Interaction of a Kinesin-like Protein with Calmodulin Isoforms from Arabidopsis*

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In Arabidopsis and other plants there are multiple calmodulin isoforms. However, the role of these isoforms in regulating the activity of target proteins is obscure. Here, we analyzed the interaction between a kinesin-like calmodulin-binding motor protein (Reddy, A. S. N., Safadi, F., Narasimhulu, S. B., Golovkin, M., and Hu, X. (1996) J. Biol. Chem. 271, 7052–7060) and three calmodulin isoforms (calmodulin-2, -4, and -6) from Arabidopsis using different approaches. Gel mobility and fluorescence shift assays revealed that the motor binds to all calmodulin isoforms in a calcium-dependent manner. Furthermore, all calmodulin isoforms were able to activate bovine calcium/calmodulin-dependent phosphodiesterase. However, the concentration of calmodulin-2 required for half-maximal activation of phosphodiesterase is 2- and 6-fold lower compared with calmodulin-4 and -6, respectively. The dissociation constants of the motor to calmodulin-2, -4, and -6 are 12.8, 27.0, and 27.8 nM, respectively, indicating that calmodulin-2 has 2-fold higher affinity for the motor than calmodulin-4 and -6. Similar results were obtained using another assay that involves the binding of 35S-labeled calmodulin isoforms to the motor. The binding saturation curves of the motor with calmodulin isoforms have confirmed that calmodulin-2 has 2-fold higher affinity to the motor. However, the affinity of calmodulin-4 and -6 isoforms for the motor was about the same. Based on these studies, we conclude that all calmodulin isoforms bind to the motor protein but with different affinities.

Plant cells elevate their cytosolic free calcium (Ca2+) in response to a variety of hormonal and environmental signals. In plants, Ca2+ mediates these signals either directly by activating a group of proteins called calcium-dependent protein kinases (1) or indirectly through Ca2+-modulator proteins such as calmodulin (CaM) and calmodulin-like proteins (2, 3). CaM is one of the well characterized multifunctional Ca2+-binding proteins that is highly conserved in eukaryotes (4, 5). It is a small acidic protein containing four Ca2+-binding motifs and is a member of the EF hand superfamily. Ca2+/CaM regulates a variety of unrelated target enzymes/proteins involved in various Ca2+-mediated signal transduction pathways in plants, including those involved in cellular and physiological processes as diverse as cell division (6) and defense responses to pathogens (5, 7, 8). However, little is known about the Ca2+/CaM target proteins involved in many of these processes.

Genes and cDNAs encoding CaM have been cloned and characterized from a variety of organisms. Studies in yeast (9) and Drosophila (10) revealed that CaM is an indispensable gene. Although there is less sequence identity between yeast and animal CaMs (60% identity) than among most animal CaMs, human CaM could functionally complement yeast CaM (11). In mouse, rat, and human, three different genes encode identical CaM proteins (12). However, a single gene encodes CaM in yeast (9) and Chlamydomonas (13). Surprisingly, studies in plants revealed the presence of multiple CaM sequences (up to 12) that encode multiple CaM isoforms and divergent CaMs (5). Although CaM isoforms and divergent CaMs contain 148 amino acids, divergent CaMs differ significantly from CaM isoforms in their amino acid sequence. CaM genes have been cloned and characterized from many plant species including Arabidopsis (5), soybean (14), potato (15), wheat (16), rice (17), apple (18), barley (19), and alfalfa (20).

