A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*

Ildoo Hwang*†, Heven Sze*, and Jeffrey F. Harper‡§

*Department of Cell Biology and Molecular Genetics, and Maryland Agricultural Experiment Station, University of Maryland, College Park, MD 20742; and ‡Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037

Edited by Roland Douce, University of Grenoble, Grenoble, France, and approved March 14, 2000 (received for review December 16, 1999)

The magnitude and duration of a cytosolic Ca^{2+} release can potentially be altered by changing the rate of Ca^{2+} efflux. In plant cells, Ca^{2+} efflux from the cytoplasm is mediated by H^+/Ca^{2+}-antiporters and two types of Ca^{2+}-ATPases. ACA2 was recently identified as a calmodulin-regulated Ca^{2+}-pump located in the endoplasmic reticulum. Here, we show that phosphorylation of its N-terminal regulatory domain by a Ca^{2+}-dependent protein kinase (CDPK isoform CPK1), inhibits both basal activity (≈10%) and calmodulin stimulation (≈75%), as shown by Ca^{2+}-transport assays with recombinant enzyme expressed in yeast. A CDPK phosphorylation site was mapped to Ser^{45} near a calmodulin binding site, using a fusion protein containing the N-terminal domain as an *in vitro* substrate for a recombinant CPK1. In a full-length enzyme, an Ala substitution for Ser^{45} (S45/A) completely blocked the observed CDPK inhibition of both basal and calmodulin-stimulated activities. An Asp substitution (S45/D) mimicked phosphoinhibition, indicating that a negative charge at this position is sufficient to account for phosphoinhibition. Interestingly, prior binding of calmodulin blocked phosphorylation. This suggests that, once ACA2 binds calmodulin, its activation state becomes resistant to phosphoinhibition. These results support the hypothesis that ACA2 activity is regulated as the balance between the initial kinetics of calmodulin stimulation and CDPK inhibition, providing an example in plants for a potential point of crosstalk between two different Ca^{2+}-signaling pathways.

Calcium is an important second messenger in plants (1, 2). Cytoplasmic Ca^{2+} spikes have been correlated with a variety of physiological stimuli such as cold, touch, light, pathogenic elicitors, and plant hormones. In principle, different messages can be encoded by changing a Ca^{2+} spike's magnitude, duration, location, or frequency (2, 3). Ca^{2+} spikes normally result from two opposing reactions, Ca^{2+} influx through channels and Ca^{2+} efflux through pumps and cotransport systems. Influx pathways have received considerable attention because their regulation is of primary importance to initiating a Ca^{2+} signal, whereas efflux is often viewed as a secondary "postsignaling" event that restores cytoplasmic $[Ca^{2+}]$ to resting levels. However, the fact that cells contain different types of Ca^{2+} pumps, which appear to be subject to regulation by different signaling pathways, suggests that efflux systems play a significant role in shaping the information content of a Ca^{2+} signal.

Plants and animals contain two distinct types of Ca^{2+} pumps, identified as type IIA and IIB, based on protein sequence identities (4). Type IIA pumps include SERCA or endoplasmic reticulum (ER)-type Ca^{2+}-ATPases. Type IIB pumps include the plasma membrane-type Ca^{2+}-ATPases (PMCAs) in animal cells and a subgroup of unique Ca^{2+} pumps recently identified in plants (5, 6). There are two features that distinguish the plant type IIB pumps from an animal PMCA. First, their regulatory domains lack significant homology to a PMCA and are located at the N-terminal instead of C-terminal end of the pump. Second, some plant isoforms have been found in non-plasma-membrane locations, such as ACA2 (ER) (7), and BCA1 (tonoplast) (5) and ACA1 (chloroplast) (8). Despite these differences, biochemical and genetic studies on ACA2 and BCA1 suggest a mechanism of autoinhibition and calmodulin activation that is analogous to a PMCA (6, 9, 10).

Of seven type IIB pumps currently identified in the model plant system *Arabidopsis* (11), ACA2 was the first to be functionally expressed in yeast and provides a model for understanding how these pumps are regulated (6). The N-terminal domain was shown to include an autoinhibitory region and a calmodulin-binding sequence. A truncated pump (ACA2-2) lacking N-terminal residues 2–80 was 4- to 10-fold more active than the full-length pump and unresponsive to further stimulation by calmodulin (i.e., "deregulated"). An inhibitory sequence was localized to a region within Val^{20}-Leu^{44}, as a peptide corresponding to this sequence lowered the V_{max} of the deregulated pump to values comparable with the basal activity of the full-length enzyme (10). A calmodulin-binding site was identified in an overlapping region between Val^{20} and Arg^{36}, as shown by binding studies with fusion proteins containing portions of the N-terminal domain (6). These studies support a model in which ACA2 is normally kept in an autoinhibited conformation and is activated when Ca^{2+} induces calmodulin to bind to the N-terminal domain. This binding of calmodulin somehow disrupts an inhibitory interaction within the pump, thereby resulting in a "release of inhibition."

Here, we report an investigation into the phosphoregulation of a plant type IIB Ca^{2+} pump. We show that a calcium-dependent protein kinase (CDPK) can inhibit the basal and calmodulin-stimulated activities of Ca^{2+} pump ACA2 through phosphorylation at position Ser^{45} in the N-terminal regulatory domain. CDPKs are multifunctional protein kinases present in plants and certain protozoans (12). They are directly activated by Ca^{2+} binding to EF hands located in their C-terminal regulatory domain. The phosphoinhibition of both ACA2's basal and calmodulin-stimulated activity makes this example of phosphoregulation distinct from that seen with PMCAs from animals. In the most analogous examples, phosphorylation of different PMCAs by a protein kinase C showed variable effects on calmodulin stimulation, but was never found to inhibit the basal activity (13, 14). The unique regulatory features identified here for ACA2 provide an example in plants of a potential point of

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ACA2, *Arabidopsis* (or autoinhibited) Ca^{2+}-ATPase isoform 2 from *Arabidopsis thaliana*; PMCA, plasma membrane-type Ca^{2+}-ATPases; CDPK, calcium-dependent protein kinase; ER, endoplasmic reticulum; GST, glutathione *S*-transferase.

