Differential regulation of Ca^{2+} /calmodulin-dependent enzymes by plant calmodulin isoforms and free Ca^{2+} concentration¹

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Multiple calmodulin (CaM) isoforms are expressed in plants, but their biochemical characteristics are not well resolved. Here we show the differential regulation exhibited by two soya bean CaM isoforms (SCaM-1 and SCaM-4) for the activation of five CaM-dependent enzymes, and the Ca^{2+} dependence of their target enzyme activation. SCaM-1 activated myosin light-chain kinase as effectively as brain CaM (K_{act} 1.8 and 1.7 nM respectively), but SCaM-4 produced no activation of this enzyme. Both CaM isoforms supported near maximal activation of CaM-dependent protein kinase II (CaM KII), but SCaM-4 exhibited approx. 12-fold higher K_{act} than SCaM-1 for CaM KII phosphorylation of caldesmon. The SCaM isoforms showed differential activation of plant and animal Ca^{2+} -ATPases. The plant Ca^{2+} -ATPase was activated maximally by both isoforms, while the erythrocyte Ca^{2+} -ATPase was activated only by SCaM-1. Plant glutamate

decarboxylase was activated fully by SCaM-1, but SCaM-4 exhibited an approx. 4-fold increase in K_{act} and an approx. 25 % reduction in V_{max} . Importantly, SCaM isoforms showed a distinct Ca^{2+} concentration requirement for target enzyme activation. SCaM-4 required 4-fold higher $[\text{Ca}^{2+}]$ for half-maximal activation of CaM KII, and 1.5-fold higher $[\text{Ca}^{2+}]$ for activation of cyclic nucleotide phosphodiesterase than SCaM-1. Thus these plant CaM isoforms provide a mechanism by which a different subset of target enzymes could be activated or inhibited by the differential expression of these CaM isoforms or by differences in Ca^{2+} transients.

Key words: Ca^{2+} signalling, differential activation, target enzyme regulation.

INTRODUCTION

Calmodulin (CaM) is a ubiquitous Ca^{2+} -binding protein which regulates many Ca^{2+} -dependent cellular processes in both plant and animal cells [1–3]. More than 50 enzymes and ion channels are regulated by CaM, and the number of CaM-modulated proteins is ever increasing. Interestingly, CaM shows contradictory behaviour in target enzyme activation, activating many enzymes that catalyse counter reactions, such as protein phosphorylation and dephosphorylation, cyclic nucleotide synthesis and breakdown, and Ca^{2+} channels and Ca^{2+} pumps. The mechanisms which allow CaM to dictate these reciprocal phenomena are currently unknown.

While CaM exists as multiple isoforms in higher plants [2,4], in mammalian cells at least three differentially regulated CaM genes exist which code for the same protein [5]. Recently, subtractive hybridization assays have identified a human CaM-like protein that is 85 % identical with CaM and whose expression in epithelial cells is dramatically decreased after cell transformation [6,7]. In

plant cells, multiple CaM genes exist which code for numerous CaM isoforms in wheat, potato, *Arabidopsis* and soya bean [2,4,8]. We have recently cloned five CaM isoforms from soya bean (SCaM-1–5) [8]. While some of these isoforms (SCaM-1–3) are more than 90 % identical with mammalian CaM, two isoforms (SCaM-4 and SCaM-5) exhibit only 78 % identity and are the most divergent isoforms reported thus far in the plant or animal kingdoms [8]. Similar divergent CaM isoforms also have been found in *Arabidopsis* and pea [9,10]. The existence of multiple divergent CaM isoforms in plants poses the question whether or not they allow differential regulation of target enzymes and can confer different Ca^{2+} sensitivity to CaM-binding enzymes or proteins.

Previously we have shown that SCaM-1 and SCaM-4 exhibit nearly identical activation of cyclic nucleotide phosphodiesterase (PDE), while only SCaM-1 activated NAD kinase [8,11]. Furthermore, we demonstrated that SCaM-1 activates calcineurin (CaN) and competitively inhibits NO synthase (NOS), while SCaM-4 activates NOS and competitively inhibits CaN [12].

Abbreviations used: CaM, calmodulin; BCaM, bovine brain CaM; SCaM, soya bean CaM; CaM KII, CaM-dependent protein kinase II; CaN, calcineurin; GAD, glutamate decarboxylase; LC₂₀, 20 kDa light-chains of myosin; MLCK, myosin light-chain kinase; NOS, NO synthase; PDE, cyclic nucleotide phosphodiesterase; SR, sarcoplasmic reticulum.

