Cytosolic Calcium Levels and Stress
Induced $\gamma$-Amino Butyrate Synthesis in Asparagus
Mesophyll Cells.

by
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Numerous investigations have demonstrated large increases in \( \gamma \)-amino butyrate (GABA) levels in response to a variety of stresses such as touch or cold shock (Wallace et al. 1984). Circumstantial evidence indicating a role of \( \text{Ca}^{2+} \) in these increases includes elevated \( \text{Ca}^{2+} \) levels in response to touch and cold shock (Knight et al. 1991), and the demonstration of a calmodulin binding domain on glutamate decarboxylase (GAD), the enzyme responsible for GABA synthesis (Baum et al. 1993). In the present study the possible role of \( \text{Ca}^{2+} \) and calmodulin in stimulation of GAD and subsequent GABA accumulation was examined using asparagus mesophyll cells. Images of cells loaded with the \( \text{Ca}^{2+} \) indicator Fluo-3 revealed a rapid and transient increase in cytosolic \( \text{Ca}^{2+} \) in response to cold shock. GABA levels increased by 106% within 15 min. of cold shock. This increase was inhibited 70% by the calmodulin antagonist W7, and 42% by the \( \text{Ca}^{2+} \) channel blocker \( \text{La}^{3+} \). Artificial elevation of intracellular \( \text{Ca}^{2+} \) by the \( \text{Ca}^{2+} \) ionophore A23187 resulted in an 61% increase in GABA levels. Stimulation of GABA synthesis by ABA resulted in an 83% increase in GABA levels, which was inhibited 55% by W7. These results support the hypothesis that cold shock stimulates \( \text{Ca}^{2+} \) entry into the cytosol of the cells which results in \( \text{Ca}^{2+} \)/calmodulin mediated activation of GAD and consequent GABA synthesis.
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Introduction.

The presence of GABA has been demonstrated in all plant species analyzed. It is a major part of the free amino acid pool. There is general agreement that GABA synthesis occurs almost exclusively through the decarboxylation of L-glutamate via a reaction catalyzed by glutamate decarboxylase (GAD). GAD is a cytosolic enzyme, thus changes in cytosolic composition may affect its activity.

Accumulation of GABA in response to various physical and chemical stress factors is well documented. The increase in GABA levels can be observed in any plant species when the amino acid pool is measured after the plant’s exposure to the stress condition. However neither the mechanism(s) nor the role(s) of GABA synthesis during adverse conditions are clearly understood.

Many stress factors affect cytosolic pH or calcium, or both. GABA synthesis in response to the cytosolic acidification is well documented (Crawford et al. 1994). Cytosolic pH is well controlled in plant cells, and acidification of the cytosol occurs when plants are exposed to adverse conditions such as anaerobiosis for prolonged lengths of time. In such cases GABA synthesis, a proton consuming reaction, may act as a mechanism of cytosolic pH regulation.

A rapid response to many stress factors is a change in cytosolic Ca\(^{2+}\) concentration. Increase in cytosolic Ca\(^{2+}\) has been
observed in response to cold, touch, fungal elicitors and plant hormones. Because GABA synthesis is stimulated by the same types of stress it was postulated that Ca$^{2+}$ ions are involved in the regulation of GAD. Calcium signals are mediated by Ca$^{2+}$-binding proteins. The most ubiquitous regulatory Ca$^{2+}$ binding protein is calmodulin. During the course of this study independent investigations demonstrated that GAD is a calmodulin (CaM)-binding protein (Baum et al. 1993), and that its activity is increased upon Ca$^{2+}$ and CaM addition (Snedden et al. 1995).

In the present study the mechanism of GABA synthesis in Asparagus sprengeri Regel mesophyll cells was investigated. The objectives of this study was to answer the following questions:

1. Can cold shock stimulate GABA accumulation?
2. Can cold shock increase cytosolic Ca$^{2+}$ levels?
3. Is there a pH change during cold shock?
4. Do the inhibitors of Ca$^{2+}$ channels inhibit GABA accumulation?
5. Does the inactivation of calmodulin inhibit GABA accumulation?
6. Can artificially increased levels of cytosolic Ca$^{2+}$ stimulate GABA accumulation?
2. Literature Review.

2.1. Calcium in Plants.

It is well established that calcium is essential for plant growth and development. Traditionally Ca²⁺ in plants has been viewed as a nutrient mineral. With respect to the whole plant, a constant supply of exogenous Ca²⁺ in the range of 1 to 10 mM is required to maintain normal plant growth (Clarkson and Hanson 1980). Ca²⁺ deficiency in soils often causes complex disorders in plants such as bitter pit of apples, cork spots of pears, blossom end root of tomatoes, and black heart of celery. In most plants Ca²⁺ is the second most abundant metal ion and the fifth most abundant element after carbon, hydrogen, oxygen, and potassium (Epstein 1972).

Traditionally the functions of Ca²⁺ revolved around its involvement in the structure of cell wall, through the cross-linking of carboxyl groups of the pectic polymers (Cleland et al. 1990). Removal of Ca²⁺ from the nutrient supply resulted in rapid death of cells in the apical meristem and cessation of growth (Epstein 1972). It is known that Ca²⁺ in plants has extremely low mobility. Plants appear unable to mobilize existing Ca²⁺ stores for apical meristem development. The transport of solutes from older leaves occurs by a pressure-flow mechanism, where solutes are loaded into the cytosol of the phloem sieve elements. Thus, this transport mechanism may be inefficient for Ca²⁺, because typically there is a low Ca²⁺
Fig. 1. Possible Ca²⁺ Concentrations and Ca²⁺ Transporters in Plant Cells.
concentration in the cytosol of the transporting cells in the range of 100 to 200 nM (Gilroy et al. 1993).

2.1.1. Calcium in the Cytosol.

Investigators generally agree that in higher plants, as in animal cells, the free cytosolic Ca\(^{2+}\) concentration is maintained below 1 μM. Absolute estimates for Ca\(^{2+}\) concentration vary with cell type and plant species, and other physiological factors such as age. There is no generally applied method for Ca\(^{2+}\) measurement available, therefore different methods often give different results. Perhaps the most sensitive and accurate methodology for measuring Ca\(^{2+}\) levels in vivo is the use of Ca\(^{2+}\) sensitive fluorescent probes. Employing fluorescent probes Ca\(^{2+}\) concentration at the level of 30 to 90 nM were found in root hair of Brassica napus and Lycopersicum esculentum using Fura-2 (Clarkson et al. 1988), Zea mays root protoplasts using Indo-1 (Lynch et al. 1989), Haemamantus Katerinae Baker wall-less endosperm cells using Quin-2 (Keith et al. 1985), and Dryopteris paleacea spores using Fura-2 (Scheuerlein et al. 1991). Higher levels of cytosolic Ca\(^{2+}\) concentrations in the range of 100 to 200 nM were found in Phaseolus mungo root tip protoplasts using Quin-2 (Gilroy et al. 1986), Hordeum vulgare mesophyll cell protoplasts using Quin-2 (Gilroy et al. 1989), Hordeum vulgare aleurone protoplasts using Indo-1 (Bush and Jones 1987), Commelina communis guard cell using Indo-1 (McAinsh et al. 1990), and Zea mays coleoptile using Fluo-3 (Gehring et al.
1990). Cytosolic Ca^{2+} concentrations at the levels of 300 to 500 nM were observed in Chara fragilis rhizoids using Indo-1 (Hodic et al. 1991), Petroselinum hortense hypocotyl and root using Fluo-3 (Gehring et al. 1990). Resting cytosolic Ca^{2+} concentrations at the level of 1 μM were observed in mature, photosynthetic cells of Mougeotia scalaris (920±29 nM using Indo-1) (Russ et al. 1991).

These low levels of Ca^{2+} concentrations in the cytosol of plant cells are maintained against the high Ca^{2+} concentrations in the intracellular compartments and in the apoplast.

2.1.2. Calcium Associated with the Plasma Membrane.

The plasma membrane (PM) of plant cells is tightly appressed to the cell wall. The presence of membrane-bound calcium was visualized in Bryonia dioica internodes using a fluorescent Ca^{2+} probe (Thonat et al. 1993). An intense yellow fluorescence connected with the PM of these parenchyma cells incubated with the Ca^{2+} indicator chlortetracycline, indicated that there is an association of Ca^{2+} with the membrane. Release of this calcium into the cytosol was also observed under stress conditions such as mechanical manipulation (Thonat et al. 1993).

Under normal conditions, Ca^{2+} has a stabilizing and tightening effect on the outer surface of the PM, ensuring maintenance of selective ionic permeability. This effect of Ca^{2+} is explained as electrostatic interactions between anionic
phospholipids and phospholipid-proteins complexes, and cationic 
Ca$^{2+}$ (Andreev 1993).

2.1.3. Calcium in the Cell Wall.

The matrices of the cell wall contain high Ca$^{2+}$ concentrations, at least $10^{-4}$ to $10^{-5}$ M (Cleland et al. 1990). The cell walls of epidermal peels isolated from the growing regions of soybean hypocotyls contain 800 to 1000 µg Ca$^{2+}$ per gram dry weight, whether the walls were prepared by freezing-thawing or boiling in methanol. Removal of protein from the walls does not lower the Ca$^{2+}$ content, indicating that calcium is not bound to these proteins (Virk and Cleland 1988).

2.1.4. Calcium in the Chloroplasts.

In mature, photosynthetic cells chloroplasts are the second largest compartment after the vacuole. In chloroplasts, calcium levels depend on the age of the tissue, and values between 4 and 23 mM have been reported (Portis and Heldt 1976). These high calcium levels are in conflict with the fact that CO$_2$ assimilation by isolated chloroplasts is inhibited 50% when 0.5 mM Ca$^{2+}$ is added to the assay medium. Possible targets for calcium inhibition of CO$_2$-fixation are the chloroplasts enzymes fructose and sedoheptulose bisphosphatases. The catalytic activity of these enzymes are completely inhibited at approximately 0.3 mM Ca$^{2+}$ (Wolosiuk et al. 1982).

As a consequence, Burchert et al. (1990) assumed that only a small portion of the total calcium in the chloroplasts is free
Ca$^{2+}$, and the majority of it has to be bound to the membranes and membrane constituents and/or to the stroma proteins and nonprotein low-MW components. To determine directly the free Ca$^{2+}$ levels in the chloroplasts, a Ca$^{2+}$ null point titration technique was applied using dark-incubated spinach chloroplasts. Titrations were performed with the addition of Ca$^{2+}$ ionophore A23187, which catalyses an electroneutral exchange of Ca$^{2+}$ for H$^+$. Upon conditions where no net movement of the calcium occurs across the chloroplast envelope in the presence of A23187, the external and internal Ca$^{2+}$ and H$^+$ is distributed according to the equation: 

$$\frac{[Ca^{2+}]_{\text{out}}}{[Ca^{2+}]_{\text{in}}} = 10^{2\Delta p\text{H}}.$$ 

Calculated values of free Ca$^{2+}$ in 12 sample from spinach leaves varied between 2.4 and 6.3 mM, when $\Delta p\text{H}=0$ across the chloroplast envelope (Burchert et al. 1990). This data suggests that CO$_2$ fixation is compartmentalized within the chloroplast or that the inhibition of CO$_2$ fixation observed by Wolosiuk et al. (1982) is compromised.

2.1.5. Calcium in the Vacuole.

The vacuole in mature plant cells represent up to 90% of the cell volume. The vacuole in higher plants plays major roles in pH and ionic regulation of the cytosol, turgor regulation, the storage and retrieval of both inorganic and organic nutrients, and the sequestration of metabolically perturbing inorganic and organic solutes. The vacuole is also the location of the storage proteins and many lysosomal-type hydrolases (Rea et al. 1987). The vacuole has been described as a reservoir of
calcium in its ionic form and very often as calcium oxalate crystals. The free Ca\(^{2+}\) concentration in the vacuole has been estimated between 1 and 10 mM (Gelli and Blumwald 1993).

### 2.1.6. Calcium Transport from the Cytosol.

To maintain low (nM range) free Ca\(^{2+}\) concentrations and support ATP-based metabolism in the cytosol of plant cells, a variety of Ca\(^{2+}\) transporters are employed. There is a large electrochemical gradient (200-300 mV) favouring Ca\(^{2+}\) entry into the cytosol across the PM, the endoplasmic reticulum (ER) or vacuolar membrane, the tonoplast. Therefore efflux of Ca\(^{2+}\) from the cytosol into extracellular space, the apoplast, or intracellular organelles requires energy, and is catalyzed by membrane proteins that utilize ATP or proton motive force to drive Ca\(^{2+}\) movement. On the other hand, influx of Ca\(^{2+}\) in to the cytosol is energetically down hill and is mediated by the opening of Ca\(^{2+}\) channels. Thus, the pathway of Ca\(^{2+}\) influx in to the cytosol is separate from efflux, and differently regulated.

### 2.1.7. Calcium Transport into the Vacuole.

The vacuole is the major intracellular storage compartment in which Ca\(^{2+}\) is being taken up. The mechanism(s) by which Ca\(^{2+}\) is mainly transported into the vacuole is based on the proton motive force and the exchange of Ca\(^{2+}\) for protons (Evans et al. 1991). The \(^{45}\text{Ca}^{2+}\) uptake into tonoplast vesicles was completely inhibited by compounds such as ionophores and protonophores; classes of compounds which dissipate the proton gradient (Chanson 1991).
In addition reports indicate that in the vesicles derived from isolated vacuoles which originated from cultured carrot cells, Ca\(^{2+}\) transport required the presence of Mg\(^{2+}\) and ATP. This system was insensitive to vanadate (P-type ATPase inhibitor) and strongly inhibited by the proton ionophore carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) (Bush and Sze 1986). This data supports the proposal for proton driven Ca\(^{2+}\) transport into the vacuole.

Similar complete inhibition of ATP-dependent Ca\(^{2+}\) transport by CCCP was observed in tonoplast enriched fractions from spinach leaves prepared by free-flow electrophoresis, but in this system Ca\(^{2+}\) transport was also strongly inhibited by vanadate and stimulated by calmodulin, suggesting the presence of calmodulin-stimulated Ca\(^{2+}\)-ATPase (Malatialy et al. 1988). In the vacuoles prepared from apple fruits, a CCCP insensitive, calmodulin stimulated Ca\(^{2+}\) transport was associated with the tonoplast (Fukumoto and Venis 1986). A low level of an ATP-dependent Ca\(^{2+}\) pump was detected in tonoplasts of maize roots (Zocchi 1988), barley roots (DuPont et al. 1990), and cauliflower (Askerlund and Evans 1992).

Thus the presence of two kinds of Ca\(^{2+}\) transporters (primary Ca\(^{2+}/H^+\) antiport, and Ca\(^{2+}\)-ATPase) at the tonoplast is indicated, implying a significant role of the vacuole in the regulation of free, cytosolic Ca\(^{2+}\) concentrations.
2.1.8. Calcium Transport into the Endoplasmic Reticulum.