†Present address: Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114.

§To whom reprint requests should be addressed. E-mail: harper@Scripps.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

crosstalk between two different Ca^{2+} signaling pathways (calmodulin and CDPKs), in this case providing a mechanism to control Ca^{2+} efflux through opposing inhibitory and stimulatory activities. Factors that shift this balance may alter the rate of Ca^{2+} efflux, and thereby alter the magnitude or duration of a Ca^{2+} signal in the microenvironment of the ER.

Materials and Methods

Bacterial and Yeast Hosts. ACA2 constructs were expressed in the *Saccharomyces cerevisiae* strain K616 (*MAT***a** *pmr1*::*HIS3 pmc1*::*TRP1 cnb1*::*LEU2, ura3*) (15). Yeast cells were grown in standard yeast extract/peptone/dextrose medium supplemented with 10 mM $CaCl_2$ for growth in the absence of a complementing pump. Yeast were transformed with the LiOAc/PEG method (16) and selected for uracil prototrophy by plating on synthetic complete medium minus uracil (SC-URA) (17). *Escherichia coli* strains BL21 (Invitrogen) or DH10α (a derivative of DH5; Stratagene) were used for propagation of plasmid constructs and bacterial expression of fusion proteins.

Plasmid Constructs. Standard PCR and subcloning procedures were used to modify ACA2 sequences in clones described below. All PCR-derived sequences were sequenced to ensure the absence of PCR mistakes. Most fusion protein constructs encoding portions of ACA2's N-terminal domain were produced as fusions to GST or maltose binding protein as previously described (6). A new construct, pACA2-N-S45/A, encodes an N-terminal fusion protein with a $Ser^{45} \rightarrow$ Ala (S45/A) and an $Asn^{43} \rightarrow$ Ile (N43/I) substitution, and was derived from pGC-1-52 by PCR mutagenesis.

All constructs for expression in yeast were made with the vector pYX112-TEV (6), which contains a *URA3* gene as a selection marker in yeast. pYX-ACA2-1 encodes a full-length ACA2, as previously described (6). This construct has two silent mutations in the coding region: CTT CG AAA CC TAT GAA GCC GCG GCG providing a *Bst*BI site for diagnostic purposes, and a *Sac*II site used for subcloning in the construction of the S45A and S45/D mutants. pYX-ACA2-3 and -4 are identical to pYX-ACA2-1 except that they encode S45/A and S45/D substitutions and different diagnostic restriction sites for S45/A (*Eae*I and Psp1406I) and S45/D (Psp1406I only). These mutations were made by PCR amplification of the N-terminal region by using mutagenic oligonucleotides S45/A [d(TGGATCCATCGCCGCGGCTTCATAACGTTTGGCCAGATT)] and S45/D [d(TGGATCCATCGCCGCGGCTTCATAACGTTTATCGAGATT)] and subcloning into the pYX-ACA2-1 to replace the sequence upstream of the introduced *Sac*II site.

Isolation of Yeast Membranes. Yeast membranes were isolated as previously described (10). Briefly, cells grown to an OD_{600} of 1–1.8 were homogenized by vortexing with glass beads in buffer containing 10% sucrose and a mixture of protease inhibitors. Membranes fractionated and collected at the interface of a 20/45% (wt/wt) sucrose step gradient were diluted and pelleted in a buffer with 10% (wt/wt) sucrose, 25 mM Hepes·BTP (pH 7.0), 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.1 mM TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone), 10 mM benzamidine, 5 μg/ml pepstatin, and 5 μg/ml leupeptin. The pellet was resuspended in 0.5 ml of the same buffer solution and stored at -80°C. Protein concentrations were determined with the Bio-Rad reagent.

Expression and Purification of CDPKs. Two recombinant versions of CDPK isoform CPK1 [KJM23–6H2 (18) and ΔNC-31 (19)] were used here to provide a constitutively active kinase that was Ca^{2+}-independent (CDPKci). Kinases were expressed in *E. coli* as previously described and purified by sequential purification for a C-terminal 6× His motif and an N-terminal GST (18). Proteins were concentrated by centrifugation in a Centricon-30 tube (Amicon) and stored at ≈60 ng/μl at -80°C in 50% glycerol/20 mM Tris·HCl (pH 7.5)/0.1 M NaCl/1 mM DTT. The kinase activities of purified enzymes were tested as previously described (18) using syntide-2 as a substrate. Enzymes used had specific activities >700 nmol/min per mg of protein in the absence of Ca^{2+}.

Ca^{2+} Transport. Calcium uptake into membrane vesicles from yeast was measured with $^{45}CaCl_2$ by the filtration method (10, 20). Briefly, standard reaction mixtures (final volume, 0.25 ml) contained 20–40 μl of vesicles (20–40 μg of protein), 200 mM sucrose, 25 mM Hepes·BTP (pH 7.0), 20 mM KCl, 0.1 mM NaN_3, 100 μM EGTA, 50 μM or 100 μM $^{45}CaCl_2$ (2 μCi/ml), 3 mM $MgCl_2$ with 1 μM bafilomycin A and 5 μM CCCP (carbonylcyanide *m*-chlorophenlyhydrazone) and were incubated for 10–15 min at room temperature. Transport was usually initiated by adding 3 mM ATP. Aliquots (0.23 ml) from duplicate reactions were filtered and washed with 2 ml of cold rinse solution [250 mM sucrose/2.5 mM Hepes·BTP (pH 7.0)/0.2 mM $CaCl_2$]. The $^{45}Ca^{2+}$ retained on the filters was determined by liquid scintillation counting. Ca^{2+}-pumping activity was calculated as vanadate-sensitive activity.

