Plant Physiol. (1995) 108: 543–549

Calcium/Calmodulin Activation of Soybean Glutamate Decarboxylase[1]

Wayne A. Snedden, Tzahi Arazi, Hillel Fromm, and Barry J. Shelp*

Department of Horticultural Science and Interdepartmental Plant Physiology Program, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (W.A.S., B.J.S.); and Department of Plant Genetics, Weizmann Institute of Science, 76100 Rehovot, Israel (T.A., H.F.)

Recently, we provided preliminary evidence for calcium (Ca^{2+})/calmodulin (CaM) stimulation of plant glutamate decarboxylase (GAD; EC 4.1.1.15). In the present study, a detailed characterization of the phenomenon is described. GAD was partially purified from various soybean (*Glycine max* L. Merr.) tissues (developing seed coat and cotyledons, leaf, and root) in the presence of EDTA by a combination of ammonium sulfate precipitation and anion-exchange fast protein liquid chromatography. GAD activity showed a sharp optimum at pH 5.8, with about 12% of maximal activity at pH 7. It was stimulated 2- to 8-fold (depending on the tissue source) in the presence of Ca^{2+}/CaM at pH 7 but not at pH 5.8. Furthermore, when the protease inhibitor phenylmethylsulfonyl fluoride was omitted from the purification procedure, GAD activity was insensitive to Ca^{2+}/CaM but was similar in magnitude to CaM-stimulated activity. The stimulation by Ca^{2+}/CaM was fully inhibited by the CaM antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and trifluoperazine. With saturating CaM or Ca^{2+}, the concentrations of Ca^{2+} and CaM required for half-maximal stimulation were about 7 to 11 μM and 25 nM, respectively. The effect of Ca^{2+} and CaM appeared to be through a 2.4-fold stimulation of V_{max} and a 55% reduction in K_m. The results suggested that GAD is activated via Ca^{2+} signal transduction.

GABA is a ubiquitous nonprotein amino acid that is produced in plants almost exclusively from an α-decarboxylation of L-Glu catalyzed by the enzyme GAD (EC 4.1.1.15) (Bown and Shelp, 1989; Satya Narayan and Nair, 1990). GABA undergoes transamination to yield succinic semialdehyde, which in turn is converted to succinate. This involves the enzymes GABA transaminase and succinic semialdehyde dehydrogenase, which together with GAD make up the GABA shunt (Bown and Shelp, 1989; Satya Narayan and Nair, 1990). Therefore, GABA is a metabolite en route from glutamate to succinate and the Krebs cycle (Tuin and Shelp, 1994).

A variety of environmental stress conditions including hypoxia, temperature shock, and mechanical manipulation induce rapid GABA accumulation (Streeter and Thompson, 1972; Wallace et al., 1984; Shelp et al., 1995). Furthermore, GABA accumulates in wheat roots in response to treatment with the stress-related phytohormone ABA (Regianni et al., 1993). The role of GABA in plants is unclear, whereas its role as an inhibitory neurotransmitter in animals is well established (Erlander and Tobin, 1991). It has been suggested that GABA is part of an adaptive response to cytosolic acidosis (Guern et al., 1986; Snedden et al., 1992; Carroll et al., 1994; Crawford et al., 1994); however, not all treatments that induce GABA synthesis are associated with declines in intracellular pH (Crawford et al., 1994). Thus, other factors appear be involved in the activation of GAD in plant cells (Fig. 1).

Interestingly, many of the same stresses that stimulate GABA synthesis in plants also cause fluxes in cytosolic Ca^{2+} (Knight et al., 1991) (Fig. 1). Ca^{2+} is an important messenger in plant signal transduction and is involved in the physiological responses to a variety of environmental stimuli (Muto, 1992; Pooviah and Reddy, 1993). Ca^{2+} exerts its modulatory properties by reversibly binding to specific target proteins, one of which is CaM. Upon binding to Ca^{2+}, CaM undergoes a conformational change that facilitates its binding to different target proteins. Because it has no known enzymatic activity of its own, it is through the stimulation of these proteins that CaM mediates the Ca^{2+} response to stimuli. Only a few CaM-regulated proteins in plants have been identified (e.g. Ca^{2+}-pumping ATPase, NAD kinase, NTPase; reviewed by Roberts and Harmon, 1992); none of these appear to be associated with a specific metabolic pathway.

Baum et al. (1993) reported that petunia GAD is a CaM-binding protein, and Ling et al. (1994) provided the first evidence that plant GAD is stimulated by Ca^{2+}/CaM. In the present study, a detailed characterization of the biochemical regulation of soybean (*Glycine max* L.) GAD by Ca^{2+} and CaM is described. In a companion paper (Arazi et al., 1995), we presented a molecular dissection of the GAD CaM-binding domain and its interaction with CaM. The data support a model of Ca^{2+}/CaM-mediated activation of GABA synthesis (Fig. 1).

[1] This work was supported by grants to B.J.S. from the Natural Sciences and Engineering Research Council (NSERC) of Canada and to H.F. from the Wolfson Research Awards administered by the Israel Academy of Sciences and Humanities. W.A.S. was the receipient of an NSERC Postgraduate Scholarship.