Ca\(^{2+}\) transport into the endoplasmic reticulum (ER) results in a decrease of cytoplasmic Ca\(^{2+}\). The experimental evidence has shown an ATP-dependent Ca\(^{2+}\) accumulation in membrane vesicles derived from ER (Gross 1982). In addition, fusion of ER vesicles with the PM has been suggested (Griffing and Ray 1979). In cultured carrot cells, Ca\(^{2+}\) transport activity into the ER was inhibited by vanadate and was insensitive to the proton ionophore CCCP (Bush and Sze 1986). The observed formation of a phosphorylated intermediate in ER from red beet roots and cauliflower suggests a P-type ATPase. The Ca\(^{2+}\)-ATPase from cauliflower was calmodulin stimulated (Gianni et al. 1987; Askerlund and Evans 1992). Calmodulin stimulation of ER Ca\(^{2+}\)-ATPase was also demonstrated in zucchini hypocotyls (Lew et al. 1986), maize roots (Brauer et al. 1990), carrot cell cultures (Hsieh et al. 1991) and cauliflower inflorescences (Askerlund and Evans 1992). In other species, for example pea roots, calmodulin did not have any effect (Butcher and Evans 1987), and in tomato the deduced amino acid sequences for the Ca\(^{2+}\)-ATPase did not contain a putative calmodulin binding domain (Wimmers 1992). These different results might arise from different mechanisms of Ca\(^{2+}\) transport into the ER for different species, or by difficulties in purification processes to completely remove bound calmodulin from CaM-binding enzymes during isolation.
2.1.9. Calcium Transport into the Apoplast.

ATP-dependent extrusion of Ca\(^{2+}\) from the cytosol to the apoplast has been demonstrated in many plant species. In many studies transport-competent membrane vesicles were used. For example, an ATP dependent \(^{45}\)Ca\(^{2+}\) transport into PM vesicles isolated from red beet storage tissue was demonstrated. The ATP hydrolysis occurred on the side of the PM facing the cytosol, corresponding to the efflux of Ca\(^{2+}\) from the cell. Only slight inhibition of Ca\(^{2+}\) translocation occurred when the ΔpH was abolished, indicating that this process is maintained primarily by ATPase activity, and not by a Ca\(^{2+}/\)H\(^{+}\) antiport. Stimulation of PM Ca\(^{2+}\)-ATPase by calmodulin was observed after repeated washing with EGTA (Briskin et al. 1990). Other studies showed that this pump is Mg\(^{2+}\)-dependent and has a high affinity for Ca\(^{2+}\) (K\(_m\) 0.1 to 6 mM), and a pH optimum of 7.0 to 7.5 (Evans et al. 1991).

2.1.10. Calcium Transport into the Chloroplasts and Mitochondria.

Isolated mitochondria accumulate large amounts of Ca\(^{2+}\) using succinate or ATP as an energy source. This accumulation can not be enhanced by calmodulin (Dieter and Marme 1980). The mitochondria isolated from maize coleoptiles exhibit an electrophoretic Ca\(^{2+}\) influx. This appears to be a Ca\(^{2+}/\)inorganic phosphate symporter, which is a ruthenium red and mersalyl sensitive (Sliva et al. 1992). The kinetic parameters of mitochondrial Ca\(^{2+}\) transport possess a relatively low affinity,
apparent $K_m$ 250 mM and relatively high $V_{max}$ 63 nmol·min$^{-1}$·mg$^{-1}$ (Dieter and Marme 1983). Comparison with the corresponding values of the microsomal Ca$^{2+}$ transport system shows that the $V_{max}$ of mitochondria is about 10-20 times higher, but the affinity is only about one tenth that of the microsome. Therefore, it seems very unlikely that under normal conditions (where Ca$^{2+}$ concentration in the cytosol is in nM range) mitochondria regulate the cytosolic Ca$^{2+}$ levels.

Calcium uptake into the chloroplast is linked to photosynthetic electron transport. Evidence for this phenomenon was provided in a study with intact spinach chloroplasts (Kreimer et al. 1985). An increase of calcium uptake into the chloroplasts upon illumination leads to the suggestion that Ca$^{2+}$ influx is a result of a uniport which is driven by a change in the membrane potential (Evans et al. 1991). Given that the observation that Ca$^{2+}$ uptake into the chloroplasts occurs mainly in the presence of light excludes therefore their role as regulatory compartments.

In conclusion it may be assumed that the low Ca$^{2+}$ concentration in the cytosol of unstimulated plant cells is maintained mainly by PM and/or ER-located, calmodulin stimulated, Mg$^{2+}$-dependent P-type ATPase, and also by tonoplast localized Ca$^{2+}$/H$^+$ antiport.
2.2. Calcium as a Second Messenger.

Without the use of neurons, plants sense and respond to environmental stimuli and internally produced hormone molecules. External stimuli such as light, gravity, pressure and temperature elicit immediate, as well as gradual, responses in eukaryotic cells. Many of the immediate responses are transient in nature, yet are key to the initiation or modulation of later events.

The evidence in support of Ca\(^{2+}\) as a messenger in the stimulus-response mechanism is unequivocal. The general mechanism by which Ca\(^{2+}\) modulates a response is through a change in its cytosolic concentration. Plant cells, like those of other organisms, maintain levels of free Ca\(^{2+}\) ions in the cytosol and nucleus that are 3 to 4 orders of magnitude lower than the levels in other cellular compartments (Felle 1988, Bush et al. 1989, Gilroy et al. 1990). There is, therefore, a large concentration gradient for calcium directed into the cytosol across most cellular membranes, a gradient that is necessary for calcium to be a signal molecule. Transport of a relatively small absolute number of Ca\(^{2+}\) into the cytosol will cause a significant increase in this ion concentration that can act as a signal.

Calcium enters the cytosol through channels. These influx transporters, which can rapidly raise cytosolic Ca\(^{2+}\) levels, have been found on PM and tonoplast membranes. At least three types of Ca\(^{2+}\)-permeable channels have been identified. These include: voltage-operated channels on the PM and tonoplast (Schroeder and
Hagivara 1990; Johanes et al. 1992), IP₃-gated channels on the tonoplast (Alexandre et al. 1990), and stretch-activated channels on the PM (Cosgrove and Hedrich 1991). Ca²⁺ transport through an open channel is very effective with an estimated ionic flux of 10⁷ ions per second per channel (Blatt and Thiel 1993).

The perception of a signal may directly cause channel opening in the PM, thereby stimulating Ca²⁺ flow down its electro-chemical gradient across the PM into the cytosol. Plant hormones are detected by plasma membrane receptors. This interaction may activate a membrane bound enzyme, PIP₂-specific phospholipase C. The PIP₂ present on the cytosolic site of PM is hydrolysed to inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Several reports indicate that DAG promotes ATP-dependent protein phosphorylation in plants (Morre et al. 1984, Shafer et al. 1987). This protein phosphorylation is phospholipid (DAG) and Ca²⁺ dependent, and is considered to be due to a C-type protein kinase. DAG binds directly to the protein kinase C and increases its affinity for Ca²⁺. This enzyme is fully activated at the resting Ca²⁺ levels below 1 µM (Hepler and Wayne 1985). IP₃ mobilizes calcium from intracellular stores. A transient change in [⁴⁵Ca²⁺] concentration was observed when zucchini microsomes were treated with 20 µM IP₃ (Drobak and Ferguson 1985). In plants the primary site for IP₃ induced calcium flux into the cytosol is the vacuolar tonoplast membrane. This was established using tonoplast vesicles. The
release of vacuolar Ca\(^{2+}\) was monitored using isolated, intact *Acer pseudoplatanus* vacuoles. A fluorescent Ca\(^{2+}\) probe Quin-2 was present in the incubation medium. Quin-2 does not cross the phospho-lipid bilayer of the membrane and can only react with the external Ca\(^{2+}\). Under these conditions any increase in fluorescence corresponds with release of the Ca\(^{2+}\) from the vacuoles to the medium (Shumaker and Sze 1987). It was also observed that IP\(_3\) controlled Ca\(^{2+}\) efflux in a dose-dependent manner (Ranjeva et al. 1988).

The molecular mechanisms by which increases in cytosolic Ca\(^{2+}\) are transduced into physiological responses include interactions with Ca\(^{2+}\) binding proteins. Two general categories of calcium-binding proteins are calcium-storage proteins and regulatory calcium-binding proteins. In animal cells a number of calcium storage proteins has been well described (calsequestrin (Hensen 1989), and reticuloplasmins (Macer and Koch 1988) as examples), however in plants calcium storage proteins are not well characterized.

Regulatory calcium-binding proteins include calcimedlins (Moore 1984), the protein family that include calmodulin (CaM) and Ca\(^{2+}\)-dependent protein kinase (CDPK).

Purified CDPK from soybean cell cultures revealed its unique molecular structure (Putnam-Evans et al. 1990). CDPK contains both a catalytic protein kinase domain and a regulatory Ca\(^{2+}\)-binding domain, similar to CaM. The regulatory domain interacts directly with Ca\(^{2+}\) (Harmon et al. 1987). Fifty to 100
fold stimulation of CDPK by μmolar free Ca\(^{2+}\) in the presence of millimolar Mg\(^{2+}\) was observed in vitro using near homogenic soybean CDPK (Putnam-Evans et al. 1990). Neither CaM, phospholipids nor diacylglycerol are required for CDPK activation (Harmon et al. 1987). Cellular targets of CDPK in plants are not well defined. The potential endogenous substrate for CDPK is nodulin-26, a nodule specific membrane protein, which was shown to be phosphorylated in vitro and in vivo by CDPK (Weaver et al. 1991). In addition the membrane associated and partially purified PM H\(^{+}\)-ATPase was phosphorylated by CDPK (Schaller and Sussman 1988, Sussman et al. 1990). Association of CDPK with pea chromatin in vivo (Li et al. 1991) and phosphorylation of Histone H1 in vitro (Polya et al. 1987) suggest that histone may be another endogenous substrate of CDPK. The ubiquitous distribution of CDPKs in the plant kingdom and its presence in several subcellular locations suggest that this enzyme may be involved in multiple signal transduction pathways.

CaM has no enzymatic activity on its own, but modulates several enzymes in a calcium-dependent manner (Klimczak and Hind 1990). CaM is thought to be fundamental component of calcium signal transduction pathways, because of its wide range of functions and its ubiquity. In yeast and fungi CaM has been shown to be essential, because deletion of the CaM-coding gene was lethal (Davis et al. 1986; Takeda and Yamamoto 1987).
Analyses of cDNA coding for CaM from different species illustrate that this protein has been highly conserved during evolution (Moncrief et al. 1990). CaM possesses four functional EF-hand calcium-binding domains with dissociation constants for Ca\(^{2+}\) in the range of \(10^{-6}\) to \(10^{-5}\) M under physiological conditions of pH and ionic strength (Klee and Vanaman 1982). Upon binding Ca\(^{2+}\), the amphipathic α-helixes of the EF-hand subdomains of CaM undergo a conformational change such that hydrophobic clefts are exposed within each globular domain (Babu et al. 1988). These hydrophobic clefts have been proposed to be involved in the binding of amphipathic amino acid sequences of target proteins. Indeed, proteins that bind CaM in vivo contain basic amphipathic α-helices that bind CaM with a \(K_m\) in the nanomolar range (O'Niel and Degrado 1990). In plants the first sequenced CaM was from spinach (Lukas et al. 1984). Since then a number of CaM sequences have been determined for different plants. These include wheat germ (Toda et al. 1985), alfalfa (Bennett and Long 1990), barley (Ling and Zielinski 1989), potato (Jena et al. 1989), and Arabidopsis thaliana (Ling et al. 1991). In Arabidopsis thaliana a number of CaM isoforms and CaM-like proteins have been observed. These proteins have a high homology to CaM, and appear to be each encoded by distinct genes. The physiological significance of CaM isoforms and CaM-like proteins is unclear, but since their expression may be differently
controlled, they may have different cell or tissue distribution (Roberts and Harmon 1992).

In plants only a few CaM regulated enzymes have been demonstrated. NAD kinase from pea was first determined to be regulated by calmodulin. This enzyme catalyzes the phosphorylation of NAD to NADP linked to the hydrolysis of ATP. During purification, NAD kinase activity was lost after anion exchange chromatography. This activity was recovered by addition of a heat stable "protein activator" (Muto and Miyachi 1977), which was later identified as CaM (Anderson and Cormier 1978). Purified NAD kinase has nearly complete dependence of the enzymatic activity on CaM, with high affinity for CaM (K_D in the range of 0.1 nM) (Harmon et al. 1984).

Another enzyme which is CaM sensitive is Ca^{2+}-ATPase. Ca^{2+}-ATPase from zucchini and maize was shown to be stimulated by CaM. The rates of ATP hydrolysis and Ca^{2+} transport into crude microsomal fractions were stimulated 2-3-fold by exogenous calmodulin (Dieter and Marme 1980). However, a high concentration of CaM was required for half-maximal activation (200 nM), and the enzyme had only a slightly lower K_m in the presence of CaM (Dieter and Marme 1983). Similarly, calcium transport in isolated maize PM was stimulated only 10 to 20% by CaM (Robinson et al. 1988).

Nuclear NTPase was demonstrated to be stimulated by CaM. An ATPase activity associated with isolated chromatin from pea nuclei was activated 15-fold by CaM in the presence of μM Ca^{2+}
concentrations (Matsumoto et al. 1984). Although this enzyme is active in the absence of CaM, addition of exogenous CaM stimulated its activity 3.5-fold in a Ca\(^{2+}\) dependent manner, with half-maximal activation at the level of 20-30 nM CaM (Chen et al. 1987).

Glutamate decarboxylase was reported to be a CaM binding protein, with a deduced putative CaM-binding domain (Baum et al. 1993). The first evidence for CaM stimulated GAD activity was presented in a study of soybean leaves (Ling et al. 1994). At pH 7.0 GAD from various soybean tissues was stimulated 2- to 8-fold (Snedden et al. 1995). This evidence supports the discovery of another enzyme regulated by Ca\(^{2+}\) and CaM in plants.

2.3. Measurements of Cytosolic Calcium Concentrations in Plants.

The measurement of cytoplasmic calcium concentration in animal cells is widely practised and, although not without problems, has provided a wealth of new information about the role of this key ion in the process of signal transduction. Various methods have been used to measure the concentration of ionic Ca\(^{2+}\) in biological systems and within the cell. The most frequently used techniques are: Ca\(^{2+}\) selective electrodes, metalochromic Ca\(^{2+}\)-indicators, NMR probe, the X-ray probe, fluorescent Ca\(^{2+}\) indicators, and Ca\(^{2+}\) sensitive proteins.

The Ca\(^{2+}\) selective electrodes consist of a layer, or column, of ion-selective ligand which separates the experimental solution from a filling solution which contains a fixed
concentration of the primary ion, such as $\text{Ca}^{2+}$. The primary ion is transported across the ion-sensitive ligand layer in accordance with the prevailing concentration gradient so that a potential is established and measured with respect to a stable reference level. Such electrodes have been used to monitor $\text{Ca}^{2+}$ flux across cell membranes and to monitor the concentration of $\text{Ca}^{2+}$ in the cell cytoplasm and in the extracellular space (Ammann 1986). Although electrodes have contributed a great deal to research of $\text{Ca}^{2+}$ metabolism, there are a few technical problems. First, the equipment used must be very precise and it varies in detail from manufacturer to manufacturer. The useful lifetime of these electrodes is from minutes to one day, especially when used on plant cells. Second, the electrode contents may leak into the cell cytoplasm after the penetration causing possible contamination. Third, they have an extremely high resistance due to the ion carrier filling solution and a lag time of 10-12 seconds before measurement. Fast changes are impossible to measure. A further disadvantage of this method is cell perturbation (Purves 1981). In plant cells, the tip of the electrode may be located in the compartment such as a vacuole, and measurements may not be representative of the average cytosolic $\text{Ca}^{2+}$ levels.