To measure Ca^{2+} uptake after phosphorylation, usually 460-μg aliquots of microsomal membranes (1.5–2 μg/μl) were incubated either without kinase (mock reaction) or with 1.8 μg of a purified CDPK (60 ng/μl) for 15 min at room temperature. The reaction mixture (450 μl) contained 20 mM Tris·HCl (pH.7.5), 5 mM $MgCl_2$, 0.6 mM ATP, and 1 mM DTT. $^{45}Ca^{2+}$ uptake into vesicles was started by adding 15 μl of the kinase or mock-treated membranes to a 235-μl Ca^{2+} transport reaction mixture containing 3 mM ATP.

Immunoblots. Membranes from yeast transformants were solubilized in an equal volume of a 2× Laemmli sample buffer, electrophoresed through an SDS/7.5% polyacrylamide gel, blotted onto Immobilon-P (Millipore), and probed with polyclonal antibody against ACA2 (6). The membrane was probed with goat anti-rabbit IgG (Calbiochem) conjugated to horseradish peroxidase. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce) and exposure to x-ray film. The relative levels of ACA2 proteins were quantitated by National Institutes of Health IMAGE software.

Results

N-Terminal Domain of ACA2 Can Be Phosphorylated at Ser^{45} by a CDPK. The N-terminal regulatory region of ACA2 contains many potential phosphorylation sites, several in close proximity to a calmodulin-binding sequence. To explore whether this region could be phosphorylated by a CDPK, we tested the ability of CPK-1 to phosphorylate a purified fusion protein (MC2-1-72) containing the first 72 residues of the N terminus. In a survey of over 100 potential substrates, the MC2-1-72 fusion protein was the most strongly phosphorylated by CPK-1 (not shown). This survey included a control fusion harboring a syntide-2 sequence. Because a syntide-2 sequence is known to be a good substrate for CPK-1 ($K_m \approx 50$ μM) (18), this comparison indicated that the N-terminal region of ACA2 contained a CDPK phosphorylation site.

To further define the location of this site, we tested a series of GST-fusion proteins as substrates (Fig. 1 *A* and *B*). Each fusion protein was progressively shorter, representing a deletion of sequence from the C terminus. A fusion protein containing residues 1–52 was a good substrate, whereas the next shorter fusion containing residues 1–44 was not. This suggested that a phosphorylation site was located in the vicinity of residues 44–52. This analysis was confirmed by a mass spectrometer study on a phosphorylated fusion protein (MC2-1-72) that identified

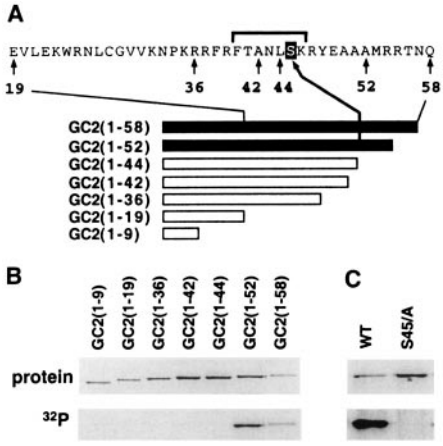

Fig. 1. N-terminal domain of ACA2–1 is phosphorylated by CDPK at Ser[45]. (*A*) Diagram of deletions used to map a CDPK phosphorylation site. The sequence is shown surrounding the phosphorylation sited mapped to Ser[45]. The solid black lines indicate fusion proteins that were phosphorylated (shown in *B*). The bracket above the sequence identifies the tryptic peptide detected as a phosphopeptide of mass 860.4 by mass spectrometry analysis. (*B*) Phosphorylation of fusion proteins showing GC2(1–52) as the smallest fusion labeled by ^{32}P. Row labeled ''Protein'': GST-fusion proteins corresponding to different lengths of the N-terminal domain are shown by Coomassie stain. Protein names indicate which ACA2 residues are present. For example, GC2(1–9) indicates GST fusion of Ca^{2+} pump ACA2 residues 1–9. All fusion proteins have an N-terminal GST, a fragment of ACA2, and a C-terminal GFP (green fluorescent protein) as described (6). Row labeled ''^{32}P'' shows the phosphorylation detected as ^{32}P-labeling during a kinase reaction. Purified fusion proteins (≈2 μg) were incubated with 90 ng of a CPK1 mutant, KJM23-6H2, and 50 μM ATP spiked with [γ-^{32}P]ATP for 60 min at 22°C in standard kinase reaction buffer without Ca^{2+}. The proteins were analyzed by SDS/PAGE and autoradiography. (*C*) A substitution of Ser[45] to Ala confirms the identification of a CPK phosphorylation site at Ser[45]. Rows correspond to markings in *B*. The protein marked WT (wild-type) contains the first 52 residues of the N terminus, GC2(1–52) from *A*. The protein marked S45/A is encoded by pACA2-N-S45/A, which is a derivative of GC2(1–52) with an Ala substitution for Ser[45]. Both proteins were subjected to a phosphorylation reaction as described in *A*. Proteins were then analyzed by SDS/PAGE and exposed to film. As a control to ensure that the kinase was active in all reactions, gels were overexposed to detect kinase autophosphorylation (not shown). Phosphorylation reactions were repeated two times with equivalent results.

