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Reciprocal Regulation of Mammalian Nitric Oxide Synthase and Calcineurin by Plant Calmodulin Isoforms†

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ABSTRACT: Calmodulin (CaM) is the primary mediator of Ca signal transduction processes in cells. Unlike animal cells, plant cells express multiple CaM isoforms. One cloned soybean CaM isoform (SCaM-4) half-maximally activated mammalian nitric oxide synthase (NOS) at 180 nM while another (SCaM-1) served as a competitive antagonist ($K_i \approx 120$ nM) of this activation. The reciprocal was true for the protein phosphatase calcineurin (CaN); SCaM-1 half-maximally activated mammalian CaN at ~12 nM, and SCaM-4 competitively antagonized ($K_i \approx 70$ nM) its activation. The reciprocal enzyme activation and competitive inhibition exhibited by these plant CaM isoforms suggest that their differential expression in cells could allow selective activation of some target enzymes and the selective inhibition of others. This may allow for a branching or bifurcation in the Ca^{2+}-CaM signal transduction pathway and to alterations in cell function.

CaM[1] is a ubiquitous Ca^{2+}-binding protein which confers Ca^{2+} sensitivity to more than 30 enzymes and ion channels (see refs *1* and *2* for reviews). Nonsense mutations of CaM are lethal (*1*), and CaM is necessary for the Ca^{2+}-dependent control of numerous cellular events including cell proliferation, cell division, smooth muscle contraction, glandular secretion, neurotransmission, plant cell growth, phototrophism, and graviotropism (*1*−*3*). CaM acivates nitric oxide synthase (NOS) and calcineurin (CaN) in both plant and animal cells. Ca^{2+}-CaM activation of NOS (*3*−*5*) results in the production of the gaseous second messenger molecule nitric oxide. Nitric oxide plays a key regulatory role in smooth muscle contraction-relaxation, neurotransmission, immune response, and plant cell growth and defense (*4*−*9*). Ca^{2+}-CaM activation of CaN has been shown to be responsible for dephosphorylation and regulation of Ca^{2+} and K channels and transcription factors in animal cells and for dephosphorylation of K channels and Ca^{2+}-induced growth inhibition in plants (*10*−*12*).

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[1] Abbreviations: CaM, calmodulin; S-CaM, soybean CaM; hCLP, human calmodulin like protein; CaN, calcineurin; NOS, neuronal nitric oxide synthase; NO, nitric oxide; PDE, phosphodiesterase; EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, 1,4-dithio-DL-threitol; MUF, 4-methyl umbelliferyl phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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SCaM-1   :  ADQLTDEQISEFKEAFSLFDKDGDGCITTKELGTVMRS  :  38
SCaM-4   :  ..I.SE...VD.....G..........VE..A..I..  :  38
SCaM-5   :  ..V.SE.....I....G..........VD..FV..I..  :  38
B-CaM    :  .....E...A..................T.........  :  38
hCLP     :  .....E..VT..................R.........  :  38

SCaM-1   :  LGQNPTEAELQDMINEVDADGNGTIDFPEFLNLMARKM  :  76
SCaM-4   :  .D.....E......S.........E.D...S...K.V  :  76
SCaM-5   :  .V.....E.........................K..  :  76
B-CaM    :  ..............................TM.....  :  76
hCLP     :  ..........R..MS.I.R....V.....GM.....  :  76

SCaM-1   :  KDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGE  :  114
SCaM-4   :  ....A........K.......Y...S.....I....  :  114
SCaM-5   :  .E..E..D.....K.......Y...S.....I....  :  114
B-CaM    :  ........IR.........G..Y.............  :  114
hCLP     :  ....N...IR.........G...V.........R...  :  114

SCaM-1   :  KLTDEEVDEMIREADVDGDGQINYEEFVKVMMA-K  :  148
SCaM-4   :  .......EQ..K...L.....V.....M..TVR  :  149
SCaM-5   :  .......EQ..E...L.....V..D...M..TIG  :  149
B-CaM    :  ..............I.....V.....QM.T.-.  :  148
hCLP     :  ..S.........A..T.....V.....R.LVS-.  :  148
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FIGURE 1: Comparison of the deduced amino acid sequences of three soybean CaMs (SCaM-1, -4, and -5), bovine brain-CaM (B-CaM), and human CaM like protein (hCLP). Shaded regions represent the four Ca^{2+}-binding loops. Dots represent identical amino acid composition as in SCaM-1.