In spite of these difficulties, Ca-sensitive microelectrodes have been used successfully in experiments with plant cells. Photosynthetically induced $\text{Ca}^{2+}$ reduction was followed using microelectrodes in the relatively large cells of
Nitellopsis (Miller and Sanders 1987). A gradient of Ca$^{2+}$ near the apex and sub apical zones of Fucus rhizoids in the range from 2.5 to 0.28 μM has been measured using microelectrodes (Brownlee and Wood 1986). The newer double-barrelled electrodes have been developed and used in the experiments with small cells of Zea mays coleoptiles and Riccia fluitans rhizoids (Felle 1988).

Metalochromic indicators, such as Arsenazo-III and Antipyrylazo-III, were extensively used for measurements of the intracellular Ca$^{2+}$ before introduction of the fluorescent indicators. These metalochromic indicators undergo an optically detectable change as a result of binding Ca$^{2+}$. The major change is an increase in absorbance at relatively long wavelengths (600-700 nm). This property is an advantage, because the requirement for long wavelength rather than UV light simplifies the optical system and greatly reduces the risk of damage to the sample. The major problems with the metalochromic indicators are their relative poor selectivity against other ions (Mg$^{2+}$ and H$^+$ in particular), and variable stoichiometry. Sometimes background absorbance of the sample in the long wavelength region interferes with the measurements (Thomas 1982).

NMR measurements of cytoplasmic free Ca$^{2+}$ concentrations have been made by using fluorine labelled indicators. The structure of $^{19}$F NMR indicator contains a component which selectively and reversibly binds Ca$^{2+}$ and this component is attached to a spectroscopic “reporter” group which senses binding of Ca$^{2+}$. This was developed from the structure of the $^{19}$F
NMR chelator BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) in which four carboxylate groups are involved in binding Ca\(^{2+}\), and the two fluorine nuclei constitute the \(^{19}\)F NMR reporter group. This indicator group has a different chemical shift for the resonances from the free and bound forms. The \(^{19}\)F NMR can be used in perfused tissues or organs where the use of fluorescent probes is restricted. In some cases \(^{19}\)F NMR indicator may not be sensitive enough to measure resting, physiological Ca\(^{2+}\) levels because its apparent dissociation constant for Ca\(^{2+}\) is 0.71 and 0.54 \(\mu\)M at 37 and 30\(^\circ\)C respectively (Metcalfe and Smith 1991).

X-ray microprobe analysis (synonymous with electron probe microanalysis, EPMA) provides measurements of not only free Ca\(^{2+}\) but also bound Ca\(^{2+}\) in the myoplasm of muscle cells as well as in the intracellular compartments. EMPA analyses of 100-200 nm thick sections of tissue were accomplished in the vacuum of the electron microscope, where an incident high-energy beam electron can ionize an inner orbit electron of the atom. The incident and the ionized electrons leave the atom, and an electron from the outer orbit drops into the vacant inner shell. In this transition energy loss is emitted as a characteristic X-ray quantum. These quanta can be collected by the detector and sorted by computer according to their energy. Each element has its own characteristic peak. The area of the peak is proportional to the number of counts and therefore to the number of atoms in the analyzed volume of the sample. The original Ca\(^{2+}\)-
distribution in the cell is preserved by rapid shock-freezing at the rate >10 000 °C/sec (Heuser et al. 1979).

In contrast, the measurement of calcium in plant cells is much more difficult. The rigid cell wall and turgor pressure are difficulties to be overcome in gaining access to the cell interior. Despite these difficulties, measurements of calcium levels in plants have been accomplished with various techniques.

2.3.1.Fluorescent Probes.

The availability of calcium sensitive and selective fluorescent probes for living cells opened new horizons in cell biology. Fluorescent probes are organic molecules containing a conjugated system of double bounds, i.e. delocalized π-electrons. When such molecules are irradiated with light, they absorb preferentially the photons whose energy corresponds to the difference between the ground state and an excited state of the π-electrons. This energy is dissipated when the electron returns to the original ground state, releasing a quantum of light. The time required for absorption is immediate, about $10^{-15}$ seconds, whereas the fluorescence life time is approximately $10^{-8}$ second. The wave length is longer for fluorescent light than for absorbed light. The differences in energy levels associated with absorption and fluorescence characterize the absorption and emission spectra. In order for a fluorescent probe to be suitable for calcium measurements, its fluorescence properties have to be altered upon binding.
calcium. For most biological applications, the following three property changes are appropriate:

1. Change in fluorescence yield.
2. Shift in excitation spectrum.
3. Shift in emission spectrum.

Almost all of the calcium indicators are fluorescent derivatives of the chelator BAPTA, which is an aromatic analogue of the calcium selective chelator EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid). In 1980 Dr Roger Tsien described a prototype calcium indicator Quin-2, followed by Fura-2 and Indo-1 in 1985, and Fluo-3 and Rhod-2 in 1989. Kuhn and colleagues in 1990 described calcium green and calcium crimson. Fura red was described by DeMarinis and coworkers in 1989 (Haugland 1989).

Fluo-3 increases its fluorescence intensity upon binding $\text{Ca}^{2+}$. This $\text{Ca}^{2+}$ indicator is very suitable for in vivo studies because of its high affinity for $\text{Ca}^{2+}$. Fluo-3 dissociation constant ($K_d = 400$ nM) is in the range of $\text{Ca}^{2+}$ concentration in the cytosol of plant cell. Therefore, this probe has a very good sensitivity. In addition, Fluo-3 has a low photobleaching rate (Graziana et al. 1993). Fluo-3 has been successfully loaded into intact plant cells (Ghering et al. 1990) and plant cell protoplast (Ranjeva et al. 1992).
2.3.2. Methods of Loading Fluorescent Probes into the Plant Cells.

The objective of loading is to introduce the probe into the cytosol of as many cells as possible in sufficient concentration to give good signal-to-noise ratio without significantly increasing the cellular ion buffering or causing toxic effects.

2.3.3. Acetoxyethyl Esters.

Esterification of the carboxyl groups with acetoxyethyl groups masks their charge and renders the probe membrane permeant. Once inside the cell, intracellular esterases hydrolyse the ester linkages and the free probe is released in active form. This technique is very successful with animal cells. With plant cells a variety of problems arise. These include the presence of cell wall associated esterases that cleave the probe before it enters the cytoplasm (Gilroy et al. 1986), no hydrolysis or incomplete hydrolysis in the cytosol (Bush and Jones 1987, 1990), rapid accumulation of probe into membrane compartments such as endoplasmic reticulum and vacuole which also have extra-cytoplasmic esterases (Hepler et al. 1987), and the release of toxic products on hydrolysis (Cork 1986).

2.3.4. Acid loading.

Acid loading refers to the procedure in which reversible protonation of the carboxyl groups is used to mask their
charge, allowing the uncharged molecule to cross the plasma membrane. This is achieved by lowering the pH of the incubation medium to 4.5-5.0. Once inside the cell the probe experiences a higher pH and dissociates into impermeable, ionic form and is thus trapped in the cytosol.

This method was first successfully applied by Bush and Jones, who loaded Indo-1 into barley aleurone protoplasts (Bush and Jones 1988).

Corn root protoplasts were loaded with Indo-1 by this technique (Lynch et al. 1989). Walled cells were also successfully loaded by acid loading (Hahm and Sanders 1991; Russ et al. 1991). The release of protons into the cytosol that follows probe loading does not alter cytosolic pH significantly (Bush and Jones 1988).

2.3.5. Electroporation.

Pores of variable size can be selectively induced in the plasma membrane of protoplasts by short, high voltage pulses. This allows the introduction of calcium probes into unwalled protoplasts. Although resealing is spontaneous, it can be slowed sufficiently at low temperatures to allow accumulation of the free probe. This method has been used to insert Quin-2 into protoplasts of *Hordeum vulgare*, *Daucus carota*, and *Phaseolus mungo* (Gilroy et al. 1986, 87, 89).

Although electroporation allows one to introduce any reporter molecule into a large number of cells, this method suffers because it requires protoplasts. Removal of the cell
wall may alter or destroy some crucial aspects of the cell’s physiology including calcium transport, polarity in growth, and non uniform distribution of cell surface proteins. Consequently, it is likely to perturb the sensitive signalling system.

2.3.6. Microinjection.

This technique allows rapid introduction of almost any molecule into the cell. The cells are impaled with a fine micropipete (0.1-0.3 μm tip) containing 0.1-1 mM of the probe. It is best suited to situations where one or a few cells are injected and individually monitored. Loading has been achieved by microionophoresis (for charged low molecular weight materials), and by pressure injection (for molecules that will not migrate sufficiently with electric field) (Callaham and Hepler 1991).

Perhaps the most elegant example of the microinjection method of loading comes from the work of Hepler and Callaham (1987). In the experiments they used stamen hair of Tradescantia, because it is made up of a single file of cells interconnected by plasmodesmata. Probe injected into one cell is carried by cytoplasmic streaming into adjacent cells. In this organ, therefore, calcium measurements can be made on single cells that have not been impaled. Unfortunately, the vacuole of the stamen cells rapidly sequests Indo-1 and Fura-
2, so calcium measurements must be made quickly after injection (Hepler and Callaham 1987).

### 2.3.7. Photoproteins.

Aequorin is the best known of the several different photoproteins that emit light upon binding calcium. Aequorin is a bioluminescent protein from the coelentrate *Aequorea victoria*. This protein consists of an apoprotein, aequorin (21 kDa) containing three calcium binding sites, and a hydrophobic luminophore coelenterazine.

This native jellyfish protein is known to be located in the cytosol, and probably lacks signal sequence for transport into vesicular cellular compartments. The blue-green light of 470 nm is emitted by aequorin upon binding calcium. This photon is generated by peroxidation of coelenterazine. This is an irreversible reaction, therefore, fresh coelenterazine is needed to be supplied, along with oxygen. The molecule is responsive to calcium in the range $10^{-7}$ to $10^{-5}$ M (Blinks 1989), which is suitable to detect physiological Ca$^{2+}$ levels.

Aequorin has multiple negative charges and therefore, does not readily cross membranes. Different techniques have been used to load aequorin into the cell. In animal cells aequorin was loaded by liposome fusion, hyperpermeabilization, scrape loading and microinjection (Blinks 1989). Plant cells have been loaded by electroporation (Gilroy et al. 1989) and microinjection (Williamson and Ashley 1982). However, these techniques are technically demanding and traumatic to the cell. A new approach
of using aequorin to detect changes in cytosolic Ca$$^{2+}$$ was developed using a recombinant DNA technology. Transgenic *Nicotiana plumbaginifolia* plants were produced which express apoaequorin (recombinant aequorin). *Agrobacterium tumefaciens* pBIN19 binary vector system, which consisted of an apoaequorin-coding region from cDNA fused to the cauliflower mosaic virus (CMV) 35S promoter, was transferred to *Nicotiana plumbaginifolia*. F$_1$ progeny from the plants transformed with the 35S-apoaequorin chimaeric gene were selfed (intercrossed) and homozygous F$_2$ progeny were used for experiments (Knight et al. 1991). This technique allows one to measure changes in cytosolic Ca$$^{2+}$$ concentration in intact plants in response to various stimuli. Photoproteins have several advantages over fluorescent indicators, including the need for much simpler recording instrumentation and freedom from factors such as autofluorescence, intracellular compartmentation, rapid leakage and buffering of calcium (Cobbold and Lee 1991). For these reasons aequorin has often been used as an indicator of intracellular calcium concentration.
2.3. GABA synthesis and metabolism.

The presence of GABA has been demonstrated in all plant tissue tested. The major pathway of GABA synthesis is α-decarboxylation of glutamate catalyzed by a pyridoxal phosphate (PLP) dependent enzyme, glutamate decarboxylase (GAD) (EC4.1.1.15). Other minor pathways that synthesize GABA have been demonstrated to exist in plants, but their physiological relevance is not yet fully understood. In one pathway GABA is produced from spermidine and putrescine with pyrroline as an intermediate (Terano and Suzuki 1978), in the other from ornithine via α-oxo-αminovalerate (Wickremasinghe and Swain 1965), and arginine is converted to GABA with γ-guanino butyrate as an intermediate (Tixier and Desmaison 1980). Pathways leading to GABA production are indicated in Fig 1.

The production of GABA through GAD catalyzed decarboxylation of glutamate has been demonstrated by the in vivo conversion of exogenous glutamate labelled at carbon 1 to labelled CO₂ and unlabelled GABA (Walker et al. 1984, Chung et al. 1992).

The conversion of glutamate to GABA may initiate the entry of glutamate carbon into the Krebs cycle, and the glutamate nitrogen into GABA and alanine through a pathway called the GABA shunt. This pathway is composed of three reactions:

1. The irreversible decarboxylation of glutamate by GAD to produce GABA.
Fig 2. Possible pathways of GABA synthesis and metabolism.

Enzymes involved in the GABA shunt:
- GDH - glutamate dehydrogenase
- GAD - glutamate decarboxylase
- GABA-T - GABA transaminase
- SSADH - succinic semialdehyde dehydrogenase.

Intermediates leading to GABA synthesis were determined by incubation of labeled substrates with crude plant extracts and recovery of labeled GABA.
2. The transamination of produced GABA by GABA-pyruvate transaminase to produce alanine and succinic semialdehyde.

3. The oxidation of succinic semialdehyde by succinic semialdehyde dehydrogenase resulting in the production of succinate with the concomitant reduction of NADP⁺ (Fig. 2).

Studies of in situ metabolism of (14-C) glutamate demonstrated that the GABA shunt operates in developing soybean cotyledons. (14-C)GABA, determined by HPLC amino acid analysis, was recovered after the metabolism of supplied (U-14-C) glutamate, but not from the metabolism of (1-14-C) glutamate. This demonstrated that GABA is produced by decarboxylation at carbon one of glutamate (Tuin and Shelp 1995a).

Using an inhibitor of PLP dependent enzymes, aminooxyacetate, it was shown that the direct decarboxylation of glutamate to GABA was 10-fold greater than glutamate deamination in developing cotyledons. Also the rapid metabolism of (14-C)GABA and the recovery of this radioactive label in protein amino acids, such as aspartate was demonstrated. This suggests that glutamate decarboxylation could contribute to the Krebs cycle derived amino acids for protein synthesis in developing cotyledons (Tuin and Shelp 1995b). Glutamate may also enter Krebs cycle via oxidation to 2-oxoglutarate in a reaction catalyzed by glutamate dehydrogenase (EC 4.1.1.15). However the purpose of having two pathways to convert glutamate to Krebs cycle intermediates, is not clear. It has been suggested that during imbibition in germinating mustard seed GAD is more active at a lower level of hydration than glutamate dehydrogenase. As
germination proceeds and the hydration level increases, glutamate dehydrogenase becomes more active, and succinate is produced via 2-oxoglutarate (Vandewalle and Olssen 1983). Thus it appears that glutamate carbon enters the Krebs cycle through different pathways under different conditions.

