A Point Mutation in a Plant Calmodulin Is Responsible for Its Inhibition of Nitric-oxide Synthase*

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Ritsu Kondo‡, Svetlana B. Tikunova‡, Moo Je Cho§, and J. David Johnson‡‡

From the ‡Department of Molecular and Cellular Biochemistry, The Ohio State University Medical Center, Columbus, Ohio 43210 and the §Department of Biochemistry, Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Chinju 660-701, Korea

The calcium/calmodulin-dependent activation of nitric-oxide synthase (NOS) and its production of nitric oxide (NO) play a key regulatory role in plant and animal cell function. SCaM-1 is a plant calmodulin (CaM) isoform that is 91% identical to mammalian CaM (wild type CaM (wtCaM)) and a selective competitive antagonist of NOS (Cho, M. J., Vaghy, P. L., Kondo, R., Lee, S. H., Davis, J. P., Rehl, R., Heo, W. D., and Johnson, J. D. (1998) Biochemistry 37, 15593–15597). We have used site-directed mutagenesis to show that a point mutation, involving the substitution of valine for methionine at position 144, is responsible for SCaM-1’s inhibition of mammalian NOS. An M144V mutation in wild type CaM produced a mutant (M144V) which exhibited nearly identical inhibition of NOS’s NO production and NADPH oxidation, with a similar Ki (<15 nm) as SCaM-1. A V144M back mutation in SCaM-1 significantly restored its ability to activate NOS’s catalytic functions. The length of the hydrophobic amino acid side chain at position 144 appears to be critical for NOS activation, presumably by influencing the function of NOS’s oxygenase domain.

A rise in cytosolic Ca2+ regulates events as diverse as muscle contraction, neurotransmission, memory, cell fertilization, cell proliferation, cell defense, and cell death (1). Calmodulin (CaM) is a ubiquitous Ca2+-binding protein that serves as a primary mediator of the Ca2+ signal (see Ref. 2 for review). Calcium binding to CaM allows it to bind and activate more than 30 target enzymes, resulting in alterations in numerous second messengers and in cell function.

CaM activates NOS in both plant and animal cells and NOS activation results in the production of the gaseous second messenger molecule NO. NO plays a key regulatory role in smooth muscle contraction-relaxation, neurotransmission, immune response, and plant cell growth and defense (3–6).

Three NOS isoforms have been identified in different tissue: neuronal NOS, endothelial NOS, and inducible NOS (7). Each isoform has an N-terminal heme-containing oxygenase domain, a CaM binding domain, and a C-terminal flavin-containing reductase domain. While both nNOS and eNOS require the Ca2+-dependent binding of CaM for activation, iNOS is fully active at low [Ca2+], because of a tightly bound CaM (8).

CaM’s activation of NOS appears to be more complex than its activation of other target enzymes, which often require the simple removal of a pseudosubstrate inhibitory domain from their active sites (9, 10). CaM binds nNOS at amino acids 725–754, between its N-terminal oxygenase domain and its C-terminal reductase domain (11, 12). CaM facilitates electron transfer at two points in NOS: 1) from NADPH to the flavins within the reductase domain and 2) from the flavins in the reductase domain to the heme in the oxygenase domain or to exogenous electron acceptors such as the heme of cytochrome c (13). Once electrons are received in the N-terminal oxygenase domain they are used for the conversion of L-Arg to citrulline and NO.

The functional unit of NOS is a dimer (see Ref. 14), and recent studies have suggested that CaM binding to inducible NOS serves to bring the flavin binding reductase domain of one monomer into close proximity with the heme binding oxygenase domain of the other monomer for optimal intermolecular electron transfer (15). The mechanisms by which CaM facilitates NOS’s intra- or intermolecular electron transfer to facilitate NO production is currently unknown.

We have recently cloned five CaM isoforms from soybean (SCaM-1 through SCaM-5) and showed that one of these isoforms, SCaM-1, was a potent and selective competitive antagonist of skeletal muscle NOS (16). Although SCaM-1 is a competitive antagonist of NOS, it activates other CaM-dependent enzymes (including PDE, NAD-kinase, CaN) as effectively as wtCaM.