In Arabidopsis, six CaM genes (AtCaM 1–6) and four CaM-related genes encoding four CaM isoforms and four CaM-like proteins, respectively, have been reported (5). CaM-like proteins contain more than 148 amino acids and a variable number (three to six) of Ca2+-binding domains (5). AtCaM2, 3, and 5 are identical, and AtCaM1 and 4 differ in a single amino acid. AtCaM6 differed by two amino acids with AtCaM2, 3, 5 and by five amino acids with AtCaM1 and 4. Interestingly, most of these changes occurred between the third and fourth Ca2+-binding motifs (5). CaM-related genes encode TCH2, TCH3 (21), CaBP-22 protein (22), and a putative Ca2+-binding protein (23) which show 44, 70, 65, and 34.8% amino acid sequence identity, respectively, with CaM2. The presence of a conserved and a divergent CaM group has been reported in soybean (14). The soybean conserved group (CaM1, 2, and 3) shows a high percentage of identity with AtCaM2. The divergent group contains two isoforms CaM4 and 5 that differ from the conserved group in 32 amino acids. These are the most divergent group of CaM isoforms identified so far in plants (14). Further, the two groups differ in their activation of pea NAD kinase, a Ca2+/CaM target protein. In potato, eight cDNAs encoding two groups of isoforms, conserved (PCaM5, 6, 7, and 8) and divergent (PCaM1) CaMs, have been reported (15). The PCaM1 differed from the conserved group by 13 amino acid substitutions scattered across the protein. About 10 CaM encoding genes were identified in hexaploid wheat (16). Of these, seven genes encode one group (TaCaM1), two genes encode the second group (TaCaM2), and a single gene encodes the third group (TaCaM3). The TaCaMIII isoform does not contain the first Ca2+-binding motif (16). Although plants and animals harbor multiple CaM genes,

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‡The abbreviations used are: CaM, calmodulin; AtCaM, Arabidopsis CaM; KCBP, kinesin-like calmodulin-binding protein; PDE, cyclic nucleotide phosphodiesterase; BSA, bovine serum albumin; DTT, dithiothreitol.

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plants, in contrast to animals that produce an identical CaM protein, possess multiple CaM isoforms (5). These findings suggest that different CaM isoforms may perform different functions in Ca²⁺-signaling pathways. In recent years, several Ca²⁺/CaM-binding proteins have been identified in plants (4, 5). However, the interaction of Ca²⁺/CaM-binding proteins with CaM isoforms from a homologous system has not been characterized. The presence of multiple isoforms of CaM and a large number of CaM target proteins in plants poses the possibility that each CaM isoform may interact with a specific set of target proteins. Alternatively, each CaM isoform may interact with all target proteins but differ in their affinities. Hence, it is necessary to determine the interaction of CaM isoforms with CaM-dependent target proteins.

Previously, we isolated a kinesin-like calmodulin-binding protein (KCBP) in a protein-protein interaction based screening of an Arabidopsis cDNA library using animal CaM as a probe (24). Using bovine CaM, the CaM-binding domain was mapped to a stretch of 23 amino acids. Furthermore, Ca²⁺/CaM has been shown to regulate the interaction of KCBP with microtubules (25–27). However, the interaction of Arabidopsis KCBP with plant CaM, especially with different isoforms of CaMs from the same system, has not been tested. To this end, we analyzed the interaction of KCBP with AtCaM isoforms (AtCaM2, 4, and 6) using a variety of approaches. First, we used a gel mobility shift assay and fluorescence spectroscopy with AtCaM isoforms and KCBP peptide corresponding to the CaM-binding region to test the binding of CaM isoforms to KCBP. We then used AtCaM isoforms in a Ca²⁺/CaM-dependent phosphodiesterase (PDE) assay in the presence and absence of KCBP CaM-binding peptide to determine activation of PDE by CaM and the dissociation constants of KCBP to AtCaMs. Finally, the binding of AtCaM isoforms to KCBP was also tested using 35S-labeled AtCaM isoforms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclic AMP, phosphodiesterase, 5'-nucleotidase, and melittin (NH₂-GIGAVLKVLTTGLPISWIKKRN-CO₂H) were purchased from Sigma. Bovine CaM, melittin, and BSA were procured from Calbiochem (La Jolla, CA). The CaM-binding 23-mer peptide of Arabidopsis KCBP was synthesized as described previously (24). Easy tag Expre³⁵S³⁵S protein labeling mix (73% L-[³⁵S]methionine) was obtained from NEN Life Science Products. Immobilon affinity membrane (Bedford, MA) used in binding of ³⁵S-labeled CaMs was used for melittin and KCBP peptides. The values obtained for bovine CaM and the two peptides were comparable between the three different methods (Bradford, gel electrophoresis, and absorption measurements) but not for the AtCaMs. This variation is likely due to extremely low number of aromatic amino acids in AtCaM isoforms, which is typical of CaMs from plants. Therefore, we used the concentration values obtained with the Bradford method and gel electrophoresis methods for AtCaMs.