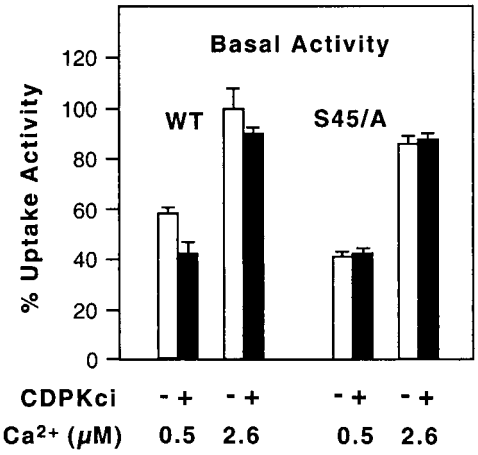

Fig. 2. Phosphorylation decreases the basal activity (without calmodulin) of wild-type ACA2, but not the mutant with an S45/A mutation. Membranes were isolated from yeast expressing a wild-type ACA2–1 (WT) or a mutant ACA2–3 (S45/A) harboring the S45/A substitution. Membranes were incubated with (+) or without (−) a Ca^{2+}-independent CPK1 mutant, ΔNC31, in the absence of Ca^{2+}. The transport assay contained 100 μM EGTA and 50 or 100 μM Ca^{2+} to give final [Ca^{2+}] of 0.5 or 2.6 μM, respectively. Activity was calculated as net vanadate-sensitive Ca^{2+} transport during the first 5 min and expressed as a percent of wild type (100% = 1.7 nmol/5 min per mg of membrane). The activity of the mutant was normalized to the equivalent amount of wild-type enzyme. Relative levels of wild type and mutant proteins were estimated by immunoblots. The average activity (±SD) from two independent experiments is shown.

the unique mass of a tryptic fragment FTANLS[45]K as being phosphorylated by CPK-1 (analysis provided by Perkin–Elmer). Because a two-dimensional phosphoamino acid analysis (21) indicated that the phosphorylated residue was a Ser (not shown), the phosphorylation site was narrowed down to Ser[45]. To verify this conclusion, Ser[45] was mutated to an Ala (S45/A) and shown to abolish the CPK1-phosphorylation of a fusion protein containing the first 52 N-terminal residues (Fig. 1*C*). Although a consensus phosphorylation site has not been identified for any specific CDPK, many of the sites appear have a hydrophobic residue in the minus 5 position, as seen here in the sequence FTANLS.

Phosphorylation by a CDPK Inhibits Basal Activity. To determine whether ACA2 could be activated by CDPK phosphorylation, as initially predicted by analogy to phosphoregulation of animal PMCAs (13, 14, 22), Ca^{2+} transport activity in microsomal membranes was assayed in the presence and absence of a constitutively active CPK1 mutant. The use of a constitutively active CPK1 was important because it allowed the kinase treatment to be completed in the absence of Ca^{2+}. The addition of Ca^{2+} was then used to start the transport assay. Pump activity was determined as vanadate-sensitive uptake of $^{45}Ca^{2+}$ in the presence of bafilomycin and CCCP, which eliminated any H^+-coupled Ca^{2+} antiport activity (10). Phosphorylation by CPK1 caused a modest but consistent (10–30%) decrease in basal activity, as measured by the accumulation of Ca^{2+} in vesicles in a 5-min transport reaction (Fig. 2). Importantly, this inhibition was not observed in the mutant pump ACA2–3 harboring an S45/A mutation. The ability of S45/A to block phosphoinhibition was confirmed by an analysis of initial uptake rates (first 40 s) (not shown). These results indicate that phosphorylation at Ser[45] inhibits basal activity of ACA2.

Phosphorylation by a CDPK Inhibits Calmodulin Activation. To determine whether CPK1 phosphorylation would also block calmodulin stimulation, we conducted similar transport assays in the presence of increasing amounts of calmodulin (Fig. 3). A preincubation of the pump with a constitutively active CPK-1 consistently reduced calmodulin stimulation at all concentrations of calmodulin tested. At 30 nM calmodulin (i.e., the K_{act}), phosphorylation reduced activation to a level only 25–35% that of an unphosphorylated control (i.e., ≈75% inhibition), as shown by three independent assays with two different enzyme preparations (not shown).

To determine whether phosphorylation of Ser[45] was responsible for the observed decrease in calmodulin stimulation, we evaluated the transport kinetics of a mutant pump harboring the S45/A mutation (ACA2–3) (Fig. 4). Like the wild-type enzyme, ACA2–3 (S45/A) was strongly stimulated by calmodulin (approximately 2-fold), although its V_{max} was slightly reduced compared with a wild-type enzyme when normalized per unit pump. However, in contrast to the wild-type enzyme, calmodulin stimulation of ACA2–3 (S45/A) was not inhibited by phosphorylation, as detected by transport assays in the presence of 0.5 μM calmodulin (Fig. 4). These results indicate that phosphorylation at Ser[45] blocks calmodulin stimulation of ACA2 activity.