Fig. 1 compares the amino acid sequence of wtCaM and SCaM-1. SCaM-1 is 91% identical to wtCaM, exhibiting only 14 different amino acids, 7 of which are nonconservative substitutions. This protein provides a unique opportunity for understanding the mechanism by which nature has designed a selective competitive antagonist of NOS. In this paper we show that the amino acid Val-144 is responsible for SCaM-1’s com-
The M144V Mutation Produces a Competitive Antagonist of NOS—to determine whether M144V, like SCaM-1, is a competitive antagonist of wtCaM activation of NOS, we examined wtCaM activation of NOS in the absence and presence of each inhibitor (M144V and SCaM-1). Fig. 3 shows a double-reciprocal plot of wtCaM activation of NOS's NADPH oxidation in the absence and in the presence of 58 and 117 nM M144V. The presence of M144V increases the slope of the double-reciprocal plot without altering the intercept on the 1/v axis. Thus, M144V is a potent competitive antagonist of NOS with a K_i of 15 nM. Nearly identical results were found for SCaM-1, and it competitively inhibited wtCaM activation of NOS with a K_i of 17 nM (data not shown). Thus, an M144V point mutation in wtCaM makes it a competitive antagonists of NOS with a similar K_i as SCaM-1.

M144V Inhibition of NOS’s NO Production—We have previously shown that SCaM-1 is a competitive antagonist of skeletal muscle NOS’s production of NO (16). We determined the effectiveness of M144V in activating NO production. Fig. 4 shows the dose-dependent activation of NO production by wtCaM, SCaM-1, and M144V. Although wtCaM half-maximally activated NOS’s NO production at 3.5 nM, neither SCaM-1 nor M144V produced any significant activation of NOS. Essentially competitive inhibition of NOS. We discuss the implications of this finding on the mechanism of CaM activation of NOS.

EXPERIMENTAL PROCEDURES

Soybean CaMs and their chimeras were cloned, expressed, and purified as described previously (17). The mutants (M144V, Q143K/M144V/T146M, and S1V144M) were constructed from the wild type CaM or SCaM-1 cDNA using primer based site-directed mutagenesis following the protocol provided by Stratagene’s (La Jolla, CA) QuikChange™ Site-Directed Mutagenesis Kit. DNA sequence analysis confirmed the correct generation of each mutant. CaM concentrations were determined by amino acid composition. Recombinant neuronal NOS was purchased from Alexis Biochemical Corp. (San Diego, CA). It had a specific activity of 68.2 nmol/min/mg for NADPH oxidation, 600 nmol/min/mg for oxygen reduction, and to 35.7 nmol/min/mg for NO synthesis when maximally stimulated by wtCaM. A >95% pure recombinant NOS (764 units/mg) was purchased from Calbiochemical Corp. NOS activities were measured by the NADPH oxidation, oxyhemoglobin, and cytochrome c reduction assays. The NADPH oxidation assay was performed as described previously (16, 18) with minor modifications. Each assay mixture contained 3 mM 1,4-dithio-DL-threitol, 1 mM CaCl_2, 1 mM ML-Arg, 6.22 mM NADPH, 0.3 mM 1,4-dithio-DL-threitol, 1 mM CaCl_2, 1 mM t-Arg, 4 mM of FAD, FMN, and H_2O_2, 10 units/ml catalase, 100 mM NADPH, in 1 ml of 50 mM HEPES at pH 7.5 and 37 °C. Assays were initiated by addition of 26 μg of NOS to obtain the basal activity, followed by addition of wtCaM, SCaM-1, or their mutants. The decrease in NADPH absorbance was monitored at 340 nm as a function of time, using ε = 6.22 mM⁻¹ cm⁻¹. For the double-reciprocal plots in the presence of inhibitors, assays were initiated with NOS in the presence of both wtCaM and the inhibitor. Oxyhemoglobin assays were performed as described previously (18). Each assay mixture contained 6 μM oxyhemoglobin, 0.3 mM 1,4-dithio-DL-threitol, 1 mM CaCl_2, 1 mM t-Arg, 4 mM of FAD, FMN, and H_2O_2, 10 units/ml catalase, 100 mM NADPH, in 1 ml of 50 mM HEPES at pH 7.5 and 37 °C. The reactions were initiated by addition of 37 μg of NOS to obtain basal activity followed by the addition of 1 μM wtCaM, SCaM-1, or their mutants. The increase in absorbance at 401 nm was monitored as a function of time using Δε = 38 mM⁻¹ cm⁻¹. Cytochrome c reduction assay was performed as described previously (19) with modifications. Each assay mixture contained 1 mM CaCl_2, 50 μM cytochrome c, 100 μM NADPH, 7.3 μg of NOS, in 1 ml of 50 mM HEPES at pH 7.5 and 37 °C. Assays were initiated with NADPH and after a basal rate was established, 1 μM of wtCaM, SCaM-1, or their mutants were added. The increase in absorbance was monitored at 550 nm, using Δε = 21 mM⁻¹ cm⁻¹. PDE and CaN were purified and assayed as described previously (16, 20).

