

Update on Biochemistry

The Metabolism and Functions of γ -Aminobutyric Acid¹

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GABA is a four-C, nonprotein amino acid found in virtually all prokaryotic and eukaryotic organisms as a significant component of the free amino acid pool. In higher plants GABA is synthesized primarily through the H⁺-consuming α -decarboxylation of L-glutamate (L-Glu) in a reaction (L-Glu + H⁺ → GABA + CO₂) catalyzed by L-Glu decarboxylase (EC 4.1.1.15). It is metabolized through a reversible transamination (GABA + pyruvate = succinic semialdehyde + Ala) catalyzed by GABA transaminase (EC 2.6.1.19). The product of transamination, succinic semialdehyde, is oxidized to succinate (succinic semialdehyde + NAD + H₂O → succinate + NADH) in an irreversible reaction catalyzed by succinate semialdehyde dehydrogenase (EC 1.2.1.16). These three reactions constitute a pathway known as the GABA shunt (Fig. 1). These and other features of GABA metabolism in higher plants have been discussed previously (Bown and Shelp, 1989; Satyanaryan and Nair, 1990, and refs. therein).

There is considerable literature demonstrating rapid and large accumulations of GABA in response to many diverse stimuli. For example, in response to cold shock or mechanical stimulation, GABA levels in soybean (*Glycine max*) leaves rise 20- to 40-fold within 5 min to 1 to 2 $\mu\text{mol g}^{-1}$ fresh weight (Wallace et al., 1984). There is a corresponding decrease in L-Glu concentration. These changes in amino acid concentration are reversed within 60 min. Other reports in various tissues indicate increases in response to mechanical stimulation, mechanical damage, cold shock, heat shock, hypoxia, cytosolic acidification, water stress, and phytohormones (for refs., see Table I). There is no evidence that rapid, stress-induced GABA synthesis involves time-consuming de novo synthesis of GAD, and increased synthesis has been ascribed to a rapid, in vivo stimulation of GAD activity (Wallace et al., 1984; Tsushida and Murai, 1987). The data raise two significant questions: what are the in vivo mechanisms activating GAD, and what are the roles of GABA accumulation? Recent reports indicate that GAD is activated by increases in the cytosolic levels of H⁺ or Ca²⁺ and that GABA accumulation is important in pH regulation, as an alternative pathway for

Glu utilization, in N storage, in plant development, and in the plant's defense against phytophagous insects.

REGULATION OF GAD

Stimulation by H⁺

In vitro GAD from a variety of tissues has a sharp, acidic pH optimum around 5.8, with 10 to 40% of maximal activity at pH 7.0 (Tsushida and Murai, 1987; Snedden et al., 1992, 1995; Shelp et al., 1995). GAD is a cytosolic enzyme (Wallace et al., 1984; Breikreuz and Shelp, 1995), and various reports have suggested that stress-induced GABA synthesis results from cytosolic acidosis and the consequent activation of GAD (Tsushida and Murai, 1987; Reggiani et al., 1988; Snedden et al., 1992). This proposal is now supported by in vivo experiments demonstrating that an increase in cytosolic H⁺ levels precedes GABA accumulation (Crawford et al., 1994; Carroll et al., 1994). These experiments also support the hypothesis that GABA synthesis in response to H⁺ is a pH-regulating mechanism. They are discussed in more detail in "Biochemical pH-Stat." Thus, both in vitro and in vivo data indicate that reduced cytosolic pH activates GAD activity and GABA accumulation (Fig. 2).

Activation by Ca²⁺/CaM

It is unlikely that the multitude of environmental factors that stimulate GABA accumulation are all mediated by a decrease in cytosolic pH. Stress factors such as touch or cold shock, which stimulate GABA levels (Wallace et al., 1984), are also known to increase cytosolic Ca²⁺ levels (Knight et al., 1991). The first direct evidence for Ca²⁺-stimulated GAD activity was obtained by screening a cDNA expression library from petunia (*Petunia* sp.) with ³⁵S-labeled CaM (Baum et al., 1993). Ca²⁺-dependent binding of CaM to a 58-kD protein was observed. The recombinant protein displayed GAD activity. Although GAD from a variety of sources has been examined, only plant GAD exhibited CaM-binding activity. The activity of partially purified GAD from bean roots was stimulated 100% after the addition of 100 μM Ca²⁺ plus 100 nM CaM (Ling et al., 1994).