In mammalian cells, at least three differentially regulated CaM genes exist which code for the same protein (see ref *1*). Recently, subtractive hybridization assays have identified a human CaM-like protein that is 85% identical to CaM and whose expression in epithelial cells is dramatically decreased after cell transformation (*13*, *14*). In plant cells, multiple CaM genes exist which code for numerous CaM isoforms in wheat (*15*), potato (*16*), Arabidopsis (*17*), and soybean (*18*). We have recently cloned 5 CaM isoforms from soybean (SCaM-1−5) (*18*). While some of these isoforms (SCaM-1, 2, and 3) are >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 kingdom (*18*). A sequence comparison of SCaM-1, SCaM-4, SCaM-5, bovine brain CaM (B-CaM), and human CaM-like protein is shown in Figure 1.

We have shown that SCaM-1 and SCaM-4 exhibit nearly identical activation of phosphodiesterase, while only SCaM-1 activated NAD kinase (*18*). To further explore this differential regulation, we examined the capacity of SCaM-1 and SCaM-4 to activate mammalian NOS and CaN. These studies show that not only do these isoforms exhibit a differential ability to activate enzymes but they can also serve as selective, competitive antagonists of particular CaM target enzymes. This may have profound implications for cellular Ca^{2+} signal transduction processes.

MATERIALS AND METHODS

Soybean CaMs were cloned, expressed, and purified as previously described (*18*). Their concentrations were determined by amino acid composition. Rabbit skeletal muscle NOS was purified by affinity chromatography using 2′,5′-ADP-Sepharose (*19*). NOS was concentrated and washed three times with 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to remove endogenous CaM. Recombinant neuronal NOS was purchased from Alexis Biochemical Corp., San Diego, CA. NOS activity was determined by the citrulline assay (*20*) or by the change in NADPH absorption at 340 nm (*21*) in the presence of 1 mM $CaCl_2$. CaN was purified from bovine brain as previously described (*22*) and its activity was monitored by the fluorescent MUF (4-methyl umbelliferyl phosphate) assay (*23*).

RESULTS

Figure 2 shows the effect of three CaM isoforms, B-CaM, SCaM-4, and SCaM-1, on mammalian NOS activity. B-CaM was the most potent activator of NOS; producing half-maximal activation at ~30 nM. SCaM-4 activated NOS to 80% of the level observed with B-CaM, and this increase in activity was half-maximal at ~180 nM. SCaM-1 was a very weak activator of NOS. It produced only 18% of the activation exhibited by B-CaM, and this activation was half-maximal at ~200 nM. Thus, while SCaM-1 produced little activation of NOS, SCaM-4 produced a similar maximal activation as B-CaM but with lower apparent affinity.

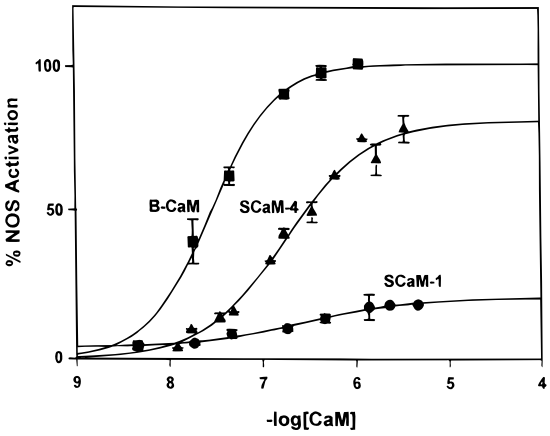

FIGURE 2: The concentration-dependent activation of mammalian NOS by B-CaM, SCaM-4 and SCaM-1. The percent activation of NOS is shown as a function of increasing concentrations of B-CaM (■), SCaM-4 (▲), and SCaM-1 (●). Activation at 100% is that achieved by 1 μM B-CaM and is equal to 591 pmol/min/mg. Each data point is the average of 3 ± SE. NOS activity was determined by a modification of the procedure of Hevel and Marletta (*20*) in a buffer containing 42 mM HEPES-NaOH, 10 mM Tris-HCl, pH 7.4, with 20 μM L-[U-^{14}C] arginine (320 mCi/mmol), 1.1 mM $CaCl_2$, 0.2 mM EDTA, 0.8 mM DTT, 1.5 mM NADPH, 10 μM tetrahydrobioperin, 2 μM flavin adenine dinucleotide, and flavin mononucleotide; and 0.1 mM phenylmethanesulfonyl fluoride, 0.2 mM iodoacetate, 0.2 μM leupeptin, and pepstatin A, 50 mM L-valine and varying concentrations of calmodulin.