* Corresponding author; e-mail bshelp@evbhort.uoguelph.ca; fax 1–519–767–0755.

Abbreviations: CaM, calmodulin; GABA, 4-aminobutyrate; GAD, L-glutamate decarboxylase; NTPase, nucleoside triphosphatase; PLP, pyridoxal-5′-phosphate; TFP, trifluoperazine; W5, *N*-(6-aminohexyl)-1-napthalenesulfonamide; W7, *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide.

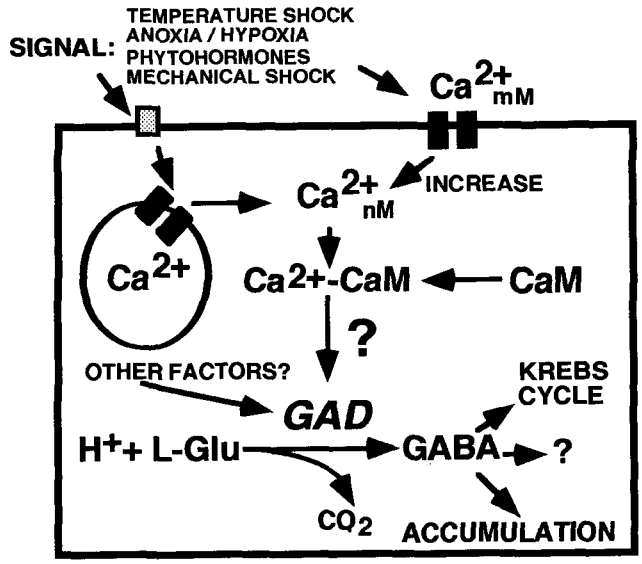

Figure 1. Model of GABA production in relation to environmental stimuli and Ca^{2+} flux. The in vivo mechanism(s) of GAD regulation and physiological role(s) of GABA remain unclear and are represented by question marks.

MATERIALS AND METHODS

Plant Materials

Methods for the growth of nonnodulated soybean (*Glycine max* L. Merr. cv Maple Arrow) plants were described previously (Tuin and Shelp, 1994). Plants were grown in 9-L pots (six plants per pot) containing Pro-mix BX (Les Tourbières Premier Lteé, Rivière du Loup, Quebec, Canada) in a naturally lighted greenhouse. During the winter months supplemental lighting was provided by high-pressure sodium vapor lamps yielding a photosynthetic photon flux of 60 μmol m^{-2} s^{-1} at pot level with a 16-h light/8-h dark period. Pots were watered twice weekly with one-quarter-strength Hoagland-type (Hoagland and Arnon, 1950) nutrient solution containing 8 mM nitrogen (as NO_3) and with tap water as required.

Partial Purification of GAD

Unless indicated otherwise, all procedures were performed at 4°C. Developing soybean fruits (approximately 26 d after anthesis) were harvested and immediately frozen in liquid nitrogen. Seed coats (5 g) were removed from the developing seeds and ground using a mortar and pestle with sterile sand and 5 volumes of 100 mM 1,3-bis-Tris-HCl (pH 7) extraction buffer containing 10% (v/v) glycerol, 1 mM DTT, 5 mM disodium-EDTA, 0.5 mM PLP, and 1 mM PMSF. The homogenate was centrifuged at 23,000*g* for 20 min and the resultant supernatant was used to prepare a 30 to 60% (w/v) ammonium sulfate pellet, which was solubilized in extraction buffer and desalted by passage through a Sephadex G-25M PD-10 column (Pharmacia). The desalted sample was spiked with PMSF to a final concentration of 1 mM and loaded onto a Waters Protein-Pak DEAE 8HR anion-exchange column (10 × 100 mm) pre-equilibrated with 50 mM bis-Tris-HCl buffer (pH 7), containing 1 mM disodium-EDTA, 1 mM DTT, and 10% (v/v) glycerol at a flow rate of 1.0 mL min^{-1}. Proteins were eluted using a 70-min linear gradient of 0 to 1 M NaCl in the column equilibration buffer described above. PLP was included in the collection tubes to give a final concentration of 0.1 mM. The fractions containing GAD activity were concentrated using a Centriprep-30 concentrator (Amicon, Beverly, MA), frozen in liquid nitrogen, and stored at −70°C until required. With this procedure, a purification of about 8-fold was obtained with approximately 75% recovery of initial GAD activity. Several independent partial purifications were carried out and pooled for GAD assays. All procedures for the partial purification of GAD from developing cotyledons, leaves, and roots were as described above. Developing cotyledons were harvested from fruits about 26 d after anthesis. First trifoliolates and main and lateral roots from 2- to 3 week-old plants were used as other sources of GAD. For determination of protein, 100 μL of sample were added to 1 mL of 12% (w/v) cold TCA and left overnight at 4°C. The protein precipitate was pelleted by centrifugation for 1 min at 10,000*g* and then dissolved in 0.1 M NaOH. Protein was measured using Bradford (1976) reagent (Pierce) with BSA as the standard.