2.3.1. Glutamate Decarboxylase.

Glutamate decarboxylase (GAD) catalyzes the essentially irreversible α-decarboxylation of L-glutamate producing GABA. GAD activity has been measured in soybean seeds (Abdul-Baki and Anderson 1975), wheat embryos (Galleschi et al. 1978), potato tubers (Satya Narayan and Nair 1986), squash fruit (Matsumoto et al. 1986), embryos and roots of barley (Inatomi and Slaughter 1975), tea leaves (Tsushida and Murai 1987), and Asparagus sprengeri mesophyll cells (Snedden et al. 1992).

GAD activity is pyridoxal phosphate (PLP) dependent. In crude extracts of barley embryos and tea leaves GAD was activated 2.5 to 3.5 times in saturating concentrations of PLP. The fact that GAD purified from potato extract absorbed strongly at 388 nm (the absorption peak for PLP) lead to a calculation indicating at least 2 molecules of PLP are bound to the enzyme (Satya Narayan and Nair 1985).

GAD was shown to be a soluble cytosolic enzyme by cellular fractionation studies involving differential centrifugation of pea extracts (Dixon and Fowden 1961), soybean (Wallance et al. 1984) and potato tubers (Satya Naryan and Nair 1986). Recently obtained data using soybean cotyledon protoplasts and gentle
osmotic lysis also indicate a cytosolic localization of GAD (personal communication, Dr. Barry Shelp, University of Guelph).

It is well known that the pH optimum for GAD activity in vitro is lower than the physiological pH in the cytosol of plant cells in vivo. It varies between 5.7 and 6.2 in sunflower cotyledons (Smith and Waygood 1961), barley tissues (Inatomi and Slaughter 1975), potatoes (Satya Narayan and Nair 1985), tea leaves (Tsuchida and Murai 1987), asparagus mesophyll cells (Snedden et al. 1992) and soybean (Snedden et al. 1995). Under in vivo conditions, where cytosolic pH values are estimated to be around 7.2 there is only 20% of the maximal activity of GAD.

Published data indicate that $K_m$ values of plant GAD for glutamate differ for embryonic and mature tissues. $K_m$ values for mature tissues are between 3 and 9 mM, and for embryonic tissues are between 22 and 25 mM (Inatomi and Slaughter 1975).

Baum et al. (1993) first reported that petunia GAD possesses a calmodulin binding domain. A petunia expression library was screened with $^{35}$S-labelled recombinant calmodulin as a probe for CaM-binding proteins. A labelled protein band of 58 kDa had a deduced amino acid sequence of 500 amino acid residues, and catalyzed conversion of glutamic acid to GABA. This protein had 67% overall amino acid homology to GAD isolated from the bacteria E. Coli. The calmodulin binding domain in petunia GAD is part of a carboxyl end extension of the enzyme. In vitro calmodulin binding to petunia GAD is calcium dependent (Baum et al. 1993). At the optimal 5.8 pH for GAD activity addition of
calcium/calmodulin was not stimulatory, but at the physiological pH of 7.0, soybean GAD is stimulated 2-to 8-fold by calcium/calmodulin with half-maximal concentrations of 5 μM and 40 nM respectively (Snedden et al. 1995).

Reports indicate that the molecular weight of GAD from potato tubers is 91 kDa, and this enzyme is a dimer composed of two identical subunits of 45.5 kDa (Satya Narayan and Nair 1985). Petunia GAD is a 58 kDa protein (Baum et al. 1993), and GAD from fava beans has an apparent molecular mass of 62 kDa (Ling et al. 1994). Anti-petunia GAD antibodies were used to detect cross-reacting proteins in potato tuber. The polyclonal antibodies for the 58 kDa GAD reacted with the 45.5 kDa protein in potato tuber extract (Baum et al. 1993). Thus plants seem to possess GAD enzymes which appear to cross-react immunologically, but which have different molecular weights. Different molecular weights may arise from estimates obtained from GAD complexed with calmodulin or in the absence of calmodulin.

Differences in specific activity of GAD from different tissues in soybean have been reported. The highest specific activity (nmol min⁻¹·mg protein⁻¹) of 1332 was observed in the developing seed coat, 9.06 and 2.94 in root and leaf, respectively, and the lowest activity of 0.53 was observed in the developing cotyledons. These results might reflect multiple forms of GAD, or different states of activation (Snedden et al. 1995). However, the significance of such high levels of GABA in the seed coat is not understood. It has been reported that two interconvertible molecular forms of GAD found in barley embryos
differ in properties from a third form of GAD present in barley roots (Inatomi and Slaughter 1975).

2.3.2. GABA Accumulation in Response to Stress.

The accumulation of GABA in response to various stress conditions is well documented (Bown and Shelp 1989, Satya Narayan and Nair 1990). These stresses include: leaf ageing (Inatomi and Slaughter 1975, Lahdesmaki 1965), γ-irradiation (Satya Narayan and Nair 1986), low temperature shock, darkness and mechanical damage or manipulation (Wallace et al. 1984), high temperature (Mayer et al. 1990), anaerobiosis (Lane and Stiller 1970, Streeter and Thompson 1972), ammonia exposure (Kishinami 1987), phytohormone (ABA) treatment (Reggiani et al. 1993), viral attack (Cooper and Selman 1974), and cytosolic acidification induced by weak acids (Crawford et al. 1994).

The rate and extent of GABA accumulation depends, however, on both the type of plant and tissue, as well as the imposed stress. For example, in Asparagus sprengerî mesophyll cells incubated with 5 mM butyrate a GABA increase of 200 to 300% was observed within 15 seconds (Crawford et al. 1994). In soybean leaves a 2000% increase in GABA levels was observed within 5 minutes of mechanical manipulation or cold stress (Wallace et al. 1984). In contrast, GABA did not accumulate until 2 hours after anoxia was imposed on radish leaves (Streeter and Thompson 1972). In maize root tips maximum GABA production was found after 12 hours of hypoxia (Roberts et al. 1992), and in roots from 3 day old wheat seedlings a 24 hour treatment with 100 μM
ABA caused a 3-fold increase in GABA levels (Reggiani et al. 1993).

Experiments performed on plants often involve mechanical manipulation, which is necessary for sampling plant material. Such procedures have been shown to result in increased GABA levels. Simple detachment of the soybean leaflet can stimulate GABA production. Rolling of leaflets six times into a coil caused a 34-fold increase in GABA levels. Crushing leaflets with mortar and pestle resulted in a 43-fold increase (Wallace et al. 1984).

Plants are often exposed to adverse conditions in their natural environment. Temperature is a variable which may fluctuate dramatically during diurnal or annual cycles. These variations in temperature were shown to influence nitrogen metabolism and to affect amino acid metabolism. It has been reported that a marked increase in GABA level has been observed in response to both an increase and decrease in temperatures (Wallace et al. 1984, Mayer et al. 1990).

Soybean leaves exhibited rapid accumulation of GABA in response to abrupt transfer to a lower temperature. GABA levels measured immediately after harvesting rose from 0.05±0.03 to 1.18±0.26 μmol*g⁻¹ fresh weight within five minutes of direct transfer of leaflets to 6 °C. The same levels of increase were observed when detached leaflets were cold shocked in the dark. When plants were slowly cooled, GABA levels did not increase to the same extent. The reversibility of these observed changes in GABA levels were also tested. One hour after being transferred
from 6 °C to 33°C in full sunlight, the leaflets reduced GABA levels to the resting values (Wallace et al. 1984).

The heat shock response was studied in suspensions of cowpea cells, which were transferred from 26 to 42 °C and maintained for 24 hours. A 64-fold increase in GABA concentration was observed within 2 hours of heat shock (Mayer et al. 1990).

Oxygen deprivation is a common biological stress that plants are often exposed to. During germination, seeds experience a brief period of hypoxia due to the rigid seed coats. Plants roots may suffer oxygen deprivation in waterlogged soil. In order to survive the oxygen deprivation, plants carry out anaerobic fermentation with the accumulation of lactic acid and ethanol. GABA accumulation was also observed in various plant species. In rice, which is resistant to hypoxia, GABA levels increased from 0.3 to 2.4 μmoles*g fresh weight. In wheat, which has low resistance to hypoxia, GABA levels increased from 0.2 to 8.5 μmoles*g fresh weight (Menegus et al. 1989).

In isolated Asparagus sprengeri mesophyll cells hypoxia resulted in an increase of 120% in the total GABA levels from 2.16 nmol of GABA/10⁶cells in the control to 4.73 nmol of GABA/10⁶cells in the experimental sample (Crawford et al. 1994).
2.3.3. Mechanisms and Possible Roles of Stimulated GABA Synthesis.

GABA accumulation in plants exposed to various, supposedly unrelated, stress conditions is well documented. However, the mechanism(s) stimulating GAD, the GABA producing enzyme, remains controversial. The literature contains different hypotheses concerning regulation of GAD. It is not clear whether a single mechanism is sufficient to explain the elevated GABA levels in response to diverse types of stress.

Mechanical damage may disrupt intracellular compartmentation, and break vacuolar membranes, the tonoplast. The vacuole in plant cells contains high H⁺ and Ca²⁺ concentrations. Its disruption will lead to high H⁺ and Ca⁺ levels in the cytosol. The lowering of cytosolic pH is known to stimulate GAD, which has an acidic pH optimum. At the same time, the elevated Ca²⁺ concentration may also activate GAD. Disruption of intracellular compartmentation may also result in the exposure of metabolically inactive pools of glutamate from the vacuole to GAD located in the cytosol. In this case increasing substrate concentration may stimulate GABA synthesis (Satya Narayan and Nair 1990).

This mechanism can not be readily applied when GABA synthesis is stimulated by lower temperatures. The interruption of intracellular compartments may occur with the formation of ice crystals, when plants are exposed to the temperatures below 0°C. However, Wallace et al. (1984) reported that exposure of soybean leaflets to low, non freezing temperatures, must cause
only minimal damage, since GABA accumulation induced by cold shock was reversed within 1 hour when the plants were returned to normal growing conditions. Cold shock is known to increase cytosolic calcium (Knight et al. 1991), which in turn may activate GAD.

Hypoxia is known to reduce intracellular pH. Cytosolic pH declined by 0.6 pH units (7.4 to 6.8) in maize root tips (Roberts et al. 1984), and 0.9 pH units (7.4 to 6.5) in tobacco cell suspensions (Wray et al. 1985). In both cases the pH decline was observed within 5 minutes of transfer to anoxic conditions. This is thought to be due to the stimulation of anaerobic lactic acid synthesis. In species resistant to hypoxia an alternative pathway may be employed which produces succinate instead of lactate. This was observed in rice where the succinate/lactate ratio was 3:1 in hypoxic conditions, and 700% GABA increase occurred. In wheat, which displays a low tolerance to hypoxia, the succinate/lactate ratio was 0.2 and a 4000% increase in GABA synthesis occurred. In this study the pH of cells sap was determined. In rice an increase in the pH was observed (6.0 to 6.2 pH units), whereas in wheat a decrease occurred (6.3 to 6.0 pH units) (Menegus et al. 1989). However, to obtain these measurements cell contents were squeezed out, and this data does not reflect changes in cytosolic pH. The hypothesis that GABA synthesis occurs in response to the cytosolic acidification was tested in a study by Crawford and coworkers (1994). Permeant weak acids, such as butyrate, were applied to isolated Asparagus sprengeri mesophyll cells. At an
external pH of 5.0, a 5 mM butyrate addition resulted in a cytosolic pH drop of 0.6 pH units within 4.2 seconds. This was determined spectrofluorimetrically using BCECF (fluorescent pH probe) loaded cells. In response to this treatment GABA levels rose from 3 nmoles GABA/10^6 cells to 18.3 nmoles GABA/10^6 cells within 5 minutes (Crawford et al. 1994).

In these conditions GABA may accumulate because of the different pH profiles of the GABA shunt enzymes. In developing soybean seeds the pH optima for GAD, GABA-T, SSADH were 5.8, 8.2, 9.5, respectively (Shelp et al. 1994). At a physiological pH of 7.0, the activities of all these enzymes were similar, but a drop in cytosolic pH might cause stimulation of enzyme responsible for GABA synthesis and inhibition of enzymes that are responsible for GABA utilization (Shelp et al. 1994).

The extent to which proton consuming GABA production may serve to regulate intracellular pH was assessed through determination of the specific acid load and the concomitant increase in GABA production. 14C butyric acid uptake was used to measure acid load, assuming that one H+ is released into the cytosol of the asparagus cell for each molecule of butyric acid. The percentage of the total acid load consumed by the decarboxylation of glutamate was calculated, knowing that production of one mole of GABA will consume one mole of protons. GABA production accounts for at least 45% of the acid load occurring 45 seconds after addition of the permeant butyric acid. This result clearly indicates that GABA production in a proton consuming reaction may function as a mechanism for
metabolic pH regulation (Crawford et al. 1994). Overall metabolism and protein synthesis are at a lower rate in stressed plants, and increased proteolysis is known to occur. This will add more glutamate to the free amino acid pool, which may be decarboxylated in the conditions that stimulate GAD activity (Satya Narayan and Nair 1989).

The role of GABA synthesis in plants remains unclear. It has been hypothesized that GABA synthesis is part of a metabolic pH-stat mechanism, an amino acid metabolite in nitrogen and carbon storage, a transport molecule, or an insect neural inhibitor.

The role of GABA as a transport molecule was discussed by Bown and Shelp (1989). It was suggested that in germinating seeds during mobilization of protein reserve arginine is degraded to urea and ornithine, with the nitrogen from urea being transferred to glutamine and asparagine, and the carbon and nitrogen from ornithine being converted via glutamate to GABA, which is then transported to the growing axis of plants (Bown and Shelp 1989). This metabolic pathway was observed in germinating chestnut (Desmaison and Tixier 1986), and mustard seeds (Vandewalle and Olssen 1983).

While there is strong evidence that GABA synthesis plays a role in resisting cytosolic acidification (Crawford et al. 1994), little is known concerning the role of the rapid increases in GABA levels in response to the stress factors that do not necessarily result in an acidification of the cytosol in the plant cells.

