Kinetic and Calcium-Binding Properties of Three Calcium-Dependent Protein Kinase Isoenzymes from Soybean†

Jung-Youn Lee,‡ Byung-Chun Yoo,‡ and Alice C. Harmon*

Program in Plant Molecular and Cellular Biology and the Department of Botany, University of Florida, P.O. Box 118526, Gainesville, Florida 32611-8526

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ABSTRACT: Calmodulin-like domain protein kinases (CDPKs) are a family of calcium- but not calmodulin-dependent protein kinases found in a wide variety of plants and in protists. CDPKs are encoded by large multigene families, and to assess whether family members play distinct or redundant roles in vivo, we characterized soybean CDPK isoforms α, β, and γ, which share 60−80% identity in amino acid sequence. RNA blot analysis showed that the three CDPKs were expressed in most plant tissues examined and in suspension-cultured soybean cells. Recombinant CDPKα, -β, and -γ phosphorylated peptide substrates containing the four-residue motif R/K-X-X-S/T, but CDPKα was the most selective for residues outside of the motif. The CDPKs were inhibited by the general protein kinase inhibitors K252a and staurosporine and by calphostin C, which is an inhibitor of protein kinase C. The calcium-binding properties of each CDPK were distinct. The K_d's for Ca^{2+} determined by flow dialysis in the absence of substrates were 51, 1.4, and 1.6 μM for CDPKα, -β, and -γ, respectively. In the presence of the peptide substrate syntide-2 the K_d of CDPKα decreased to 0.6 μM. Also, the sensitivity of this isoenzyme's activity to calcium varied with protein substrate. The concentrations of Ca^{2+} required for half-maximal activity ($K_{0.5}$) for each CDPK with syntide-2 as substrate were 0.06, 0.4, and 1 μM, respectively. These results show that members of the CDPK family differ in biochemical properties and support the hypothesis that each isoform may have a distinct role in calcium signal transduction.

CDPKs[1] (Ca^{2+}-dependent protein kinases or calmodulin-like domain protein kinases) are a large family of protein kinases regulated by calcium but not by calmodulin (*1*). CDPK was first described (*2*), highly purified (*3*), and cloned (*4*) from soybean. The catalytic domain of CDPK isoenzyme α is most closely related to that of CaMKII, and its C-terminal regulatory domain, like that of calmodulin, has four EF-hands (Ca^{2+}-binding motifs). CDPKs also contain a short junction domain that links the catalytic and calmodulin-like domains and functions as an autoinhibitor (*5*−*7*). Numerous DNA sequences have been reported for CDPKs from plants (8−16) and the protist *Plasmodium falciparum* (*17, 18*). These CDPKs vary in length, but all contain catalytic, junction, and calmodulin-like domains. The amino acid sequences of the conserved domains range from 50% to 95% in identity.

Recently, two classes of protein kinases have been described that have catalytic domains related to those of CDPKs but differ in the regulatory domains. The CDPK-related protein kinases from carrot (*19*) and maize (*20*) have no functional EF-hands in their carboxyl terminal domains. CCaMK from lily anthers has a regulatory domain that contains three predicted EF-hands and is more similar to visinin than to calmodulin (*21*). Its activity, unlike that of CDPKs, is stimulated by calmodulin (*21, 22*).

While knowledge of the genes encoding the CDPK family of plant protein kinases is growing, information regarding the physiological roles of these enzymes is limited. It is likely that calcium-sensitive CDPKs are involved in mediating many of the diverse responses of plant cells that depend on changes in intracellular free Ca^{2+} concentrations. Evidence is accumulating for involvement of CDPKs in regulation of metabolism and gene expression (*23*−*25*), but with the exception of one study (*25*), specific roles for individual isoenzymes have not been identified.

Since differences in biochemical properties, Ca^{2+} sensitivity, and substrate specificity could contribute to specific roles for the CDPK isozymes, and because little information regarding the properties of CDPK isoenzymes from a single plant species is available, we undertook characterization of three recombinant soybean CDPK isoforms, CDPKα (*4*), -β, and -γ (*26*). These CDPKs are 508, 490, and 538 residues in length, respectively. CDPKβ and CDPKγ are 76% and

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* To whom correspondence should be addressed. Telephone: 352-392-3217. Fax: 352-392-3993. E-mail: harmon@botany.ufl.edu.

‡ Current address: Section of Plant Biology, University of California, Davis, CA 95616.

[1] Abbreviations: CDPK, calcium-dependent protein kinase, or calmodulin-like domain protein kinase; CaMK, calcium/calmodulin-dependent protein kinase; PKA, protein kinase A; PKC, protein kinase C; MLCK, myosin light chain kinase; CCaMK, chimeric calcium/calmodulin-dependent protein kinase; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethyl sulfoxide; IPTG, isopropyl β-D-thiogalactopyranoside.

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58% identical, respectively, to CDPKα. The most notable differences in the primary structures of the three kinases are that CDPKγ has the longest (83 residues) and most basic amino-terminal domain, and it has eight extra amino acids between EF-hands III and IV in the calmodulin-like domain. The results reported here show that the three CDPKs differ in kinetic properties, substrate preferences, and Ca^{2+}-binding properties. These observations suggest that these CDPKs may play distinct roles in the cell.

EXPERIMENTAL PROCEDURES

Materials. Membranes (Hybond-N^+ and -N) for plaque and RNA blotting were purchased from Amersham. Syntide-2 and oligonucleotides were synthesized by the Protein Chemistry and DNA Synthesis Core Laboratories, University of Florida. Autocamtide and skeletal and smooth muscle myosin light chain kinase substrates were purchased from BACHEM California. Protein kinase inhibitors were purchased from Calbiochem (H8, ML7, KN62, and staurosporine), Kamiya Biochemical Company (calphostin C, and K-252a), and Seikagaku America, Inc. (KN93). Analytical standards of 100 mM $CaCl_2$ and 1 M $MgCl_2$ were purchased from Orion and Fluka, respectively. Solutions of $^{45}CaCl_2$ (29.6 Ci/g, 10 mCi/mL) and [γ-^{32}P]ATP were supplied by DuPont NEN. Spectra/Por Macrodialyzer and dialysis tubing (molecular cutoff ~3500) for flow dialysis were obtained from Spectrum Co. Chelex 100 was purchased from Bio-Rad. Fluo-3 pentapotassium salt was from Molecular Probes. All other chemicals were reagent grade or higher.