GAD Assays

Pooled sample was assayed for GAD activity as L-[1-^{14}C]Glu-dependent $^{14}CO_2$ production (Snedden et al., 1992). Unless indicated otherwise, samples were incubated at 30°C for 30 min in 34-mL sealed serum vials (Wheaton, Millville, NJ) containing a CO_2 trap of 0.5 mL of 0.1 N NaOH (prepared fresh daily). Enzyme activity was linear with time and sample volume (20–200 μL). The reaction medium consisted of a 50-μL sample (containing 0.01 to 0.5 mg of protein depending on tissue source), 100 mM bis-Tris-HCl buffer (pH 7), 1 mM DTT, 10 mM L-glutamate (2.5 μCi $mmol^{-1}$, Amersham), 0.5 mM PLP, and 10% (v/v) glycerol in a final volume of 2 mL. Ca^{2+} (as $CaCl_2$) and bovine brain CaM (Sigma) were included as required at final concentrations of 500 μM and 200 nM, respectively. When required, CaM antagonists (W5, W7, and TFP) were present at 100 μM; W5 and W7 were initially dissolved in 95% (v/v) ethanol and then added at time zero to the reaction mixture at a final ethanol concentration of 1% (v/v). EGTA or EDTA were not included because these chelators were found to competitively inhibit GAD activity in this assay system (W.A. Snedden and B.J. Shelp, unpublished data). The reaction was terminated by the injection of 0.1 mL of 18 N H_2SO_4 into the reaction medium. Vials were left at 4°C overnight before the ^{14}C content of the CO_2 trap was determined using liquid scintillation spectrometry. Assays were usually conducted in triplicate; results presented represent a typical experiment. Kinetics data were analyzed using both half-reciprocal and direct-linear plots, which are considered more accurate than conventional double-reciprocal plots (Wharton and Eisenthal, 1981)

RESULTS

GAD Activity as a Function of Ca^{2+}/CaM and PMSF during Purification

When PMSF was omitted from the purification procedure, Ca^{2+}/CaM stimulated seed coat GAD activity at pH 7 by only 20% (Fig. 2). With PMSF, the activity was reduced by about 80% but was restored with the addition of Ca^{2+}/CaM. The activity of GAD prepared with PMSF and assayed in the presence of Ca^{2+}/CaM was similar to that of GAD prepared without PMSF. PMSF included in the GAD assays did not affect the activity of either control or Ca^{2+}/CaM-stimulated samples (data not shown). The addition of other protease inhibitors (leupeptin, pepstatin A, and antipain, each at 2.5 μg mL^{-1}) during the purification procedure did not enhance the response to Ca^{2+}/CaM (data not shown).

GAD Activity as a Function of pH and the Presence of CaM Antagonists

The pH response of partially purified soybean GAD from developing cotyledons or leaves was investigated using overlapping buffer systems. In both cases, a sharp pH optimum was found at pH 5.8, with about 12% of maximal activity at pH 7 (data not shown).

At pH 7, in the presence of 500 μM Ca^{2+} and 200 nM CaM, seed coat GAD activity was more than 3-fold greater than controls lacking Ca^{2+}/CaM (Fig. 3A). Samples containing CaM but lacking Ca^{2+} displayed similar activity to controls lacking both Ca^{2+} and CaM. A similar result was observed using GAD from leaves (data not shown). At pH 5.8, no significant stimulation of GAD activity by Ca^{2+}/CaM was observed. However, activity at pH 5.8 was greater than controls and Ca^{2+}/CaM-stimulated GAD at pH 7 by about 10- and 1.6-fold, respectively.

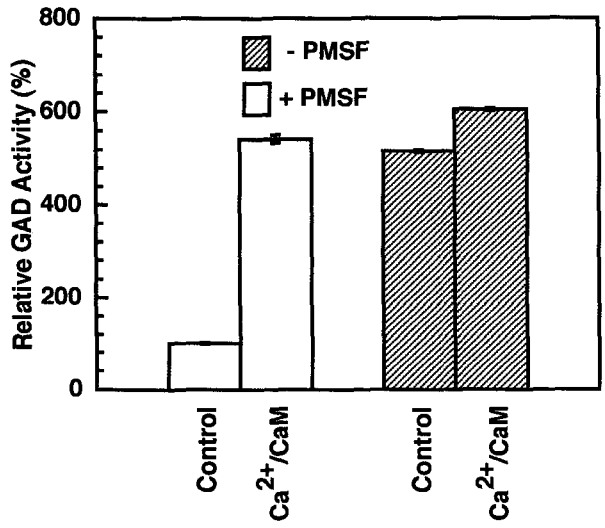

Figure 2. Ca^{2+}/CaM activation of the activity at pH 7 of seed coat GAD that was partially purified in the absence or presence of the protease inhibitor PMSF (1 mM). Ca^{2+} and CaM when present were included at final concentrations of 500 μM and 200 nM, respectively. Values are shown as a percentage of activity observed in control samples extracted without PMSF and represent the means ± SE.

