of [32P]PO4 incorporation (autophosphorylation) in the presence of Ca2+ to the [32P]PO4 incorporation in the absence of Ca2+. The data were plotted using SIGMAPLOT software (Jandel Scientific).

**CaM-binding Assay**—CCaMK was incubated with or without autophosphorylation and spotted to nitrocellulose filters (Millipore). CaM binding was assayed by incubating these nitrocellulose filters containing CCaMK with 25-S-CaM (0.5 × 10⁶ cpm/μg) in binding buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1% (w/v) nonfat dry milk), plus either 1 mM Ca2+ or 5 mM EGTA as described previously (9).

**Preparation and Purification of Dansyl CaM (CaM-D)**—Dansyl CaM was prepared as described (12). Briefly purified CaM (PDM1) was incubated with an approximately 4-fold molar excess of dansyl chloride (Pierce) for 45 min at room temperature (12) (10 mg/ml CaM, 50 mM KCl, 20 mM HEPES, pH 9.0). Unreacted dansyl chloride was then separated from dansylated CaM by dialysis (Spectrapor 1, Spectrum Medical Industries) against dialysis buffer (50 mM Tris-Cl, pH 8.0, containing 0.2 mM NaCl and 0.1 mM EGTA). CaM species with different amounts of labeling were separated by fast protein liquid chromatography (Macro Prep t-Butyl HIC column, Bio-Rad) in an ammonium sulfate gradient (1.2 to 0 m; 100 mM sodium phosphate, pH 7.0, present in both buffers). Fractions were collected, and the concentration of CaM-D was estimated by absorbance and the Bradford method. Each fraction was tested for Ca2+ binding-induced changes in anisotropy (see below). The fractions that showed changes in anisotropy upon Ca2+ binding were designated as CaM-D and used for all studies. The CaM-D was used in substrate phosphorylation experiments using histone II AS substrate as described previously (9) and found to be capable of stimulating kinase activity.

**Anisotropy Measurements and Data Analysis**—All reagents used for fluorescence measurements were screened for fluorescence impurities, treated with CaM-Sepharose (Amersham Pharmacia Biotech) to deplete endogenous Ca2+, and filtered using a 0.2-μm filter. Autophosphorylated CCaMK for anisotropy measurements was prepared as described above (12). Briefly purified CaM (PCM1) was incubated with an approximately 4-fold molar excess of dansyl chloride (Pierce) for 45 min at room temperature (12) (10 mg/ml CaM, 50 mM KCl, 20 mM HEPES, pH 9.0). Unreacted dansyl chloride was then separated from dansylated CaM by dialysis against dialysis buffer (50 mM Tris-Cl, pH 8.0, containing 0.2 mM NaCl and 0.1 mM EGTA). CaM species with different amounts of labeling were separated by fast protein liquid chromatography (Macro Prep t-Butyl HIC column, Bio-Rad) in an ammonium sulfate gradient (1.2 to 0 m; 100 mM sodium phosphate, pH 7.0, present in both buffers). Fractions were collected, and the concentration of CaM-D was estimated by absorbance and the Bradford method. Each fraction was tested for Ca2+ binding-induced changes in anisotropy (see below). The fractions that showed changes in anisotropy upon Ca2+ binding were designated as CaM-D and used for all studies. The CaM-D was used in substrate phosphorylation experiments using histone II AS substrate as described previously (9) and found to be capable of stimulating kinase activity.

**Anisotropy Measurements and Data Analysis**—All reagents used for fluorescence measurements were screened for fluorescence impurities, treated with CaM-Sepharose (Amersham Pharmacia Biotech) to deplete endogenous Ca2+, and filtered using a 0.2-μm filter. Autophosphorylated CCaMK for anisotropy measurements was prepared as described above (12). Briefly purified CaM (PCM1) was incubated with an approximately 4-fold molar excess of dansyl chloride (Pierce) for 45 min at room temperature (12) (10 mg/ml CaM, 50 mM KCl, 20 mM HEPES, pH 9.0). Unreacted dansyl chloride was then separated from dansylated CaM by dialysis against dialysis buffer (50 mM Tris-Cl, pH 8.0, containing 0.2 mM NaCl and 0.1 mM EGTA). CaM species with different amounts of labeling were separated by fast protein liquid chromatography (Macro Prep t-Butyl HIC column, Bio-Rad) in an ammonium sulfate gradient (1.2 to 0 m; 100 mM sodium phosphate, pH 7.0, present in both buffers). Fractions were collected, and the concentration of CaM-D was estimated by absorbance and the Bradford method. Each fraction was tested for Ca2+ binding-induced changes in anisotropy (see below). The fractions that showed changes in anisotropy upon Ca2+ binding were designated as CaM-D and used for all studies. The CaM-D was used in substrate phosphorylation experiments using histone II AS substrate as described previously (9) and found to be capable of stimulating kinase activity.