When NOS was activated by 45 nM B-CaM or 260 nM SCaM-4, SCaM-1 produced a concentration-dependent inhibition of NOS activity (Figure 3A). SCaM-1 half-maximally inhibited B-CaM and SCaM-4 activation of NOS at ~165 and ~115 nM, respectively. This suggests that SCaM-1 can bind to NOS and inhibit its activation by both B-CaM and SCaM-4. Figure 3B shows a Lineweaver−Burk double reciprocal plot of B-CaM activation of NOS in the presence and absence of 1 μM SCaM-1. Clearly, SCaM-1 increased the slope of the curve without affecting the V_{max}. This is characteristic of a competitive inhibitor and suggests that this naturally occurring isoform serves as a competitive antagonist of B-CaM activation of NOS with a K_i of ~120 nM.

SCaM-1 was also found to inhibit SCaM-4 activation of a recombinant neuronal NOS enzyme. Figure 4 shows this using a continuous NOS assay based on the decrease in NADPH absorption (at 340 nm) that occurs upon NOS mediated oxidation (*21*). Addition of recombinant NOS produced a slight increase in the rate of NADPH oxidation, and the subsequent addition of SCaM-4 produced a dramatic (~8-fold) increase in the rate of NADPH oxidation. The subsequent addition of SCaM-1 inhibited this SCaM-4 activation of NOS. Addition of SCaM-1, by itself, produced little activation of NOS. These data are consistent with the results of the citrulline NOS assay and confirm that SCaM-4 can activate NOS and that this activation can be prevented by SCaM-1.

The SCaM-1 isoform is unique because, while it has a high homology to mammalian CaM and can bind and activate some CaM target enzymes (NAD kinase and PDE), its binding to NOS is nonproductive. Since SCaM-1 is a competitive antagonist of NOS, it may provide a unique, natural tool for inhibiting nitric oxide (NO) production in