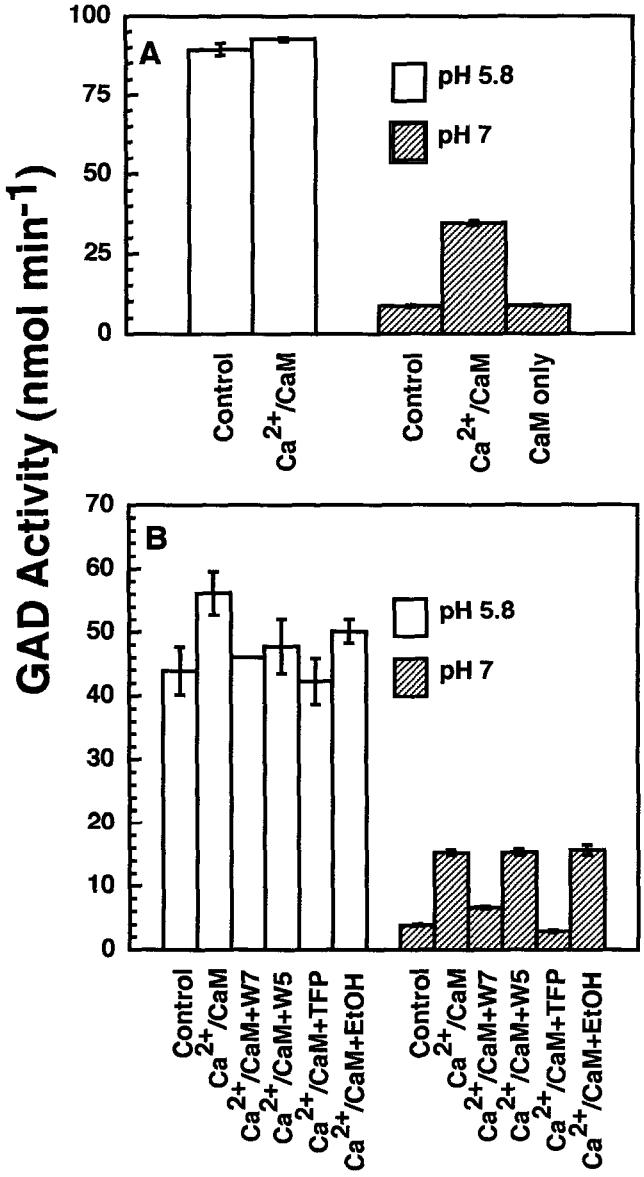

Figure 3. Effects of pH (A and B) and CaM antagonists (B) on Ca^{2+}/CaM activation of seed coat GAD activity at pH 7. Ca^{2+} and CaM when present were included at final concentrations of 500 μM and 200 nM, respectively. Also shown is the effect of CaM on GAD activity (A) in the absence of Ca^{2+} at pH 7. As an additional control, ethanol (B), the solvent for W7 and W5, was also included with a Ca^{2+}/CaM treatment. Values represent the means ± SE of at least three determinations except for the sample containing W7 (B), which are the means ± SE of two determinations.

The influence of the CaM antagonists TFP and W7 (Asano and Hidaka, 1984) and the inactive W7 analog W5 on GAD stimulation by Ca^{2+}/CaM is presented in Figure 3B. In the presence of 100 μM W7 or TFP, Ca^{2+}/CaM failed to stimulate GAD activity at pH 7. However, neither W5 nor ethanol affected the stimulation of GAD activity by Ca^{2+}/CaM. At pH 5.8, no significant differences between any of the treatments were observed.