¹ This paper is dedicated to the late Dr J. David Johnson.

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These results suggest that plant CaM isoforms not only have differential ability but also serve as competitive antagonists in target enzyme activation. Also, these studies point out the importance of evaluating CaM-dependent enzyme activation in the context of CaM isoform, especially for plant enzymes that are mostly tested simply using bovine brain CaM (BCaM). To further explore this differential and/or reciprocal regulation of CaM target enzymes by CaM isoforms, we examined the capacity of SCaM-1 and SCaM-4 to activate five additional enzymes: myosin light-chain kinase (MLCK), Ca^{2+} /CaM-dependent protein kinase II (CaM KII), plant glutamate decarboxylase (GAD), and plasma membrane-type Ca^{2+} -ATPases from animal and plant sources. We also examined the Ca^{2+} -dependent activation of two enzymes by these CaM isoforms, which is, to our knowledge, the first demonstration for plant CaM isoforms. Our studies show that these isoforms exhibit differences in the Ca^{2+} -dependence of their activation of target enzymes and differential and/or reciprocal activation of these additional target enzymes. The differential expression of these plant CaM isoforms provides a mechanism for altering Ca^{2+} signal transduction in plants and may provide an intrinsic means of selectively activating or inhibiting specific CaM target enzymes.

EXPERIMENTAL

SCaMs were over-expressed in *Escherichia coli* and purified as described previously [8,11]. For the Ca^{2+} -dependent activation of PDE and CaM KII, the purified CaM isoforms were dialysed exhaustively against deionized water, and the free $[\text{Ca}^{2+}]$ was controlled by adding a given amount of CaCl_2 to the 200 mM Mops (pH 7.0)/90 mM KCl/2 mM EGTA/3 mM MgCl_2 buffer, as previously described [13]. The Ca^{2+} stock solutions and Ca^{2+} buffer were calibrated by the Ca^{2+} -dependent increase in Quin-2 fluorescence occurring at pCa 6.86 with a Hill coefficient of 1.0. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (> 5000 Ci/mmol) was purchased from Amersham International. BCaM was purchased from Sigma, and autocamtide 2 substrate (a peptide substrate of CaM KII) with the sequence KKALRRQETVDAL and $[\text{Ala}^9]\text{autocamtide 2}$ (a CaM KII inhibitor) were from American Peptide Co. (Sunnyvale, CA, U.S.A.). Myosin [14], MLCK [15], and CaM KII [16], co-purified with caldesmon) were purified from chicken gizzard. One unit of CaM KII is the amount of kinase catalysing the incorporation of 1 nmol P_i /min into caldesmon under standard conditions including 1 μM CaM. The CaM KII preparation was free of other kinases since the phosphorylation of caldesmon was dependent on both Ca^{2+} and CaM, and was inhibited by $[\text{Ala}^9]\text{autocamtide 2}$. CaM KII activity was 1.07 nmol P_i /ml per min in the presence of Ca^{2+} and CaM, 0.04 nmol P_i /ml per min in the presence of EGTA and CaM, 0.05 nmol P_i /ml per min in the presence of Ca^{2+} alone, 0.03 nmol P_i /ml per min in the presence of EGTA alone, and 0.05 nmol P_i /ml per min in the presence of Ca^{2+} , CaM and $[\text{Ala}^9]\text{autocamtide 2}$. The 20-kDa light-chains of myosin (LC_{20}) were isolated from purified myosin using a modification of the procedure of Hathaway and Haeberle [17]. Activation of sarcoplasmic reticulum (SR)-associated CaM KII was tested using the SR preparation from rabbit cardiac muscle as described [18,19]. Bovine PDE and red cell Ca^{2+} -ATPase were purified and assayed as previously described [20,21]. Radish seedling Ca^{2+} -ATPase was purified and its CaM-dependent activation was determined as described [22]. Recombinant GAD activity was measured using a radiometric assay measuring conversion of $[\text{C}^{14}]\text{glutamate}$ into $[\text{C}^{14}]\gamma\text{-aminobutyric acid}$, as described in [23,24]. All experiments were performed at least in triplicate for each enzyme. Data points and error bars represent means and S.E.M. respectively.