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Abbreviations: CaM, calmodulin; GABA, γ -aminobutyric acid; GAD, L-glutamate decarboxylase.

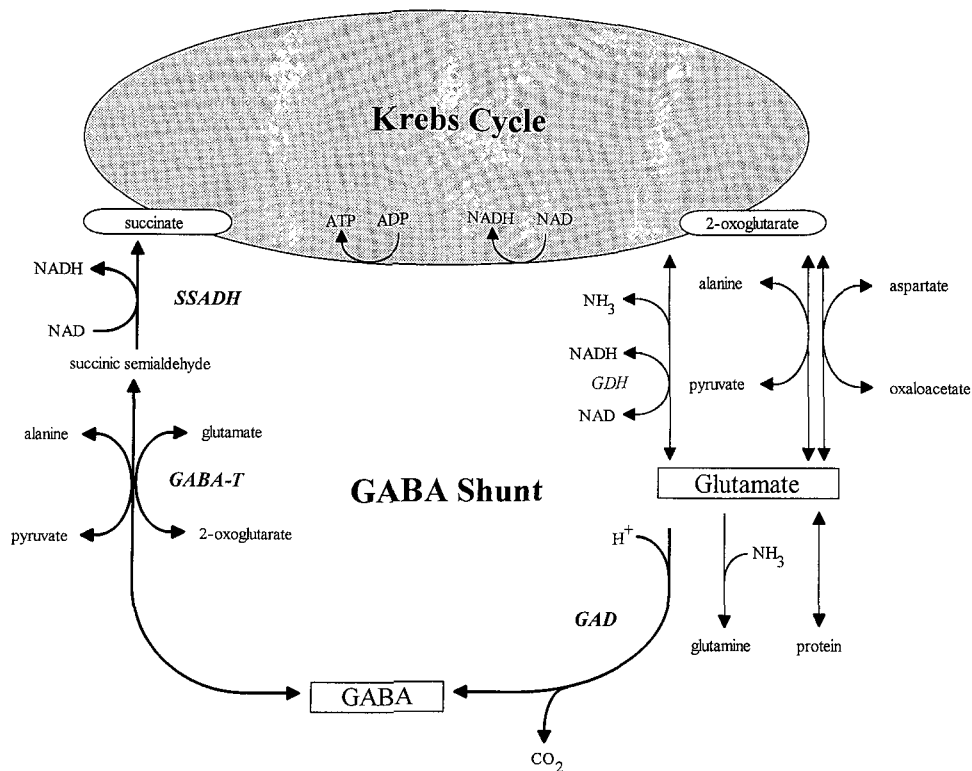


Figure 1. The GABA shunt and its relationship to other metabolic pathways. Enzymes are indicated in italics; those associated exclusively with the GABA shunt are in boldface. *SSADH*, Succinic semialdehyde dehydrogenase; *GABA-T*, GABA transaminase; *GDH*, L-glutamate dehydrogenase.

GAD from a variety of soybean tissues was used to investigate the relationship between H⁺ and Ca²⁺/CaM activation of GAD (Snedden et al., 1995). Activity was maximal at pH 5.8, with 12% of maximal activity at pH 7.0. The addition of 500 μM Ca²⁺ plus 200 nM CaM stimulated activity at pH 7.0 by 2- to 8-fold, but did not stimulate activity at pH 5.8. CaM antagonists eliminated the stimulation caused by Ca²⁺/CaM addition. Differences in the level of Ca²⁺/CaM activation of GAD from different tissues were interpreted as resulting from contamination by CaM or by proteolysis of the CaM-binding domain during GAD isolation. To eliminate these difficulties, the recombinant petunia GAD has been produced in *Escherichia coli*, which lacks CaM (Snedden et al., 1996). The purified 58-kD

enzyme was inactive at pH 7.0 when tested in the absence of Ca²⁺ or CaM. The addition of CaM ($K_{0.5}$ of 15 nM) plus Ca²⁺ ($K_{0.5}$ of 0.8 μM) resulted in levels of activity similar to those obtained at pH 5.8 in the absence of CaM and Ca²⁺. In addition, a monoclonal antibody specific for the 26-amino acid, CaM-binding, carboxyl-terminal region fully activated GAD in the absence of Ca²⁺/CaM. These experiments demonstrate that Ca²⁺/CaM or antibody binding to an autoinhibitory domain activates GAD.