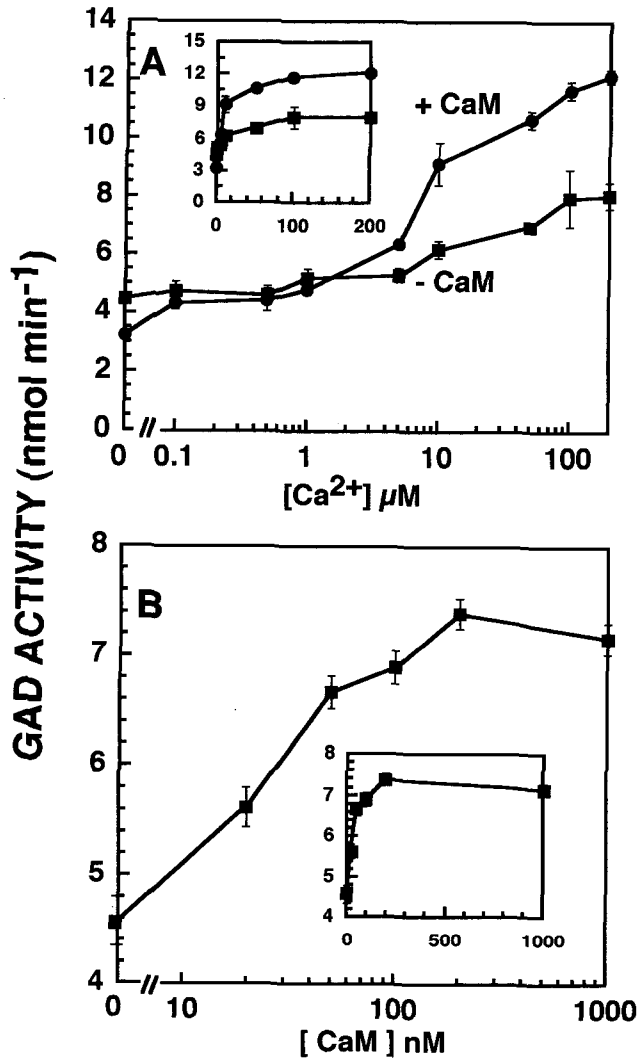

Figure 4. Seed coat GAD activity at pH 7 as a function of Ca^{2+} (A) and CaM concentrations (B). The data are shown on both semi-log and linear plots. The CaM (A) and Ca^{2+} concentrations (B) were 200 nM and 500 μM, respectively. Note that in B the *y* axis does not begin at zero; the control activity was 1.86 ± 0.15 nmol min^{-1}. Values represent the means ± SE; SE is not presented when smaller than the symbol.

GAD Activity as a Function of Ca^{2+} and CaM Concentrations

GAD activity at pH 7 displayed a hyperbolic response to increasing levels of Ca^{2+}, saturating between 50 and 100 μM Ca^{2+} in the presence or absence of 200 nM CaM (Fig. 4A). In the presence of CaM, activity increased by about 3-fold with increasing Ca^{2+} levels. The most dramatic response was observed between 0 and 10 μM Ca^{2+}, with half-maximal stimulation at about 7 μM Ca^{2+}. In the absence of CaM, activity increased less than 1-fold over the range of Ca^{2+} levels tested, with half-maximal stimulation at about 11 μM Ca^{2+}. GAD activities at 500 μM Ca^{2+} were similar to those found at 200 μM (data not shown). Another divalent cation, magnesium (as $MgCl_2$), at a final concentration of 500 μM, had no effect on GAD activity (data not shown).

GAD activity in the presence of saturating (500 μM) Ca^{2+} increased very rapidly with CaM concentrations from 0 to 50 nM and saturated at about 200 nM; half-maximal stimulation was observed at about 25 nM (Fig. 4B). Even in the absence of added CaM, a stimulation of about 2-fold was observed in the presence of 500 μM Ca^{2+}, a result consistent with those of Figure 4A.

GAD Activity as a Function of Ca^{2+}/CaM and Glutamate Concentration

A half-reciprocal plot of GAD activity in the presence or absence of 500 μM Ca^{2+} and 200 nM CaM is presented in Figure 5. When Ca^{2+}/CaM was absent, calculated values of K_m and V_{max} were about 21 mM and 324 nmol min^{-1} mg^{-1} protein, respectively. However, in the presence of added Ca^{2+}/CaM, K_m and V_{max} values were about 9 mM and 1110 nmol min^{-1} mg^{-1} protein, respectively. Analyses using direct linear plots gave similar values. This represents a 55% decrease in K_m and a 242% increase in V_{max} as a consequence of Ca^{2+}/CaM.

GAD Activity in Various Tissues

Of the tissues examined, the highest specific activity was observed in developing seed coat and the lowest in developing cotyledons (Table I). Regardless of the source, a stimulation by Ca^{2+}/CaM, which varied from 250 to 836%, was observed.

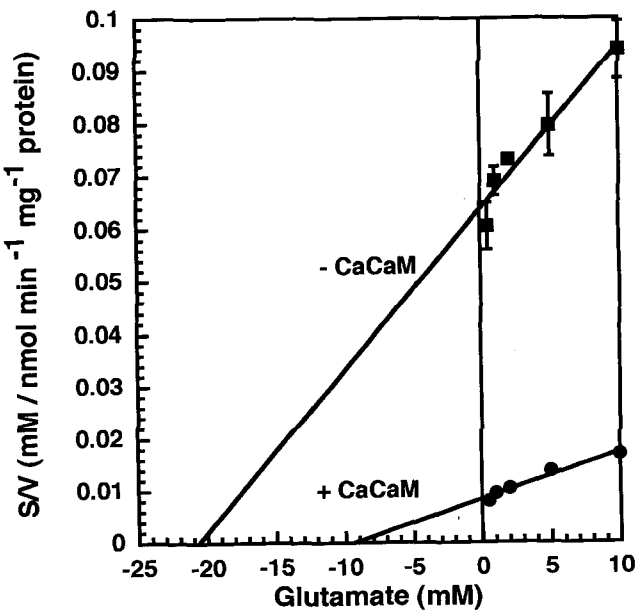

Figure 5. Ca^{2+}/CaM activation of partially purified seed coat GAD activity at pH 7 as a function of L-Glu concentration. Ca^{2+} and CaM when present were included at final concentrations of 500 μM and 200 nM, respectively. The assay duration was 15 min. Values represent the means ± SE; where SE is not shown it is smaller than the symbol. Lines were best-fitted by linear-regression techniques.

Table I. *Tissue comparisons of GAD activities and response to Ca^{2+}/CaM*

Each value represents the mean ± SE of at least three determinations. Assay conditions were as described in "Materials and Methods."