RESULTS

Activation of MLCK by SCaM isoforms

Figure 1 shows that BCaM and SCaM-1 half-maximally activated MLCK with K_{act} of 1.7 nM and 1.8 nM respectively. SCaM-4 produced essentially no activation of MLCK, even at 1 μM concentration. Thus while SCaM-1 activated MLCK as

Figure 1 CaM-isoform activation of MLCK

MLCK activity was monitored with increasing concentrations of BCaM (■), SCaM-1 (○) or SCaM-4 (▲). MLCK (0.05 mg/ml) was incubated at 30 °C for 10 min with LC_{20} (10 μM) in 25 mM Tris/HCl (pH 7.5), 60 mM KCl, 4 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1% (v/v) Tween 80, and 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approx. 200 c.p.m./pmol) in the presence of the indicated concentrations of CaM isoforms. Reactions were started by addition of radiolabelled ATP and stopped by spotting 20 μl of the reaction mixture on squares (1 cm $\times$ 1 cm) of P81 phosphocellulose paper (Whatman) and immersing in a beaker containing 0.5% (v/v) H_3PO_4 . Papers were washed three times with 500 ml of 0.5% (v/v) H_3PO_4 (5 min each) and once with 500 ml of acetone (5 min), air-dried, and ^{32}P was quantified by Cerenkov counting. Values represent means $\pm$ S.E.M. of three independent experiments, each done in duplicate.

Figure 2 CaM-isoform activation of chicken gizzard CaM KII

CaM KII activity was monitored as a function of increasing concentrations of BCaM (■), SCaM-1 (○) or SCaM-4 (▲). CaM KII (0.11 unit/ml) was incubated at 30 °C for 10 min with caldesmon (0.2 mg/ml) in 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 , 0.2 mM CaCl_2 , 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approx. 350 c.p.m./pmol), 0.1% (v/v) Tween 80 in the presence of the indicated concentrations of SCaM-1, BCaM or SCaM-4. Phosphate incorporation was quantified as described in the legend to Figure 1. Values represent the means of three independent experiments (four for BCaM) each done in triplicate, and the error bars indicate S.E.M.

Figure 3 Activation of rabbit cardiac SR-associated CaM KII δ by SCaM isoforms

Effects of varying concentrations of SCaM isoforms on CaM KII δ -mediated protein phosphorylation in rabbit cardiac SR were monitored. The SR-associated CaM KII δ phosphorylates three substrate proteins in the SR: (A) Ca^{2+} -release channel (CRC), (B) Ca^{2+} -ATPase (Ca^{2+} pump), and (C) phospholamban (PLB). Reactions were performed by incubating assay mixtures of 50 mM Hepes (pH 7.4), 10 mM MgCl_2 , 0.8 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 0.2 mM EGTA, 0.2 mM CaCl_2 , and SR membrane (25 μg), for 2 min at 37 $^{\circ}\text{C}$, in the presence of the indicated amounts of each CaM isoform. ^{32}P -incorporation was determined by counting radioactivity after excision of the phosphorylated bands from the gel. Each data point represents the mean of three independent assay results $\pm$ S.E.M.

effectively as BCaM, SCaM-4 failed to produce activation of this enzyme.

Isoform activation of CaM KII

We determined the ability of SCaM-1 and SCaM-4 to activate a purified CaM KII-calmodulin preparation from chicken gizzard [16], and a SR membrane-associated CaM KII from rabbit

Figure 4 Differential activation of Ca^{2+} -ATPases by CaM isoforms

(A) CaM isoform activation of red blood cell Ca^{2+} -ATPase. Ca^{2+} -ATPase (2 $\mu\text{g}/\text{ml}$), purified from human red blood cells, was incubated with BCaM (■), SCaM-1 (○) or SCaM-4 (▲), at the indicated concentrations in a reaction mixture containing 50 mM Tris/maleate (pH 7.4), 80 mM KCl, 8 mM MgCl_2 , 3 mM ATP, 0.15 mM C_{12}E_8 , 1 mM EGTA, and CaCl_2 yielding 100 nM free Ca^{2+} . The Ca^{2+} -ATPase reaction was started with ATP and carried out for up to 30 min at 37 $^{\circ}\text{C}$. Aliquots were withdrawn at various times for colorimetric P_i measurements with ammonium molybdate/metavanadate. Steady-state velocities were obtained from plots of P_i production, which were linear with time. Each point represents the mean of 2–4 independent experiments $\pm$ S.E.M. The specific activity was 25 μmol of P_i/mg per h in the absence of CaM, and 150 μmol of P_i/mg per h (100%) in the presence of optimal BCaM concentration (pCaM 6.5). (B) Radish seedling Ca^{2+} -ATPase activation. CaM-dependent activation was measured as Ca^{2+} -dependent $\text{Mg} \cdot \text{ITP}$ hydrolysis by the Ca^{2+} -ATPase (50 $\mu\text{g}/\text{ml}$) at 25 $^{\circ}\text{C}$ for 1 h in an assay medium containing 40 mM Bis-Tris propane/Hepes (pH 7), 50 mM KCl, 3 mM MgSO_4 , 0.1 mM ammonium molybdate, 75 $\mu\text{g}/\text{ml}$ Brij 58 and 1 mM ITP in the presence of 30 μM free Ca^{2+} .