**Mobility Shift Assay**—The interaction of the AtCaM isoforms with the Arabidopsis KCBP synthetic peptide (CaM-binding region of KCBP) was analyzed by electrophoretic mobility shift assay in urea containing gels (33). Each of the AtCaM isoforms and the bovine CaM (178 pmol) was incubated with KCBP synthetic peptide (712 pmol) in the presence of 4 mM urea, 100 mM Tris-HCl, pH 7.5, and 1 mM CaCl₂ or 5 mM EGTA at room temperature for 1 h in a total volume of 20 μl. Then 10 μl of sample buffer (0.375 M Tris-HCl, pH 6.8, 30% glycerol, and 0.025% bromphenol blue) (34) was added to the samples and electrophoresed in 12% polyacrylamide gels containing 4% urea, 0.375 mM Tris, pH 8.8, and either 1 mM CaCl₂ or 5 mM EGTA. The gels were run at a constant voltage of 25 V/gel in an electrode buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and either 1 mM CaCl₂ or 5 mM EGTA). The gels were stained for 24 h with 0.25% Coomassie Blue R-250 in 7.5% acetic acid and 50% methanol for 1 h and then destained with 30% methanol and 7% acetic acid.

**Fluorescence Spectroscopy Assay**—Trp-based fluorescence was performed in a 600-μl reaction on a Hitachi-F-3010/4010 spectrofluorimeter as described previously (24) except that the concentration of peptide and AtCaMs was 200 μM. Before measuring the fluorescence, the solutions were equilibrated for 1 h at room temperature (25 °C) in a cuvette in free and AtCaM-bound synthetic 23-mer KCBP peptide was excited at 290 nm, and the emission values were recorded from 300 to 450 nm wavelengths (peak at 328 nm) with a bandwidth of 5 nm in a 5-mm quartz cell at 25 °C. The values obtained for free 23-mer KCBP peptide and free CaM isoforms alone were subtracted from the values of AtCaM-KCBP peptide complex.

**Competitive 3'-5'-Cyclic AMP Phosphodiesterase Assay**—Cyclic AMP phosphodiesterase was assayed as described earlier (35) with several modifications. The initial 100-μl reaction volume contained buffer (20 mM Tris-HCl, pH 7.0, 5 mM MgSO₄, 0.3 mM DTT), increasing concentrations of CaM (5–500 nM), 2 mM cAMP, 2 mM CaCl₂ (or 1 mM EGTA in control reactions) and the presence (100 nM) or absence of peptide (23-mer KCBP peptide or melittin). The reaction was started with the addition of 0.05 units of phosphodiesterase (30 units/mg). The reaction without CaM was used as a control. After incubation at 30 °C for 30 min, the reaction was stopped by placing the reaction tubes in a boiling water bath for 3 min. Following a brief spin and re-equilibration to 30 °C, 20 μl of 3'-5'-nucleotidase assay mixture (containing 0.2 units of the 5'-nucleotidase (94 units/mg) and 8 μl of MnCl₂) was added and further incubated at 37 °C for a further 5 min. The reaction was stopped by adding 80 μl of 0.75 mM perchloric acid. The precipitates were briefly spun down and the supernatant (100 μl) was assayed for P₁ as described (36). The dissociation constants (Kᵣ) of bovine CaM and AtCaMs for KCMB and melittin were calculated from the concentration of CaM (nm) required to obtain half-maximal (50%) P₁ activity in the presence (100 nM) and absence of peptide. The following equation was used (33).
to calculate dissociation constants. $K_D = \frac{[P][1] + K - [CaM]}{K}$, where $[P]$ is the total concentration of peptide added, and $[CaM]$ and $K$ are the concentrations of CaM required to obtain half-maximal activation of PDE in the presence and absence of peptide, respectively.