In vivo evidence for Ca²⁺/CaM activation of GAD has been obtained with Ca²⁺ channel blockers and CaM antagonists. Treatment of rice (*Oryza sativa*) roots for 1 h with these agents blocked the GABA accumulation normally observed during a subsequent 3-h period of anoxia (Au-

Table 1. Treatments stimulating GABA accumulation

Treatment	Tissue	Reference
Mechanical stimulation	Soybean leaves	Wallace et al., 1984
Mechanical damage	Soybean leaves	Ramputh and Bown, 1996
Cold shock	Soybean leaves	Wallace et al., 1984
	Asparagus mesophyll cells	Cholewa et al., 1997
Heat shock	Cowpea cell cultures	Mayer et al., 1990
Hypoxia	Tea leaves	Tsushida and Murai, 1987
	Rice roots	Reggiani et al., 1988; Aurisano et al., 1995a
Cytosolic acidification	Asparagus mesophyll cells	Crawford et al., 1994
	Carrot cell suspensions	Carroll et al., 1994
Water stress	Tomato leaves and roots	Bolarin et al., 1995
Phytohormones	Root cultures	Ford et al., 1996

risano et al., 1995b). A more detailed study investigated the role of $\text{Ca}^{2+}/\text{CaM}$ in cold-shock-stimulated GABA accumulation in isolated mesophyll cells (Cholewa et al., 1997). That study satisfied the criteria for establishing Ca^{2+} involvement in a physiological response. A fluorescent indicator demonstrated an increase in cytosolic Ca^{2+} levels within 2 s of cold shock. Detectable GABA accumulation occurred within 1 min. In the absence of cold shock, a Ca^{2+} ionophore stimulated cytosolic Ca^{2+} levels and GABA synthesis. Ca^{2+} channel blockers or CaM antagonists inhibited cold-shock-stimulated GABA synthesis. Thus, both in vitro and in vivo data indicate that elevated Ca^{2+} levels stimulate GABA synthesis (Fig. 2).

GAD activation through increases in H^+ or Ca^{2+} levels indicates two independent signal transduction pathways leading to GABA accumulation. Alternatively, an increase in the level of one ion that results in an increase in the other could indicate a correlation only between GAD activity and ion level. However, the in vitro data illustrate that although $\text{Ca}^{2+}/\text{CaM}$ stimulates activity at pH 7.0, stimulation was not observed at the optimal pH of 5.8 (Snedden et al., 1995). In addition, cold-shock-induced GABA synthesis was accompanied by an increase in Ca^{2+} levels without an increase in H^+ levels, and GABA synthesis in response to cytosolic acidification was not blocked by Ca^{2+} channel blockers or CaM antagonists (Cholewa et al., 1997). Consequently, independent in vivo stimulation of GAD by H^+ and Ca^{2+} is indicated. Increases in either can be regarded as a sufficient but not a necessary requirement for GABA synthesis (Fig. 2).

ROLES OF GABA SYNTHESIS

Biochemical pH-Stat

GAD activity is not only stimulated by H^+ but also consumes H^+ ($\text{L-Glu} + \text{H}^+ \rightarrow \text{GABA} + \text{CO}_2$). Thus, it has been suggested that stress-induced GABA synthesis can contribute to pH regulation (Tsushida and Murai, 1987; Reggiani et al., 1988; Snedden et al., 1992). This proposal is

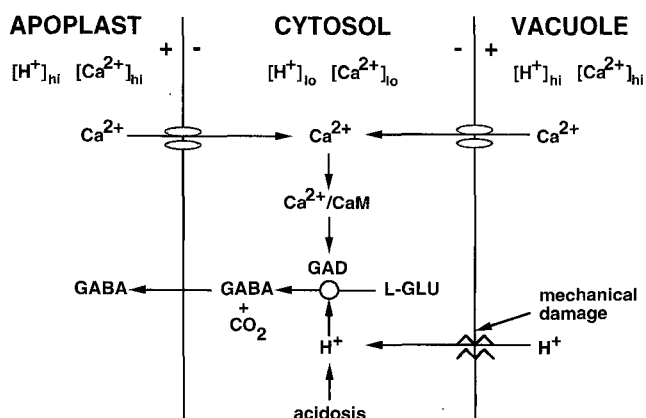


Figure 2. The stimulation of GABA synthesis through increases in cytosolic H^+ or Ca^{2+} levels. Ca^{2+} entry into the cytosol is mediated by Ca^{2+} channels located in the plasma membrane or tonoplast. Opening of these channels may occur in response to cold shock, mechanical stimulation, or the binding of ligands.