cardiac muscle [18,19]. Both enzymes consist mainly of the δ -isoform of CaM KII. Figure 2 shows that BCaM, SCaM-1 and SCaM-4 half-maximally activated chicken gizzard CaM KII with K_{act} values of 58 nM, 22 nM and 275 nM respectively. Thus SCaM-1 was a better activator than BCaM, and SCaM-4 was approx. 12-fold less effective than SCaM-1 in activating this kinase.

The SR-membrane associated CaM KII phosphorylates three endogenous substrates: the cardiac Ca^{2+} -release channel, SR Ca^{2+} -ATPase, and phospholamban in SR-enriched membrane preparations [18,19]. Figure 3(A) shows that SCaM-1 and SCaM-4 half-maximally activate CaM KII phosphorylation of the cardiac Ca^{2+} -release channel at 63 nM. Figure 3(B) shows that SCaM-1 and SCaM-4 half-maximally activate CaM KII phosphorylation of the Ca^{2+} -ATPase at 63 nM and 158 nM respectively. Figure 3(C) shows that SCaM-1 and SCaM-4 half-

Figure 5 Activation of recombinant petunia GAD by SCaM isoforms

GAD activity was determined in a 0.2 ml reaction mixture of 100 mM Bis-Tris propane/HCl, pH 7.3, 1 mM dithiothreitol, 5 mM glutamate containing L-[U- 14 C]glutamate (0.5 μ Ci/ml), 0.2 mM pyridoxal phosphate, 500 μ M free Ca^{2+} , 10% (v/v) glycerol, 0.4 μ g of purified GAD, with various amounts of CaM at 30 °C for 60 min. Reactions were terminated by adding 350 μ l of chloroform and 800 μ l of methanol, and activities were determined by counting [14 C] γ -aminobutyric acid radioactivity following TLC. Data represent the means of three independent assays $\pm$ S.E.M.

maximally activate CaM KII phosphorylation of phospholamban at 56 nM and 158 nM respectively. Thus both SCaM-1 and SCaM-4 activate CaM KII phosphorylation of each of these substrate proteins, but it generally takes approx. 2–3-fold more SCaM-4 for maximal activation. Interestingly, for CaM KII phosphorylation of the cardiac Ca^{2+} -release channel, the activation profiles are similar for both isoforms, but SCaM-4 shows an approx. 25% reduction in V_{max} .

Isoform activation of animal and plant Ca^{2+} -ATPases

We determined the ability of SCaM-1 and SCaM-4 to activate two plasma membrane-type Ca^{2+} -ATPases, one from animal and the other from plant [21,22]. As shown in Figure 4(A), the erythrocyte Ca^{2+} -ATPase was half-maximally activated by BCaM at approx. 52 nM. SCaM-1 also activated this enzyme, but its K_{act} was increased by approx. 7-fold (390 nM) relative to BCaM. SCaM-4 produced little activation of this enzyme even at 1 μ M concentration. In contrast with these results, all three CaM isoforms produced maximal activation of radish seedling Ca^{2+} -ATPase. As shown in Figure 4(B), BCaM, SCaM-1 and SCaM-4 half-maximally activated this plant Ca^{2+} -ATPase at 12.5 nM, 8.3 nM and 155 nM respectively. While SCaM-1 exhibits a similar activation of the plant enzyme as BCaM, SCaM-1 has a 7.5-fold higher K_{act} for the mammalian Ca^{2+} -ATPase than BCaM. In addition, while SCaM-4 does not activate the mammalian Ca^{2+} -ATPase, it does activate the plant Ca^{2+} -ATPase, but with an approx. 19-fold higher K_{act} than SCaM-1. Thus these CaM isoforms exhibit very different activation profiles for the mammalian Ca^{2+} -ATPase than the plant Ca^{2+} -ATPase.