Binding of $^{35}$S-Labeled CaM to 1.5 C KCBP—220 pmol of 1.5 C KCBP peptide (equivalent to 10 pmol binding respect to CaM-binding domain in KCBP) was immobilized on Immobilon affinity membranes using a slot-blot apparatus. The membranes containing the KCBP were cut into small, equal size pieces and were subjected to the following treatments at room temperature. After rinsing in phosphate-buffered saline containing 0.1% Tween-20 for 5 min, the membranes were incubated for 2 h in 10% monoethanolamine in 1 m NaHCO$_3$ to block negative amino groups on the membrane and then rinsed again in phosphate-buffered saline two times each for 10 min. Then they were incubated in blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$, and 1% nonfat milk) for 1 h and transferred into a new tube containing increasing concentration of $^{35}$S-labeled CaM (0.5–200 nM). The membranes were incubated for 12 h at room temperature with gentle shaking (60 rpm) and then given three 15-min washes in blocking buffer without nonfat milk. The washed membranes were submerged individually in 4 ml of scintillation fluid, and counts were recorded on 1217 Rackbeta liquid scintillation counter. Membranes without KCBP were also processed as above to measure nonspecific binding. Prior to incubation of membranes with $^{35}$S-labeled CaM, an aliquot (10 µl) from each sample was used to determine total counts.

Statistics and Graphical Presentation—All assays were performed in triplicate. Each experiment was repeated at least three times. Average values obtained in triplicate were analyzed for standard deviation using Microsoft Excel and transferred to KaleidaGraph 3.0 software for graphical presentation.

RESULTS

Binding of KCBP Synthetic Peptide to AtCaM Isoforms—In a protein-protein interaction-based screening with animal CaM as a probe, we isolated a novel CaM-binding domain containing kinesin motor protein from Arabidopsis (24). Further characterization of this protein using bovine CaM showed regulation of this motor function by Ca$^{2+}$/CaM through its CaM-binding domain (25, 27). It was not known, however, whether KCBP binding and regulation by bovine CAM are the same as with plant CaM. So far all functional studies with KCBP were carried out using nonplant CaM. In our effort to further characterize the KCBP, we have analyzed the interaction of KCBP with different isoforms of plant CaMs. In Arabidopsis, molecular and biochemical analyses led to the identification of at least four distinct groups of CaM isoforms that differ in their deduced amino acid sequences (5). To test whether or not KCBP is capable of interacting with these different AtCaM isoforms we selected AtCaM2, 4, and 6 isoforms, representative members of three of the four isoforms.

Initially, we tested the binding of AtCaM isoforms to KCBP using gel mobility shift assay. In these assays, if a peptide binds to a CaM, the peptide complex migrates differently from that of free CaM in polyacrylamide gels containing 4% urea. In our earlier analysis of KCBP, we showed that a 23-amino acid KCBP synthetic peptide bound to bovine CaM and retarded its mobility in urea-containing gels (24). Here, we applied the same method to test whether KCBP peptide binds to AtCaM isoforms. We incubated 3 µg of each AtCaM isoform or bovine CaM with KCBP peptide. As shown in Fig. 1, KCBP peptide retarded the mobility of AtCaM isoforms and bovine CaM compared with the free CaMs. These results show that all isoforms of Arabidopsis CaM bind to the KCBP peptide. However, CaM-KCBP complexes are not formed in the presence of 1 mM EGTA, indicating that the interaction between CaM and peptide is Ca$^{2+}$-dependent (data not shown). Furthermore, the formation of complexes between CaM isoforms and KCBP peptide in the presence of 4% urea suggests that the dissociation constant ($K_D$) of KCBP to AtCaM isoforms is likely to be 100 nM or less (33). However, the gel mobility shift assay does not permit analysis of KCBP affinity for CaM isoforms.