now supported by two independent investigations that monitored changes in cytosolic pH and rates of GABA accumulation. A fluorescent pH probe and an enzymatic assay for GABA were employed to measure cytosolic pH changes and GABA accumulation in photosynthetic asparagus (*Asparagus sprengeri*) cells exposed to permeant weak acids (Crawford et al., 1994). A cytosolic pH decrease of 0.6 occurred with a half-time of 2 s, and a 200 to 300% increase in GABA levels was observed within 15 s. It was calculated that after 45 s of weak acid treatment, H^+ -consuming GABA production accounted for approximately 50% of the imposed acid load. In vivo ^{31}P - and ^{15}N -NMR spectroscopy has been employed to monitor cytosolic pH and GABA levels in aerated, cultured carrot (*Daucus carota*) cells (Carroll et al., 1994). The initiation of ammonium assimilation caused a cytosolic pH decline of 0.2 unit, followed by an accumulation of GABA. GAD activity increased with reduced pH and declined as the pH recovered. Thus, two independent reports indicate that GABA accumulation, by consuming H^+ , ameliorates cytosolic acidification (Fig. 2).

Krebs Cycle Bypass

Glutamate has several potential metabolic fates (Joy, 1988; Tuin and Shelp, 1994), some of which are shown in Figure 1. The C skeleton of Glu may enter the Krebs cycle as 2-oxoglutarate or succinate. It has been suggested that the GABA shunt to succinate assumes significance when the metabolism of 2-oxoglutarate to succinate is restricted (for refs., see Satyanarayan and Nair, 1990). However, recent evidence indicates that GABA shunt activity is not a response to low rates of 2-oxoglutarate metabolism or glutamate deamination/transamination (Shelp et al., 1995; Tuin and Shelp, 1996). Because the metabolism of 2-oxoglutarate requires NAD and is tightly regulated by ADP (Wiskich, 1980), a stress such as hypoxia, which reduces respiration and the NAD/NADH ratio, can restrict the production of succinate. Such a stress may also contribute to the accumulation of GABA by reducing the oxidation of succinic semialdehyde to succinate. Following the removal of the stress, GABA provides an immediate substrate for the Krebs cycle (Wallace et al., 1984). It is noteworthy that the metabolism of glutamate to succinate via the GABA shunt is energetically less favorable (1 NADH) than via the Krebs cycle (1 NADH plus 1 ATP). If L-glutamate dehydrogenase (EC 1.4.1.2) provides a major entry point to the Krebs cycle (Melo-Oliveira et al., 1996), thereby generating an additional NADH, the energetics for the GABA shunt are relatively less favorable. This proposal remains speculative.

N Storage

Glutamate to GABA conversion is increased under conditions that inhibit Gln synthesis, reduce protein synthesis, or enhance degradation (for refs., see Satyanarayan and Nair, 1990). This has prompted the hypothesis that GABA is a temporary N store. Developing excised soybean coty-

ledons rapidly metabolize Glu to GABA (Micallef and Shelp, 1989a). The disappearance of N from these two compounds within 3 h accounts for 49% of the N required for protein synthesis during this same period (Micallef and Shelp, 1989b). Furthermore, the Glu flux through the GABA shunt is comparable to direct incorporation of Glu into protein (Tuin and Shelp, 1996). Evidence also indicates that Glu and GABA are produced during protein storage and mobilization as a means of recycling Arg-derived N and C (Bown and Shelp, 1989; Micallef and Shelp, 1989b). Thus, Glu metabolism via the GABA shunt may be of considerable importance in the N economy of plants.