Plant Materials. Soybean cell suspension cultures (*Glycine max* L.) were maintained and prepared as described previously (*6*). Soybean seeds (*G. max* L. cv. Cobb) were imbibed for 4 h, disinfected in 10% Clorox for 10 min, rinsed several times with water, sown in perlite, and grown for 3 weeks in a greenhouse.

RNA Analysis. Total RNA was isolated from soybean cell suspension cultures, 8-day-old seedlings, and 3-week-old plants as described elsewhere (*27*). RNA was blotted onto nitrocellulose membranes by downward alkaline capillary transfer (*28*), and hybridization was performed by following protocols recommended by the membrane supplier in 50% formamide at 42 °C. Specific DNA probes for each isoform (the 5′ untranslated regions from the cDNA clones encoding CDPKα and -γ and the 3′ untranslated region from cDNA clone encoding CDPKβ) were generated using PCR. After hybridization, the blots were briefly washed several times in 2× SSC and 0.1% (w/v) SDS at room temperature, twice for 10 min each in the same buffer, and twice in 1× SSC and 0.1% (w/v) SDS at 42 °C for 15 min. Due to high background, the blots hybridized with the CDPKβ-specific probe were washed further in 0.1× SSC and 0.1% (w/v) SDS at 65 °C for 10 min.

Construction of Plasmids. The vector pGEX-KG (*29*) was used to express GST fusion proteins of full-length CDPKα, -β, and -γ and an N-terminal deletion mutant of CDPKγ containing amino acid residues 66−538, CDPKγ(66−538) in *Escherichia coli*. pGST−CDPKα contained the full-length cDNA insert cut from pHis1530 (*6*). The 5′-end of the cDNA encoding CDPKβ was amplified by PCR using 5′-GCTCTAGACCATATGCAGAAGCATGGT-3′ and 5′-GCCTTGTATCTGGACAACG-3′ as primers. The amplified DNA was digested with *Xba*I and *Hin*dIII and subcloned into *Xba*I-digested pGEX-KG together with an *Xba*I/*Hin*dIII double-digested cDNA clone of CDPKβ in pBluescript. For full-length CDPKγ, the 5′-end of the cDNA clone encoding CDPKγ was amplified by PCR with the primers 5′-GCTCTAGACCATATGGTTACAGACATGCT-3′ and 5′-GGAATTCTTAAAGTGTGTGGAACTGCT-3′. The fragment was digested with *Xba*I and *Sph*I, ligated to the *Sph*I-digested full-length cDNA clone of CDPKγ, and then digested with *Xba*I. The resulting fragment was subcloned in *Xba*I-digested pGEX-KG. To make the N-terminal deletion mutant of CDPKγ, DNA amplified using the primers 5′-GCTCTAGACCATATGGGTGTTAGGCAAGAC-3′ and 5′-TAATCCATGGGTGCTCAA-3′ was digested with *Sst*I and *Xba*I and subcloned into *Xba*I-digested pGEX-KG together with an *Sst*I/*Xba*I double-digested cDNA clone of CDPKγ. Constructs expressing active recombinant proteins were selected and confirmed to be error-free by DNA sequencing.

Expression and Purification of GST Fusion Proteins. A colony of transformed *E. coli* cells (PR745) was grown overnight at 37 °C in 2 mL of LB/ampicillin (0.1 mg/mL), transferred to 1 L of M9TB/ampicillin (*30*), and further cultured until the OD_{600} was about 0.5. Expression of recombinant protein was induced by adding IPTG (0.4 mM) at room temperature.

Cells expressing CDPKβ were collected, resuspended in lysis buffer containing 1 mM PMSF, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM DTT, 50 mM Tris, pH 7.5, and 150 mM NaCl, and centrifuged after sonication (*7*). The supernatant was loaded onto a glutathione−agarose column equilibrated with 50 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM DTT and washed with the same buffer. Protein was eluted with 50 mM Tris, pH 8.0, 1 mM DTT, and 10 mM glutathione. Fractions containing kinase activity were pooled and loaded onto Mono-Q equilibrated in 20 mM Tris, pH 7.2, 1 mM $CaCl_2$, 2% (w/v) betaine, and 1 mM DTT. CDPKβ was eluted with a gradient of 0−0.5 M NaCl in equilibration buffer. Fractions containing kinase activity were pooled and further purified by chromatography on Mono-Q in the presence of 2.5 mM EDTA instead of calcium.

The procedure for purification of CDPKα, -γ, and -γ(66−538) was as follows. Cell lysis and affinity chromatography were performed in buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, and 2 mM DTT). The debris from the lysed cells was pelleted by centrifugation, and the supernatant was loaded onto a glutathione−agarose column. The column was washed with buffer A, and protein was eluted with buffer B (50 mM Tris, pH 8.0, 10 mM glutathione, 10 mM EDTA, and 2 mM DTT). Fractions containing kinase activity were pooled and loaded onto a column of Mono-Q equilibrated in buffer C (20 mM Tris, pH 8.0, 2.5 mM EDTA, 5% (w/v) betaine, and 2 mM DTT). Enzymes were eluted with a gradient of 0 to 0.5 M KCl in buffer C. Purified recombinant CDPKα and -β were dialyzed against 20 mM Tris, pH 8.0, and 14.4 mM 2-mercaptoethanol and stored at −80 °C in 50% glycerol (v/v). CDPKγ was labile to freeze/thaw, so eluted fractions with the highest purity were stored at 4 °C.

Determination of Kinetic Parameters. Enzyme activity assays were performed by a modification of the procedure

described previously (*6*). The reaction mixture contained 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA, 1.1 mM $CaCl_2$, 2 mM DTT, 0.1 mg/mL BSA, the indicated amount of substrates, 5 nM recombinant CDPK, and 60 μM [γ-^{32}P]ATP (500 cpm/pmol). The concentrations of synthetic peptides (syntide-2, autocamtide, and skeletal and smooth muscle myosin light chain kinase substrates) and histone IIIS in the assays were 100 μM and 0.5 mg/mL, respectively. Kinetic parameters (apparent K_m and V_{max}) were determined from double-reciprocal plots. Concentrations of synthetic peptides were determined from amino acid composition analyses carried out by the Protein Chemistry Core Facility, University of Florida. Each kinetic parameter was determined by averaging at least four independent assay results.