Replacing Ser[45] with Asp[45] Mimics the Phosphorylated Enzyme. A mutant pump was engineered with an S45/D mutation

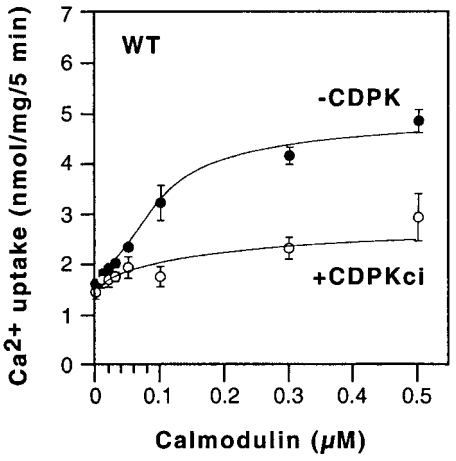

Fig. 3. Calmodulin stimulation of ACA2 is decreased by a CDPK phosphorylation. Membranes isolated from yeast expressing ACA2–1 were incubated with ΔNC31 kinase as in Fig. 2. Net Ca^{2+} uptake (after 5 min) was determined by adding the CDPK-treated membranes to reaction mixtures containing 0–0.5 μM calmodulin. The average (±SD) from three independent determinations of two different membrane preparations is shown.

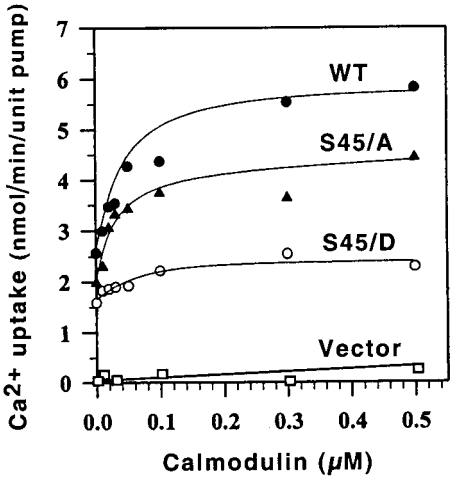

Fig. 5. The S45/D mutant is only weakly activated by calmodulin. Membranes isolated from yeast expressing ACA2–1 (WT), ACA2–3 (S45/A), ACA2–4 (S45/D), or vector only were used to determine the initial rate of uptake (after first 40 s) as a function of calmodulin at a free [Ca^{2+}] of 2.6 μM. The maximum initial activity of the wild-type enzyme was 5.8 nmol/min per mg. The activity of mutant enzymes was normalized to the same amount of wild-type enzyme. Average of three independent assays is shown.

(ACA2–4) to determine whether phosphoinhibition was because of a negative charge being added at the Ser^{45} position. Similar to the phosphoinhibition observed for a wild-type enzyme, the calmodulin stimulation of mutant ACA2–4 (S45/D) was severely inhibited (by ≈80%) (Fig. 5). In these assays, the half-maximal activation (K_{act}) by calmodulin was difficult to estimate for ACA2–4 (S45/D) because its degree of stimulation was so low. In comparison, the K_{act} values for a wild-type and phosphoinsensitive mutant ACA2–3 (S45/A) were similar at 30–40 nM and 25–30 nM, respectively. The basal activity of the S45/D mutant also appeared to be inhibited by approximately 30%. Thus, the introduction of a negative charge at position Ser^{45} (i.e., S45/D mutation) appears to mimic the phosphoinhibition of both basal and calmodulin-stimulated activities.

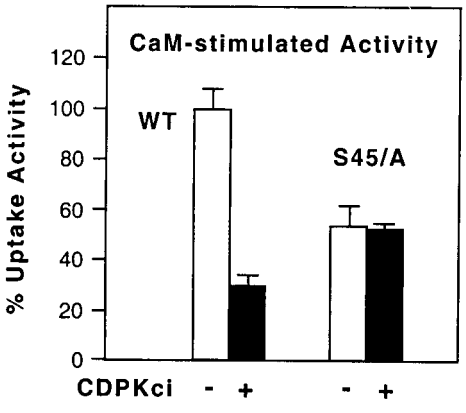

Fig. 4. Phosphoinhibition of calmodulin stimulation is prevented by an S45/A mutation in ACA2. Membranes isolated from yeast expressing either ACA2–1 (WT) or ACA2–3 (S45/A) were incubated with kinase as described in Fig. 2. Net uptake (after 5 min) was determined with or without calmodulin (0.5 μM) in reaction mixtures containing 2.6 μM free Ca^{2+}. Activity is presented as a percent of calmodulin-stimulated activity of wild-type (100% = 2.6 nmol/5 min per mg). The activity of the mutant enzyme was normalized to the equivalent amount of wild-type enzyme. The average (±SD) from two independent assays is shown. Assays on a second independent set of membrane preparations gave similar results.

Phoshorylation of Ser^{45} Fails to Disrupt Calmodulin Binding. To examine whether phosphorylation at Ser^{45} disrupts a calmodulin-binding site, we tested the ability of calmodulin to bind to a fusion protein containing the first 72 residues of the N-terminal domain (MC2-1-72) after phosphorylation. Fig. 6 shows that phosphorylation had no detectable effect on calmodulin binding, as indicated by a calmodulin gel overlay analysis.

Bound Calmodulin Can Block Phosphorylation of Ser^{45}. To examine whether calmodulin binding to the N-terminal region interferes with phosphorylation of Ser^{45}, we tested the ability of fusion protein MC2-1-72 to be phosphorylated by a CDPK in the presence of calmodulin. A preincubation of MC2-1-72 with calmodulin was done with and without Ca^{2+} to generate bound and unbound complexes, respectively. Fig. 7 shows that phosphorylation was nearly abolished after a preincubation with Ca^{2+}/calmodulin, whereas a parallel treatment with apocalmodulin (i.e., no Ca^{2+}) resulted in only slight inhibition of phosphorylation. The kinase used here was a constitutively active mutant version of CPK1 (23). This mutant kinase allowed us to compare the phosphorylation of the fusion protein in the presence and absence of Ca^{2+}, thereby allowing Ca^{2+} to be used