3.1. Materials.

3.1.1. Cell Isolation and Incubation

<table>
<thead>
<tr>
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<th>Supplier</th>
</tr>
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<tr>
<td>Acetone</td>
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<tr>
<td>ABA (±)-cis,trans-abscisic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>A23187-hemicalcium salt</td>
<td>Sigma</td>
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<tr>
<td>Calcium Sulphate</td>
<td>BDH</td>
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<td>Citric acid</td>
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<tr>
<td>Dimethyl sulfoxide</td>
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</tr>
<tr>
<td>MES 2-(n-morpholino)ethanesulphonic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>W5 N-(6-aminohexyl)-1-naphtalenesulfoamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>W7 N-(-aminohexyl)-1-naphtalene-5-chlorosulfoamide</td>
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3.1.2. GABA Extraction and Determination

<table>
<thead>
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<th>Supplier</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Chloroform</td>
<td>BHD</td>
</tr>
<tr>
<td>Methanol</td>
<td>BHD</td>
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<td>Glycerol</td>
<td>BHD</td>
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<tr>
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<td>Sigma</td>
</tr>
<tr>
<td>γ-aminobutyric acid (GABA)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Nicotamine adenine dinucleotide phosphate(NADP⁺)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>Kodak Eastman</td>
</tr>
</tbody>
</table>
3.1.3. Fluorescence Studies

BCECF-AM(2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester  Molecular Probes
Fluo-3, pentapotassium salt  Molecular Probes
Dimethyl sulfoxide  Calbiochem
Polylysine  Sigma
A23187, 4-bromo  Sigma

3.1.4. Photographic Supplies

Kodak 400SA slide film  Kodak

3.1.5. Other

Ethylene diamine tetraacetic acid (EDTA)  Sigma
H$_2$SO$_4$  BDH
NaOH  BDH
HCl  BDH

3.1.6. Plant Material

Asparagus sprengeri Regel plants were used for cell isolation. Plants were grown in the greenhouse under natural day-length light conditions, in a slightly acidic mixture of vermiculite and sphagnum peatmoss (40:60 v/v pH 5.0 to 6.5). The plants were watered daily and fertilized weekly (N:P:K-30:10:10).

3.2.1. Cell Isolation.

Asparagus sprengeri Regel mesophyll cells were mechanically isolated using a method similar to that described by Colman et al. (1979). On the day of each experiment four to five mature, dark green, glossy asparagus fronds were harvested from different plants in the greenhouse. Cladophylls were stripped of the middle branches of the frond and collected in a Buchner funnel. Any contaminants present were removed by rinsing collected cladophylls in running tap water. Clean cladophylls were cut into approximately 5 mm segments with a razor blade and collected in 100-200 ml of 1 mM CaSO₄, pH 5.5 (adjusted with NaOH).

Fresh 1 mM CaSO₄, pH 5.5 was vacuum infiltrated into cladophyll segments for 5 minutes to replace air from apoplastic space and facilitate mechanical isolation. Infiltrated cladophylls were transferred to a mortar with the addition of fresh 1 mM CaSO₄, pH 5.0. Cells were mechanically pushed out of the cladophyll segments by application of gentle pressure with the pestle. The presence of cells in the medium was observed as the appearance of a green coloured suspension. This cell suspension was transferred with a Pasteur pipette into 30 ml Corex centrifuge tubes through two layers of cheese cloth to remove large pieces of cladophylls. The resulting cell suspension was centrifuged at 550g for 2 minutes, supernatant fluid containing cell debris was
discarded, and the green pellet of cells was resuspended in fresh CaSO₄.

Fresh medium was added to the mortar for further cell isolation. The process of isolating cells was repeated no more than 5 times, depending on the number of the cells required for the experiment. Isolated cells in each repetition were combined together to give a stock suspension for further experimental procedures.

3.2.2. Cell Examination.

After mechanical isolation, the cell density and the percentage of damaged cells were determined using a Spencer Bright Line haemocytometer and a Wild Light microscope.

Approximately 1 to 2 ml of well shaken stock cell suspension was incubated for 20 minutes with a drop of Evan’s blue dye (20% w/v) solution. Evan’s blue will not penetrate into physiologically competent cells having an intact polarized plasma membrane (Graff and Okong’o-ogola 1971). Therefore, only physiologically compromised cells, with a disrupted plasma membrane, appear blue after dye accumulation.

Cell suspensions with less than 20% of damaged cells were used for experiments. The density of cells in the stock cell suspension varied from 2 to 5 million cells per ml.

Stock cell suspensions were stored at room temperature (21±1 °C) in the dark. Each experiment was completed within
four hours from frond harvesting to minimize any possible effects of cell storage.

3.2.3. Cell Incubation.

For all experiments a cell suspension density of 4X10^6 cells/ml was used. The required number of cells was spun down and resuspended in 5 mM MES/1 mM CaSO₄ pH 5.0 or 6.0, depending on the experiment. Volumes of cell suspension varied from 5 to 30 ml. For a typical experiment 2X10^7 cells were resuspended in 5 ml MES/CaSO₄ buffer and incubated in different conditions for 15 minutes.

The same number of cells was always incubated without any treatment to serve as a control for GABA levels in unstimulated cells and was compared to the experimental samples. One stock cell suspension was used to give one replication of the experiment. For following replications of an experiment the process of cell isolation, which began with frond harvesting, was repeated and new stock suspensions of cells were used.

Temperature shock was applied by resuspending a pellet of 2X10^7 cells with 5 ml of 5 mM MES/1 mM CaSO₄ pH 6.0 at different temperatures and stirring with a magnetic bar for fixed times of incubation. Constant temperatures from 40 to 10°C were maintained in a water jacketed incubation vessel. Cold shock was applied by resuspension of the cell pellet with 5 ml of 5 mM MES/ 1 mM CaSO₄ pH 6.0 at a temperature of 1°C.
This temperature was maintained by incubation of the suspension on an ice bath.

Depending on the experiment, sodium butyrate (5 mM), glutamic acid (5 mM), abscisic acid (1 to 100 µM), A23187 (25 µM), Lanthanum chloride (100 µM), W5 or W7 (1 mM), was added to the experimental cell suspension.

3.2.4. GABA Extraction.

i. From Isolated Cells

GABA was extracted from the cells following the procedure described by Chung et al. (1992).

After treatment, the 5 ml cell suspension was transferred to 5 ml 100% methanol, and incubated for 30 minutes on ice with stirring. This was followed by sequential addition of 10 ml chloroform (20 minutes on ice incubation with stirring), and 5 ml of H₂O (15 minutes on ice with stirring).

In some experiments the medium containing extracellular GABA was separated from the cells by centrifugation at 2500g for 2 minutes. Samples of the medium were dried in a filtered stream of air at 60 °C prior to further analysis. The cell pellet was solubilized by sequential addition and shaking with 2.5 ml 100% methanol for 30 minutes, 5 ml chloroform for 20 minutes, and 2.5 ml of distilled water for a further 15 minutes.
The aqueous phase, which contained GABA, was separated from the chloroform phase by centrifugation at 2800g for 10 minutes.

Eighty percent of the aqueous phase, representing 16 million cells was removed and dried out under a filtered air stream at 60°C. Collected dried cell extracts were stored in the refrigerator for further GABA determination.

**ii. From Intact Tissue.**

Stimulation of GABA synthesis in intact cladophylls by cold shock was investigated by stripping 200-300 cladophylls (approximately 1g of fresh weight tissue) directly from the plant and floating them on 1 mM CaSO₄ pH 5.5 at 1°C for 15 min. Control samples of cladophylls from the same frond were incubated similarly at 21±1°C.

After cold shock application experimental and control cladophylls were dried quickly using a cheese cloth sieve and boiled in 25 ml of 100% methanol for 10 min. Boiled cladophylls were ground in a mortar and pestle with the remaining methanol. An additional 5 ml of methanol was used.

This tissue slurry was transferred to a 30 ml Corex centrifuge test tube and incubated with 10 ml of chloroform and 5 ml of H₂O for 30 minutes on ice with occasional shaking.

The aqueous fraction was separated by centrifugation from the chloroform fraction and collected for GABA determination. The pellet of tissue debris was washed 3 times by the addition
of 2 ml of H₂O and centrifugation each time. The resulting aqueous supernatant fluids were combined into one sample and dried out under a filtered air stream at 60 °C.

Dried samples were redissolved in 1 ml of 5 mM MES pH 6.0. The resulting extract was purified prior to GABA determination by ion exchange chromatography using AG 50W-X8(H⁺) resin (bed size 0.8X4cm, 200-400; mesh BioRad Laboratories). Columns were equilibrated with 30 ml of H₂O, prior to loading the cladophyll extract. After sample application, organic acids were washed through the column with 20 ml of water and amino acids were eluted with 20 ml of 4 N NH₄OH. The eluted amino acids were dried out as described previously, and resuspended in 0.5 ml of 5 mM MES pH 6.0, for GABA determination.

3.2.5. GABA Determination.

Dried samples of plant extract were dissolved in 500 µl of 1 mM potassium pyrophosphate buffer pH 8.6. GABA levels in the sample were assayed spectrophotometrically using a coupled enzyme assay (GABAse, Sigma Chemical Company, St. Louis, MO, USA). A quartz cuvette (1 cm light path) containing 650-700 µl 1 mM potassium pyrophosphate buffer pH 8.6, 150 µl 0.04 M NADP⁺, 50 µl GABAse, and 50 or 100 µl of sample was placed in a DU-50 spectrophotometer. An initial absorbance was recorded. The reaction was initiated by the addition of 50 µl 0.02 M α-keto-glutarate, and the increase in absorbance at 340 nm was
monitored for up to 45 minutes, or until the reaction reached equilibrium.

In this assay GABA is consumed in 1:1 ratio as NADPH is produced according to reactions:

1. \[ \text{GABA} + \alpha\text{KG} \leftrightarrow \text{L-GLU}^- + \text{SUCCINATE SEMIALDEHYDE} \]

2. \[ \text{SUCCINATE SEMIALDEHYDE} + \text{NADP}^+ \rightarrow \text{SUCCINATE} + \text{NADPH} \]

The enzyme catalyzing the first reaction is GABA transaminase (EC 2.6.1.19), when the second reaction is catalyzed by succinic semialdehyde dehydrogenase (EC 1.2.1.16). The enzymatic activity and number of active units of enzymes were assayed at the beginning and end of the analysis series. In each assay more than 0.07 active units of enzymes were present.

3.2.6. Preparation of a Calibration Curve for GABAAse Assay.

To recapitulate the experimental procedure, seven test tubes containing 0 to 100 nmoles standard GABA in 1 ml of 5 mM MES, pH 5.0 were prepared. Samples were dried under a filtered air stream at 60 °C. Prior to analysis samples were redissolved in 500 μl of 1 mM potassium pyrophosphate buffer, pH 8.6. For GABAAse analysis 100 μl of sample was used. The change in absorbance at 340 nm due to NADPH production was recorded and plotted against GABA concentrations. The resulting best fitted line had a regression factor of \( R^2 = 0.9902 \). GABA levels present
in samples of cells extracts were calculated using the following equation for a straight line (Fig. 3) \[ \text{GABA} = -3.8368 + 162.93 \times \Delta \text{Absorbance}. \]

GABA levels present in the experimental samples were expressed in nmoles per million cells.

### 3.2.7. Recovery of Standard GABA Added During GABA Extraction from the Cells.

To investigate any possible effects of experimental procedure on GABA extraction from asparagus cells, standard GABA was added to the cell suspension at the stage of methanol addition.

To the 5 ml of $2 \times 10^7$ cell suspension in 1 mM CaSO$_4$/ 5mM MES, 50 nmoles of standard GABA was added simultaneously with 5 ml of methanol. GABA extraction was performed as described previously. From the resulting 15 ml of supernatant fluid a 12 ml sample equivalent to 16 million cells was pipetted and dried. Dried samples were resuspended in 500 µl of pyrophosphate buffer, and 50 µl of this extract was used for analysis, containing approximately 4 nmoles of standard GABA. Standard GABA present in the cuvette was expected to give an additional $\Delta$ absorbance of 0.048 units, similar to that found in the unstimulated, control samples of the cells. This experiment was run in triplicate and 92% of the standard GABA was recovered (Table 1).
### TABLE 1. Recovery of the Standard GABA Added to the Cell Suspensions at the Stage of Methanol Addition.

In each experiment 20 million cells were suspended in 5 ml 5 mM MES / 1 mM CaSO₄. Fifty nmoles standard GABA in 100 μl 5 mM MES / 1 mM CaSO₄ was added to the samples simultaneously with the 5 ml methanol. GABA was extracted and assayed as in "Methods". Duplicate GABAse assays were performed in each trial.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control</th>
<th>+GABA</th>
<th>% Recovery</th>
</tr>
</thead>
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<tr>
<td></td>
<td>nmoles GABA / 10⁶cells</td>
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</tr>
<tr>
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</tr>
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<tr>
<td>Mean</td>
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<td>7.18</td>
<td>95</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.51</td>
<td>±0.44</td>
<td>±2</td>
</tr>
</tbody>
</table>
Fig. 3. Calibration Curve for GABA Assay.

Each point is the mean of three separate GABAse analyses, each with duplicate assays. Standard error is indicated. GABA = \(-3.8368 + 162.83 \times \Delta\) Absorbance. Standards were prepared as described in "Methods".
3.2.8. Chlorophyll Determination.

GABA levels in intact tissue were expressed as μmoles of GABA per mg chlorophyll. The procedure for chlorophyll determination was based on a spectrophotometric assay of Arnon (1949). Fifty or 100 μl of chloroform chlorophyll extract were mixed with the 80% acetone to give a total of 3 ml volume in a quartz cuvette. Absorbance at 663 and 645 nm was determined in a DU-50 spectrophotometer. Chlorophyll concentration in the cuvette was calculated in units of milligrams per liter according to the equation (Aron 1949):

\[ C = 20.2 \times A_{645} + 8.02 \times A_{663} \text{ (mg/l)} \]

Knowing the concentration of chlorophyll in the 3 ml solution in the cuvette, the total chlorophyll content in the plant sample was determined including calculation of all dilutions during the procedure.

3.2.9. Monitoring Changes in Cytosolic H⁺ and Ca²⁺ Concentrations.

i. Loading Cells with the Fluorescent Probes.

To monitor changes in intracellular pH, cells were loaded with BCECF-AM using a method described by Crawford et al. (1994). This pH probe is a membrane permeant, lipophilic ester. Inside the cell it is hydrolyzed by endogenous esterases to BCECF, the free acid form. Hydrolyzed BCECF is trapped as an impermeant anion in the cytosol of the cell, and
exhibits pH-dependent fluorescence. The probe was initially dissolved (1mg/ml) in dry dimethyl sulfoxide and stored desiccated at -20°C. Suspensions of asparagus cells at a density of 5X10^6 cells per ml, in 1 mM CaSO_4, pH 5.5, were incubated with 6 mM BCECF-AM for 30 min. After incubation any extracellular BCECF-AM or BCECF was removed from the medium by centrifugation (at 550g for 1.5 min.) and resuspension in fresh medium three times. Changes in cytosolic Ca^{2+} were observed by loading cells with the fluorescent calcium indicator Fluo-3. Fluo-3, pentapotassium salt, was dissolved in 5 mM MES, pH 6.5, and stored desiccated at -20°C. Cells at a density of 5X10^5 per ml were incubated in 10 mM citric acid buffer, pH 4.5, with 50 mM Fluo-3 for 60 minutes. Incubation was carried out in ambient light at room temperature (21±1°C) with gentle stirring with a magnetic bar. The low pH was used to protonate the negative carboxyl groups of Fluo-3 in the medium, outside the cells. The protonated, uncharged, probe is able to cross the plasma membrane. Inside the cell Fluo-3 dissociates at the higher pH (around 7.2), and is trapped in the ionic, calcium sensitive form in the cytosol. This loading procedure resulted in 50% of the cells exhibiting Fluo-3 uptake, as determined with a Wild Light Microscope. The loaded cells were distinct from unloaded cells, as they exhibited the orange colour of Fluo-3.
Cells loaded with fluorescent probes were observed under a Leitz Diaplan microscope equipped with epifluorescence optics (100x objective lens, oil immersion; 10x eyepiece lens). Light was supplied by high pressure mercury lamp (HBO 50W) and the combination filters for excitation were BP 450-490, and for emission RKP 510 and LP 515. Photographs were taken using 400 ASA Kodak Ectachrome colour film. To eliminate red autofluorescence of the chlorophyll, a green coloured glass filter (peak transmission 535 nm) was employed. Localization of the probe was determined using the fluorescent Leitz Diaplan microscope. It was observed that cells exhibited a red autofluorescence of the chloroplasts which was overwhelmed by the yellow-green fluorescence of the Fluo-3. Observations of fluorescence from loaded mature cells with developed vacuoles revealed a dark spot in the cell region where the vacuole was located. This verified that Fluo-3 was not sequestered into the vacuole. Using a green glass filter with a transmission peak of 535 nm on the detection side, the autofluorescence of the chloroplasts was eliminated. Observations of dark spots in the cell regions where chloroplasts were located confirmed that Fluo-3 was absent from the chloroplasts. These observations indicate that the only compartment in which Fluo-3 is located is the cytosol of asparagus cells. Any changes in the fluorescence intensity from Fluo-3 loaded cells should therefore reflect changes in free cytosolic Ca$^{2+}$ concentration in asparagus cells.
Fig. 4. Fluorescent Micrographs of Asparagus sprengeri Regel Mesophyll Cells.