Determination of IC_{50} Values with Protein Kinase Inhibitors. Stocks of protein kinase inhibitors (1 mg/mL) were prepared in DMSO or water as recommended by the suppliers. For inhibitors dissolved in DMSO, assays were performed in a final concentration of DMSO of 0.01% (v/v). The concentration of syntide-2 was 100 μM. Values of IC_{50} were determined from plots of the mean of enzyme activities from at least four determinations versus inhibitor concentration.

Removal of Contaminating Calcium. Glass containers were avoided in the experiments involving calcium binding measurements. Plastic bottles were filled with deionized water and autoclaved. Small containers and graduated cylinders were soaked in 0.1 M HCl and rinsed thoroughly with deionized water. Water and solutions used for flow dialysis were decalcified by passage through a Chelex-100 column (*31*). The membranes for the flow dialysis were stored in 10 mM EGTA at 4 °C and thoroughly rinsed with deionized water followed by final washes in Chelex-treated water prior to flow dialysis. Stock solutions of enzymes were dialyzed in Chelex-treated buffer A (50 mM HEPES, pH 7.5, and 100 mM KCl) in the presence or absence of 14.4 mM 2-mercaptoethanol at 4 °C overnight and passed through a Chelex column equilibrated with buffer A (*32*). Following the Chelex treatment, the protein samples were concentrated using a centrifugal concentrator (Centricon-10, Amicon) which was prewashed with Chelex-treated buffer A.

Free Calcium Measurements. Contaminating Ca^{2+} in water, buffers, and protein samples was measured using the fluorescent Ca^{2+} indicator Fluo-3 (33), as described elsewhere (*34*, *35*). Fluorescence was measured with a Perkin-Elmer LS5 spectrofluorometer. Calcium calibration buffers from Molecular Probes were used for generating a standard curve following the recommendations of the supplier. Fluo-3 (0.5−5 μM) was added to a 2-mL sample for the measurement of free calcium. A fluorometer cuvette was soaked in 0.1 M HCl for at least 30 min and rinsed thoroughly with deionized water before the fluorescence measurement. Free calcium concentrations in Ca^{2+}/EGTA buffers containing <1 μM free calcium were verified also by using Fluo-3. The contaminating calcium concentration in Chelex-treated flow dialysis buffer (50 mM HEPES, pH 7.5, and 100 mM KCl) and protein solutions was less than 0.3 μM according to the fluorescence measurement with Fluo-3 and atomic absorption spectrometry. The residual bound Ca^{2+} in CDPK samples after passage through a Chelex-100 column was less than 0.3 mole of Ca^{2+} per mole of enzyme. Concentrations of various $CaCl_2$ stock solutions that were made by diluting 100 mM $CaCl_2$ in Chelex-treated water and kinase assay buffers containing >1 μM free calcium were confirmed by atomic absorption spectrometry.

Calcium Binding Studies. The calcium binding properties of CDPK isoenzymes were studied using flow dialysis method (*36*−*38*). The experiments were performed using a dialysis apparatus (Spectra/Por Macrodialyzer) at room temperature (24 ± 2 °C). The apparatus consisted of two dialysis cells that were separated by a dialysis membrane. The upper cell chamber contained 7−30 μM metal-free protein sample in 1 mL of buffer A (50 mM HEPES, pH 7.5, and 100 mM KCl), and the lower chamber was filled with 1 mL of buffer A and was continuously pumped to the effluent collector at a flow rate of 3 mL/min. Enzymes used for the calcium binding studies were purified as described above except that betaine was omitted from the buffers for the anion-exchange chromatography.

The Ca^{2+} titration was initiated by adding an aliquot of 1.5−7.5 μM $^{45}CaCl_2$ to the protein sample in the upper chamber and continued by adding 1.25−4 μL of different $CaCl_2$ stock solutions in each cycle. The final chase-out was carried out with 4 mM $CaCl_2$. The effluent was collected in 1.5-mL fractions in microcentrifuge tubes every 30 s. Each was mixed with 2 mL of SintiVerse (Fisher) for the radioactivity measurement in a scintillation counter (Beckman). Steady states of radioactivity were reached at the third fraction of each cycle. The average counts from the last two fractions (third and fourth) of each cycle were taken to calculate the free calcium concentration from the known initial calcium concentration. The moles of bound calcium per mole of protein was calculated by combining the concentration of bound calcium and protein concentration as described (*36*). Control experiments in the absence of protein sample confirmed that a steady state was reached within 1.5 min and showed that the loss of $^{45}CaCl_2$ by diffusion during flow dialysis through the membrane was negligible (∼5%).

The calcium binding data were processed according to the Hill model (*39*, *40*) using KaleidaGraph. The Hill model provides information regarding the degree of cooperativity (Hill constant, α), the maximum number of Ca^{2+} binding sites (n), and the apparent dissociation constant (K_d) according to the equation

$$r = \frac{nx^{\alpha}}{K_d{}^{\alpha} + x^{\alpha}}$$

where r and x denote the average number of moles of Ca^{2+} bound per mole of protein and the free Ca^{2+} concentration, respectively (*36*, *40*, *41*).

Protein Concentration Determination. Protein concentrations were determined by the BioRad dye-binding assay (*42*) using bovine serum albumin as a standard or by absorbance at 280 nm using extinction coefficients calculated for each isoenzyme (*43*). Protein concentrations determined by either method were in good agreement.

Effects of Calcium on Enzyme Activity and Autophosphorylation. Kinase assays were performed in the presence or absence of calcium as described above, with the following changes. The assays were carried out in a buffer containing 50 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 2 mM

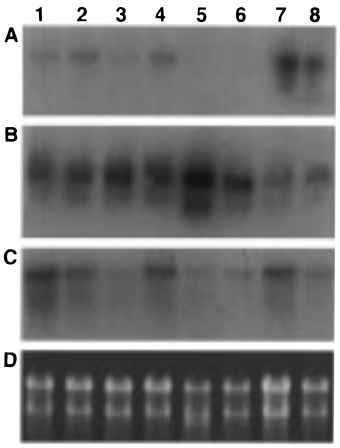

FIGURE 1: RNA blot hybridization analyses of the three soybean CDPKs. Total RNA (10 μg) from 3-week-old soybean plants (lanes 1−5), 8-day-old seedlings, (lane 6), and soybean cell cultures (lanes 7−8) was fractionated in a formaldehyde−agarose gel, blotted, and hybridized with specific probes for CDPKα (A), CDPKβ (B), and CDPKγ (C) as described in Experimental Procedures. The ethidium bromide stained gel (D) demonstrates that equal amounts of RNA were loaded in each lane. Samples: lane 1, root; lane 2, stem; lane 3, shoot tip; lane 4, petiole; lane 5, leaf; lane 6, whole seedling; lane 7, 3-day-old cell culture; lane 8, 7-day-old cell culture. The relative amounts of each CDPK mRNA were determined by analysis of the digitized image by the computer program NIH Image 1.61. The values were adjusted for RNA loading, and the highest amount of each mRNA was assigned a value of 1.00. The relative amounts in lanes 1−8 were as follows: CDPKα, 0.35, 0.40, 0.17, 0.35, 0.33, 0.02, 0.99, 1.00; CDPKβ, 0.41, 0.35, 0.46, 0.46, 1.00, 0.55, 0.15, 0.21; CDPKγ, 1.00, 0.48, 0.25, 0.40, 0.19, 0.12, 0.35, 0.24.