RESULTS

Determination of Amino Acid Residue(s) Responsible for SCaM-1’s Inhibition of NOS—Since SCaM-1 has only 14 amino acids that are different from those in wtCaM (see Fig. 1), we mutated wtCaM so that it would have some of the nonconservative mutations found in SCaM-1 and determined the effect of these mutants on NOS activation. We made a T70N/M71L mutation in the second EF-hand of wtCaM and a I85L/R86K mutation in the third EF-hand of wtCaM. This mutant (T70N/M71L/I85L/R86K) exhibited normal activation of NOS’s NADPH oxidation (data not shown). We next mutated wtCaM so that it contained three amino acids that are found in helix 8 of SCaM-1’s fourth EF-hand. This mutant (Q143R/M144V/T146M) exhibited impaired activation of NOS. Fig. 2 shows the dose-dependent activation of NOS’s NADPH oxidation by wt-CaM, Q143R/M144V/T146M, and SCaM-1. WtCaM half-maximally activated NOS at 3.2 mM. The Q143R/M144V/T146M mutant and SCaM-1 both activated NOS’s NADPH oxidation to about 25% of the level produced by wtCaM with a similar dose dependence (half-maximal activation at 4.6 and 4.4 nM, respectively). Fig. 2 also shows that the point mutation, M144V in wtCaM, produced a mutant that activates NOS to only 24% of the level produced by wtCaM and that this activation was half-maximal at 6.4 nM. Thus, this point mutation in wtCaM produces a mutant that acts essentially identical to SCaM-1 with respect to activation of NOS’s NADPH oxidation.

The M144V Mutation Produces a Competitive Antagonist of NOS—To determine whether M144V, like SCaM-1, is a competitive antagonist of wtCaM activation of NOS, we examined wtCaM activation of NOS in the absence and presence of each inhibitor (M144V and SCaM-1). Fig. 3 shows a double-reciprocal plot of wtCaM activation of NOS’s NADPH oxidation in the absence and in the presence of 58 and 117 nM M144V. The presence of M144V increases the slope of the double-reciprocal plot without altering the intercept on the 1/v axis. Thus, M144V is a potent competitive antagonist of NOS with a K_i of 15 nM. Nearly identical results were found for SCaM-1, and it competitively inhibited wtCaM activation of NOS with a K_i of 17 nM (data not shown). Thus, an M144V point mutation in wtCaM makes it a competitive antagonists of NOS with a similar K_i as SCaM-1.