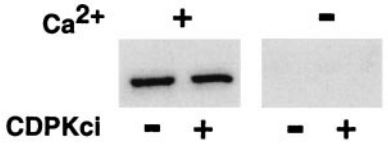

Fig. 6. Phosphorylation of Ser^{45} does not disrupt calmodulin binding to the N-terminal domain. Fusion protein MC2-1-72 (0.3–3 μg) containing the first 72 residues of ACA2 was subjected to phosphorylation by a reaction with 90 ng of a Ca^{2+}-independent CDPK mutant [CDPKci; either KJM23-6H2 (18) or ΔNC-31 (19)], and 300 μM ATP for 60 min at 22°C in a standard kinase reaction buffer without Ca^{2+} (18). The proteins were then tested for changes in calmodulin binding, as determined by gel overlay analysis in the presence (+) or absence (−) of 0.5 mM Ca^{2+}, as described in ref. 23. Bound calmodulin was detected by enhanced chemiluminescence and exposure to x-ray film. One example is shown of three independent experiments. Equivalent results were obtained by using 6 or 60 nM biotinylated bovine calmodulin.

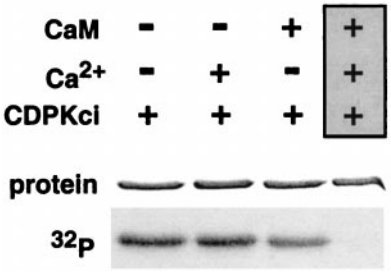

Fig. 7. Phosphorylation of Ser^{45} is blocked by calmodulin binding. Fusion protein MC2-1-72 (2.5 μg) was preincubated in standard kinase reaction buffer with or without Ca^{2+} (100 μM) and calmodulin (CaM, 5 μM) for 15 min. Kinase reactions were started by adding 90 ng of a Ca^{2+}-indpendent CDPK mutant, KJM23-6H2, and 50 μM ATP spiked with [γ-^{32}P]ATP. Reactions were incubated for 60 min at 22°C. The proteins were subjected to SDS/PAGE and exposed to x-ray film. Row marked ''protein'' shows fusion proteins detected by Coomassie stain. Row marked ''^{32}P'' shows an autoradiogram detecting the level of phosphorylation.

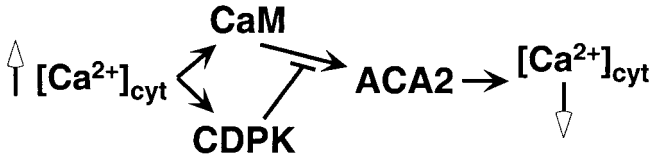

Fig. 8. Diagram illustrating the opposing activities of calmodulin (CaM) and CDPKs on the activity of Ca^{2+} pump ACA2.

to specifically induce calmodulin binding without changing the activity of the kinase. The kinase activity itself was not sensitive to calmodulin, as indicated by normal levels of autophosphorylation (not shown). Thus, these results indicate that the formation of a Ca^{2+}/calmodulin complex with the N-terminal regulatory domain can block the ability of CPK-1 to phosphorylate Ser^{45}.

Discussion

ACA2 Is Inhibited by Phosphorylation. Three lines of evidence indicate that phosphorylation of Ca^{2+} pump ACA2 at Ser^{45} inhibits both basal and calmodulin-stimulated transport activities. First, phosphorylation by a CDPK produced a modest ≈10% decrease in basal activity and a more dramatic ≈75% decrease in calmodulin-stimulated activity (Figs. 2 and 3). Second, the S45/A substitution of a phosphorylation site mapped to this position completely prevented phosphoinhibition of both activities (Figs. 2 and 4). Third, an S45/D substitution mimicked the phosphoinhibited state (Fig. 5). These results indicate that a phosphorylation of Ser^{45} introduces a negative charge that reduces basal activity and severely disrupts calmodulin activation.

Mechanism of Phosphoinhibition? The inhibition of both basal and calmodulin-stimulated activities makes the phosphoregulation of ACA2 distinct from that observed with animal PMCAs. Phosphoregulation of isoforms rPMCA 2a and 3a by a protein kinase C appear to be the most analogous to ACA2, because they show a similar phosphoinhibition of calmodulin-stimulated activity (≈50%) (14). However, these animal pumps differ because they also show a modest phosphostimulation of basal activity (≈10–20%).

For rPMCA2a and -3a, the phosphorylation sites that mediate phosphoregulation have not been identified. Thus, mutational studies have not been possible to evaluate whether the same phosphorylation site mediates both stimulation and inhibition. In contrast, genetic evidence presented here for ACA2 clearly demonstrates that a single phosphorylation site, Ser^{45}, provides a mechanism to inhibit both basal and calmodulin-stimulated activities, as shown by the complete absence of phosphoinhibition of a mutant pump harboring an S45/A substitution. This genetic evidence also provides an important control that argues against the possibility that a reduction in Ca^{2+} transport occurred as an artifact of phosphorylating some other protein or transporter in the assay mix.