DTT, 60 μM [γ-^{32}P]ATP (about 500 cpm /pmol), 100 μM syntide-2, and 10 ng of each isoenzyme at 24 ± 2 °C for 6 min in the presence of free calcium at varying concentrations from 0.1 to 100 μM or in the absence of calcium (1 mM EGTA). Ca^{2+}/EGTA buffers containing 0.1−1 μM free calcium in the presence of 0.2 mM free EGTA were prepared according to Tsien and Pozzan (*44*). Assay buffers containing free calcium from 5 to 100 μM were made by direct dilution from 0.1 M $CaCl_2$ standard solution (Orion) as recommended by Bers et al. (*45*). Activity assays of CDPKα using protein substrates were performed in the same assay mixture except that 2 μg/mL enzyme was used and 0.5 mg/mL histone IIIS or 0.2 mg/mL recombinant soybean serine acetyltransferase (SAT) was substituted for peptide substrates. Autophosphorylation of each isoenzyme was performed with 2.5 μg of CDPK in 25 μL of assay buffer for 15 min at room temperature.

RESULTS

RNA Expression Pattern of CDPK Isoforms. As a step toward understanding the roles of CDPK family members in soybean, the expression pattern of mRNA encoding each isoform was determined. Blots of total RNA from different organs of soybean plants or from suspension-cultured cells were hybridized to DNA probes specific for genes encoding each of the CDPK isoenzymes (Figure 1). Transcripts of CDPKα, -β, and -γ were expressed in roots, leaves, petioles, stems, and shoot tips of 3-week-old plants, in 8-day-old seedlings, and in suspension cell cultures of different ages. However, the relative distribution of each transcript differed, as indicated in the caption of Figure 1. CDPKα mRNA was highest in cell culture and lowest in seedlings and shoot tips. CDPKβ, which was originally isolated from a plumule library, was highest in leaves and lowest in cell culture. CDPKγ, which was originally isolated from a library made from tissues enriched in nitrogen-fixing root nodules, was most highly expressed in roots and was lowest in leaves and seedling.

Expression of CDPK Fusion Proteins in E. coli and Purification. The three full-length enzymes and an amino-terminal deletion mutant of CDPKγ, CDPKγ(66−538), were expressed as glutathione *S*-transferase (GST) fusion proteins. Recombinant enzymes were highly purified by affinity chromatography on glutathione−agarose and anion-exchange chromatography (data not shown). The activities of CDPKα and -β were stable in the absence of reducing agents and at low ionic strength, and these enzymes could be stored in 50% (v/v) glycerol at −80 °C without appreciable loss of activity. In contrast, CDPKγ and CDPKγ(66−538) required reducing agents and ≥300 mM KCl or NaCl during dialysis and could not be frozen without almost complete loss of activity. These enzymes were also expressed as His_6 fusion proteins, but because His_6−CDPKγ required high ionic strength to maintain solubility and activity, further purification of this protein by anion-exchange chromatography was not possible. In addition, His_6−CDPKγ(66−538) bound to phenyl-Sepharose at low ionic strength in the presence of Ca^{2+}, but it could be eluted only by 6 M urea.

Substrate Specificities and Kinetic Parameters. The activities of soybean CDPKs with various synthetic peptides and histone IIIS were determined in the presence or absence of Ca^{2+} (Table 1). Histone IIIS, a good substrate for CDPK purified from soybean cell culture (*3*), was phosphorylated by all three isozymes, but CDPKα was 10-fold less active than the other isoenzymes with this substrate (Table 1). Other proteins such as casein and bovine serum albumin were not phosphorylated by any of the recombinant isoenzymes (data not shown).

Substrate peptides containing the motif basic-X-X-Ser/Thr, in which the basic residue is arginine or lysine, X is any residue, and serine or threonine is the phosphorylated residue, are good substrates for CDPKs (*1*). The specific activities of the soybean CDPKs with 100 μM of each of four peptides containing this motif, syntide-2, autocamtide, and substrate peptides of skeletal and smooth muscle myosin light chain kinases, were compared (Table 1). Phosphorylation of the peptide substrates by CDPKα and CDPKβ with these substrates was stimulated 22−30-fold by Ca^{2+}, whereas the activities of CDPKγ and the amino-terminal deletion mutant CDPKγ(66−538) were stimulated 52−480-fold. There was little difference in the activities of CDPKγ and CDPKγ-(66−538), showing that the N-terminal 65 residues of this isoenzyme are not required for catalytic activity. All four peptides were good substrates for CDPKγ and CDPKγ(66−538), and the maximal activity of these enzymes with each peptide varied <1.4-fold. The maximal activities of CDPKα and CDPKβ varied over wider ranges, 12- and 3.6-fold, respectively. While syntide-2 and autocamtide were good substrates for both CDPKα and CDPKβ, MLCK substrate peptides were good substrates for CDPKβ, but not for CDPKα. These results show that the CDPK isoenzymes exhibit substrate selectivity with both protein (histone IIIS) and peptide substrates.