**Anisotropy Measurements and Data Analysis**—All reagents used for fluorescence measurements were screened for fluorescence impurities, treated with CaM-Sepharose (Amersham Pharmacia Biotech) to deplete endogenous Ca2+, and filtered using a 0.2-μm filter. Autophosphorylated CCaMK for anisotropy measurements was prepared as described above using 2 μM ATP or AMP-PNP. Fluorescence anisotropy was measured in a T-format using an SLM 4800 spectrofluorometer. The excitation wavelength was 335 nm, and the excitation bandpass was 4 nm. Emitted light was collected without a monochromator through a 400-nm Schott low cut-off filter. Experiments were performed at 30 °C in 100-μl cuvettes. Anisotropy was calculated using SLM software. Data plotted are the values obtained after about 10 consecutive averages (integrated for 1 s).

We used fluorescence anisotropy as an index of the fraction of fluorescent calmodulin (CaM-D) bound to the kinase. The equations used for this analysis were taken from Ref. 13 to determine the value of K, the equilibrium association constant for a simple non-cooperative binding isotherm. At any kinase concentration (L), F abs = fraction of CaM-D bound to the kinase can be obtained from Equation 1.

\[
F_b = (A - A_f)/(A_b - A_f) 
\]

(Eq. 1)

The measured anisotropy (A) is from a titration where CaM-D concentration is kept constant (10 nM) and less than the binding constant for the kinase while the kinase concentration (L) is varied. A is the anisotropy of the free CaM-D, and Aτ is the anisotropy of bound CaM-D. Aτ was determined in an independent experiment where the concentration of the kinase was sufficient to >95% saturate the CaM-D. In our case, the quantum yield of bound CaM-D is not equal to the quantum yield of the free CaM-D. Therefore, Equation 1 must be modified to account for this (Equation 2).

\[
F_b = (A - A_f)/(A_b - A_f + A - A_f) 
\]

(Eq. 2)

X is the ratio of quantum yield of the bound to the quantum yield of the free CaM-D. X was determined in an independent experiment to be 1.9 by the following procedure. The fluorescence intensities for free and bound CaM-D (see above) were determined at two different emission polarizer settings, 90° and 0°, to obtain Iperpendicular and Iparallel, respectively. The absolute intensities for each sample were then calculated from Equation 3.

\[
I_{absolute} = I_{parallel} + 2I_{perpendicular} 
\]

(Eq. 3)

The ratio of bound to free (Iabsolute bound/W[absolute free]) = X.

Equation 4 can be used for a simple binding isotherm, where L approximates Iabs, which is true in our case because the CaM-D concentration (10 mM) is less than the binding constant for the kinase.

\[
F_s = KL/(1 + KL) 
\]

(Eq. 4)

Therefore, the relationship between A and L, in terms of K, can be obtained by rearranging Equation 2 and substituting in Equation 4, as shown below.

\[
A = F_s(X_A - A_f) + A_f 
\]

(Eq. 5)

The anisotropy (A) versus kinase concentration (L) plots were fit to this equation to obtain K, A0, and Aτ by giving initial parameter estimates of these variables. The fitted values for A0 and Aτ were in reasonable agreement with those determined experimentally. The fits to the data did not appreciably change by fixing A0 and Aτ to the experimentally determined values.

**RESULTS**

**Autophosphorylation Analysis of CCaMK and Deletion Mutants**—The role of the neural visinin-like domain in regulating CaMK was investigated. Here we report the effect of successive EF-hand deletions from the COOH terminus of the visinin-like domain on autophosphorylation and CaM activation of CCaMK. The autophosphorylation of three mutants lacking one, two, or three EF-hands was performed as described and analyzed using the parameter SAP. This is the ratio of [32P]PO4 incorporation (autophosphorylation) in the presence of Ca2+ to that in the absence of Ca2+. See Table 1 for values. B, schematic diagrams of the wild type and mutant constructs of CCaMK used for the autophosphorylation analysis.