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FIG. 1. Comparison of the deduced amino acid sequence of wtCaM and SCaM-1. The four Ca^2+ binding loops are indicated. Residues different from the corresponding residues in wtCaM are indicated by their letter amino acid codes. Dots represent identical amino acids that are different from those in wtCaM (see Fig. 1), we mutated wtCaM so that it would have some of the nonconservative mutations found in SCaM-1 and determined the effect of these mutants on NOS activation. We made a T70N/M71L mutation in the second EF-hand of wtCaM and a I85L/R86K mutation in the third EF-hand of wtCaM. This mutant (T70N/M71L/I85L/R86K) exhibited normal activation of NOS’s NADPH oxidation (data not shown). We next mutated wtCaM so that it contained three amino acids that are found in helix 8 of SCaM-1’s fourth EF-hand. This mutant (Q143R/M144V/T146M) exhibited impaired activation of NOS. Fig. 2 shows the dose-dependent activation of NOS’s NADPH oxidation by wt-CaM, Q143R/M144V/T146M, and SCaM-1. WtCaM half-maximally activated NOS at 3.2 mM. The Q143R/M144V/T146M mutant and SCaM-1 both activated NOS’s NADPH oxidation to about 25% of the level produced by wtCaM with a similar dose dependence (half-maximal activation at 4.6 and 4.4 nM, respectively). Fig. 2 also shows that the point mutation, M144V in wtCaM, produced a mutant that activates NOS to only 24% of the level produced by wtCaM and that this activation was half-maximal at 6.4 nM. Thus, this point mutation in wtCaM produces a mutant that acts essentially identical to SCaM-1 with respect to activation of NOS’s NADPH oxidation.

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identical results were obtained when these experiments were conducted using a 6.6 nM amount (1.05 μg/ml) of a highly purified (>95% pure, specific activity 764 units/mg) recombinant NOS. With B-CaM half-maximal activation occurred at ~2 nM, and M144V produced no activation of NO production even at 1 μM concentration. Thus, the M144V mutation in SCaM-1 is responsible for its inactivation of NOS’s NO production and NADPH oxidation.

To further confirm that Val-144 is primarily responsible for SCaM-1’s competitive antagonism of NOS we used site-directed mutagenesis to produce a V144M back mutation in SCaM-1 (S1V144M). Fig. 4 also shows the dose dependent activation of NO production is shown as a function of increasing concentrations of wt-CaM (■), S1V144M (○), SCaM-1 (●), and M144V(△). Each data point represents the average of 3 ± S.E. NO production was measured by the oxyhemoglobin assay as described under “Experimental Procedures.”

Fig. 3. Lineweaver-Burk double-reciprocal plots of wtCaM activation of NOS in the absence ( ■) and in the presence of 58 nM (●) and 117 nM (△) M144V. NOS activity was determined using the NADPH oxidation assay.

M144V Activation of PDE and CaN—Although SCaM-1 was a competitive antagonist of NOS, we have previously shown that it exhibits full activation of other CaM target enzymes, including PDE, CaN, and NAD-kinase (16, 17). Similarly, M144V exhibited a normal dose-dependent activation of PDE and CaN (data not shown). Half-maximal activation of PDE was observed at 13 nM wtCaM and at 18 nM M144V. Half-maximal activation of CaN was observed at 14 nM wtCaM and at 21 nM M144V. Thus, methionine at position 144 in wtCaM may have a specific role in the activation of NOS.

Comparative Ability of CaM Mutants to Activate NOS Catalytic Functions—Our studies of SCaM-1 and M144V activation of NOS’s NADPH oxidation and NO production show that valine at position 144 completely inhibits NOS’s NO production, while it reduces NOS’s NADPH oxidation to ~25% of that observed with wtCaM. Fig. 6 compares the ability of wtCaM, M144V, SCaM-1, and S1V144M to stimulate NOS’s NADPH oxidation, cytochrome c reduction, and NO synthesis. wtCaM stimulated all three activities 100% (control). M144V stimulated NADPH oxidation to 24 ± 2%, cytochrome c reduction to 72 ± 2% and NO synthesis to only 3.5 ± 2.2% of control (wtCaM) values. M144V produced similar percent activation of each of these NOS activities when a >95% pure NOS was used. SCaM-1 produced similar results as M144V on the oxygenase domain-dependent functions of NOS, stimulating NADPH oxidation to 25 ± 1.7% and NO synthesis to only 1.8 ± 1.1% of control values. Thus, the M144V point mutation produces virtually all of the impaired activation of NOS’s oxygenase domain-dependent functions (NADPH oxidation and NO production) exhibited by SCaM-1. NOS’s reductase domain-specific function (cytochrome c reduction) was more impaired with SCaM-1 (39 ± 2.9%) than with M144V (72 ± 2%). Mutations in SCaM-1, other than M144V, must be affecting its activation of cytochrome c reduction.