AtCaM Isoforms Bind to KCBP Peptide and Increase Fluorescence—To investigate further the binding of each AtCaM isoform to the KCBP synthetic peptide, we employed Trp-based fluorescence change of the AtCaM-KCBP complexes in the presence of Ca$^{2+}$. Fluorescence spectroscopy is a convenient method to determine the CaM-peptide complex formation. It has been used to show CaM-binding property of several synthetic and natural peptides including LK1, LK2, melittin, and hemolysin to bovine CaM (37). The presence of one Trp residue in KCBP peptide and the absence of Trp in the AtCaMs or bovine CaM make this assay feasible to determine the interaction of KCBP peptide with AtCaM isoforms. Previously, we showed that the fluorescence of the KCBP synthetic peptide was shifted to a shorter wavelength (328 nm) with higher intensity upon its binding to bovine CaM (24). Based on this observation, we conducted fluorescent spectroscopy experiments with KCBP and AtCaMs to show peptide-CaM complex formation at equivalent amounts (200 pmol) of CaM and KCBP. The fluorescence intensity of AtCaM-KCBP complexes was shifted to a lower wavelength (328 nm) with increased fluorescence. The KCBP peptide, which fluoresces in the absence of CaM at 350 nm with less intensity than that of a CaM-KCBP complex, was used as a control. The KCBP peptide when complexed with BCaM, AtCaM2, AtCaM4, and AtCaM6 showed 3.8-, 6.1-, 3.8-, and 2.2-fold increase in fluorescence emission, respectively. These results revealed that each of the AtCaM isoforms binds to KCBP.

AtCaM Isoforms Stimulate the Mammalian Phosphodiesterase Activity—To further quantify the differences in binding of AtCaM isoforms to KCBP, we examined the CaM-dependent enzyme activity of bovine brain PDE using AtCaM isoforms as activators. Fig. 2 shows the effect of different CaMs on the phosphodiesterase activity. Because bovine CaM is a known activator of PDE, we used it as a positive control in the PDE assay (37). As shown in Fig. 2, the AtCaM isoforms activated mammalian PDE with different half-maximal velocities. Bovine CaM activated the PDE activity with a half-maximal activity at 20 nM. The most potent activator among the AtCaM isoforms was AtCaM2 with a half-maximal activation at 4.8 nM. In contrast to AtCaM2, AtCaM6 with a half-maximal activation at 29 nM was the least effective in activating the PDE. AtCaM4 also stimulated the PDE activity with a half-maximal activation in between those of AtCaM2 and AtCaM6 at 10 nM. The concentration of AtCaM2 required for half-maximal activation of PDE was 2- and 6-fold less than that of AtCaM4 and 6, respectively. These results revealed that the order of affinity of AtCaMs to CaM-binding site of PDE is AtCaM2 followed by AtCaM4, bovine CaM, and AtCaM6 (Table 1). In fact, AtCaM2 was a more potent activator of mammalian PDE than bovine CaM by as much as 4.1-fold. Furthermore, the enhanced PDE activity by each CaM tested is Ca$^{2+}$-dependent because the PDE activity is reduced to the basal level in the presence of 1
The affinity of melittin for AtCaMs was 1.5- to 4-fold higher than that of KCBP. In summary, the competitive PDE assay results suggest that KCBP has 2-fold greater affinity for AtCaM2 than to the other two AtCaMs. However, KCBP exhibits nearly equal affinity for AtCaM4 and 6.

**Quantitative Differences in Binding of 35S-Labeled AtCaM Isoforms to 1.5 C KCBP**—In the previous section we have inferred the affinity of KCBP for different AtCaMs using a competitive PDE assay. To further confirm the differences in the affinity between KCBP and AtCaMs, we tested directly the binding of 35S-labeled AtCaM isoforms to KCBP. The binding of AtCaMs to KCBP can be determined directly by measuring the radioactive counts from the radioactive CaM-KCBP complex. To carry out this assay, we used a C-terminal domain of KCBP (1.5 C) expressed in E. coli (27) and 35S-labeled AtCaM isoforms in binding reactions. The KCBP was immobilized on membranes, which were then incubated with increasing concentrations of 35S-labeled AtCaM isoforms, until binding was saturated. As shown in Fig. 4, the binding of radioactive AtCaMs to KCBP saturated at 70 ± 2 (AtCaM2), 160 ± 3 (AtCaM4), and 150 ± 2.8 nm (AtCaM6). The dissociation constants of AtCaM2, 4, and 6 isoforms were calculated from the Scatchard plots as 20 ± 0.04, 49 ± 0.96, and 49 ± 1.2 nm, respectively (insets in Fig. 4). Although the dissociation constants of KCBP with AtCaM isoforms slightly varied in two different assays (PDE and 35S-CaM binding), these results demonstrate that KCBP has higher affinity for AtCaM2 by over 2-fold compared with AtCaM 4 or 6. Further, the binding of AtCaMs to KCBP was completely inhibited by substituting Ca2+ with 1 mM EGTA (data not shown). These results are in agreement with that of the PDE assay.