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Autophosphorylation analysis of wild type and EF-hand deletions in the visinin-like domain of CCaMK. A, autophosphorylation analysis of wild type (WT) and various deletion mutants (M1, M2, M3). Ca2+-SAP index (Ca2+-stimulated autophosphorylation) is the ratio of amount of [32P]PO4 incorporation in the presence of Ca2+ to that in the absence of Ca2+. See Table 1 for values. B, schematic diagrams of the wild type and mutant constructs of CCaMK used for the autophosphorylation analysis.
phosphorylation assay mixture abolished Ca$^{2+}$-dependent autophosphorylation of wild type CCaMK.

Fluorescence Emission Anisotropy Measurements Using Dansylated Calmodulin—Previous studies from our laboratory (14) have shown that binding of Ca$^{2+}$/CaM releases autoinhibition (intrasteric regulation) making CCaMK fully active. The effect of autophosphorylation of CCaMK on interaction with CaM was investigated (Fig. 2). Equilibrium binding of CaM-D was monitored by fluorescence emission anisotropy measurements in solution. The rotational correlation time of free CaM-D (mass 17 kDa) is increased upon binding of CCaMK (mass 57 kDa). This change in correlation time is reflected in the anisotropy values (Fig. 2) compared with those in the absence of ATP (Fig. 2). The dissociation constants were calculated to fit the anisotropy data as described under “Experimental Procedures.” This showed that autophosphorylation tightens the binding by approximately a factor of 8 (see Table II). Competitive binding studies using unlabeled CaM showed a large decrease in anisotropy (Fig. 2) in the presence of excess (1:100) unlabeled CaM, 0.2 mM Ca$^{2+}$, 2 μM ATP, and autophosphorylated CCaMK.

We used an unhydrolyzable ATP analog (AMP-PNP) to study whether the increase in affinity for CaM-D is due to binding of ATP to the kinase active site or due to the consequent autophosphorylation of the kinase. Incubation of CCaMK with 0.2 mM Ca$^{2+}$ and 2 μM AMP-PNP in the assay mixture containing 10 nM CaM-D did not show any significant changes in anisotropy values (Fig. 2) compared with those in the absence of ATP (assay mixture containing 10 nM CaM-D and 0.2 mM Ca$^{2+}$).

Deletion mutants of CCaMK were used to test whether Ca$^{2+}$-dependent autophosphorylation is a prerequisite for the variable affinity for CaM and to study contributions of visinin-like domain. The three deletion mutants (Fig. 1) were used to carry out fluorescence anisotropy measurements (Fig. 4, A–C). Interestingly, none of the three EF-hand deletion mutants showed any increase in affinity for CaM-D in the presence of 0.2 mM Ca$^{2+}$ and 2 μM ATP (Table II). These mutants showed Ca$^{2+}$-dependent interaction with CaM-D, suggesting a functional CaM binding site.

Filter Binding Experiments Using 35S-Labeled CaM—As an additional measure to test the autophosphorylation-dependent changes in the affinity for CaM by CCaMK, we performed filter-binding experiments using 35S-labeled CaM as described. Our data suggested a significant increase in CaM binding upon autophosphorylation of CCaMK (Fig. 3) compared with unphosphorylated controls.