The V144M back mutation in SCaM-1 markedly restored each NOS activity. S1V144M stimulated NADPH oxidation to 65 ± 4.1%, cytochrome c reduction to 70 ± 4% and NO synthe-
Figure 6. The relative stimulation of NOS's NADPH oxidation, cytochrome c reduction, and NO production by 1 μM wtCaM, M144V, SCaM-1, or S1V144M. Each NOS activity was assayed as described under "Experimental Procedures." 100% corresponds to the activation produced by wtCaM and corresponds to 68.2 nmol/min/mg for NADPH oxidation, 600 nmol/min/mg for cytochrome c reduction, and to 35.7 nmol/min/mg for NO synthesis.

sis to S58 ± 6.3% of control values. Thus, Met-144 in CaM plays a critical role in it's activation of NOS's NADPH oxidation and NO production and a less important role in activation of NOS's reductase domain reduction of cytochrome c.

**DISCUSSION**

We have previously shown that SCaM-1 is a competitive antagonist of skeletal muscle NOS (nNOSμ) with a K_i of ~120 nM (16). The present studies show that SCaM-1 is an even more effective competitive antagonist of recombinant neuronal NOS exhibiting a K_i of ~17 nM. The K_i we determined for wtCaM activation of nNOS (3.2 nM) is in excellent agreement with previously reported values of 2–4 nM (9, 10). The 10-fold higher apparent affinity of wtCaM and SCaM-1 for nNOS than nNOSμ could result from the additional 34 amino acids near nNOSμ CaM binding site (21), or it could be a consequence of the lower specific activity of the skeletal muscle isoform relative to the more purified nNOS recombinant isoform used in this study. Clearly, SCaM-1 is a powerful competitive antagonist of both NOS isoforms.

Our results indicate that the M144V point mutation is primarily responsible for SCaM-1's selective competitive antagonism of mammalian NOS. An M144V point mutation in wtCaM produced a mutant that failed to activate and served as a competitive antagonist of NOS with a K_i (~15 nM) similar to SCaM-1. Furthermore, the V144M back mutation in SCaM-1 greatly enhanced its activation of NOS's NO production. The M144V mutation had little effect on CaM's affinity for NOS, since both SCaM-1 and M144V bind with similar affinity as wtCaM, but fail to activate NOS. Clearly, Met-144 must play a pivotal role in CaM's ability to control electron flow and regulate NOS's production of NO.

Currently we do not know why the amino acid at position 144 is so critical to NOS activation. The fact that M144V does not activate, while M144L does activate, indicates that the length of the side chain at position 144 is critical to NOS activation. Consistent with this finding the M144F mutant that places the longer more hydrophobic phenylalanine residue at this position activates NOS. Clearly, the sulfur group is neither necessary nor sufficient for NOS activation because M144C fails to activate. Perhaps methionine's flexible unbranched side chain allows it to make a specific contact with a some key regulatory residues in the oxygenase domain of NOS. These interactions must still occur in mutants like M144L and M144F and probably result from some specific hydrophobic interactions of the amino acid at position 144 of CaM with particular amino acids residues of NOS.

X-ray studies of CaM-peptide complexes suggest that CaM's Met-144 makes a critical contact with a tryptophan residue in smooth muscle MLCK (22). Sequence alignment of the CaM binding sequence of smooth and skeletal muscle MLCK with NOS, suggest that Met-144 of CaM would interact with Phe-731 in nNOS (11, 23). Our results suggest that a critical hydrophobic interactions between M144 of CaM and P731 of NOS are important in CaM activation of NOS's heme domain function and NO production. It has been suggested that CaM may interact with regions of nNOS and iNOS other than its canonical CaM binding sequence to produce enzyme activation (9, 10, 24). If this is the case, then it may be difficult to speculate as to which specific residue(s) of NOS interact with M144 of CaM to produce NOS activation and NO production.