The fate of GABA N remains uncertain (Fig. 1). GABA-transaminase isolated from various plant sources is 0.5 to 19 times more active with pyruvate than with 2-oxoglutarate as the amino acceptor (Satyanarayan and Nair, 1990; Shelp et al., 1995). With pyruvate the resultant amino acid is Ala, which may serve as an amino donor for the synthesis of Gly and Ser (Kleczkowski and Givan, 1988). In contrast, with 2-oxoglutarate the amino group is cycled between Glu and GABA. This "futile" cycling of the amino group in the GABA shunt may function to rid plants of excess C.

Plant Development

Evidence suggests that GABA accumulation and efflux are part of an intercellular signal transduction pathway leading to the regulation of growth and development. GAD is one of the most abundant soluble proteins in plants with a Ca^{2+} /CaM-binding domain (Baum et al., 1993, 1996), it is found in all plant tissues, and its level is regulated during development by transcription or posttranscriptional processes (Chen et al., 1994). Transgenic tobacco plants expressing a mutant GAD that lacks the auto-inhibitory CaM-binding domain exhibit higher GABA levels, lower Glu levels, and less stem elongation (Baum et al., 1996). Presumably, in these plants intracellular Ca^{2+} pools are unaltered and growth inhibition may result from altered GABA metabolism.

Separate investigations showed that mechanical manipulation transiently increases cytosolic Ca^{2+} (Knight et al., 1991) and GABA (Wallace et al., 1984; Ramputh and Bown, 1996), and inhibits stem elongation (Jones and Mitchell, 1989). Growth inhibition in response to manipulation is blocked by Ca^{2+} chelators and CaM antagonists. The hormones α -NAA and kinetin induce dedifferentiation of root tissue (Ford et al., 1996). ^{15}N -NMR studies demonstrated that these changes were accompanied by enhanced GABA and reduced Glu levels. GABA accumulation is accompanied by efflux from the cell, suggesting that it may function as an intercellular signaling molecule (Chung et al., 1992). The emerging literature suggests that environmental stresses elevate cytosolic Ca^{2+} , which in turn activates GAD and GABA synthesis, thereby regulating growth and development. However, the plant equivalent of the GABA_A and GABA_B receptors found associated with the plasma membrane of animal cells has not been identified.

Plant Defense

In animals GABA is an inhibitory neurotransmitter. It hyperpolarizes the neural membrane by stimulating Cl^- influx through GABA-gated Cl^- channels. Phytophagous activity by insects and other invertebrates destroys vacuolar compartmentation, increases H^+ levels in the cytosol, and stimulates GABA synthesis (Fig. 2). The hypothesis that the consequent ingestion of GABA inhibits normal growth and development has been investigated (Ramputh and Bown, 1996). Simulation of the mechanical damage resulting from phytophagous activity increases soybean GABA levels 10- to 25-fold within approximately 2 min to values around $2 \mu\text{mol GABA g}^{-1}$ fresh weight. This level of GABA introduced into a synthetic diet reduces the rates of growth and development and the survival of phytophagous larvae of the oblique-banded leaf roller (*Choristoneura rosaceana*). In addition, the larvae frequent terminal, light-green, expanding leaves, which produce lower GABA levels than mature leaves when damaged. In insect larvae the GABA-gated Cl^- channels in the neuromuscular junctions of body wall muscles are exposed to solutes in the hemolymph and are not protected by a blood-brain barrier. Many commercially employed insecticides are antagonists and agonists of the GABA-gated Cl^- current and are thought to inhibit normal neuromuscular activity; ingested GABA may have a similar effect.

CONCLUSIONS AND PERSPECTIVES

During the last decade research on GABA has uncovered some of the most exciting findings in the field of plant biology. Evidence indicates that stress initiates a signal transduction pathway in which increased cytosolic Ca^{2+} stimulates Ca^{2+} /CaM-dependent GAD activity and GABA synthesis. It is clear that GAD activity is also stimulated by H^+ . Increases in either Ca^{2+} or H^+ can be regarded as a sufficient, but not a necessary requirement for GABA synthesis. Control of GABA accumulation is likely mediated primarily via GAD, although more information is needed on the role of GABA-transaminase and succinic semialdehyde dehydrogenase. Experimental support for the involvement of GABA synthesis and the GABA shunt in short-term pH regulation, N storage, plant development, and plant defense is available. Future research will determine whether GABA is involved in these processes or if its major role remains to be elucidated.

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