DISCUSSION

Ca$^{2+}$-stimulated autophosphorylation of CCaMK and different EF-hand deletion mutants were analyzed using SAP index. We found this index very useful to compare the extent of stimulation of different mutants by Ca$^{2+}$. The SAP index values for all the EF-hand deletion mutants were almost unity (Fig. 1, Table I). However, wild type CCaMK showed a 4.8-fold increase in autophosphorylation in the presence of Ca$^{2+}$ and ATP. The results of the successive deletions in the visinin-like domain suggest that the third EF-hand is absolutely essential for the functioning of the visinin-like domain. We expected that the mutant M1 with all three EF-hands deleted would show Ca$^{2+}$/CaM-dependent autophosphorylation similar to CaMK II (15) because the kinase domain and CaM-binding domain of CCaMK and CaMK II are highly similar (9) at the amino acid level (although they have different C-terminal domains). In CaMK II, deletion of the C-terminal association domain does not have any effect on Ca$^{2+}$/CaM-dependent autophosphorylation. Interestingly, the Ca$^{2+}$-stimulated autophosphorylation was absent in this mutant, indicating a different mechanism of autophosphorylation operating for CCaMK unlike other CaM-dependent kinases. The PROFILE scan analysis on Ca$^{2+}$-binding sites of the C-terminal visinin-like domain (normalized scores: EF hand 1: 7.6, EF hand 2: 9.3, EF hand 3: 10.3) suggest that different EF-hands may have different affinities for Ca$^{2+}$. However, we did not detect any intermediary levels of autophosphorylation (in terms of SAP index values) with different EF-hand deletions under the conditions tested (Fig. 1). Reduction in Ca$^{2+}$-stimulated autophosphorylation upon deletions in the visinin-like domain (VLD) could be explained by either the loss of autophosphorylation sites due to deletions in the VLD or due to lack of Ca$^{2+}$ binding to VLD. We have observed from the
The visinin-like domain of CCaMK acts as a Ca\(^{2+}\) sensor. These domains show 52–54% sequence similarity and 32–35% sequence identity compared to CCaMK with neural visinin-like calcium-binding protein domains. The sequence of the visinin-like domain (amino acids 338–520) of CCaMK is well known. Visinin-like Ca\(^{2+}\)-binding proteins in plants or visinin-like domains in plant proteins is not as well known. Visinin-like Ca\(^{2+}\)-binding proteins are members of a family of Ca\(^{2+}\)-sensitive regulators, which include frequenin, neurocalcin, and hippocalcin and are activated at nanomolar concentrations of Ca\(^{2+}\). Comparison of amino acid sequence of the visinin-like domain (amino acids 338–520) of CCaMK with neural visinin-like calcium-binding protein shows 52–54% sequence similarity and 32–35% sequence identity. Our autophosphorylation data (Fig. 1) suggests that the visinin-like domain of CCaMK acts as a Ca\(^{2+}\)-sensitive switch and that the third EF hand is important in regulating Ca\(^{2+}\)-stimulated autophosphorylation.

We performed fluorescence emission anisotropy measurements using dansylated calmodulin to quantify the interaction between calmodulin and phosphorylated and nonphosphorylated forms of the kinase. In the presence of 0.2 mM Ca\(^{2+}\), addition of nonphosphorylated kinase to the assay mixture containing 10 nM CaM-D resulted in a significant increase in anisotropy indicating Ca\(^{2+}\)-dependent interaction between CaM-D and nonphosphorylated CCaMK (Fig. 2). Such an increase in anisotropy was absent when nonphosphorylated CCaMK was added to the assay mixture containing 2.5 mM EGTA. In the presence of 0.2 mM Ca\(^{2+}\) and 2 \(\mu\)M ATP, addition of autophosphorylated kinase resulted in a very large increase in anisotropy (Fig. 2). The anisotropy data were analyzed as described and the dissociation constants were calculated (Table II). The dissociation constant for CaM-D was changed from 55.3 nM to 6.5 nM upon autophosphorylation of CCaMK. This significant increase in anisotropy due to the addition of autophosphorylated kinase is Ca\(^{2+}\)-dependent and reversible as evident from the large decay in anisotropy when the free Ca\(^{2+}\) was lowered to less than 100 nM by the addition of EGTA (data not shown). Competitively binding studies using unlabeled CaM showed a large decrease in anisotropy (Fig. 2) in the presence of excess (1:100) unlabeled CaM, 0.2 mM Ca\(^{2+}\), 2 \(\mu\)M ATP, and autophosphorylated CCaMK. This demonstrates that the increase in anisotropy is due to the specific interaction between CaM-D and autophosphorylated kinase. Filter binding experiments using biosynthetically labeled CaM (\(^{35}\)S-CaM) suggested that CaM could distinguish between phosphorylated and nonphosphorylated forms of the kinase (Fig. 3).

We used an unhydrolyzable ATP analog (AMP-PNP) to study whether the increase in affinity for CaM is due to binding of ATP to the kinase active site or due to the consequent autophosphorylation of the kinase. Incubation of CCaMK with AMP-PNP (Fig. 2) did not show any significant changes in anisotropy values compared with those in the absence of ATP. Thus, the increased affinity could not be induced with the unhydrolyzable ATP analog. This suggests that an ATP-mediated modification (autophosphorylation) is absolutely necessary for increased affinity for CaM. This is a novel finding that autophosphorylation-dependent affinity for CaM could function as a molecular switch in regulating plant CCaMK.