Soybean Tissue	Specific Activity		
	Control	+Ca^{2+}/CaM	Percent Control
	nmol min^{-1} mg^{-1} protein		
Developing seed coat	133 ± 5	721 ± 42	542
Developing cotyledons	0.53 ± 0.06	4.96 ± 0.02	936
Root	9.1 ± 0.4	68.9 ± 0.6	757
Leaf	2.9 ± 0.2	10.3 ± 1.0	355

DISCUSSION

Baum et al. (1993) first reported that petunia GAD possesses a CaM-binding domain but did not detect any effect of Ca^{2+}/CaM on GAD activity at pH 5.8. Ling et al. (1994) also demonstrated that *Vicia fava* GAD is a CaM-binding protein and then presented preliminary evidence that its activity at pH 7 is stimulated by Ca^{2+}/CaM. In the present study, we provide evidence that soybean GAD is stimulated (2- to 8-fold) by Ca^{2+}/CaM ($K_{0.5}$ values of 7–11 μM and 25 nM, respectively) at pH 7 (Figs. 2–5, Table I), but not pH 5.8 (Fig. 3), and may be sensitive to proteolysis during partial purification (Fig. 2). GAD that was not protected from proteolysis during partial purification was as active as the Ca^{2+}/CaM-stimulated enzyme but was insensitive to Ca^{2+}/CaM (Fig. 2). However, an indirect effect of proteolysis on GAD activity cannot be ruled out at this point. The stimulation by Ca^{2+}/CaM occurred primarily by increasing V_{max} (2.4-fold, Fig. 5) and was independent of the soybean tissue examined (Table I). The data indicate that plant GAD may be regulated by Ca^{2+}/CaM.

Activation of GAD at pH 7 is consistent with the neutral pH of the cytosol (Felle, 1988b; Horn et al., 1992), the subcellular location of GAD (Wallace et al., 1984; Satya Narayan and Nair, 1986; Breitkreuz and Shelp, 1995). However, plant GAD displays a sharp response to pH (see "Results"; Satya Narayan and Nair, 1985; Snedden et al., 1992), and environmental stimuli known to cause acidosis may, therefore, increase GABA synthesis even in the absence of Ca^{2+}/CaM. Furthermore, there is evidence suggesting that cytosolic Ca^{2+} levels are responsive to changes in cellular pH (Felle, 1988a; Bush, 1993), as well as environmental stimuli (Knight et al., 1991). It is noteworthy that pH-independent stimulation of plant GAD activity has been observed (Crawford et al., 1994). Thus, it is possible to conclude only that several factors may act in concert to regulate GAD activity in response to external stimuli.

Ca^{2+} stimulation of GAD even in the absence of added CaM (Fig. 4) may be interpreted as a CaM-independent process or as evidence for bound CaM. Despite efforts to remove CaM bound to GAD during the purification procedure, a monoclonal antibody raised against a recombinant plant CaM detected contaminating CaM in our enzyme preparations (data not shown). Other research on plant CaM-dependent enzymes has been complicated by difficulty in removing endogenous CaM (Collinge and Trewavas, 1989; Rasi-Caldogno et al., 1993). The presence of contaminating CaM and possibly Ca^{2+} (either free or bound to CaM) provides an explanation for the background (control level) activities observed in the absence of added CaM and suggests that potential CaM-stimulated activity was underestimated and the $K_{0.5}$ values for both Ca^{2+} and CaM concentrations were overestimated. Nevertheless, the Ca^{2+} and CaM concentrations required for half-maximal stimulation of GAD activity are similar to those reported for other plant CaM-binding proteins and fall within physiological range (Roberts and Harmon, 1992). Estimates of K_m for Glu in the absence of Ca^{2+}/CaM (21 mM, Fig. 5) are similar to values reported for plant GAD from other embryonic sources, whereas estimates of K_m in the presence of Ca^{2+}/CaM (9 mM, Fig. 5) resemble values reported for mature tissues (Bown and Shelp, 1989; Satya Narayan and Nair, 1990).

The differences observed in specific activity of GAD from different tissues (Table I) may reflect different states of activation, multiple forms of GAD, or differing degrees of contamination by CaM or proteolysis of GAD. For example, in the present study CaM activation of soybean GAD appears to be Ca^{2+} dependent, whereas petunia GAD possesses some Ca^{2+}-independent activation by CaM (Arazi et al., 1995). Different forms of GAD with distinct properties and perhaps metabolic roles have been reported for barley embryos and roots (Inatomi and Slaughter, 1975) and animal cells (Nathan et al., 1994). Although plant GAD appears to be unique among eukaryotes in possessing a CaM-binding domain, one form of brain GAD has been shown to associate peripherally with intracellular membranes in a Ca^{2+}-dependent manner (Salganicoff and De Robertis, 1965; Nathan et al., 1994).