**DISCUSSION**

In the model plant *Arabidopsis*, four CaM isoforms were reported (5). Group I isoform (CaM2, 3, and 5) differed by two (T117S and K126R) and four (D7E, R74K, D122E, and K126R) amino acid substitutions from group II (CaM6) and group III (CaM1), respectively. The group IV isoform (CaM4) is identical to group III but has a substitution of a basic amino acid (His) at 138th position in place of an aromatic amino acid (Tyr). Based on these amino acid differences we used CaM1, 2, 4, and 6 in the present analysis.

Using gel mobility shift assay, fluorescence spectroscopy, competitive PDE assay, and direct binding studies with KCBP, we have demonstrated that all three AtCaM isoforms interact with the *Arabidopsis* microtubule motor protein, KCBP. Although the AtCaM isoforms (2, 4, and 6), upon binding to KCBP peptide, showed similar altered electrophoretic mobilities (Fig. 1), they showed differential activation of PDE. By comparing concentrations of AtCaM isoforms required to obtain maximal and half-maximal activation of PDE, we found that AtCaM2 is more efficient activator than are AtCaM4, AtCaM6, and bovine CaM.

The concentrations of AtCaM2, 4, and 6 required for half-maximal activation of PDE were 4.8, 10, and 29 nM (Fig. 2), whereas the pea NAD kinase required 1.24, 1.83, and 2.23 nM of the same isoforms for half-maximal activation (28). CaM2 is most effective and CaM6 is least effective in activating both PDE and NAD kinase activities. However, PDE required 3.8, 5.4, and 13.0-fold higher concentration of AtCaM2, 4, and 6, respectively, for half-maximal activation as compared with NAD kinase. Furthermore, in comparison to AtCaM2, about 5-fold higher concentration of bovine calmodulin was required to obtain 50% PDE activity (Table I). The difference in the
concentration of CaM required to activate PDE and NAD kinase by the same isoforms of Arabidopsis CaMs reflects different affinities of CaM isoforms for these target proteins and supports our hypothesis that each CaM isoform may interact with various target proteins but with different affinities.

The results reported here differ somewhat from previous work that compared soybean CaM isoforms (SCaM1 and SCaM4), which are much more diverged in their sequences than are the AtCaM isoforms. SCaM1 and 4 activated PDE to very similar extents, but SCaM4 failed to activate NAD kinase (14, 17). The differences between SCaM1 and SCaM4 were shown to be attributable to Ca$^{2+}$-binding domain I. The AtCaM isoforms, on the other hand, differ primarily in domain IV. Domain IV is also the location of the majority of amino acid sequence differences between AtCaMs and bovine CaM (5). Furthermore, SCaM1 specifically activates calcineurin and competitively inhibits nitric-oxide synthase where as SCaM4 activates nitric-oxide synthase and competitively inhibits calcineurin (38).

These studies have shown that CaM isoforms and divergent CaMs are likely to interact with a different set of target proteins, suggesting that the specificity of CaMs for target proteins depends on the extent of sequence similarity among CaMs and their relative levels of accumulation in different cell types or organs (5). Our studies show that CaM isoforms interact with the same target protein but with different affinities. SCaM4-specific antibody immunologically cross-reacted with an approximately 17-kDa protein from Arabidopsis extracts, suggesting the presence of divergent CaMs in Arabidopsis (14).

Recently, a gene encoding a homolog of SCaM4/5 has been identified in Arabidopsis. It would be interesting to test the interaction of KCBP with this divergent CaM isoform.