Deletion mutants of CCaMK were also used to test whether Ca\(^{2+}\)-stimulated autophosphorylation is a prerequisite for the variable affinity for CaM and to study contributions of C-terminal visinin-like domain. The three deletion mutants were used to carry out fluorescence anisotropy measurements (Fig. 4, A–C). Interestingly none of the three EF-hand deletion mutants showed any increase in affinity for CaM-D in the presence of 0.2 mM Ca\(^{2+}\) and 2 \(\mu\)M ATP (Table II). However, these mutants showed a Ca\(^{2+}\)-dependent interaction with CaM-D (Fig. 4, A–C) evident from the increase in anisotropy in the presence of 0.2 mM Ca\(^{2+}\), suggesting a functional CaM-binding site. The increase in anisotropy was not observed when the mutant kinases were added to the assay mixture containing 2.5 mM EGTA. The increase in anisotropy obtained in the presence of Ca\(^{2+}\) could be reversed by adding 2.5 mM EGTA (data not shown). These mutants also did not show any Ca\(^{2+}\)-stimulated autophosphorylation (Fig. 1). This suggests that autophosphorylation of CCaMK mediated by the visinin-like domain is a prerequisite for increased affinity for CaM by CCaMK.

Our results provide the first biochemical mechanism for CaM regulation of a plant protein kinase. Nonphosphorylated CCaMK has low affinity (\(K_d = 55.3\) nM), whereas autophosphorylated form of CCaMK has significantly increased affinity for CaM (\(K_d = 6.5\) nM) (Fig. 2, Table II). The autophosphorylation-dependent increase in affinity for CaM acts as a switch for maximal kinase activity. The state of high affinity for CaM due to Ca\(^{2+}\)-stimulated autophosphorylation could also be physiologically significant in plants. This mechanism may help to maintain kinase activity when the free CaM concentration is limiting. This condition could result from Ca\(^{2+}\) concentrations that do not saturate CaM or from CaM concentrations that are locally lower than the concentration of CaM-binding sites. Phosphorylation-dependent affinity changes for CaM is a new mechanism of signal transduction in plants. It is necessary to explore the properties of the Ca\(^{2+}\)-messenger system in detail to understand how Ca\(^{2+}\) regulates diverse cellular processes. The ability to transmit information by Ca\(^{2+}\)-messenger system is greatly enhanced through the use of modulation (AM) and frequency modulation (FM). It has been shown that the mammalian Ca\(^{2+}\)/CaM-dependent protein ki-

---

\(2^\text{P. V. Sathyarayanan, W. F. Siems, and B. W. Poovaliah, manuscript in preparation.}

---

**TABLE I**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Autophosphorylation</th>
<th>SAP index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1142 ± 28</td>
<td>238 ± 9</td>
</tr>
<tr>
<td>M1(1–356)</td>
<td>41 ± 6</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>M2(1–438)</td>
<td>73 ± 7</td>
<td>72 ± 9</td>
</tr>
<tr>
<td>M3(1–482)</td>
<td>166 ± 8</td>
<td>159 ± 8</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Unphosphorylated + 0.2 mM Ca(^{2+}), 2 (\mu)M ATP</th>
<th>Autophosphorylated + 0.2 mM Ca(^{2+}), 2 (\mu)M ATP</th>
<th>Control + 2.5 mM EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>55.3 (0.3)</td>
<td>6.5 (0.4)</td>
<td>&gt;400</td>
</tr>
<tr>
<td>M1(1–356)</td>
<td>61.9 (0.2)</td>
<td>55.6 (0.6)</td>
<td>&gt;400</td>
</tr>
<tr>
<td>M2(1–438)</td>
<td>51.5 (0.4)</td>
<td>56.4 (0.4)</td>
<td>&gt;400</td>
</tr>
<tr>
<td>M3(1–482)</td>
<td>49.7 (0.4)</td>
<td>50.1 (0.4)</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>
Fig. 4. Visinin-like domain of CCaMK regulates CaM affinity. Equilibrium binding constants of CaM-D for different deletion mutants of CCaMK were calculated from fluorescence emission anisotropy measurements. All plots were obtained at 10 nM CaM-D. See Fig. 1B for descriptions of M1, M2, and M3 mutants. Unless otherwise noted, the data points are simply connected by lines. A, M1 mutant: open circles, with solid line representing the fit to data, in the presence of 0.2 mM Ca\(^{2+}\), 2 μM ATP; and solid circles, with dotted lines representing the fit to data in the presence of 0.2 mM Ca\(^{2+}\). The dashed line connecting solid squares in the presence of 2.5 mM EGTA. B, M2 mutant: solid circles, with solid line representing the fit to data points, in the presence of 0.2 mM Ca\(^{2+}\), 2 μM ATP; and open circles, with dotted lines representing the fit to data in the presence of 0.2 mM Ca\(^{2+}\). The dashed line connecting diamonds in the presence of 2.5 mM EGTA. C, M3 mutant: open circles, with dotted line representing the fit to data, in the presence of 0.2 mM Ca\(^{2+}\), 2 μM ATP; and solid circles, with solid lines representing the fit to data in the presence of 0.2 mM Ca\(^{2+}\). The dashed line connecting triangles in the presence of 2.5 mM EGTA.
nase, CaMK II, can decode the frequency of \( \text{Ca}^{2+} \) oscillations (12, 18 and 19). It may be possible that the visinin-like domain can mediate some kind of signal modulation (FM or AM) regulating the kinase activity in the target tissues. CaMK is highly tissue-specific, developmentally regulated, and found to be expressed in dividing cells like pollen mother cells in anther where it is expressed during the onset of meiosis through the tetrad stage (8).