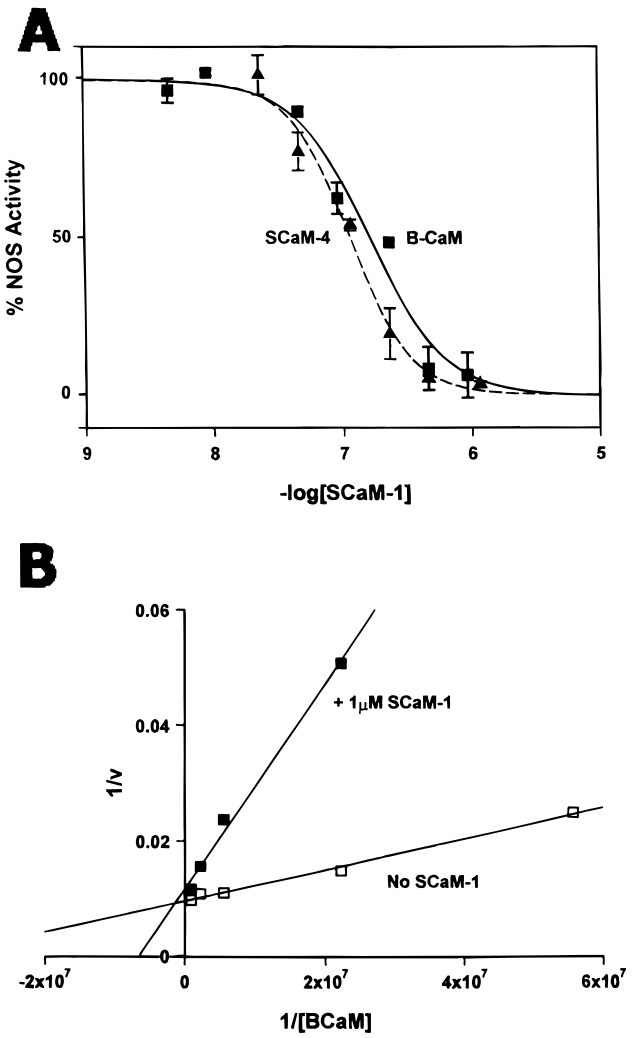

FIGURE 3: (A) SCaM-1 inhibition of B-CaM or SCaM-4 activation of NOS. The effect of increasing concentrations of SCaM-1 on NOS activity is shown when NOS was activated (defined as 100% activity) by either 45 nM B-CaM (■) or by 260 nM SCaM-4 (▲). Activity at 0% is define as the activation produced by 2 μM SCaM-1 alone. B. A Lineweaver−Burk double reciprocal plot of B-CaM activation of NOS in the absence (□) or presence (■) of 1 μM SCaM-1. V = the velocity of the reaction. Assay conditions are as described in Figure 2.

mammalian cells and this could facilitate our understanding of NO's role in cell function.

To further explore the divergent nature of these isoforms we examined the activation of mammalian CaN by SCaM-1 and SCaM-4. Figure 5A shows the effect of increasing concentrations of SCaM-1 and SCaM-4 on CaN activity. B-CaM (not shown) and SCaM-1 produced an ~4-fold increase in activity, which was half-maximal at 12 nM. SCaM-4 produced only a 2-fold increase in activity which was half-maximal at ~20 nM. Figure 5B shows that 100 nM SCaM-1 produced a greater activation of CaN than 10 μM SCaM-4. After activation by SCaM-1, the addition of 10 μM SCaM-4 suppressed CaN activity back to the level produced by SCaM-4 alone. This suggests that, while SCaM-1 exhibits a similar activation of CaN as B-CaM, SCaM-4 inhibits SCaM-1 activation of CaN. Lineweaver−Burk double reciprocal plots of SCaM-1 activation of CaN in the presence and absence of 200 nM SCaM-4 showed that SCaM-4 competitively inhibited SCaM-1 activation of

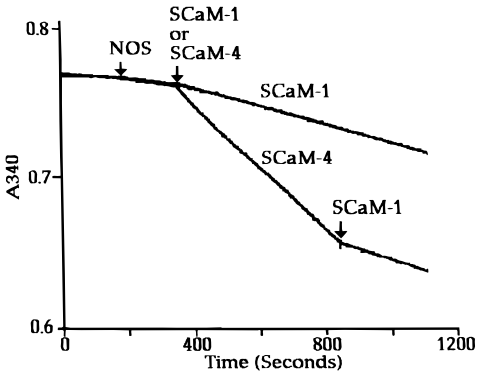

FIGURE 4: Demonstration of SCaM-1 inhibition of SCaM-4 activated recombinant neuronal NOS using a continuous assay. The decrease in NADPH absorption at 340 nm is shown as a function of time after the addition of recombinant neuronal NOS (45 μg), SCaM-4 (50 nM), and the subsequent addition of SCaM-1 (2 μM). In a second assay, neuronal NOS (45 μg) was added, followed by the addition of SCaM-1 (2 μM). Assays were conducted in 1 mL of 50 mM Hepes, pH 7.5, with 3 mM DTT, 1 mM L-arginine, 4 μM FAD, FMN, and tetrahydro-L-biopterin, 1 mM $CaCl_2$, 10 units of catalase, and 0.1 mM NADPH at 37 °C. Addition of 2 mM EGTA completely inhibited the NOS-dependent decrease in NADPH absorption.

CaN with a K_i of ~70 nM (data not shown). Thus, SCaM-4 binding to CaN produces little activation and actually serves to competitively inhibit CaN activation by SCaM-1.

DISCUSSION

We have previously shown that SCaM-1 and SCaM-4 exhibit differential enzyme regulation. Both isoforms activated PDE while only SCaM-1 activated NAD-kinase (*18*). CaM isoforms from yeast (*24*), chicken (*25*), and human (*14*) have also been shown to exhibit differential activation of specific CaM target enzymes when compared to mammalian CaM. For example, yeast CaM is a poor activator of most mammalian enzymes (*24*), and a chicken CaM isoform produced normal activation of MLCK, only half-maximal activation of CaN and CaM kinase II, and no activation of phosphorylase kinase (*25*). A human CaM isoform (hCLP) exhibited normal activation of CaM kinase II, weaker activation of PDE, and no activation of MLCK, CaN, or NOS (*14*).