Further evidence in support of the hypothesis that CaM isoforms interact with similar target proteins with different affinities comes from the studies with human CaMs. In human, the presence of a conserved CaM and another CaM-like protein (hCLP) have been reported (11). The conserved CaM activates most target proteins with different affinities. However, hCLP selectively activates CaM targets, showing normal activation of CaM kinase II, moderate activation of PDE, and no activation of MLCK, calcineurin, or nitric-oxide synthase (11). Yeast and chicken CaMs also showed differential activation of most of mammalian enzymes (39, 40). However, most of these interaction studies were performed with CaMs and target proteins from heterologous systems. Therefore, the information concerning the interaction of CaM isoforms with target proteins from homologous systems is important in elucidating the function of CaM isoforms in plants.

The results obtained in the competitive PDE (Fig. 3 and Table I) and $^{35}$S-CaM binding assays (Fig. 4) have allowed us to understand better the interaction of KCBP with AtCaM isoforms. Our studies show that the AtCaM2 has over 2-fold higher affinity for KCBP as compared with AtCaM4 and 6 (Table I and Fig. 4). Because CaM2 differs from CaM6 in only

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**TABLE I**

Summary of half-maximal PDE activity by AtCaM isoforms and BCaM in the presence and absence of KCBP and melittin peptides and their dissociation constants

| Activator CaM | Half-maximal PDE activity$^a$ | $K_d$$^b$ |
|---------------|-------------------------------|-----------|
|               | Presence of peptide           | Presence of peptide |
|               | KBP                          | Melittin   | KBP      | Melittin |
| BCaM          | 20.0 ± 1.0                    | 60.0 ± 3.1 | 1.2 ± 1.1| 17.2 ± 1.1|
| AtCaM2        | 04.8 ± 0.1                    | 32.0 ± 6.6 | 042.0 ± 1.1| 12.8 ± 0.7| 08.1 ± 0.6|
| AtCaM4        | 00.0 ± 0.2                    | 37.0 ± 0.8 | 050.0 ± 1.5| 27.0 ± 0.3| 15.0 ± 0.5|
| AtCaM6        | 29.0 ± 0.2                    | 80.0 ± 1.2 | 110.0 ± 1.0| 27.0 ± 0.8| 06.8 ± 0.6|

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$^a$ Increasing concentrations of CaM as indicated on the abscissa in fig. 3 were used to activate PDE.

$^b$ The concentration of CaM (in nm) required to reach half-maximal activity of PDE.

$^c$ 100 nm concentration of KBP and melittin peptides were used in the assay.

$^d$ The dissociation constants for KBP and melittin were calculated (in nm) using the formula described under “Experimental Procedures.”

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**Fig. 3. Inhibition of AtCaMs/bovine CaM stimulated PDE activity by KCBP and melittin peptides.** The PDE activity was assayed as a function of the increasing concentrations of CaM alone (●) or CaM in the presence of 100 nM KBP (▲) or melittin (▼). A, bovine CaM; B, AtCaM2; C, AtCaM4; D, AtCaM6. Each data point is an average ± S.D. of three assays. The percentage of activity of PDE was calculated as described in Fig. 2. In the presence of KBP and melittin, the CaM concentration required to obtain 50% activity of PDE was calculated from these curves and represented in Table I. The $K_d$ values were calculated from the half-maximal activities of PDE in the presence and absence of peptides as described under “Experimental Procedures” and represented in Table I.
two amino acids (threonine to serine at position 117 and lysine to arginine at position 126), it is likely that these substitutions contribute to the difference in the affinity for KCBP. Similarly, AtCaM4, AtCaM6, and bovine calmodulin are less effective in activating PDE. These CaMs differ from CaM2 in 2–14 amino acid residues including a lysine to arginine substitution at position 126 that is common in the three CaMs with lower affinity for KCBP. This substitution may play a critical role in stabilizing the CaM-KCBP complex. Previous studies with CaM mutants and isoforms have demonstrated that minor changes, including conservative amino acid substitutions, influence the CaM’s target specificity or affinity for target proteins (17, 28). For example, a single substitution (M36I) in SCaM1 reduces its ability to activate NAD kinase by about 20%. Hence, our result that CaM2, which differs from CaM6 in two conservative substitutions, is more efficient activator of PDE and has 2-fold high affinity for KCBP is not surprising. Studies with yeast mutants have indicated that different regions of CaM contribute to the specificity of its interaction with different target proteins (41).