Here we describe a new mechanism of regulation of a chimeric kinase by \( \text{Ca}^{2+} \) and \( \text{Ca}^{2+}/\text{CaM} \) previously not known to operate for protein kinases from plants or animals or microbial systems. In animals visinin-like \( \text{Ca}^{2+} \) sensing proteins and \( \text{Ca}^{2+}/\text{CaM} \)-dependent protein kinases are encoded by two different genes, whereas in plants it appears that these two gene products exist as a chimeric kinase. We have compared the \( \text{Ca}^{2+} \) binding properties of the \( E. \ coli \) expressed visinin-like domain protein of CCaMK to CaM. Our results have revealed that the visinin-like domain protein binds \( \text{Ca}^{2+} \) at nanomolar range where as CaM binds at micromolar range. A possible mechanism of CaMK activation that arises from consideration of this paper and previously published work from our laboratory can be summarized as follows (Fig. 5). In the ground state the kinase is autoinhibited and inactive. The first event in the kinase cycle could be the perception of \( \text{Ca}^{2+} \) signals by the visinin-like domain at the C-terminal (could serve as a molecular antenna for the kinase domain). In the presence of ATP, \( \text{Ca}^{2+} \) binding to the visinin-like domain results in autophosphorylation of the kinase, and an increased affinity for CaM. \( \text{Ca}^{2+}/\text{CaM} \) binding to the CaM-binding domain displaces the autoinhibitory region interacting with kinase domain and releases autoinhibition (14), thus stimulating the kinase activity. At this stage the CCaMK is maximally active. In the case of CaMK II, the autophosphorylation and release of autoinhibition mediated by \( \text{Ca}^{2+} \) (15, 24 and 25). In the case of CCaMK these are two separate processes, autophosphorylation mediated by visinin-like domain and release of autoinhibition mediated by \( \text{Ca}^{2+}/\text{CaM} \) (Fig. 5). Previous reports from this laboratory (9) indicated that incubation of 1 \( \mu \text{M} \) CaM in the autophosphorylation assay mixture decreased autophosphorylation. This suggests that CaM binding may reduce access to the autophosphorylation sites.

Our results suggest that autophosphorylation and CaM binding are two separate steps in the activation of CCaMK, one leading to the other. Autophosphorylation regulated variable affinity for CaM mediated by the neural visinin-like domain of plant CCaMK could be a mechanism of transducing \( \text{Ca}^{2+}/\text{CaM} \) signals into precise phosphorylation signals. Elucidating the precise molecular nature of the information transfer leading to the autophosphorylation of CCaMK and variable CaM affinity should provide valuable insights into the mechanisms of the \( \text{Ca}^{2+}/\text{CaM} \)-mediated signal transduction in plants.

Acknowledgment—We thank Matt Ladon for helping with the fast protein liquid chromatography purification of CaM-D.

REFERENCES

Plant Chimeric Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase: ROLE OF THE NEURAL VISININ-LIKE DOMAIN IN REGULATING AUTOPHOSPHORYLATION AND CALMODULIN AFFINITY
P. V. Sathyanarayanan, Christine R. Cremo and B. W. Poovaiah

doi: 10.1074/jbc.M000771200 originally published online June 5, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000771200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 13 of which can be accessed free at http://www.jbc.org/content/275/39/30417.full.html#ref-list-1