Many manmade CaM mutants have been shown to exhibit altered activation of NOS. Su et al. (9) have replaced the individual EF-hands (or their subdomains) of CaM with the corresponding EF-hand of cardiac TnC to show that the latch domain comprised of helix 2 in site 1 and helix 6 in site 3 is critical for NOS activation. Replacing CaM's first EF-hand with TnC's first EF-hand produced a mutant (CaM (1 TnC)) that functioned as a potent competitive antagonist of NOS and smooth muscle MLCK (9). Perschini et al. (25) have interchanged CaM's first and third or second and fourth EF-hand to probe which functional determinants might affect the activation of smooth and skeletal MLCK and NOS. They found that when the first EF-hand was replaced with the third EF-hand, NOS activation was reduced by ~55%, and smooth muscle MLCK activation was totally inhibited. These results suggest that the first EF-hand of CaM may have specific determinants that are required for NOS and smooth muscle MLCK activation. Furthermore, while replacement of CaM's fourth EF-hand with the second EF-hand (which has a Met corresponding to Met-144) had little effect on NOS activation, it reduced activation of skeletal muscle MLCK by 60% (25). Stevens-Truss et al. (10) have used CaM mutants with mutations in each EF-hand to show that some of the EF-hand Ca²⁺ binding sites of CaM play a more important role in regulating NOS oxygenase domain dependent functions than reductase domain-specific functions. Mutation of CaM's first EF-hand (site 1) had equally devastating effects on NOS's oxidation of NADPH, reduction of cytochrome c, and formation of citrulline. Mutation of Ca²⁺ binding site 3 had little effect on any NOS activity. Site 2 or 4 mutations inhibited NOS activity by affecting N-terminal heme-dependent activities more than the heme-independent reductase domain activity of cytochrome c reduction. These studies suggest that site 2 and 4 play a more important role in regulating NOS's heme domain. They proposed a model where CaM's C-terminal contacts NOS's oxygenase domain and CaM's N-terminal contacts the reductase domain. Gachhui et al. (26) have used CaM-TnC chimeras to show that the first, third, and fourth EF-hand of CaM are important in regulating NOS's heme domain function. For example, replacing the fourth EF-hand of CaM with the fourth EF-hand of TnC produced a mutant, CaM-TnC, which exhibited ~60% maximal activation of NOS's reduction of cytochrome c and only 13% maximal activation of NO synthesis. This is similar to our M144V mutant, which exhibits 72% maximal activation of NOS's cytochrome c reduction and less than 4% maximal activation of NO synthesis.

The above studies suggest that many regions and EF-hands
Calmodulin Activation of NOS

of CaM are important for its activation of NOS. Our studies are consistent with the above studies because they implicate the fourth EF-hand of CaM in the regulation of NOS’s heme domain function. Our studies extend the finding that the fourth EF-hand is important by showing that a particular amino acid, methionine at position 144, plays a critical role in facilitating electron transfer to the heme in the oxygenase domain of NOS and/or its production of NO from L-Arg. While the M144V mutation has its most dramatic inhibitory effects on the oxygenase domain of NOS, it also produces a small (28%) decrease in cytochrome c reduction.

Although M144V and SCaM-1 inhibit NOS, they exhibit normal activation of PDE and CaN. Consistent with this observation Edwards et al. (27) and Chin and Means (28) have demonstrated that a M144L mutation and a M144Q mutation in CaM had little effect on the activation of CaN and smooth muscle MLCK. Thus, while the amino acid at CaM’s position 144 plays a critical role in NOS activation, it may have little effect on the activation of numerous other target enzymes. Consistent with this concept, we have examined SCaM-1 for its ability to activate eight target enzymes, including phosphodiesterase, calcineurin, MLCK, red cell and plant Ca-ATPases, CaM kinase II, NAD kinase and plant glutamate decarboxylase. SCaM-1 activates each of these enzymes as effectively as mammalian CaM. Of all the enzymes examined, NOS is the only enzyme that is competitively inhibited by SCaM-1. A manuscript describing SCaM-1 and SCaM-4’s activation of these other enzymes has just been submitted for publication.2