Table 1: Activity of CDPK Isoforms with Various Substrates[a]

enzyme	Ca^{2+}	histone IIIS	syntide-2	autocamtide	MLCKsk	MLCKsm
CDPKα	+	0.045	1.67	0.81	0.15	0.14
	−	nd[b]	0.075	0.025	0.005	0.005
CDPK*β*	+	0.45	3.55	2.41	0.98	1.82
	−	0.02	0.14	0.07	0.03	0.08
CDPK*γ*	+	0.25	1.86	1.82	1.92	1.57
	−	nd[b]	0.03	0.01	0.004	0.03
$\gamma_{(66-538)}$	+	0.28	1.89	2.12	1.65	1.56
	−	nd[b]	0.02	0.008	0.007	0.008

[a] Enzyme activities (μmol min^{-1} mg^{-1}) were measured as described in Experimental Procedures with 0.5 mg/mL histone IIIS or 100 μM synthetic peptide in the presence of 1 mM EGTA ($-Ca^{2+}$) or 1 mM EGTA plus 1.1 mM Ca^{2+} ($+Ca^{2+}$). The standard error of each mean value of enzyme activities was less than 10% of the tabulated mean value. [b] nd, not detectable.

Table 2: Kinetic Parameters of CDPK Isoforms with Two Peptide Substrates

	syntide-2			MLCKsk		
enzyme	V_{max}	K_m	$\frac{V_{max}}{K_m}$	V_{max}	K_m	$\frac{V_{max}}{K_m}$
CDPKα	2.4 ± 0.3	18.2 ± 1.5	0.13	5.7 ± 2.5	3700 ± 1900	0.001
CDPK*β*	5.5 ± 0.7	34.2 ± 1.0	0.16	3.8 ± 0.8	282 ± 46	0.013
CDPK*γ*	2.5 ± 0.1	16.6 ± 0.7	0.15	2.0 ± 0.1	27 ± 0.8	0.074

[a] Values of apparent V_{max} (μmol min^{-1} mg^{-1}) and K_m (μM) were determined from double-reciprocal plots.

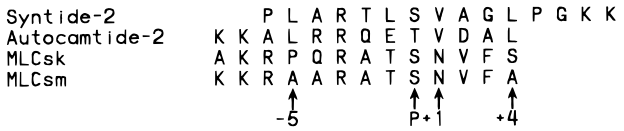

FIGURE 2: Sequences of substrate peptides. Residues are numbered relative to the phosphorylated residue at position P. Residues amino-terminal to P have negative numbers, and those carboxyl-terminal to P have positive numbers.

To examine further the difference in substrate preference, we determined the kinetic parameters for the CDPKs with two of the peptide substrates (Table 2). When syntide-2 was the substrate, values of apparent K_m and V_{max} for each CDPK varied by only 2-fold. Comparison of the ratio of V_{max} to K_m, which is an indicator of catalytic efficiency, shows that syntide-2 is an equally good substrate for all three isoenzymes. In contrast, skeletal muscle myosin light chain kinase peptide is a good substrate for only CDPK*γ*. Values of apparent V_{max} varied only 2−3-fold, while values of apparent K_m varied 10−140-fold. Comparison of the ratios of V_{max} to K_m shows that CDPKα has a strong preference for syntide-2, while CDPK*β* has some preference and CDPK*γ* has only a slight preference for this substrate. Syntide-2 and autocamtide contain aliphatic amino acid residues at positions P−5, P+1, and P+4, whereas the MLCK peptides do not (Figure 2). One or more of these residues may be determinants for phosphorylation of a site by CDPKα.

pH Optima. The effect of pH 6−10 on phosphorylation of syntide-2 by the three CDPKs was determined (data not shown). The activity of each isoenzyme was maximal between pH 7 and 8, but CDPKα and -*β* were more tolerant of pH between 8 and 9. The broad pH optimum of CDPKα and -*β* is similar to that of CDPK purified from soybean (*3*).

Effect of Protein Kinase Inhibitors . The effect of several classes of protein kinase inhibitors on the activity of soybean CDPKs was determined. Control assays containing 0.01% (v/v) DMSO did not affect kinase activity. Staurosporine, an inhibitor of broad specificity that is suggested to interact with an essential region of the catalytic domain of protein kinases (*46*), inhibited the CDPKs with IC_{50}'s between 70 and 120 nM (Table 3). K-252a, another general inhibitor of protein kinase (*47*) which competes with ATP, inhibited the CDPKs, with IC_{50}'s between 300 and 800 nM (Table 3). The IC_{50}'s for both of these inhibitors were 1−2 orders of magnitude higher than those observed with PKC, PKA, or MLCK (*46*−*48*). KN62, a compound that inhibits CaMKII competitively with respect to calmodulin, did not inhibit the CDPKs significantly when added at a concentration (10 μM) that inhibits over 80% of CaMKII activity. Other inhibitors that had little or no effect on the CDPKs were 50 μM H8 (*49*), 30 μM KN93 (*50*), and 50 μM ML7 (*51*). Calphostin C, which is reported to specifically inhibit protein kinase C through interaction with its regulatory domain, inhibited all three CDPK isoforms (Table 3), but the IC_{50} values were 1−2 orders of magnitude greater than those for protein kinase C (*46*).

Table 3: Effect of Protein Kinase Inhibitors on CDPK Isoenzymes[a]

enzyme	staurosporine	K-252a	calphostin C
CDPKα	0.11	0.8	9.0
CDPK*β*	0.12	0.8	5.0
CDPK*γ*	0.07	0.3	1.6

[a] IC_{50} (μM) was determined from a plot of CDPK activity versus concentration of inhibitor.

Ca^{2+} Binding Properties of CDPKs. Binding of Ca^{2+} to CDPK isoenzymes in the absence of substrates or Mg^{2+} was measured by flow dialysis as shown in Figure 3 A-C. Binding of Ca^{2+} to CDPK*β*, but not to the other CDPKs, saturated in the concentration range tested. Nonspecific binding of Ca^{2+} at around 100 μM free Ca^{2+} was observed for all three enzymes. This may be attributed to the limitation of the flow dialysis method (*36*, *52*), and data for $[Ca^{2+}]_{free}$ >100 μM were ignored in the fitting of the data to the Hill model.

The values of the Hill parameters K_d, n (number of binding sites), and α (Hill constant) determined from the best-fit model for CDPK*β* were 1.4 μM, 3.2, and 1.6, respectively. Although CDPK*β* contains four predicted EF-hands, the 4-site Hill model gave a poorer fit for the data (Figure 3B). The value of α greater than 1 indicates that CDPK*β* binds Ca^{2+} with positive cooperativity.