Activation of plant GAD

GAD catalyses conversion of glutamate into γ -aminobutyric acid. Activation of the plant GAD is dependent on Ca^{2+} /CaM, unlike its mammalian counterpart [23,24]. As shown in Figure 5, SCaM-1 half-maximally activated recombinant petunia GAD at

Figure 6 Ca^{2+} -dependent activation of PDE and CaM KII by SCaM isoforms

(A) Ca^{2+} -dependence of PDE activation. These assays were conducted in 1 ml of 200 mM Mops (pH 7.0), 90 mM KCl, 2 mM EGTA, 3 mM MgCl_2 , containing 8 μ M methylanthraniloyl-cGMP, 100 nM of BCaM (■), SCaM-1 (○), or SCaM-4 (▲) and 20 μ g/ml PDE. CaCl_2 was added to each 1 ml reaction mixture to achieve the indicated free [Ca^{2+}] concentration ($\text{pCa} = -\ln[\text{Ca}^{2+}]$). 100% activity corresponds to the rate of cGMP hydrolysis in the presence of 100 nM BCaM at pCa 4.0, and this rate was 42-fold faster than the basal rate (0% activity) of hydrolysis in the absence of CaM. Each point represents the mean of three determinations $\pm$ S.E.M. (B) Ca^{2+} -dependence of chicken gizzard CaM KII activation. CaM KII (0.11 units/ml) was incubated at 30 °C for 5 min, with 0.1 mM autocalcine 2 in 0.2 M Mops (pH 7.0), 90 mM KCl, 3 mM MgCl_2 , 2 mM EGTA, 0.2 mM [γ - 32 P]ATP (~ 150 cpm/pmol) and varying amounts of CaCl_2 to give the indicated free [Ca^{2+}] in the presence of 2 μ M BCaM (■), SCaM-1 (○) or SCaM-4 (▲). Phosphate incorporation was quantified as described in the legend to Figure 1.

24 nM, which is similar to BCaM activation of this enzyme [23]. In contrast, SCaM-4 was less effective than SCaM-1 for GAD activation; K_{act} increased 3.8-fold (93 nM) and only approx. 75% of the maximal activation was achieved.

Ca^{2+} -dependent activation of CaM KII and PDE by SCaM isoforms

Figure 6(A) shows the Ca^{2+} dependence of SCaM-1, BCaM and SCaM-4 activation of PDE. SCaM-1, BCaM and SCaM-4 half-maximally activated this enzyme at pCa 5.55, 5.8 and 5.39 respectively. Interestingly, the Hill coefficient for the Ca^{2+} -dependent activation of PDE by SCaM-1 was 4.0, while the Hill coefficient for activation by SCaM-4 was approx. 1.6. Thus approx. 1.5-fold higher [Ca^{2+}] is required for half-maximal activation of PDE by SCaM-4, and the activation by SCaM-4 shows much less co-operativity when compared with activation

by SCaM-1. Figure 6(B) shows the Ca^{2+} -dependence of SCaM-1, BCaM and SCaM-4 activation of CaM KII. SCaM-1, BCaM and SCaM-4 half-maximally activated this enzyme at pCa 6.6, 6.4 and 6.0 respectively. Thus 4-fold higher $[\text{Ca}^{2+}]$ is required for half-maximal activation of CaM KII by SCaM-4 than by SCaM-1.

DISCUSSION

We have previously shown that SCaM-1 and SCaM-4 exhibit similar activation of PDE, but very different activation profiles for NAD kinase, NOS and CaN. These studies led us conclude that SCaM-1 activates PDE, NAD kinase and CaN, but is a competitive antagonist of NOS [8,12]. SCaM-4, on the other hand, activates NOS and PDE, but is a competitive antagonist of CaN and NAD kinase [11,12]. In the present paper we show that both SCaM-1 and SCaM-4 activate plant GAD, plant Ca^{2+} -ATPase and CaM KII. In general it takes more SCaM-4 than SCaM-1 to produce half-maximal activation of these enzymes. For example, 3.8-fold more SCaM-4 is required for half-maximal activation of GAD, and 18.7-fold more SCaM-4 is required for half-maximal activation of radish Ca^{2+} -ATPase. For CaM KII the differences in K_{act} depend on the substrate of the kinase. It takes 12 times more SCaM-4 than SCaM-1 for half-maximal activation of CaM KII catalysed phosphorylation of caldesmon, yet only approx. 2–3-fold more SCaM-4 for half-maximal activation of CaM KII catalysed phosphorylation of the SR Ca^{2+} -ATPase or phospholamban. This is consistent with the notion that the affinity of CaM for its kinases can be altered by the presence of substrate [25]. Apparently, different substrates can alter the affinity of SCaM-1 and SCaM-4 for CaM KII.