Top panel – a population of cells viewed through epifluorescence optics. Acid loading of asparagus cells resulted in approximately 50% loaded cells. Cells loaded with Fluo-3 exhibit yellow fluorescence of the probe. Unloaded cells exhibit red autofluorescence of the chloroplasts (magnification 2000x).

Bottom left panel – bright field photograph of a mature asparagus mesophyll cell with developed chloroplasts and vacuole (magnification 4000x).

Bottom right panel – the same cell viewed through epifluorescence optics. The Fluo-3 is localized in the cytosolic strands around chloroplasts and vacuole. Autofluorescence of the chloroplasts is overwhelmed by fluorescence from Fluo-3 located in the cytosol around them. The dark central area of the cell indicates that Fluo-3 was not taken up by the vacuole (magnification 4000x).
iii. Image Analysis.

Image analysis were performed using an MCID M2 Microprocessor Controlled Imaging Device (Imaging Research Inc. Brock University, Ontario).

Cells loaded with fluorescent probes were plated onto 22 mm diameter glass coverslips. To facilitate cell adhesion, coverslips were coated with polylysine (5 µg per ml). The positive charge of the polylysine may form an electrostatic interaction with the negatively charged cell wall. This causes cell immobilization.

The cover slips with adhered cells were transferred to a flow chamber (flow rate 1-2 ml/min) on the stage of a Ninon Diaphod 300 inverted microscope equipped with a x100 fluorescence objective (N.A.1.3.) and continuously perfused with 5 mM MES/1 mM CaSO₄ buffer, pH 6.0.

Fluo-3 was excited at a single wavelength using a software-controlled Stuffer filter (495 nm DF25), positioned between a 100W Xeon light source and the microscope. The emitted fluorescent light was monitored at 535 nm and images acquired using a Hamamatsu C4880, Fast Cooled Integrating (FCI) camera.

BCECF was excited at two wavelengths using 440 and 495 nm DF20 filters. Emission of fluorescent light was monitored at 535 nm with a Df10 filter. The fluorescence emission from excitation at 495 nm is pH dependent, whereas emission from
440 nm is unaffected by changes in pH. This allows one to monitor changes in pH through the $I_{495}/I_{440}$ ratio. Images of this ratio were recorded every second using the camera described above.

Experiments were carried out with the continuous perfusion of buffer at room temperature ($21\pm2^\circ C$), except for the cold shock application when 5 mM MES/1 mM CaSO$_4$ at 1°C was perfused.

3.2.10. Spectrofluorimetry.

Changes in the intracellular Ca$^{2+}$ upon the treatment with A23187,4-bromo, in Fluo-3 loaded cells were measured in a Perkin-Elmer LS-50 spectrofluorimeter. Cells were loaded with Fluo-3 as described previously. Cells were washed three times by centrifugation at 550g for 2 minutes and resuspended in fresh medium to remove any extracellular probe. A cuvette containing the Fluo-3 loaded cell suspension (200,000 cells/ml) in 2 ml of 5 mM citric acid buffer pH 5.0, was placed in a thermally regulated analysis chamber in the spectrofluorimeter, where it was maintained at 25 °C with gentle stirring. Additions were made directly to the cuvette, while located in the chamber.

Fluo-3 was excited at 506 nm and fluorescence intensity was measured at 526 nm with slit width set at 5 nm. An increase in fluorescence intensity was indicative of an increase in intracellular free Ca$^{2+}$ concentration.
4. Results.


4.1.1. GABA Accumulation in Response to Different Temperatures.

The production and efflux of GABA in asparagus cells exposed to different temperature gradients was examined. The extracellular (in the medium) and intracellular (in the cells) GABA levels were measured in this experiment as described in "Methods". The most dramatic increase in GABA accumulation was observed in response to cold shock. Fifteen minutes incubation of 20 million cells at 1°C resulted in an increase of GABA levels from 3.41 nmoles GABA / million cells in the control, to 5.57 nmoles GABA / million cells in experimental samples. The Wilcoxon test revealed a 0.05 probability that observed difference between these two samples are due to the chance alone. The cold shock application resulted in a 106 % increase in GABA levels.

A 10 degree drop in temperature from 20 to 10°C, resulted in a 26% increase in GABA levels.

Heat shock did not stimulate GABA synthesis to the same extent as a cold shock. Compared to the control, GABA levels were elevated by about 20%, after 15 minutes incubation at 40 °C.
Table 2. GABA Accumulation in Response to Different Temperatures.

GABA levels are mean values from 8 experiments. Standard error is indicated. Approximately 20 million cells were resuspended in 1 mM CaSO₄/5 mM MES, pH 6.0, at the temperatures indicated, and incubated for 15 minutes. For each experiment duplicate GABAse assays were performed.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Cells</th>
<th>Medium</th>
<th>Total</th>
<th>%Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles GABA/10⁶ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1°</td>
<td>4.86</td>
<td>0.71</td>
<td>5.57</td>
<td>106</td>
</tr>
<tr>
<td>S.E.</td>
<td>±1.24</td>
<td>±0.39</td>
<td>±1.34</td>
<td>±52</td>
</tr>
<tr>
<td>10°</td>
<td>2.88</td>
<td>0.53</td>
<td>3.41</td>
<td>26</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.71</td>
<td>±0.25</td>
<td>±0.72</td>
<td>±13</td>
</tr>
<tr>
<td>20°</td>
<td>2.34</td>
<td>0.35</td>
<td>2.69</td>
<td>control</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.69</td>
<td>±0.20</td>
<td>±0.72</td>
<td></td>
</tr>
<tr>
<td>40°</td>
<td>2.46</td>
<td>0.78</td>
<td>3.23</td>
<td>20</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.51</td>
<td>±0.35</td>
<td>±0.38</td>
<td>±13</td>
</tr>
</tbody>
</table>
4.1.2. Cold Shock Stimulated GABA Accumulation as a Function of Time.

The time course of cold shock stimulated GABA accumulation revealed an increase in GABA levels within 1 minute of cold shock application. GABA levels found in the cold shocked cells were 2.94±0.56 nmoles/million cells as compared to control values of 2.03±0.36 nmoles/million cells. These values are an average of 3 experiments and represent a 45% increase in GABA levels upon cold shock treatment. Elevated GABA levels after only 1 minute of cold shock application suggest that GABA synthesis is a fast response of asparagus cells to the temperature drop (Fig. 5).

The accumulation of GABA in cells incubated at 1°C was observed during the time span of the experiment, with increases of 61% at 2 minutes, 84% at 4 minutes and a maximum increase of 118% after 8 minutes incubation. At 16 minutes of incubation an average increase of 103% was observed (Fig. 5).
Fig. 5. Time Course for Cold Shock Stimulated GABA Accumulation.

Each point is a mean of three separate experiments. Standard error is indicated. Approximately 100 million cells in 25 ml of 1 mM CaSO₄/5 mM MES were used for each experiment. Cold shock was applied by cell incubation at 1°C. A 5 ml of cell suspension was removed at the times indicated for GABA determination. In each case control experiment was performed simultaneously using cells from the same stock suspension.
4.1.3. Inhibition of Cold Shock Stimulated GABA Accumulation by the Calmodulin Antagonist W7.

The mechanism of cold shock stimulated GABA accumulation was investigated in cells incubated with the CaM antagonist W7. Cell suspensions were preincubated at 21±1 °C with W7 for 60 minutes prior to cold shock application. The presence of W7 during incubation of cells at 1°C for the subsequent 15 minutes resulted in a decrease in GABA accumulation. Upon cold shock treatment, in the presence of W7, GABA levels were 1.93 nmoles GABA/ million cells, as compared to 6.54 nmoles GABA/ million cells in the controls. This represents a 70% inhibition of cold shock induced GABA accumulation (Table 3). The Wilcoxon test revealed a statistical difference between these samples with the value of P<0.014.

W5, an inactive analog of W7 did not affect GABA synthesis. This control experiment ruled out any non specific, pharmacological effects of the CaM antagonist on GABA metabolism in asparagus cells (Table 3).

Incubation of 20 million cells in 5 ml 1 mM CaSO₄, 5 mM MES for 60 minutes, with constant stirring, resulted in elevated GABA levels (Table 12). Subsequent to this treatment cold shock stimulated GABA synthesis did not approach levels obtained in previous experiments (Table 2, Fig. 5).
The possibility of inhibition of the enzymes present in the GABA\textsubscript{e} assay (GABA transaminase and glutamate dehydrogenase) by the CaM antagonist W7 was investigated. Standard GABA was added to the assay when reduced GABA levels upon treatment with CaM antagonists were observed. Addition of GABA resulted in a rapid increase of NADPH production, which was determined as an increase of the absorbance at 340 nm. Therefore CaM antagonists did not inhibit the enzymes in the GABA\textsubscript{e} assay. Thus the reduced GABA levels in the samples treated with W7 are the result of \textit{in vivo} inhibition of GABA synthesis.

The results of this experiment suggest that CaM is involved in the mechanism of cold shock induced GABA synthesis.
Table 3. Stimulation of GABA Accumulation by Cold Shock in the presence of Active (W7) or Inactive (W5) Calmodulin Antagonists.

Twenty million cells in 5 ml of 1 mM CaSO₄, 1 mM MES pH 6.0 were used for each experiment. Preincubation with CaM antagonists for 60 minutes. Cold shock was applied by resuspending centrifuged cells in 1°C fresh medium (minus W7 or W5), and incubating for 15 minutes. Each value represents a mean from four (±W7) or two (±W5) experiments, with duplicate GABAse assays for each trial.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1°C</th>
<th>21°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles GABA/ million cells</td>
<td></td>
</tr>
<tr>
<td>+W7</td>
<td>1.93</td>
<td>1.38</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.77</td>
<td>±0.66</td>
</tr>
<tr>
<td>-W7</td>
<td>6.54</td>
<td>4.95</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.62</td>
<td>±0.02</td>
</tr>
<tr>
<td>+W5</td>
<td>6.59</td>
<td>5.78</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.06</td>
<td>±0.71</td>
</tr>
<tr>
<td>-W5</td>
<td>6.56</td>
<td>5.41</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.075</td>
<td>±0.335</td>
</tr>
</tbody>
</table>
4.1.4. Inhibition of Cold Shock Stimulated GABA Accumulation by Lanthanum, a Ca$^{2+}$ Channel Blocker.

To investigate the role of external Ca$^{2+}$ in cold stimulated GABA accumulation, asparagus cells were incubated with 1 mM LaCl$_3$, 5 mM MES pH 6.0. Lanthanum is a known inhibitor of Ca$^{2+}$ channels at the PM level, and may prevent Ca$^{2+}$ entry into the cytosol during cold shock (Knight et al. 1992).

Preincubation of asparagus cells for 5 minutes with La$^{3+}$ resulted in a 42% inhibition of GABA accumulation during 15 minutes of a subsequent cold shock application. GABA levels were reduced from 5.181 nmoles / million cells with Ca$^{2+}$ present in the medium, to 2.989 nmoles / million cells with La$^{3+}$ present in the medium. The Wilcoxon test indicated that there is a 0.075 probability that the observed difference between these two samples is due to chance, and Student t-test (P=0.05) revealed a significant difference between these two samples. GABA levels in control experiments were not affected by La$^{3+}$ to the same extent as during cold shock. However GABA levels were reduced by 14%.

This result suggests that external Ca$^{2+}$ plays a role during cold shock induced GABA accumulation.
Twenty million cells were used for each experiment. Cells suspensions were preincubated in 1 mM LaCl₃, 5 mM MES or 1 mM CaSO₄, 5 mM MES pH 6.0 for 15 minutes prior to cold shock application. Centrifuged cells were resuspended in the above buffers at the temperatures indicated, and incubated for 15 min. Each value is an average of at least three experiments. Duplicate GABAse assays were performed for each experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>+Ca²⁺</th>
<th>+La³⁺</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles GABA /million cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1°C</td>
<td>5.18</td>
<td>2.99</td>
<td>42%</td>
</tr>
<tr>
<td>S.E.</td>
<td>±1.21</td>
<td>±0.24</td>
<td></td>
</tr>
<tr>
<td>21°C</td>
<td>2.33</td>
<td>2.01</td>
<td>14%</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.72</td>
<td>±0.68</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Inhibition of Cold Shock Stimulated GABA Accumulation by Lanthanum.
4.1.5. Stimulation of GABA Synthesis by Cold Shock in Intact Cladophylls.

Cold shock stimulated GABA accumulation was observed in isolated asparagus cells (Table 2, Fig. 5). The response of intact asparagus cladophylls to the cold shock treatment was investigated in this experiment. In all experimental trials a consistent increase in GABA levels was observed. This increase was variable from 11% to 86%. The Wilcoxon test indicated that there is a 0.1 probability that the observed increase in GABA levels was due to chance alone.
Table 5. Stimulation of GABA Accumulation by Cold Shock in Intact Asparagus Cladophylls.

For each experiment cladophylls were stripped directly from the plant. Cold shock was applied by floating cladophylls on 1 mM CaSO₄ at 1°C, controls at 22±1°C. Each experimental value is the mean of duplicate GABAse assays. GABA and chlorophyll content was determined as described in "Methods".

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control 22±1°C (µmoles GABA/mg chlorophyll)</th>
<th>Experimental 1°C</th>
<th>Increase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.442</td>
<td>0.504</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>0.380</td>
<td>0.447</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>0.288</td>
<td>0.320</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>0.342</td>
<td>0.514</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>0.103</td>
<td>0.139</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>0.196</td>
<td>0.364</td>
<td>86</td>
</tr>
<tr>
<td>7</td>
<td>0.496</td>
<td>0.537</td>
<td>14</td>
</tr>
<tr>
<td>Mean</td>
<td>0.321</td>
<td>0.404</td>
<td>32.43</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.054</td>
<td>±0.052</td>
<td>±10.42</td>
</tr>
</tbody>
</table>
4.1.6. Continuous Measurements of the Cytosolic Ca\textsuperscript{2+} Levels in Response to Cold Shock.