Because full-length CDPK*γ* precipitated when dialyzed overnight against the buffer used for Ca^{2+} binding measurements, the N-terminal deletion mutant CDPK*γ*(66−538),

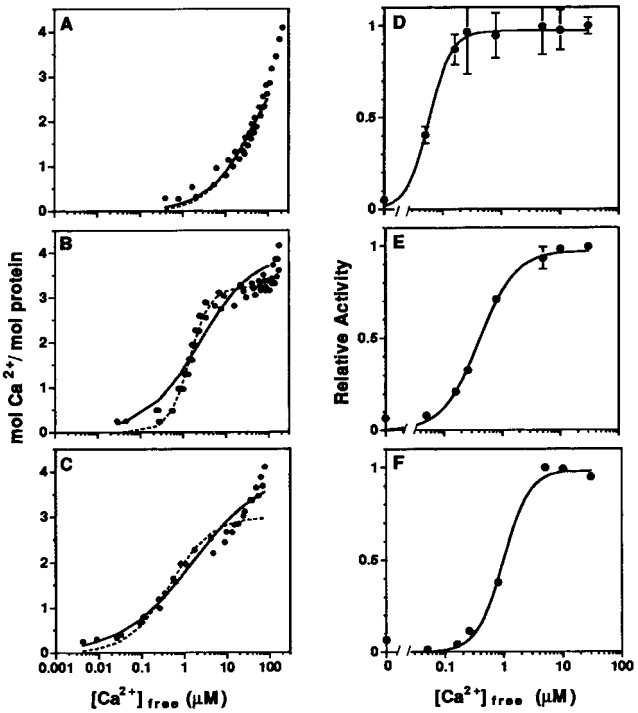

FIGURE 3: Ca^{2+} binding and activation of CDPKs. Direct Ca^{2+} binding (A−C) and the effect of Ca^{2+} on the activities (D−F) of CDPKα (A and D), CDPKβ (B and E), and CDPKγ (C and F) were determined as described in Experimental Procedures. For determination of Ca^{2+} binding by flow dialysis, the protein concentration was 20 μM in 50 mM HEPES, pH 7.5, containing 100 mM KCl at 24 ± 2 °C. For CDPKγ, 2 mM DTT was added for enzyme stability. Combined data from at least two determinations for each isoenzyme are shown. The data were fit to 3-site (dashed lines in panels A and C) and 4-site (solid lines) Hill models by the program KaleidaGraph. The dashed line in panel B is for a 3.2-site Hill model that gave the best fit of the data. Phosphorylation of syntide-2 was determined at various free Ca^{2+} concentrations that were controlled by calcium buffers or by the presence of 1 mM EGTA (indicated as zero Ca^{2+} on the abscissa). Data from three independent determinations for each isoenzyme are shown. Error bars indicate standard deviations.

which is more stable when freshly prepared, was used for the calcium binding measurements. These two enzymes did not differ in the dependence of their activities on various concentrations of Ca^{2+} (data not shown). The data were better fit by a 4-site rather than a 3-site binding model (Figure 3C). For the 4-site model the values of K_d and α were 1.6 μM and 0.5, respectively. The K_d was similar to that of CDPKβ, but in sharp contrast to CDPKβ the value of α was less than 1. This suggests that CDPKγ bound Ca^{2+} with negative cooperativity.

CDPKα bound Ca^{2+} with a K_d much higher than those of the other two isoenzymes. This CDPK is predicted to have four functional EF-hands, and the 4-site Hill model (R^2 = 0.94) gave only a slightly better fit than the 3-site model (R^2 = 0.91). For the 4-site model the values of K_d and α were 51 μM and 0.8, respectively. This value of K_d is similar to the value of 80 μM (n = 3.9) determined by equilibrium dialysis for PfCDPK (*53*).

Activation of CDPKs by Ca^{2+}. To examine how Ca^{2+} binding affects kinase activity, the kinase activity in the presence of various Ca^{2+} concentrations was determined (Figure 3, panels D−F). The Ca^{2+} concentrations for half-maximal activity ($K_{0.5}$) for CDPKβ and -γ were 0.4 and 1 μM, respectively. These values were lower than the K_d's, and comparison of the curves for Ca^{2+} activation and Ca^{2+} binding for these isoenzymes (Figure 3) suggested that binding of 1−2 calcium ions per molecule of enzyme is sufficient for significant activation. Another contributing factor could be that the presence of ATP and peptide substrate in the activity assay may affect the calcium binding properties of these isoenzymes, as is the case for CDPKα (see below).

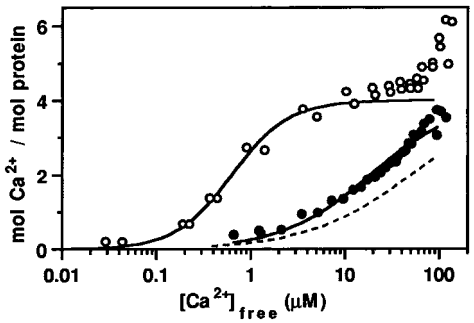

FIGURE 4: Binding of Ca^{2+} to CDPKα in the presence of substrates. Flow dialysis was performed to examine the effect of syntide-2 and/or ATP on the binding of Ca^{2+} by CDPKα. The sample in the upper chamber contained 7 μM CDPKα and 1.5 μM $^{45}CaCl_2$ in dialysis buffer (50 mM HEPES, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$) plus either 60 μM ATP (●) or 100 μM syntide-2 and 60 μM ATP (○). The curves drawn with solid lines were determined by fitting the data to a 4-site Hill model. The control experiment (- - -) performed in 50 mM HEPES, pH 7.5, and 100 mM KCl (Figure 3A) is shown for comparison.

The $K_{0.5}$ of CDPKα was 0.06 μM (Figure 3D). This value was 10-fold lower than the $K_{0.5}$'s of the other two enzymes and nearly 3 orders of magnitude lower than the K_d for Ca^{2+} of CDPKα (Figure 3A). Moreover, the saturation of kinase activity of CDPKα occurred at 0.3 μM free Ca^{2+}, which is far below the concentration at which 1 mole of calcium is bound per mole of CDPKα. The basis for the difference between K_d and $K_{0.5}$ was examined in experiments described below.