Only a few plant CaM-stimulated enzymes are known (reviewed by Roberts and Harmon, 1992). After controlled proteolysis, the Ca^{2+}-pumping ATPase of radish is activated but is no longer sensitive to CaM (Rasi-Caldogno et al., 1993). It displays maximal stimulation (2- to 3-fold; Dieter and Marmé, 1981, 1983; Rasi-Caldogno et al., 1993) at pH 7 (Askerlund and Evans, 1992) and half-maximal saturation at Ca^{2+} and CaM concentrations of 5 to 10 μM (Brauer et al., 1990; Askerlund and Evans, 1992; Rasi-Caldogno et al., 1993) and 160 to 175 nM (Dieter and Marmé, 1981; Askerlund and Evans, 1992), respectively. A variety of CaM-binding proteins from animals also lose their CaM responsiveness and yet retain substantial activity upon proteolysis of the CaM-binding domain (Colbran et al., 1988; Hubbard and Klee, 1988; Carafoli, 1991; Sheta et al., 1994). Interestingly, the CaM-binding domain of the plasma membrane Ca^{2+}-pumping ATPase from animal cells is also an autoinhibitory domain, which when cleaved increases enzyme activity but renders it insensitive to stimulation by CaM (Carafoli, 1991). Despite precautions in the present study to prevent partial proteolysis of GAD, it cannot be excluded and may have contributed to background activity. It is not clear whether susceptibility to partial proteolysis has any physiological relevance or is a general characteristic of plant CaM-binding proteins.

A nuclear NTPase from pea has recently been cloned

(Hsieh and Roux, 1994) and is stimulated 3.5-fold by Ca^{2+}/CaM, with half-maximal stimulation occurring at 20 to 30 nM CaM (Chen et al., 1987). NAD kinase appears to be almost entirely dependent on added Ca^{2+}/CaM (Harmon et al., 1984). In both the NTPase and the Ca^{2+}-pumping ATPase, Ca^{2+}/CaM has a much greater effect on V_{max} than on K_m (Chen et al., 1987; Askerlund and Evans, 1992).

Our finding that Ca^{2+}/CaM activates GAD activity in vitro, together with evidence for rapid Ca^{2+} fluxes (Knight et al., 1991), suggests that environmental stimuli and stress conditions influence GABA synthesis via a Ca^{2+}/CaM signal transduction pathway (Fig. 1). Whether other factors are involved in the stimulation (or deactivation) of GAD is unknown. Because GAD catalyzes the first step in the GABA shunt (glutamate → GABA → succinic semialdehyde → succinate) and is an irreversible reaction, it may represent the key, regulatory point of this pathway in plants. To the best of our knowledge, it does represent in plants the first Ca^{2+}/CaM-stimulated enzyme reported to be associated with a specific metabolic pathway. Moreover, the present biochemical characterization of GAD, combined with molecular (Baum et al., 1993; Chen et al., 1994; Ling et al., 1994; Arazi et al., 1995) and physiological studies (Crawford et al., 1994), makes GAD one of the most thoroughly studied Ca^{2+}/CaM-binding enzymes of higher plants.

Received September 9, 1994; accepted February 2, 1995.

LITERATURE CITED

Arazi T, Baum G, Snedden WA, Shelp BJ, Fromm H (1995) Molecular and biochemical analysis of calmodulin interactions with the calmodulin-binding domain of plant glutamate decarboxylase. Plant Physiol **108:** 551–561

Asano M, Hidaka H (1984) Biopharmacological properties of napthalenesulfonamides as potent calmodulin antagonists. *In* W Cheung, ed, Calcium and Cell Function, Vol 5. Academic Press, Toronto, pp 123–164

Askerlund P, Evans DE (1992) Reconstitution and characterization of a calmodulin-stimulated Ca^{2+}-pumping ATPase purified from *Brassica oleracea* L. Plant Physiol **100:** 1670–1681

Baum G, Chen Y, Arazi T, Takatsuji H, Fromm H (1993) A plant glutamate decarboxylase containing a calmodulin binding domain. J Biol Chem **268:** 19610-19617

Bown A, Shelp B (1989) The metabolism and physiological roles of 4-aminobutyric acid. Biochem (Life Sci Adv) **8:** 21–25

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brauer D, Schubert C, Tsu S-I (1990) Characterization of a Ca^{2+}-translocating ATPase from corn root microsomes. Physiol Plant **78:** 335–344

Breitkreuz KE, Shelp BJ (1995) Subcellular compartmentation of the 4-aminobutyrate shunt in protoplasts from developing soybean cotyledons. Plant Physiol **108:** 99–103

Bush DS (1993) Regulation of cytosolic calcium in plants. Plant Physiol **103:** 7–13

Carafoli E (1991) Calcium pump of the plasma membrane. Physiol Rev **71:** 129–153

Carroll AD, Fox GG, Laurie S, Phillips R, Ratcliffe RG, Stewart GR (1994) Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell suspensions. Plant Physiol **106:** 513–520

Chen Y, Baum G, Fromm H (1994) The 58-kD calmodulin-binding glutamate decarboxylase is a ubiquitous protein in petunia organs and its expression is developmentally regulated. Plant Physiol **106:** 1381–1387

Chen Y-R, Datta N, Roux SJ (1987) Purification and partial characterization of a calmodulin-stimulated nucleoside triphosphatase from pea nuclei. J Biol Chem **262:** 10689-10694

Colbran RJ, Fong Y-L, Schworer CM, Soderling TR (1988) Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem **34:** 18145-18151

Collinge M, Trewavas AJ (1989) The location of calmodulin in the pea plasma membrane. J Biol Chem **262:** 8865–8872