SCaM-1 and M144V are different from any of the manmade competitive antagonists of NOS because they use different amino acid substitutions, and only SCaM-1 and M144V are selective competitive antagonists of NOS. This feature of SCaM-1 and M144V might make them very useful tools for understanding the role of NOS and nitric oxide in biological systems. It is likely that transfection of cells with SCaM-1 could selectively inhibit nNOS while still allowing the normal activation of other 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 that recognize specific CaM target enzyme complexes (9, 25, 29). Persechini et al. (25) suggest that calmodulin activation of many of its target enzymes “is dependent upon a distinct pattern of unique determinants in the four EF-hands of calmodulin.” Our study is in agreement with this, because it shows that a specific amino acid at position 144 in CaM’s fourth EF-hand is a specific functional determinant for CaM activation of NOS. Unlike any of the manmade mutant CaM’s, SCaM-1 with its M144V mutation is a selective competitive antagonist of NOS. This points out the potential of having CaM mutants or isoforms that can activate and/or competitively inhibit specific subsets of CaM target enzymes.

Nitric oxide is known to play an important role in mediating plant disease resistance, and plants have a Ca2+/CaM-stimulated NOS that is similar to neuronal NOS (5, 6, 30–33). For example, Ribeiro et al. (33) used Western blots with a neuronal NOS antibody to show that an ~166-kDa NO is present in the cytosol, nucleus, and in the elongation zone of maize root tips. Furthermore, maize NOS, like mammalian NOS, was a Ca2+-dependent enzyme that was inhibited by inhibitors of mammalian NOS (33). Ribeiro et al. (33) conclude the existence of a homologous NOS enzyme in this plant species with antigenic epitopes common to mammalian NOS isoforms. Thus, plants like maize, tobacco, soybeans, and wheat have a NOS that is similar to the Ca2+/CaM-sensitive NOS found in mammalian cells.

Many plants also have CaM isoforms with an M144V mutation. A search of the Scripp’s calcium-binding protein data bank indicates that wheat, potato, spinach, corn, barley, apple, and alfalfa have CaM isoforms with valine at position 144. We tested wheat germ-CaM and a potato CaM isoform that have a V144 and found that they were both competitive antagonist of NOS. Since many plants have CaM isoforms like SCaM-1 and a NOS like mammalian NOS, it is possible that plants may use the M144V mutation to produce a competitive antagonist of NOS and alter cell function.

We have recently shown that while SCaM-1 is expressed in healthy soybeans and tobacco plants, SCaM-4 expression is rapidly induced when plants are challenged by fungal elicitors or bacterial pathogens (34). SCaM-4 expression correlated with the expression of several protective pathogen-resistant proteins. We have previously predicted that if SCaM-1 inhibits plant NOS, then pathogens could perhaps induce NOS activation by expression of SCaM-4 (16). Recent studies have shown that when soybean (6) and tobacco (7) cells are exposed to pathogens, a Ca-dependent NOS is activated producing a 4–5-fold increase in NO production. In both plants, NO induced the synthesis of pathogen-resistant proteins and hypersensitive cell death to protect the plant from pathogen attack. Mammalian NOS inhibitors prevented the hypersensitive disease resistance response to pathogen, resulting in a promotion of the disease and bacterial growth (6, 7). The introduction of recombinant mammalian NOS to tobacco plants stimulated the production of pathogen-resistant proteins (7). If SCaM-1 inhibits plant NOS like it inhibits mammalian NOS, then it is likely that it could prevent NO production and hypersensitive cell death in healthy plants. The SCaM-4 isoform activates NOS (16), and its expression when a plant is challenged by a pathogen could result in the activation of plant NOS, the production of NO and pathogen-resistant proteins to protect the plant from pathogen attack.

These speculations can be tested by determining the effect of SCaM-1 and SCaM-4 on activation of soybean NOS. If soybean NOS is competitively inhibited by SCaM-1 and M144V, then it would appear that nature has designed this M144V point mutation in SCaM-1 to produce a selective competitive antagonist of NOS.

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