Effect of ATP and Peptide Substrate on K_d for Ca^{2+} of CDPKα. The composition of the enzyme mixtures used for the calcium binding and activity assays differed in the presence of DTT, Mg^{2+}, ATP, and peptide substrate. To examine whether interaction of CDPKα with these compounds was the basis for the great sensitivity of CDPKα to low Ca^{2+} concentration in activity assays, they were added alone or in combination to CDPKα, and Ca^{2+} binding was measured by flow dialysis as described in Experimental Procedures. $MgCl_2$ and DTT did not affect the Ca^{2+} binding of CDPKα (data not shown), and they were added to all experiments involving substrates. Addition of ATP decreased the K_d to 19 μM (Figure 4). An even more dramatic shift in K_d to 0.6 μM was observed when the peptide substrate syntide-2 and ATP were added (Figure 4) or when syntide-2 was added alone (data not shown). These results demonstrate that the activation of CDPKα by Ca^{2+} correlates well to the enzyme's binding of Ca^{2+} in the presence of substrates. In addition, because Ca^{2+} binding saturated in these conditions, the data showed that CDPKα binds 4 moles of Ca^{2+} per mole of protein with high affinity.

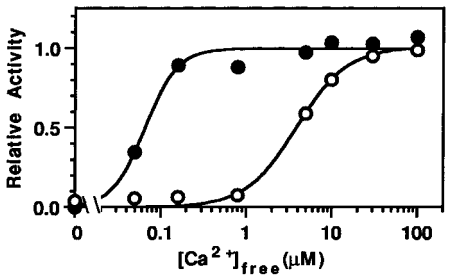

FIGURE 5: Effect of protein substrates on the calcium sensitivity of CDPKα. The activity of CDPKα was determined at various concentrations of free Ca^{2+} with either 0.2 mg/mL SAT (●) or 0.5 mg/mL histone IIIS (○) as a substrate.

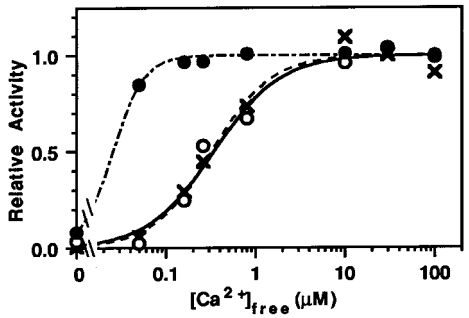

FIGURE 6: Effect of Ca^{2+} on autophosphorylation of CDPKs. Autophosphorylation of CDPKα (●), CDPKβ (○), and CDPKγ (×) in the presence of various concentrations of free calcium was determined as described in Experimental Procedures.

$K_{0.5}$ of CDPKα in the Presence of Protein Substrates. To ask whether protein substrates also affect the calcium sensitivity of the activity of CDPKα, assays were performed with the substrates histone IIIS and serine acetyltransferase (SAT). Histone IIIS is not a preferred substrate of CDPKα (Table 1), but phosphorylation occurred sufficiently well to detect the effect of Ca^{2+} on the kinase activity. The $K_{0.5}$ for histone IIIS phosphorylation by CDPKα was about 4 μM (Figure 5), which was 67-fold higher than that for syntide-2 phosphorylation ($K_{0.5} = 0.06$ μM). Saturation of histone IIIS phosphorylation occurred at ~30 μM free Ca^{2+}. When soybean SAT [a CDPK substrate identified by interaction cloning (B.-C. Yoo and A. C. Harmon, submitted)] was used as a protein substrate for CDPKα, the $K_{0.5}$ was 0.07 μM. These results show that phosphorylation of certain substrates can occur at very low concentrations of free calcium despite CDPKα's intrinsically low affinity for binding Ca^{2+}. In vivo the response of CDPKα to changes in free Ca^{2+} could be greatly influenced by the substrates that are present in the cell.

Autophosphorylation of CDPKs in Response to Ca^{2+}. The effect of Ca^{2+} on autophosphorylation of the soybean CDPKs was determined (Figure 6). The values of $K_{0.5}$ for CDPKα, -β, and -γ were 0.03, 0.3, and 0.3 μM, respectively. These values were similar to the respective values of $K_{0.5}$ (0.06, 0.4, and 1 μM) for phosphorylation of syntide by each isoenzyme. It should be noted that, in measurements of Ca^{2+} binding to CDPKα in the presence of Mg^{2+}·ATP, no high-affinity calcium binding site was observed (Figure 4). If the calcium contamination in the flow dialysis buffers was as high as the limit of detection (0.3 μM), then a site having a K_d below 0.1 μM would not have been observed in our experiments.

DISCUSSION

In animals, cellular responses to calcium are brought about (in part) by two families of protein kinases, protein kinase C (PKC) and the calmodulin-dependent protein kinases. Activation of conventional PKCs (isoforms α, β, and γ) is dependent upon phosphatidylserine, diacylglycerol, and the binding of Ca^{2+} to the C2 domain. Activation of the calmodulin-dependent protein kinases (phosphorylase kinase, myosin light chain kinases, and CaMKs I−IV) occurs through the binding of calcium to calmodulin. PKCs and several of the calmodulin-dependent kinases are multifunctional enzymes that have broad substrate specificity (*54*, *55*). They are widely distributed in various tissues and cell types, and they are activated by a variety of external signals. The work reported here indicates that CDPKs also have many of these characteristics. An important question under active investigation is how different stimuli are able to bring about specific responses while using calcium as the second messenger and a common set of calcium-regulated kinases. One answer suggested for PKC is that cellular signals direct the translocation and compartmentalization of specific isotypes and thus target them to the locations of substrates involved in specific physiological functions (*56*).

The CDPKs examined in this study are present in numerous parts of soybean plants grown in standard conditions and in suspension-cultured soybean cells. Some members of the CDPK family are expressed in a single tissue (*14*) or in a developing seed (*13*), while expression of others is induced by environmental stress (*9*), mechanical strain, or the hormone auxin (*15*). Since the expression patterns of the soybean CDPKs overlap, and since each CDPK is present in normal growth conditions (Figure 1), they may represent the constitutively expressed CDPKs, and it is possible that they play roles early in signal transduction pathways.

To ask whether the CDPKs have distinct or overlapping roles in vivo, we characterized the substrate specificity and the kinetic and calcium binding properties of the isoenzymes. All three phosphorylated peptides contain a basic-X-X-Ser motif, but each isoenzyme has different values of apparent K_m and V_{max} for each of the peptides. While CDPKγ phosphorylated four test peptides equally well, CDPKα preferentially phosphorylated syntide-2. Recently, Bachmann et al. (*57*) showed that serine-543 in nitrate reductase is phosphorylated by partially purified CDPK from spinach. Analysis of synthetic peptides in which residues surrounding the phosphorylation site were varied showed that the motif preferred by the spinach CDPK was hydrophobic-X-basic-X-X-Ser, where the hydrophobic residue at P−5 was leucine. These observations agree with our conclusion that the presence of a branched-chain aliphatic amino acid at one or more of the positions P−5, P+1, and P+4 is a determinant for phosphorylation by some CDPK isoenzymes. However, a systematic analysis of phosphorylation motifs is needed to define the importance of the hydrophobic residues for each isoenzyme.