In contrast with the above enzymes, SCaM-1 and SCaM-4 exhibited more extreme differential regulation of MLCK and erythrocyte Ca^{2+} -ATPase. While SCaM-1 activated both enzymes, SCaM-4 activated neither. We have previously shown that amino acid residues in the first EF hand of SCaM-4 are responsible for its failure to activate NAD kinase [11]. Van Berkum and Means [26] have used mutant CaMs to demonstrate the importance of the first EF hand of CaM in the activation of MLCK. Consistent with this, we have found that SCaM-4 is a competitive antagonist of CaM activation of MLCK, and that mutations in the first EF hand of SCaM-4 are responsible for this inhibition (J. D. Johnson, M. J. Cho and M. P. Walsh, unpublished work). In this context, it would be very interesting to determine whether the first EF hand of SCaM-4 is also responsible for the differential regulation of erythrocyte Ca^{2+} -ATPase. Our studies with these CaM isoforms have shown that different regions of CaM are often involved in their differential regulation of different target enzymes. For example, we have shown that a single amino acid, valine-144, in the fourth EF hand of SCaM-1 is responsible for its competitive inhibition of NOS [27]. Therefore distinct domains or residues in these CaM isoforms may be responsible for their differential regulation of various target enzymes.

The Ca^{2+} -ATPases purified from red blood cells and radish seedlings exhibited a completely different activation profile by SCaM-1 and SCaM-4. The radish Ca^{2+} -ATPase was activated by both isoforms, with an approx. 19-fold higher K_{act} for SCaM-4, while the red-cell Ca^{2+} -ATPase was only activated by SCaM-1. The plant and red-cell Ca^{2+} -ATPases have approx. 50% sequence identity [28], and their different activation profiles presumably result from structural differences. CaM activates both Ca^{2+} pumps by removing an autoinhibitory domain from their Ca^{2+} -binding site [3,29]. This autoinhibitory domain is in the C-terminal region of the red-cell pump and in the N-terminal

region of the plant Ca^{2+} -ATPase [30,31]. In addition, there is little amino acid sequence similarity in the CaM-binding domain and autoinhibitor region between the plant and animal enzymes. Therefore it is possible that the nature and location of the CaM-binding domain and its associated inhibitory domain may be responsible for the differential regulation of these enzymes by SCaM isoforms. These results suggest that enzymes from different sources that have similar function can be differentially regulated by plant CaM isoforms, and that the activation profiles may be different for some mammalian and plant cell enzymes.

The biochemical characteristics of SCaM isoform regulation of nine CaM-dependent enzymes are summarized in Table 1. The activation characteristics can be categorized into three different types: (1) enzymes activated by both SCaM-1 and SCaM-4; (2) enzymes activated only by SCaM-1, with SCaM-4 often serving as a competitive antagonist of enzyme activation; and (3) enzymes activated only by SCaM-4, with SCaM-1 often serving as a competitive antagonist of enzyme activation. Type 1 enzymes include PDE, plant Ca^{2+} -ATPase, plant GAD, and CaM KII. Type 2 enzymes include CaN, MLCK, plant NAD kinase, and possibly red-cell Ca^{2+} -ATPase. Thus far, NOS is the only Type 3 enzyme, and SCaM-1 appears to be a selective competitive antagonist of mammalian NOS.

In plants, numerous CaM isoforms are expressed from a small multigene family within a single organism. For example, at least four expressed CaM isoforms are present in soya bean [8], ten different CaM genes are known to produce three CaM isoforms in wheat [32], at least four different CaM isoforms are produced in *Arabidopsis* [33], and eight genomic clones of CaM have been found in potato [34]. It is likely that the number of CaM isoforms found in plants will increase when genomic level sequencing is completed. Furthermore, studies by Zielinski and co-workers [33] and Poovaiah and co-workers [35] have shown that CaM isoforms from *Arabidopsis* and potato exhibit differential regulation of NAD kinase and tobacco CaM kinase respectively. The biological consequences of the expression of CaM isoforms which exhibit differential regulation of target enzymes could provide a primary mechanism for altering the Ca^{2+} signal.

We have previously shown in soya bean that SCaM-4 exhibits different transcriptional regulation from SCaM-1. SCaM-1 is expressed at relatively high levels in most soya bean tissues, but SCaM-4 exhibits lower levels of basal expression [8]. However, Ca^{2+} signals induced by pathogen infection or fungal elicitors produce > 10-fold increase in the SCaM-4 mRNA expression [36]. In plants, the biological availability of CaM isoforms can be modulated by alterations in gene expression, including tissue/cell-specificity [2,4] and signal-induced changes [36], and by altering the subcellular localization of isoforms [37]. We proposed that, since these isoforms exhibit a reciprocal activation and inhibition of some CaM target enzymes, a particular arm of the Ca^{2+} /CaM signal-transduction pathway might be activated, while other arms are inhibited, when a particular isoform is expressed [12]. This could provide the opportunity for the expression of certain CaM isoforms, and the execution of their specific Ca^{2+} /CaM signal-transduction pathway, during a particular stage of plant cell growth or development.