The analyses of the fluorescence intensities from Fluo-3 loaded asparagus cells were performed using a MCID M2 Microprocessor Controlled Imaging Device. Fluo-3 was excited at 495 nm, and fluorescence intensity was recorded at 535 nm (as described in "Methods"). Cold shock was applied after recording initial fluorescence levels for 3 seconds, and continued throughout the experiment, as indicated in Fig. 7. In all 8 experiments perfusion of 1 mM CaSO\textsubscript{4} / 5 mM MES at 1°C resulted in an increase in fluorescence intensity. When fluorescence was measured from the whole cell, variable increases from 2.07 to 9.47 fluorescence levels (arbitrary units) were observed. However, when discrete areas of the cells were chosen for measurements, greater localized increases were observed in the cytosolic regions of cells.

For example, cold shock application to the cell shown in Fig. 6 resulted in an increase in fluorescence intensity of 4.49 fluorescence levels (arbitrary units), when the whole cell fluorescence was measured. However, measurements in the cytosolic region of the cell (as indicated in Fig. 6) revealed an increase of 17 fluorescence levels (arbitrary units). This increase was observed within 2 seconds. The peak of the fluorescence intensity was observed within 5 seconds of the cold shock application. For the following 10
seconds fluorescence intensity was above the control measurements, but decreasing. This decrease continued until the fluorescence intensity was below initial and control levels (Fig. 7).

A smaller increase in fluorescence intensity was observed in the vacuolar area of the cell (as indicated in Fig. 6 and 7). This is probably due to a very low fluorescence from the thin layer of cytosol between the PM and the tonoplast.

Asparagus cells subjected to repetitive cold shock after 5 minute perfusion intervals at 21°C were able to give the same transient increase in fluorescence intensity.

Perfusion of 5 mM butyric acid or 5 mM glutamic acid in 1 mM CaSO₄/5 mM MES, pH 5.0, did not increase fluorescence intensity. However, these cells responded to subsequent cold shock application. In these experiments with butyric or glutamic acid, cold shock served as a control to indicate the cell’s ability to increase cytosolic Ca²⁺ levels.

Results of this experiment indicate that asparagus cells respond to cold shock by an increase in cytosolic free Ca²⁺ concentration. This increase is localized, being greater in some areas of the cell. Two reasons may contribute to this phenomenon: unequal distribution of cytosol in the cell, and/or localized higher concentration of free Ca²⁺ in distinct regions of the cytosol.
Fig.6. Computer Generated Images of Fluo-3 Loaded Asparagus Cells During Cold Shock Application.

Top left - phase image of asparagus cells. The left cell is loaded with Fluo-3, the right cell is partially loaded. The cell at the right top corner contains no Fluo-3. The autofluorescence of its chloroplasts is excluded and does not contribute to the measurements.

Fluo-3 was excited at 495 nm, and fluorescence intensity was measured at 535 nm. Measurements were obtained from two discrete regions of the cell as indicated in the top right panel where:

C - cytosolic region
V - vacuolar region.

The levels of fluorescence intensity are presented as a pseudo-colour scale where blue indicates low and red indicates high fluorescence intensities. These fluorescence intensities are representative of free cytosolic $\text{Ca}^{2+}$ concentrations.

Initial resting $\text{Ca}^{2+}$ levels (top right image) were recorded at time 0. Two discrete areas of high intensity of fluorescence were observed. An increase in cytosolic $\text{Ca}^{2+}$ during cold shock application (bottom left image, time 10 sec.), were observed in these discrete areas of the cell as colours changed from green to red. At 40 seconds fluorescence intensity decreased in these areas and changes from red to yellow were observed (bottom right image, time 40 sec). The result shown here is representative of 8 experiments.
Fig. 7. Continuous Recordings of Fluorescence Levels from Fluo-3 Loaded Cell During Cold Shock Application.

1-Fluorescence levels from the cytosolic region of the cell during perfusion of 1 mM CaSO₄/5 mM MES, pH 6.0 at 1°C.
2-Fluorescence levels from cytosolic regions of the cell during perfusion of the same buffer at 25°C.
3-Fluorescence levels from the vacuolar region during cold shock application.
4-Fluorescence levels from the vacuolar region during perfusion of 25°C buffer. Measurements were obtained from the cell exhibited in Fig. 6. The regions of measurements are indicated in Fig. 6. Cold shock was applied at the 3 seconds and carried out through the experiment. Fluorescence levels are represented by arbitrary units.
4.1.7. Continuous Measurements of Cytosolic H⁺ Levels in Response to Cold Shock.

To investigate the role of protons in cold shock stimulated GABA accumulation, fluorescence ratios $I_{501}/I_{435}$ of BCECF loaded asparagus cells were measured using a MCID M2 Microprocessor Controlled Imaging Device (as described in "Methods"). Five experiments were performed. The measurements were obtained from whole cell areas. Cold shock was applied at 5 seconds and carried out for an additional 10 seconds as indicated in Fig 9. Perfusion of 1 mM CaSO₄, 5 mM MES at 1°C did not cause a drop in the fluorescence ratio. This indicates that no cytosolic acidification occurs in asparagus cells during cold shock.

Variable increases in the fluorescence ratio from 0.1 to 0.5 units were observed in these experiments indicating that alkalinization of the cytosol may take place during cold shock application. The fluorescence ratio did not decrease when cold shock was removed, indicating that the ratio change was not due to a physical effect of temperature on BCECF fluorescence.

The results of this experiment excluded the role of protons in cold shock stimulated GABA synthesis.
Fig. 8. Computer Generated Images of BCECF-Loaded Asparagus Cells During Cold Shock Application.

Top left - phase image of the cells. Three cells in the center are loaded with BCECF. Some intact non-loaded cells and damaged cells observed in the phase image, did not exhibit any fluorescence. Thus red autofluorescence of the chloroplasts is excluded and does not contribute to the measurements.

BCECF was excited at 501 and 435 nm. Fluorescence was measured at 527 nm. Measurements were obtained from the whole cell area.

The fluorescence ratio $I_{501}/I_{435}$ is represented by a pseudo-colour scale, where blue represents low- and red-high ratios. This reflects intracellular pH, where acidic pH is represented by blue colour and alkaline pH by red colour.

Initial $I_{501}/I_{435}$ ratios were recorded at the beginning of the experiment (right top image). Cold shock was applied at 5 seconds of the experiment and continued for an additional 10 seconds. An increase in the $I_{501}/I_{435}$ was observed from discrete regions of cells, as colours changed from green to orange and red (bottom left image). These changes were visible after the removal of cold shock at 14 seconds to the end of experiment (bottom right image). The result shown is representative of five experiments.
Fig. 9. Continuous Recordings of Fluorescence Ratio $I_{501}/I_{435}$ from a BCECF Loaded Cell During Cold Shock Application.

1—Fluorescence ratio $I_{501}/I_{435}$ from a whole cell during cold shock application by perfusion of 1 mM CaSO$_4$ / 5 mM MES, pH 6.0 at 1°C. 2—Control fluorescence ratios from the same cell during perfusion of the same buffer at 21°C. Measurements were performed using the cell exhibited in Fig. 8. (Left-bottom cell). Cold shock was applied at 5 sec. and carried out through an additional 10 sec. Fluorescence ratios are represented by arbitrary units.
4.2. The Influence of the Ca\textsuperscript{2+} Ionophore A23187 on GABA Accumulation and Intracellular Ca\textsuperscript{2+} Levels.

4.2.1. GABA Accumulation in Response to Treatment with the Ca\textsuperscript{2+} Ionophore A23187.

Incubation of 20 million cells with 25 µM A23187 for 15 minutes resulted in increased GABA levels in all trials. Compared to the control samples, this increase ranged from 31 to 117%, with an average of 61%. Upon treatment with A23187 more GABA was found in the experimental medium, with an average of 0.94 nmoles GABA/10\textsuperscript{6} cells, compared to 0.23 nmoles GABA/10\textsuperscript{6} cells in control medium. In most control trials GABA concentration was below detectable levels.

The majority of synthesized GABA remained within the cells; an average of 7.32 nmoles GABA/ million cells was found in the experimental cells, as compared to 4.9 nmoles GABA/ million cells in control cells.

The results of this experiment indicate a large variance in GABA levels in different cell preparations. Statistical analysis of the difference in total GABA levels using Wicoxon test revealed a value of P<0.02. There is, therefore, a significant difference between control and treated samples.
Table 6. Stimulation of GABA Accumulation by Treatment with the Ca²⁺ Ionophore A23187.

For each experiment 20 million cells were used. Incubation medium was 5 mM MES, 1 mM CaSO₄, pH 5.0. Incubation time was 15 minutes at 21±1 °C, with ambient light, constant stirring. Final concentration of A23187 25 μM. Duplicate GABAse assays were performed in each trial. N.D. not detectable. GABA levels below 0.2 nmoles per million cells.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control</th>
<th>Treated</th>
<th>Total</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># Cells</td>
<td>Medium</td>
<td>Cells</td>
<td>Medium</td>
</tr>
<tr>
<td></td>
<td>(nmoles GABA/10⁶ cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.119</td>
<td>N.D</td>
<td>5.64</td>
<td>1.34</td>
</tr>
<tr>
<td>2</td>
<td>5.411</td>
<td>N.D.</td>
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<td>0.79</td>
</tr>
<tr>
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<td>1.25</td>
</tr>
<tr>
<td>4</td>
<td>6.440</td>
<td>N.D.</td>
<td>8.27</td>
<td>1.25</td>
</tr>
<tr>
<td>5</td>
<td>1.995</td>
<td>N.D.</td>
<td>3.71</td>
<td>N.D.</td>
</tr>
<tr>
<td>6</td>
<td>6.496</td>
<td>N.D.</td>
<td>8.27</td>
<td>0.95</td>
</tr>
<tr>
<td>Mean</td>
<td>4.498</td>
<td>0.234</td>
<td>7.32</td>
<td>0.936</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.69</td>
<td>±0.234</td>
<td>±1.02</td>
<td>±0.21</td>
</tr>
</tbody>
</table>
4.2.2. The Influence of the Ca\(^{2+}\) Ionophore A23187(4-bromo) on Intracellular Ca\(^{2+}\) Levels.

A23187 was shown to stimulate GABA accumulation (Table 6). The presumed mechanism was GAD activation via an increase in cytosolic Ca\(^{2+}\) concentration. This was tested by placing 2\times10^5 cells loaded with Fluo-3 in a cuvette with 2 ml of 5 mM citric acid buffer with 25 \(\mu\)M CaSO\(_4\) in the spectrofluorimeter, and measuring fluorescence intensity (as described in "Methods").

An increase in fluorescence intensity upon treatment with a brominated, non fluorescent form of A23187(4-bromo) was observed. This increase ranged from 1 to 4 intensity units in different experiments. In each experiment, the increase was observed within 2 seconds, followed by a slower, progressive augmentation. Addition of the Ca\(^{2+}\) chelator EDTA abolished the increase in fluorescence and brought fluorescence intensity to the initial levels (Fig. 10). Since EDTA is thought to readily penetrate into the cells (Mehra and Deiman 1983), it was expected to chelate extracellular and intracellular Ca\(^{2+}\).

Addition of A23187,4-bromo or EDTA to control, non loaded cells did not cause any change in fluorescence. This confirms that both compounds do not fluoresce under the conditions of the experiment (excitation 506 nm, emission
526 nm), and did not contribute to the changes in fluorescence intensity observed during the experiments.

The results of this experiment suggest that the observed increase in fluorescence intensity was due to an increase in the intracellular Ca\(^{2+}\) concentration upon treatment with the ionophore. Thus, the stimulation of GABA accumulation upon treatment with A23187 may be mediated through increases in cytosolic Ca\(^{2+}\) concentration (Table 6).
Fig 10. The Influence of the Ionophore A23187 on Intracellular Calcium

A representative result of five experiments is shown. 200 000 cells were suspended in 2 ml of 5 mM citric acid buffer pH 5.0. External calcium concentration was 0.025 mM. The initial fluorescence intensity was recorded. Additions of 0.025 mM A23187, 4 brommo and 0.25 mM EDTA were made directly to the cuvette as indicated.
4.3. GABA Accumulation in Response to Treatment with Glutamate.

4.3.1. Stimulation of GABA Accumulation by Glutamate in the Presence of Active (W7) and Inactive (W5), Calmodulin Antagonists.

Glutamate uptake and GABA synthesis in asparagus cells are well documented (Chung et al. 1992, Snedden et al. 1992). The issue of how GAD activity is stimulated during glutamate treatment has not been resolved. No significant acidification of the cytosol during glutamate uptake was reported, excluding the role of H⁺ ions in stimulation of GABA synthesis by glutamate (Crawford 1993, MSc thesis).

The objective of this experiment was to assess the role of CaM during glutamate stimulation of GABA synthesis. Five minutes of incubation of asparagus cells with 5 mM glutamate, at pH 5.0 resulted in an increase in GABA levels from 3.76 to 10.81 nmoles GABA/million cells. Preincubation with CaM antagonists did not result in any inhibition of glutamate stimulated GABA accumulation. Neither the active compound W7, nor its inactive analog W5 affected GABA levels during treatment with glutamate. Using the Wilcoxon test a P=0.65 was obtained when samples treated with glutamate in the presence and absence of W7 were compared. Thus no significant difference was found.
These findings support the hypothesis that in asparagus cells GAD activity may be regulated by more than one mechanism. This experiment suggests that neither H⁺ (Crawford et al. 1994) nor CaM has any effect on glutamate stimulated GABA synthesis.
Table 7. Stimulation of GABA Accumulation by Glutamate in the Presence of Active (W7), or Inactive (W5), Calmodulin Antagonists.

Twenty million cells suspended in 1 mM CaSO₄/5 mM MES pH 5.0, were used in each trial. Cells suspensions were preincubated with 1 mM W7 or W5 for 60 minutes prior to glutamate treatment. Preincubated cells were treated with 5 mM glutamate for 5 minutes. Each experimental value is a mean of duplicate GABAse assay.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control</th>
<th>+W7</th>
<th>+W5</th>
<th>+Glu</th>
<th>+W7+Glu</th>
<th>+W5+Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles of GABA/10⁶ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.80</td>
<td>1.64</td>
<td>6.85</td>
<td>8.27</td>
<td>11.70</td>
<td>10.84</td>
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<td>2</td>
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<td>4.82</td>
<td>5.38</td>
<td>12.26</td>
<td>11.76</td>
<td>12.46</td>
</tr>
<tr>
<td>3</td>
<td>4.07</td>
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<td>3.91</td>
<td>11.91</td>
<td>12.97</td>
<td>11.81</td>
</tr>
<tr>
<td>Mean</td>
<td>3.76</td>
<td>3.38</td>
<td>5.38</td>
<td>10.81</td>
<td>12.14</td>
<td>10.71</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.49</td>
<td>±0.97</td>
<td>±0.85</td>
<td>±1.36</td>
<td>±0.397</td>
<td>±0.47</td>
</tr>
</tbody>
</table>
4.3.2 Stimulation of GABA Accumulation by Glutamate in the Presence of Lanthanum or Calcium in the Media.