Our studies show that two soybean CaM isoforms exhibit the opposite activation and competitive inhibition of two CaM target enzymes. SCaM-1 activates CaN and competitively inhibits NOS while SCaM-4 activates NOS and competitively inhibits CaN. This is, to our knowledge, the first example of two naturally occurring CaM isoforms which exhibit not only the opposite activation but also the opposite competitive inhibition of two CaM target enzymes. These soybean CaM isoforms are unique in that they differentially regulate target enzymes not only by their selective activation of specific enzymes but also by their capacity to act as competitive antagonists of other enzymes.

Site-directed mutagenesis of mammalian CaM has yielded a number of man-made mutant CaMs which can act as competitive antagonists of CaM activation of target enzymes. For example, Van Berkum and Means (*26*) have produced a triple mutant of CaM (E14A, T34K, and S38M) which activated PDE like wild-type CaM but served as a potent (K_i = 38 nM) competitive antagonist of MLCK. Furthermore, Persechini et al. (*27*) have produced a mutant CaM which activated NOS but served as a competitive antagonist of MLCK. These studies with man-made mutants have clearly shown that CaM binding is necessary but sometimes not sufficient for enzyme activation. When mutant CaMs bind with high affinity and do not activate, they can serve as effective competitive antagonists of target enzyme activation. Nature has apparently taken advantage of this possiblity in the design of SCaM-1 and SCaM-4, since these isoforms exhibit reciprocal regulation of CaM-dependent enzymes not only by their ability to activate specific enzymes but also by their ability to competitively inhibit the activation of others.

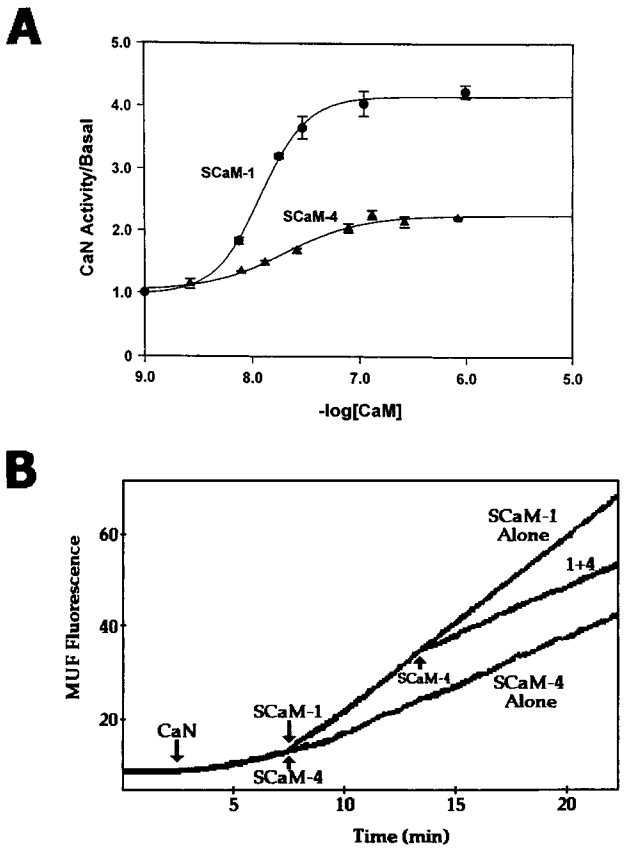

FIGURE 5: (A) The concentration-dependent activation of mammalian CaN by SCaM-1 and SCaM-4. The fold activation of CaN is shown as a function of increasing concentration of SCaM-1 (●) or SCaM-4 (▲). Fold activation was determined by dividing the rate of MUF hydrolysis in the presence of SCaM-1 or SCaM-4 by the rate of MUF hydrolysis in the absence of CaM (basal activity). Each assay was conducted in 1 mL of 50 mM Tris-HCl, pH 7.4, 200 μM EGTA, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$ and 100 μM MUF, and 4 μg of CaN, at 20 °C. (B) SCaM-4 inhibition of SCaM-1 activation of CaN. The fluorescence of MUF is shown upon addition of CaN (basal activity) and upon the subsequent addition of 100 nM SCaM-1 (SCaM-1 Alone curve). In a second experiment, the increase in MUF fluorescence is shown after the addition of CaN followed by the addition of 100 nM SCaM-1 and the subsequent addition of 10 μM SCaM-4 (1+4 curve). In a third experiment, the increase in MUF fluorescence is shown after the addition of CaN and after the subsequent addition of 10 μM SCaM-4.