This hypothesis is represented in Scheme 1. Depending on the relative abundance (i.e. biological availability) of a specific CaM isoform in cells, a given Ca^{2+} signal might produce different biochemical consequences by virtue of its CaM isoform-dependent selective activation/inhibition of particular target enzymes. This might play a pivotal role in evoking different cellular responses as cytosolic $[\text{Ca}^{2+}]$ rises. In support of the idea that the expression of different CaM isoforms can activate

Table 1 Summary of CaM-dependent enzyme activation by soya bean CaM isoforms

The ability of CaM isoforms to activate ten CaM-dependent enzymes is listed. The sources of tested enzymes are as follows: PDE and CaN from bovine brain; NOS from rabbit skeletal muscle; MLCK from chicken gizzard; Ca^{2+} -ATPase from red blood cells (animal) or radish seedlings (plant); CaM KII from chicken gizzard and rabbit cardiac muscle SR; NAD kinase from pea seedlings; and GAD from petunia (recombinant enzyme). N.D., not determined. CRC, Ca^{2+} release channel; PLB, phospholamban.

CaM-dependent enzymes	SCaM-1		SCaM-4		B-CaM		Reference
	% Maximal activation	K_{act} (nM)	% Maximal activation	K_{act} (nM)	% Maximal activation	K_{act} (nM)	
Animal enzymes							
Phosphodiesterase	100	7	104	6	100	2	[8]
Calcineurin	100	12	33	20	100	12	[12]
NOS	18	200	80	180	100	30	[12]
MLCK	100	1.8	No activation		100	1.7	Present study
Ca ²⁺ -ATPase	93	390	No activation		100	52	Present study
CaM KII (chicken gizzard)							
Caldesmon	100	22	83	275	100	58	Present study
CaM KII (rabbit cardiac muscle)							
CRC	100	63	75	63	N.D.		Present study
Ca ²⁺ -ATPase	100	56	92	158	N.D.		Present study
PLB	100	63	100	158	N.D.		Present study
Plant enzymes							
NAD kinase	100	7	No activation		40*	7	[8]
GAD	100	24	67	93	100	15	Present study, [23]
Ca ²⁺ -ATPase	100	8.3	100	155	100	12.5	Present study

* The lower maximal activity of brain CaM for NAD kinase activation is due to the methylation of CaM at lysine-113 (see [8]).

Scheme 1 Schematic representation of how the expression of different CaM isoforms could produce a bifurcation of the Ca^{2+} signal

If SCaM-4 is expressed, then as cytosolic $[\text{Ca}^{2+}]$ rises and binds to SCaM-4 it would activate a specific subset of CaM target enzymes (Type 1 and Type 3 enzymes), and competitively inhibit CaN, NAD kinase, MLCK and perhaps red-cell Ca^{2+} -ATPase (Type 2 enzymes). If SCaM-1 is expressed, then as cytosolic Ca^{2+} rises and binds to SCaM-1 it would activate a distinct subset of CaM target enzymes (Type 1 and Type 2 enzymes) and competitively inhibit NOS (Type 3 enzyme). Thus depending on which CaM isoform is expressed, different target enzymes would be activated and/or inhibited and the Ca^{2+} signal would be modified.

different cellular responses, we recently reported completely different cellular responses upon constitutive expression of the more divergent CaM isoforms in tobacco cells [36]. In non-stimulated cells, SCaM-4 is expressed at very low levels compared

with the more conserved SCaM-1 isoform. Pathogen infection produces an increase in $[\text{Ca}^{2+}]$, and this increases the expression of SCaM-4. SCaM-4 then activates a cellular signalling pathway which increases the expression of systemic-acquired-resistance-associated genes, and provides for the plant's defence from pathogens [36]. We currently do not know whether SCaM-4 inhibits signalling pathways involving Type 2 enzymes, or if it selectively activates a defence signalling pathway by activating Type 3 enzymes, which are normally suppressed by SCaM-1. These results clearly indicate that expression of specific CaM isoforms can produce a completely different response to a given Ca^{2+} signal. This may provide plant cells with a sophisticated means of modulating Ca^{2+} /CaM signalling pathways and of altering Ca^{2+} signal transduction and cell function.