Crawford LA, Bown AW, Breitkreuz KE, Guinel FC (1994) The synthesis of gamma-aminobutyric acid in response to treatments reducing cytosolic pH. Plant Physiol **104:** 865–871

Dieter P, Marmé D (1981) A calmodulin-dependent, microsomal ATPase from corn (*Zea mays* L.). FEBS Lett **125:** 245–248

Dieter P, Marmé D (1983) The effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and microsomal calcium-ion transport system from corn. Planta **159:** 277–281

Erlander MJ, Tobin AJ (1991) The structural and functional heterogeneity of glutamic acid decarboxylase: a review. Neurochem Res **16:** 215–226

Felle H (1988a) Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: interaction of Ca^{2+} and pH? Planta **176:** 248–255

Felle H (1988b) Short-term pH regulation in plants. Physiol Plant **74:** 583–591

Guern J, Mathieu Y, Pean M, Pasquier C, Boloeil J, Lallemand J (1986) Cytoplasmic pH regulation in *Acer pseudoplatanus* cells. I. A ^{31}P NMR description of acid-load effects. Plant Physiol **82:** 840–845

Harmon AC, Jarret HW, Courmier MJ (1984) An enzymatic assay for calmodulins based on plant NAD kinase activity. Anal Biochem **141:** 168–178

Hoagland DR, Arnon DI (1950) The water culture method of growing plants without soil. Calif Agric Exp Stn Circ 347

Horn MA, Meadows RPM, Apostol I, Jones CR, Gorenstein DG, Heinstein PF, Low PS (1992) Effect of elicitation and changes in extracellular pH on the cytoplasmic and vacuolar pH of suspension-cultured soybean cells. Plant Physiol **98:** 680–686

Hsieh H-J, Roux SJ (1994) Cloning and expression of a gene encoding a nuclear-envelope associated calmodulin-regulated nucleoside triphosphatase (abstract No. 373). Plant Physiol **105:** S-75

Hubbard MJ, Klee CB (1988) Functional domain structure of calcineurin A: mapping by limited proteolysis. Biochemistry **28:** 1868–1874

Inatomi K, Slaughter JC (1975) Glutamate decarboxylase from barley embryos and roots. Biochem J **147:** 479–484

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effect of touch and cold shock and elicitors on cytoplasmic calcium. Nature **352:** 524–526

Ling V, Snedden WA, Shelp BJ, Assman SM (1994) Analysis of a soluble calmodulin binding protein from fava bean roots: identification of glutamate decarboxylase as a calmodulin-activated enzyme. Plant Cell **6:** 1135–1143

Muto S (1992) Intracellular Ca^{2+} messenger system in plants. Int Rev Cytol **142:** 305–345

Nathan B, Hsu C-C, Bao J, Wu R, Wu J-Y (1994) Purification and characterization of a novel form of brain L-glutamate decarboxylase. J Biol Chem **269:** 7249–7254

Pooviah BW, Reddy ASN (1993) Calcium and signal transduction in plants. Crit Rev Plant Sci **12:** 185–211

Rasi-Caldogno F, Carnelli A, De Michelis MI (1993) Controlled proteolysis activates the plasma membrane Ca^{2+} pump of higher plants. Plant Physiol **103:** 385–390

Regianni R, Aurisano N, Mattana M, Bertani A (1993) ABA induces 4-aminobutyrate accumulation in wheat seedlings. Phytochemistry **34:** 605–609

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu

Rev Plant Physiol Plant Mol Biol **43:** 375–414

Salganicoff L, De Robertis E (1965) Subcellular distribution of the enzymes of the glutamic acid, glutamine and gamma-aminobutyric acid cycles in rat brain. J Neurochem **12:** 287–309

Satya Narayan V, Nair PM (1985) Purification and characterization of glutamate decarboxylase from *Solanum tuberosum*. Eur J Biochem **150:** 53–60

Satya Narayan V, Nair PM (1986) The 4-aminobutyrate shunt in *Solanum tuberosum*. Phytochemistry **25:** 997-1001

Satya Narayan V, Nair PM (1990) Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. Phytochemistry **29:** 367–375

Shelp BJ, Walton CS, Snedden WA, Tuin LG, Oresnik IJ, Layzell DB (1995) Gaba shunt in developing soybean seed is associated with hypoxia. Physiol Plant **94**: (in press)

Sheta EA, McMillan K, Masters BSS (1994) Evidence for a biodomain structure of constitutive cerebellar nitric oxide syntase. J Biol Chem **269:** 15147-15153

Snedden WA, Chung I, Pauls RH, Bown AW (1992) Proton/L-glutamate symport and the regulation of intracellular pH in isolated mesophyll cells. Plant Physiol **99:** 665–671

Streeter JG, Thompson JF (1972) Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). Plant Physiol **49:** 572–578

Tuin LG, Shelp BJ (1994) *In situ* [^{4}C]glutamate metabolism by developing soybean cotyledons. I. Metabolic routes. J Plant Physiol **143:** 1–7

Wallace, Secor J, Schrader L (1984) Rapid accumulation of gamma-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol **75:** 170–175

Wharton CW, Eisenthal R (1981) Molecular Enzymology. Blackie, Glasgow, Scotland, pp 275–285