The mechanism by which phosphorylation inhibits ACA2 activity is not clear. For rPMCA2a and -3a, an apparent block of calmodulin binding accompanied phosphoinhibition of calmodulin stimulation, as indicated by the poor retention of phosphorylated pumps on a calmodulin-Sepharose column (14). This apparent disruption of a calmodulin-binding site provides an obvious mechanism for blocking calmodulin stimulation. However, for ACA2, an alternative mechanism of phosphoinhibition needs to be considered for two reasons. First, unlike the animal pumps, the basal activity of ACA2 was inhibited by phosphorylation. Because this phosphoinhibition occurs in the absence of calmodulin, it cannot be explained by an interference with calmodulin binding. Second, we failed to detect a phospho-dependent disruption in calmodulin binding to ACA2's regulatory domain, as indicated by binding studies with an N-terminal domain fusion protein (Fig. 6). However, because these binding studies were limited to an interaction with isolated regulatory domain, they leave open the possibility that calmodulin binding may still be disrupted in the context of a holo-enzyme. Nevertheless, for ACA2, the simplest explanation of our results favors a single mechanism of phosphoinhibition in which a phosphorylation at position Ser^{45} somehow stabilizes an autoinhibitory conformation, rather than two separate inhibitory mechanisms, one of which would involve an indirect interference with calmodulin binding. We offer the speculation that a phospho-Ser^{45} may decrease the flexibility of a hinge structure located in this region, and thereby uncouple the binding of calmodulin from a subsequent conformational change that displaces the autoinhibitor from its interaction with some other region of the pump.

Crosstalk Between CDPKs and Calmodulin. In animal cells, there is evidence that Ca^{2+}-pump activity can be regulated by two types of Ca^{2+}-activated kinases, PKCs and CaMKs (13, 24). However, orthologs of these kinases appear to be rare or nonexistent in plant cells (25). Instead, plants have a large family of CDPKs, which are activated directly through Ca^{2+} binding to a calmodulin-like regulatory domain (12). More than 40 isoforms are predicted in a model plant system *Arabidopsis*. Different isoforms have been shown to be activated by different thresholds of Ca^{2+} (26), with some isoforms synergistically activated by putative lipid second messengers (27–29). Here, we demonstrated that CDPK isoform CPK1 could inhibit the calmodulin activation of ACA2, as indicated by *in vitro* transport assays. Although it is not known whether CDPKs actually provide this phosphoregulation *in vivo*, several isoforms have been found associated with different membrane systems (12). Other non-CDPK kinases may also help regulate pump activity.

The observed inhibition of ACA2 by a CDPK provides an example of an ion transport system potentially regulated by a CDPK pathway. Other examples include a Cl^- channel (30), a K^+ channel (31), a plasma membrane H^+-ATPase (32), and aquaporins (33, 34). The addition of ACA2 to this list is significant because it provides the first example in plants (to our knowledge) of (*i*) a Ca^{2+} transporter regulated by phosphorylation and (*ii*) crosstalk between two independent Ca^{2+} signaling pathways (i.e., activation by calmodulin and inhibition by a CDPK) (Fig. 8).

The opposing actions of a CDPK and calmodulin may provide an important strategy for regulating Ca^{2+} signals in plant cells. In theory, the activity of a Ca^{2+} pump can alter the magnitude

or duration of a Ca^{2+} signal by changing the rate of Ca^{2+} efflux after the initiation of a Ca^{2+} release (35, 36). Thus, any shift in the relative activities of calmodulin and CDPKs may alter the dynamic form of a Ca^{2+} signal. A reduction in pump activity may result from the synergistic activation of CDPKs by lipid signals (12) or 14-3-3 (37). Alternatively, an increase in pump activity could result from increased expression levels of calmodulin, the activation of phosphatases, or production of endogenous CDPK inhibitors.

It is noteworthy that the ability of a CDPK to phosphorylate Ser^{45} can be blocked if calmodulin is allowed to bind before the kinase reaction (Fig. 7). This interference means that once a pump is switched on by calmodulin, it likely adopts a stably activated state that is relatively insensitive to phosphoinhibition. Conversely, if phosphorylation occurs before calmodulin binding, the enzyme is likely to form a stable low-activity state. Thus, the degree of pump activity may be set by the initial kinetics of two opposing regulatory pathways. A subsequent resetting of ACA2's activity level may first require the dissociation of the calmodulin/pump complex.

ACA2 has been identified as the first calmodulin-stimulated type IIB pump to be located in the ER membrane of any eukaryote (7). Thus, phosphoinhibition of ACA2 may have unique significance to modulating Ca^{2+} fluxes in the vicinity of the ER in plant cells. Interestingly, the plant ER also contains a type IIA pump that is not directly stimulated by calmodulin, as shown by the characterization of ECA1 from *Arabidopsis* (17, 38). Thus, the discovery of two types of ER-localized pumps with different regulatory properties emphasizes the importance of Ca^{2+} transport at this subcellular location in plant cells.

Finally, the phosphorylation site that makes ACA2 sensitive to phosphoinhibition is present only in a subset of plant type IIB isoforms. An alignment of nine pumps from plants reveals that only the three isoforms most closely related to ACA2 have a Ser at or near the position equivalent to Ser^{45} (Fig. 9). This variation between isoforms supports a hypothesis that different type IIB pumps in plants are independently regulated to provide specialized functions at multiple subcellular locations (39).

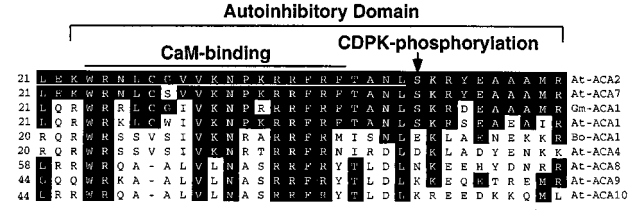

Fig. 9. Alignment of ACA2-like pumps showing that a regulatory phosphorylation site at position Ser^{45} is not present in all pumps. Sequences war taken from Geisler *et al.* (11) with the addition of Gm-ACA1 from Moo Je Cho and Woosik Chung (personal communication). There is evidence from studies on ACA2 for a calmodulin-binding site in the region identified immediately upstream of Ser^{45} (6) and an autoinhibitory sequence contained within residues 20–44 (white line) (10). The potential limits of a functional autoinhibitory domain are marked based on mutations found in this region that disrupt autoinhibition (unpublished observations).