Interestingly, although CaM4 and CaM6 isoforms differ from each other in six residues including a nonconserved substitution (Tyr to His) in the fourth calcium-binding domain, these two isoforms did not show any significant differences in their affinity for KCBP (Table I). Differential activation of pea NAD kinase by AtCaM isoforms was also reported recently (28), where CaM2 has been shown to be the potent activator when compared with CaM4 and 6. Our results, together with a recent report on pea NAD kinase activation by AtCaM isoforms (28), support our hypothesis that closely related CaM isoforms bind to different target proteins with different affinities. We have also used melittin, a CaM-binding synthetic peptide in competitive PDE assay as a positive control in our assays. As shown in Table I, melittin exhibited higher affinity to AtCaMs than that of KCBP peptide. Furthermore, AtCaM6, in contrast to the results obtained with KCBP, showed the highest affinity to melittin (Table I), further supporting the conclusion that the same isoform may interact differentially with different target proteins.

Recently, CCaMK, a chimeric Ca$^{2+}$/CaM-dependent protein kinase, from tobacco (42) and lily (43) has been shown to undergo autophosphorylation in the presence of Ca$^{2+}$ alone. However, calcium and CaM inhibit autophosphorylation and promote substrate phosphorylation Liu et al. (42) have tested the effect of two isoforms of potato calmodulin (PCaM1 and PCaM6) on the activity of CCaMK from tobacco and lily. Interestingly, the PCaM1 acts as a more potent inhibitor of autophosphorylation and activator of substrate phosphorylation of CCaMK than PCaM6. However, these CaM isoforms did not show this differential regulation of lily CCaMK and pea NAD kinase activity (42). Comparison of these interaction studies from homologous (our study) and heterologous (42) systems revealed differential roles of CaM isoforms in regulating target proteins (Table I).

Although the level of different CaM isoforms at the protein level in different tissues is not known in Arabidopsis, Northern analyses with gene-specific probes indicate that CaM isoforms are differentially expressed in various tissues and in response to external signals (5). For example, AtCaM1 (group III) is constitutively expressed in all tissues, whereas AtCaM2 and AtCaM3 (group I) are expressed abundantly in leaves, flowers, and siliques. In addition, AtCaM2 and AtCaM3 (group I) mRNA is induced by a touch stimulus several-fold more than group II CaMs (5). Differential regulation of CaM isoforms has been reported in other plant systems also (4, 5, 15). Taken together, these data suggest that the level of different isoforms is likely to vary in different tissues. Hence, it is likely that different affinities of the CaM isoforms to a target protein could reflect different concentrations of these isoforms in the cell. Alternatively, at in vivo concentrations the low affinity of some of the CaM isoforms for a particular target protein may not permit interaction of those isoforms with the target protein.

In addition to four CaM isoforms, four CaM-like proteins have also been cloned and biochemically characterized in Arabidopsis. These are TCH2, TCH3 (44), CaBP-22 (22), and a putative Ca$^{2+}$-binding protein (23). The CaM-like proteins, which are specific to plants, are different in size and contain...
three to six EF hand motifs compared with the highly conserved CaM that contains 148 amino acids and four EF hand motifs. It will be interesting to study the interaction of these CaM-like proteins with KCBP.

In conclusion, our results demonstrate that the AtCaM isoforms bind KCBP. However, CaM2 showed 2-fold higher affinity to KCBP as compared with CaM4 and 6, suggesting that CaM isoforms differ significantly in their interaction with target proteins. This is the first study where interaction of plant CaM isoforms has been investigated with a target protein from the same species. It would be interesting to examine the interaction of AtCaM isoforms with other CaM-regulated target proteins from *Arabidopsis*, but only a few CaM-binding proteins have been identified and characterized from this system (4). Further elucidation of precise roles of each CaM isoform in regulating CaM target proteins would require *in vivo* interaction studies with CaM isoforms and other CaM target proteins. Future studies using the yeast two-hybrid system or fluorescence resonance energy transfer (45) will help to assess *in vivo* differences in CaM-target protein interactions that are predicted from the results of *in vitro* experiments such as those reported here.

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