The soybean CDPKs differed in their susceptibility to inhibition by the protein kinase inhibitors staurosporine, K-252a, and calphostin C, with CDPKγ being the most sensitive to inhibition by all three compounds (Table 3). The CDPKs were less sensitive than PKC to calphostin C and

less sensitive than PKA, PKC, MLCK, or CaMKII to the general protein kinase inhibitors. The IC_{50}'s for staurosporine and K-252a observed in our experiments are similar to the concentrations of these inhibitors needed for inhibition of various responses of plant cells to stimuli (*58*, *59*). Since the PKC inhibitor calphostin C and the calmodulin antagonists trifluoperazine (*60*) and W-7 (*2*) inhibit CDPKs, experiments using these compounds with plant cells must be interpreted with caution.

The three CDPKs differed dramatically in calcium binding properties in the absence of substrates. While the mean K_d's of CDPKβ and -γ were similar (1.4 and 1.6 μM, respectively), the shapes of the Ca^{2+} binding isotherms of these enzymes were distinctive (Figure 3). The values of K_d for these two isoenzymes are similar to the K_d's reported for calmodulin (1.5−5 μM) (*36*). In contrast, CDPKα had a much higher K_d of 51 μM, which is similar to the K_d (80 μM) reported for PfCDPK (*53*). The remarkable differences between the K_d's of these CDPKs is especially interesting in view of the degree of similarity in the sequences of the calcium binding domains of these enzymes. CDPKα and CDPKβ are 80% identical with each other, and each is about 55% identical with CDPKγ. CDPKα is only 32% identical with PfCDPK.

The effects of calcium on activities of the three CDPKs also differed. With syntide-2 as substrate, the $K_{0.5}$'s for CDPKβ and -γ were similar to each other but an order of magnitude greater than that for CDPKα. In addition, the value of $K_{0.5}$ for each CDPK was lower than its respective value of K_d. The most dramatic difference was the nearly 900-fold difference between the K_d and $K_{0.5}$ values of CDPKα. Inclusion of syntide-2 with CDPKα during Ca^{2+} binding measurements reduced its K_d 85-fold and showed that peptide substrate affected the calcium binding properties of the enzyme. The $K_{0.5}$ (15 μM) for CDPK from *P. falciparum* was also observed to be lower than the enzyme's K_d (80 μM) determined by equilibrium dialysis in the absence of substrates (*53*). Because 1 mole of Ca^{2+} was bound per mole of PfCDPK when the free calcium concentration was 15 μM, it was concluded that occupation of only one of the enzyme's four calcium binding sites was sufficient for activation. It is possible, however, that the calcium binding affinity of PfCDPK is increased in the presence of substrate, as is the case for soybean CDPKα.

The $K_{0.5}$ of CDPKα varied 55-fold with two protein substrates, histone IIIS and SAT (Figure 5). These results show that substrates affect the calcium sensitivity of CDPK to different degrees. The inducible Ca^{2+} affinity of CDPKα by certain substrates could serve as another mechanism controlling the CDPK activity in vivo, since such substrates could be phosphorylated at resting levels of free calcium, which have been measured in living plant cells to be 100−200 nM.

This striking shift of the K_d of CDPKα observed in the presence of syntide-2 resembles the change in the K_d of calmodulin induced by complex formation with its numerous target proteins or peptides such as MLCK, cyclic nucleotide phosphodiesterase (*61*), caldesmon, mastoparan (*62*), and calmodulin binding peptides derived from plasma membrane calcium pump (*63*) and calcineurin (*32*). The effect of syntide-2 on the K_d of CDPKα could be explained in two ways. Syntide-2 could interact directly with the calmodulin-like domain (CLD) of CDPKα, or it could act indirectly by promoting interaction between CLD and the enzyme's autoinhibitory domain. The autoinhibitory domain contains a pseudosubstrate site and a CLD binding site. The binding of Ca^{2+} to CLD is proposed to release the autoinhibitory domain from the catalytic domain by a mechanism that involves (in part) binding of the CLD to the autoinhibitory domain, thus activating the enzyme (*5*−*7*, *64*). If syntide-2 binds to the enzyme's active site in the absence of calcium, the autoinhibitory domain would be free to interact with CLD. Support for this mechanism comes from the observation that the activity of CDPKα with syntide-2 is significantly greater than zero in the absence of Ca^{2+} (Table 1). This observation shows that substrate peptides are able to bind to the active site in the absence of calcium.

Autophosphorylation and phosphorylation of peptide and protein substrates by CDPKs are clearly stimulated by calcium, but the concentrations of calcium required for stimulation of these activities are lower than the K_d's for Ca^{2+} measured in the presence of substrates. Several factors could individually or collectively contribute to these observations. Binding of Ca^{2+} to only one high-affinity site may be all that is required for activation. The detection limits of our procedures may have precluded detection of a binding site with a K_d below 0.1 μM. It is also possible that autophosphorylation that occurs at low concentrations of calcium affects the K_d and/or the $K_{0.5}$ for Ca^{2+}. While autophosphorylation does not cause the activity of CDPKs to become independent of calcium (unpublished observations), it could shift the K_d for Ca^{2+} downward. The low values of $K_{0.5}$ could result from a combination of substrate binding and autophosphorylation. Our observations suggest that regulation of CDPKs in vivo is more complicated than simple binding of Ca^{2+} and that factors such as the types of substrates present may be involved.

Our results demonstrate that three soybean CDPK isozymes differ in their RNA expression patterns, biochemical and kinetic properties, and Ca^{2+} binding properties. Although all three CDPKs phosphorylate peptides having the same core motif, each isoform differs in its selectivity for substrate peptides varying in sequence surrounding this motif. The CDPKs also differ in basal activity, fold-activation by Ca^{2+}, K_d, and $K_{0.5}$ for Ca^{2+}. These results support the hypothesis that CDPKα, -β, and -γ are multifunctional protein kinases that may play distinct roles in mediating responses to Ca^{2+} signals. Identification of the endogenous substrates of these protein kinases is in progress in our laboratory.

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