We thank Drs. Jooyeon Yoo, Robyn Stoddard, and Hong Qing Guo for technical assistance. This work was supported by Department of Energy grants to H.S. (DE-FG02-95ER20200) and J.F.H. (DE-FG03-94ER20152), and from a joint grant by the National Aeronautics and Space Administration and National Science Foundation to J.F.H. (IBN-9416038).

1. Bush, D. S. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 95–122.
2. Sanders, D., Brownlee, C. & Harper, J. F. (1999) *Plant Cell* **11,** 691–706.
3. Ghosh, A. & Greenberg, M. E. (1995) *Science* **268,** 239–247.
4. Axelsen, K. B. & Palmgren, M. G. (1998) *J. Mol. Evol.* **46,** 84–101.
5. Malmstrom, S., Askerlund, P. & Palmgren, M. G. (1997) *FEBS Lett.* **400,** 324–328.
6. Harper, J. F., Hong, B., Hwang, I., Guo, H. Q., Stoddard, R., Huang, J. F., Palmgren, M. G. & Sze, H. (1998) *J. Biol. Chem.* **273,** 1099–1106.
7. Hong, B., Ichida, A., Wang, Y., Gens, J. S., Pickard, B. G. & Harper, J. F. (1999) *Plant Physiol.* **119,** 1165–1176.
8. Huang, L., Berkelman, T., Franklin, A. E. & Hoffman, N. E. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10066–10070.
9. Malmstrom, S., Akerlund, H.-E. & Askerlund, P. (2000) *Plant Physiol.* **122,** 517–526.
10. Hwang, I., Liang, F., Harper, J. F. & Sze, H. (2000) *Plant Physiol.* **122,** 157–167.
11. Geisler, M., Axelsen, K., Harper, J. F. & Palmgren, M. G. (2000) *Biochim. Biophys. Acta* **1465,** 52–78.
12. Harmon, A. C., Gribskov, M. & Harper, J. F. (2000) *Trends Plant Sci.* **5,** 154–159.
13. Enyedi, A., Verma, A. K., Filoteo, A. G. & Penniston, J. T. (1996) *J. Biol. Chem.* **271,** 32461–32467.
14. Enyedi, A., Elwess, A., Filoteo, A. G., Verma, A. K., Paszty, K. & Penniston, J. T. (1997) *J. Biol. Chem.* **272,** 27525–27528.
15. Cunningham, K. W. & Fink, G. R. (1994) *J. Cell Biol.* **124,** 351–363.
16. Becker, D. M. & Guarente, L. (1991) *Methods Enzymol.* **194,** 182–187.
17. Liang, F., Cunningham, K. W., Harper, J. F. & Sze, H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8579–8584.
18. Harper, J. F., Huang, J. F. & Lloyd, S. J. (1994) *Biochemistry* **33,** 7267–7277.
19. Vitart, V., Christodoulou, J., Huang, J. F. & Harper, J. F. (2000) *Biochemistry*, in press.
20. Hwang, I., Ratterman, D. M. & Sze, H. (1997) *Plant Physiol.* **113,** 535–548.
21. Duclos, B., Marcandier, S. & Cozzone, A. J. (1991) *Methods Enzymol.* **201,** 10–21.
22. Hofmann, F., Anagli, J., Carafoli, E. & Vorherr, T. (1994) *J. Biol. Chem.* **269,** 24298–24303.
23. Huang, J. F., Teyton, L. & Harper, J. F. (1996) *Biochemistry* **35,** 13222–13330.
24. Odermatt, A., Kurzydlowski, K. & MacLennan, D. H. (1996) *J. Biol. Chem.* **271,** 14206–14213.
25. Hardie, D. G. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 97–131.
26. Lee, J. Y., Yoo, B. C. & Harmon, A. C. (1998) *Biochemistry* **37,** 6801–6809.
27. Harper, J. F., Binder, B. M. & Sussman, M. R. (1993) *Biochemistry* **32,** 3282–3290.
28. Binder, B. M., Harper, J. F. & Sussman, M. R. (1994) *Biochemistry* **33,** 2033–2041.
29. Farmer, P. K. & Choi, J. H. (1999) *Biochim. Biophys. Acta* **1434,** 6–17.
30. Pei, Z. M., Ward, J. M., Harper, J. F. & Schroeder, J. I. (1996) *EMBO J.* **15,** 6564–65746.
31. Li, J., Lee, Y. R. & Assmann, S. M. (1998) *Plant Physiol.* **116,** 785–795.
32. Lino, B., Baizabal-Aguirre, V. M. & Gonzalez de la Vara, L. E. (1998) *Planta* **204,** 352–359.
33. Lee, J. W., Zhang, Y. X., Weaver, C. D., Shomer, N. H., Louis, C. F. & Roberts, D. M. (1995) *J. Biol. Chem.* **270,** 27051–27057.
34. Johansson, I., Karlsson, M., Shukla, V. K., Chrispeels, M. J., Larsson, C. & Kjellbom, P. (1998) *Plant Cell* **9,** 5–19.
35. Penniston, J. T. & Enyedi, A. (1998) *J. Membr. Biol.* **165,** 101–109.
36. Palmgren, M. G. & Axelsen, K. B. (1998) *Biochim. Biophys. Acta* **1365,** 37–45.
37. Camoni, L., Harper, J. F. & Palmgren, M. G. (1998) *FEBS Lett.* **430,** 381–384.
38. Liang, F. & Sze, H. (1998) *Plant Physiol.* **118,** 817–825.
39. Sze, H., Liang, F., Hwang, I., Curran, A. C. & Harper, J. F. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51,** in press.