Alterations in intracellular [Ca^{2+}] play a fundamental role in plant growth and development and in a plant's response to touch, wind, rain, pathogens, and graviotropic and phototropic responses (*3*). The response of plant cells to an increase in cytosolic Ca^{2+} can be very different, suggesting that bifurcations occur in the Ca^{2+} signal transduction

pathway (*3*). In plants, as in animals, CaM is a pivotal mediator of the Ca^{2+} signal and multiple CaM isoforms exist in many plants. Our demonstration that soybean CaM isoforms exhibit differential transcriptional regulation (*18*) and can produce reciprocal activation and inhibition of mammalian CaM target enzymes suggests that differential expression of particular CaM isoforms could also contribute to the diverse responses which are observed upon [Ca^{2+}] elevation in plants.

It is quite remarkable that the SCaM-1 isoform, which is 91% identical to mammalian CaM, was a potent competitive antagonist of NOS. This suggests that the expression of SCaM-1 might allow the normal activation of some CaM target enzymes (PDE, NAD kinase, and CaN) and the inhibition of NOS. Furthermore, the expression of SCaM-4 might allow the Ca^{2+}-dependent activation and inhibition of other distinct subsets of CaM target enzymes. We believe that the differential expression of these isoforms and their differential ability to activate and/or inhibit specfic target enzymes could produce a bifurcation or branching in Ca^{2+}-CaM signal transduction processes in cells. Depending on which isoform is expressed, particular subsets of CaM-dependent enzymes could be activated and inhibited, perhaps resulting in a reprogramming of the Ca^{2+} signal.

While neither NOS nor CaN have been purified from plants, ample evidence suggests that plants have both enzymes and that they are similar to the mammalian enzymes (*7*−*9*, *11*, *12*, *28*, *29*). In fact, selective inhibitors of NOS and CaN have dramatic effects on plant cell function (*8*, *9*, *11*, *12*, *28*, *29*).

Nitric oxide is known to play an important role in plant disease resistance (*7*−*9*). Recent studies have shown that when tobbaco (*28*) and soybean (*29*) cells are exposed to pathogens, a Ca^{2+}-dependent NOS is activated, producing a 4−5-fold increase in NO. In both plants, NO induced the synthesis of natural protective products and hypersensitive cell death to protect the plant from pathogen attack (*28*, *29*). We have recently found that, while SCaM-1 is expressed in normal healthy plants, SCaM-4 expression is rapidly induced when plants are challenged by fungal elicitors or bacterial pathogens. Furthermore, SCaM-4 induces the expression of several protective pathogen resistant proteins [Heo, W. D., Lee, S. H., Kim, M. C., Kim, J. C., Chung, W. S., Chun, H. J., Lee, K. J., Kim, J. S., Choi, J. Y., and Cho, M. J. (1998) Plant Disease Resistance Response Mediated by Divergent Calmodulin Isoforms. *Proc. Natl. Acad. Sci. U.S.A.* (submitted for publication)]. If SCaM-1 acts as a competitive antagonist of plant NOS, as it does with mammalian NOS, then SCaM-1 production in healthy cells could inhibit NOS and prevent NO production and hypersensitive cell death. When challenged by a pathogen, SCaM-4 expression could result in NOS activation, and NO production could trigger hypersensitive cell death and the production of protective products to prevent the spread of the pathogen. These speculations will require confirmation at the molecular level by Western blotting and overexpression and/or knockout of these CaM isoforms. The information generated by these studies should give a clear indication of how both of these isoforms activate or inhibit specific cellular processes to affect cell function.

Clearly both SCaM-1 and SCaM-4 have the capacity to activate one subset of CaM target enzymes and competitively inhibit another subset of target enzymes in a reciprocal fashion. In collaboration with others, we are currently examining the ability of these CaM isoforms to activate and/or inhibit seven other CaM target enzymes. This should allow us to characterize each isoform with respect to the subset of CaM target enzymes that they activate or inhibit.

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