One extremely interesting feature of these CaM isoforms is that they often bind to specific target enzymes without activating. When this is the case, they act as competitive antagonists of target enzyme activation. We have demonstrated that this is the case for SCaM-1 inhibition of NOS, and for SCaM-4 inhibition of CaN [12]. Studies using CaM gel overlays with plant CaM-binding proteins suggest that SCaM-1 and SCaM-4 both bind to the majority of plant CaM target proteins [38]. Since both of these soya bean CaM isoforms are expressed at different levels in the same soya bean cells, depending on the pathogen infection, it is likely that they compete with one another for activation/inhibition of target enzymes [38]. This finding is in agreement with our *in vitro* enzyme assays which show that these isoforms often bind without activating specific target enzymes. Thus when these isoforms bind target enzymes and do not activate, they serve as competitive antagonists of enzyme activation. This feature of these CaM isoforms gives them their unique ability to activate one subset of target enzymes, while competitively inhibiting another subset of target enzymes.

While there are similarities in the way that CaM binds and activates various target enzymes, it is becoming increasingly evident that CaM is capable of unique interactions with many of

these enzymes. This concept has been supported by several mutagenesis studies and by the production of CaM antibodies which recognize specific CaM target enzyme complexes [39,40]. For example, Persechini et al. [40] suggest that CaM activation of many of its target enzymes 'is dependent upon a distinct pattern of unique determinants in the four EF hands of calmodulin'. Our studies are in agreement with this, because we are finding that the differential activation and/or inhibition of target enzymes by SCaM isoforms does indeed involve unique and specific changes in amino acid sequence and sometimes even a point mutation [27].

Previous studies with numerous man-made CaM mutants have shown that particular mutants can activate some target enzymes and competitively inhibit other enzymes [1,26,40]. Our studies demonstrate the same effect with naturally occurring CaM isoforms. These studies point out the possibility that a unique conformation of CaM may be required to activate each of its target enzymes. Clearly, isoforms which have slight differences in their amino acid sequence may not be able to achieve the particular conformation that is required for activation of some target enzymes. If they bind and do not activate they can serve as a competitive antagonist of enzyme activation. Because these CaM isoforms are quite similar to mammalian CaM, they provides a unique opportunity to determine which amino acid substitutions are responsible for their failure to activate a particular enzyme. These studies should give important new insights into the mechanism by which CaM activates its numerous cellular target enzymes. In addition, these isoforms could potentially be used to alter Ca^{2+} /CaM signal-transduction processes and cell function by their over-expression in both plant and animal cells.

Plants use Ca^{2+} as a signal transducer for recognizing and responding to numerous external stimuli, including hormones, light, gravity and stress [41–43]. The amplitude, duration and frequency of the Ca^{2+} signal are different with different stimuli [44,45]. Here, we show that SCaM isoforms have different Ca^{2+} concentration requirements for target enzyme activation, which still fall well into a physiological Ca^{2+} concentration range (0.5–5 μM). SCaM-4 requires 1.5-fold higher $[\text{Ca}^{2+}]$ than SCaM-1 for activation of PDE, and exhibits a dramatically reduced Hill coefficient for its Ca^{2+} -dependent activation of this enzyme. This suggests that although both isoforms activate PDE, higher $[\text{Ca}^{2+}]$ would be required for SCaM-4 activation than for SCaM-1 activation of PDE. Furthermore, 4-fold higher $[\text{Ca}^{2+}]$ is required for SCaM-4 activation of CaM KII than for SCaM-1. These differences in the Ca^{2+} -dependent activation of target enzymes suggest that these isoforms may have different Ca^{2+} -binding properties, and these studies are in progress. Similarly, approx. 8-fold higher $[\text{Ca}^{2+}]$ is required for saturation of human CaM-like protein than for BCaM [46]. Given the diverse nature of plant Ca^{2+} signals, including their spatio-temporal distribution, these differences in Ca^{2+} sensitivity of CaM isoforms could be an important factor in determining their biological activity in response to various cytosolic Ca^{2+} signals.

In summary, because they are differentially expressed and exhibit differential activation/inhibition of specific target enzymes with different Ca^{2+} sensitivities, these CaM isoforms may play a pivotal role in modulating a cell's Ca^{2+} /CaM signal-transduction pathways. As observed with the over-expression of SCaM-4, this may represent an important cellular mechanism, linking various stimuli and their accompanying Ca^{2+} signals to specific cellular responses in plants.

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