The role of external Ca\(^{2+}\) in the glutamate induced GABA accumulation was investigated in this experiment. Twenty million cells were preincubated with CaSO\(_4\) or LaCl\(_3\) for 15 minutes. Lanthanum was expected to block Ca\(^{2+}\) channels at the PM, therefore excluding entry of Ca\(^{2+}\) into the cytosol from the medium. GABA synthesis was stimulated by 5 mM glutamate in both conditions. With Ca\(^{2+}\) present in the medium GABA levels increased from 3.41 nmoles/10\(^6\) cells in the controls to 8.33 nmoles/10\(^6\) cells treated with glutamate. The presence of lanthanum in the medium did not inhibit glutamate-induced GABA synthesis. An increase from 3.48 to 7.36 nmoles GABA/10\(^6\) cells was observed (Table 8). Using the Wilcoxon test samples treated with glutamate in the presence of Ca\(^{2+}\) or La\(^{3+}\) were compared. A value of P<0.5 was obtained, indicating that there is no difference between these two samples.
Table 8. Stimulation of GABA Accumulation by Glutamate in the Presence of Lanthanum or Calcium.

For each experiment twenty million cells were resuspended in the following incubation media: 5 mM MES/1 mM CaSO₄ pH 5.0, or 5 mM MES/1 mM LaCl₃ pH 5.0. Cells were preincubated in these buffers for 15 minutes. GABA synthesis was stimulated by incubation with 5 mM glutamic acid for 5 minutes. Duplicate GABAse assays were performed for each experiment.

<table>
<thead>
<tr>
<th>Trial</th>
<th>+CaSO₄</th>
<th>+CaCl₂+Glu</th>
<th>+LaCl₃</th>
<th>+LaCl₃+Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles GABA/10⁶ cells</td>
<td>nmoles GABA/10⁶ cells</td>
<td>nmoles GABA/10⁶ cells</td>
<td>nmoles GABA/10⁶ cells</td>
</tr>
<tr>
<td>3</td>
<td>3.311</td>
<td>5.94</td>
<td>3.175</td>
<td>6.234</td>
</tr>
</tbody>
</table>

Mean: 3.413, 8.333, 3.484, 7.356
S.E.: ±0.06, ±1.18, ±0.18, ±1.0
4.4. GABA Accumulation in Response to Butyrate.

4.4.1. Stimulation of GABA Accumulation by Butyrate in the Presence of the Calmodulin Antagonists.

Addition of permeant weak acid to asparagus cell suspensions, such as butyrate, resulted in a reduction of cytosolic pH by 0.6 units with a half time of approximately 2 sec. This cytosolic pH drop resulted in rapid GABA accumulation (Crawford et al. 1994). GAD has a sharp pH optimum around 6.0 (Snedden et al. 1992). Therefore reduction of cytosolic pH was implicated in the stimulation of GABA synthesis in response to the butyric acid treatment (Crawford et al. 1994). The objective of this experiment was to investigate the possible role of CaM in butyric acid stimulated GABA accumulation. A five minute incubation with butyrate at pH 5.0 resulted in an increase in GABA levels from 3.77 nmoles GABA/10⁶ cells in controls, to 11.11 nmoles of GABA/10⁶ cells in the experimental trials. This confirmed earlier findings (Crawford et al. 1994). Preincubation of cell suspensions with CaM antagonists did not significantly inhibit butyrate-induced GABA accumulation. Acidification of the cytosol of asparagus cells is sufficient to stimulate GAD and induce GABA synthesis and accumulation.
In each experiment 20 million cells were suspended in 1 mM CaSO₄/5 mM MES pH 5.0. Cells were preincubated with CaM antagonists for 60 minutes prior to butyric acid treatment. Cells were incubated with 5 mM butyric acid at pH 5.0 for 5 minutes. Each experimental value is a mean of duplicate GABAse assay.

Table 9. Stimulation of GABA Accumulation by Butyric Acid in the Presence of Active (W7) or Inactive (W5) Calmodulin Antagonists.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control</th>
<th>+W7</th>
<th>+W5</th>
<th>+But</th>
<th>+W7+But</th>
<th>+W5+But</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.80</td>
<td>1.64</td>
<td>6.85</td>
<td>10.74</td>
<td>9.38</td>
<td>9.23</td>
</tr>
<tr>
<td>1</td>
<td>4.42</td>
<td>4.83</td>
<td>5.38</td>
<td>10.74</td>
<td>13.42</td>
<td>13.42</td>
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<tr>
<td>2</td>
<td>4.07</td>
<td>4.37</td>
<td>3.92</td>
<td>11.86</td>
<td>10.74</td>
<td>10.74</td>
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<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.77</td>
<td>3.61</td>
<td>5.38</td>
<td>11.11</td>
<td>11.18</td>
<td>10.54</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.49</td>
<td>±0.99</td>
<td>±0.85</td>
<td>±0.37</td>
<td>±1.14</td>
<td>±1.53</td>
</tr>
</tbody>
</table>

nmoles of GABA/10⁶cells
4.4.2. Stimulation of GABA Accumulation by Butyrate in the Presence of Lanthanum or Calcium.

To investigate the role of exogenous Ca\textsuperscript{2+} in butyric acid induced GABA synthesis asparagus cells were suspended in two different media: 5 mM MES/ 1 mM CaSO\textsubscript{4} or 5 mM MES/ 1 mM LaCl\textsubscript{3}, pH 5.0. After 15 minutes preincubation, GABA synthesis was stimulated by addition of 5 mM butyric acid at pH 5.0. This resulted in an increase in GABA levels in all experiments as compared to the control samples. With Ca\textsuperscript{2+} present in the medium GABA levels increased from 1.76 to 8.74 nmoles/10\textsuperscript{6} cells. With La\textsuperscript{3+} present in the medium GABA levels increased from 2.5 to 7.4 nmoles/10\textsuperscript{6} cells. The presence of lanthanum in the media, which is known to inhibit Ca\textsuperscript{2+} channels at the plasma membrane, did not significantly inhibit butyrate-induced GABA accumulation in asparagus cells.
Table 10. Stimulation of GABA Accumulation by Butyrate in the Presence of Lanthanum or Calcium.

In each experiment twenty million cells were resuspended in the following incubation media: 5 mM MES/1 mM CaSO₄ pH 5.0, or 5 mM MES/1 mM LaCl₃ pH 5.0. Cells were preincubated in above buffers for 15 minutes. GABA synthesis was stimulated by incubation with 5 mM butyric acid at pH 5.0 for 5 minutes. Each experimental value is a mean of duplicate GABAse assays.

<table>
<thead>
<tr>
<th>Trial</th>
<th>CaSO₄</th>
<th>CaSO₄+butyrate</th>
<th>LaCl₃</th>
<th>LaCl₃+butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles GABA/10⁶ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.412</td>
<td>12.31</td>
<td>3.816</td>
<td>12.19</td>
</tr>
<tr>
<td>2</td>
<td>0.277</td>
<td>6.951</td>
<td>3.0</td>
<td>5.382</td>
</tr>
<tr>
<td>3</td>
<td>1.591</td>
<td>6.959</td>
<td>0.681</td>
<td>4.625</td>
</tr>
<tr>
<td>Mean</td>
<td>1.76</td>
<td>8.74</td>
<td>2.502</td>
<td>7.398</td>
</tr>
<tr>
<td>S.E.</td>
<td>±0.91</td>
<td>±1.78</td>
<td>±0.94</td>
<td>±2.41</td>
</tr>
</tbody>
</table>
4.5. GABA Accumulation in Response to ABA.

4.5.1. Stimulation of GABA Accumulation by ABA.

The production of GABA in response to treatment with ABA was investigated in detail. Time and concentration courses were performed. Maximum GABA production of 6.04 nmoles/million cells was obtained by the incubation of cells with 100 μM ABA. As compared to the control this represents a 58% increase in GABA levels upon treatment. Lower concentrations of 1 and 10 μM ABA in the incubation media resulted in a similar increase in GABA levels of 5.76 and 5.99 nmoles/million cells respectively (Fig 12). The Wilcoxon test revealed that there is a significant difference between control and all experimental samples, with a value P<0.028.

Examination of the time course of ABA induced GABA synthesis showed that maximal GABA levels of 7 nmoles/million cells occurred after 5 minutes of incubation with 100 μM ABA. This represents an 83% increase as compared to controls. GABA levels at 10 and 20 minute cell incubation with ABA were 6.5 and 5.7 nmoles/million cells, respectively (Fig. 11). All experimental samples differ significantly from control sample as P<0.014 was revealed using the Wilcoxon test.
Fig. 11. Time Course for ABA Stimulated GABA Accumulation.

Approximately 80 million cells in 20 ml of 1 mM CaSO₄ / 5 mM MES were used for each experiment. ABA was added to the experimental trials to the final concentration of 100 μM. About 20 million cells in 5 ml of cells suspension were removed for GABA determination at the times of 0, 5, 10 and 20 minutes. Each point is an average of three experiments. Standard error is indicated. Duplicate GABAse assays were performed in each assay.
Fig. 12. GABA Accumulation as a Function of ABA Concentration.

Each bar is a mean of three separate experiments. Standard error is indicated. Approximately $2 \times 10^7$ cells were incubated for 10 minutes with indicated final ABA concentration. GABA extracted and determined as described in "Methods". Duplicate GABAse assays performed for each experiment.
4.5.2. Stimulation of GABA accumulation by ABA in the presence of Calmodulin antagonists W7 and W5.

The mechanism of ABA stimulated GABA accumulation was investigated using calmodulin antagonists W7 and W5. W7 was shown to be effective in inhibition of cold shock stimulated GABA synthesis (Table 3). In this experiment, W5 served as a control to rule out possible pharmacological effects of these compounds on asparagus cells.

A ten minute incubation of asparagus cells with 100 μM ABA resulted in accumulation of GABA from 3.69 nmoles/million cells in controls, to 6.23 nmoles/million cells in experimental samples treated with ABA, which represents a 68% increase in GABA levels. The Wilcoxon test revealed a difference in the GABA levels in these samples with P<0.028.

This increase was inhibited 55% by the active CaM antagonist W7. The difference between samples treated with ABA in the presence or absence of W7 is statistically significant as Wilcoxon test revealed P<0.014.

The less potent compound W5 did not have any effect on ABA induced GABA accumulation. These results strongly suggest that calmodulin is involved in ABA induced GABA synthesis.
Table 11. Stimulation of GABA Accumulation by ABA in the Presence of Active (W7) or Inactive (W5) Calmodulin Antagonists.

Twenty million cells in 5 ml of 1 mM CaSO₄, 5 mM MES pH 5.0 were used in each experiment. Cell suspensions were preincubated with 1 mM W7 or W5 for 60 minutes prior to ABA treatment. Cell suspensions were incubated for 10 minutes with 100 μM ABA. Duplicate GABAse assays were performed in each experiment.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control</th>
<th>+ABA</th>
<th>+W7+ABA</th>
<th>+W5+ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles GABA / million cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.64</td>
<td>6.42</td>
<td>2.89</td>
<td>5.97</td>
</tr>
<tr>
<td>2</td>
<td>5.12</td>
<td>5.69</td>
<td>4.10</td>
<td>5.74</td>
</tr>
<tr>
<td>3</td>
<td>3.85</td>
<td>8.35</td>
<td>2.23</td>
<td>7.76</td>
</tr>
<tr>
<td>4</td>
<td>2.16</td>
<td>4.45</td>
<td>1.85</td>
<td>4.27</td>
</tr>
<tr>
<td>Mean</td>
<td>3.69</td>
<td>6.23</td>
<td>2.77</td>
<td>5.94</td>
</tr>
<tr>
<td>±SE</td>
<td>±0.61</td>
<td>±0.82</td>
<td>±0.49</td>
<td>±0.72</td>
</tr>
</tbody>
</table>
4.6. The Influence of a 60 Minutes Cell Incubation on GABA Levels in the Presence of the Calmodulin Antagonist W7.

This experiment was performed to investigate the effect of a 60 minute incubation of 20 million cells in 5 ml of 1 mM CaSO₄, 5 mM MES pH 6.0. GABA was extracted from control samples within 1 hour from cell isolation. Experimental samples were exposed to a further 60 minute incubation with constant stirring with a magnetic bar, in conditions of ambient light and 21±1 °C, in the presence or absence of 1 mM W7.

A sixty minutes incubation in the above conditions resulted in elevated GABA levels in trials not treated with W7. A 47% increase in GABA levels was observed as compared to immediate GABA extraction. The presence of the CaM antagonist W7 in the media resulted in inhibition of GABA synthesis by 65%. GABA levels in the trials treated with W7 were lower than controls with immediate GABA extraction. The elevated GABA levels in control samples, extracted immediately after cell isolation may be due to mechanical manipulation of the asparagus samples during cell isolation. This suggests that W7 is inhibiting GABA synthesis, but not GABA metabolism.
Table 12. The Influence of a 60 Minutes Cell Incubation on GABA Levels in the Presence of the Calmodulin Antagonist W7.

In control trials GABA was extracted from 20 million cells immediately after cell isolation and counting. In experimental trials cells were incubated for 60 minutes with constant stirring in 1 mM CaSO$_4$ / 5 mM MES pH 6.0, in the presence or absence of 1 mM W7. Duplicate GABAse assays were performed for each experiment.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control</th>
<th>60 min. inc. (+W7)</th>
<th>60 min. inc. (-W7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmoles GABA/10$^6$cells</td>
<td>nmoles GABA/10$^6$cells</td>
</tr>
<tr>
<td>1</td>
<td>2.67</td>
<td>1.38</td>
<td>4.95</td>
</tr>
<tr>
<td>2</td>
<td>2.80</td>
<td>1.64</td>
<td>5.38</td>
</tr>
<tr>
<td>3</td>
<td>3.01</td>
<td>2.56</td>
<td>3.86</td>
</tr>
<tr>
<td>4</td>
<td>1.42</td>
<td>1.02</td>
<td>4.72</td>
</tr>
<tr>
<td>Mean</td>
<td>2.47</td>
<td>1.65</td>
<td>4.72</td>
</tr>
<tr>
<td>±SE</td>
<td>±0.36</td>
<td>±0.41</td>
<td>±0.32</td>
</tr>
</tbody>
</table>
4.7. GABA Induced Increases in Cytosolic Ca^{2+}.

The measurement of the fluorescence intensity from Fluo-3 loaded cells was performed using a MCID M2 Microprocessor Controlled Imaging Device (as described in "Methods"). Fluo-3 was excited at 495 nm and fluorescence intensity was recorded at 535 nm. Loaded cells were treated with 10 mM GABA for 15 seconds. Measurements were obtained from the whole cell area. An increase in fluorescence intensity was observed in 5 experiments performed. This increase varied from 2.22 to 28.2 arbitrary fluorescence units. The increases in fluorescence intensities are indicative of elevated cytosolic Ca^{2+} levels.

The mechanism of this GABA-induced Ca^{2+} increase is unknown, but these results indicate a possible role for GABA as a signaling molecule in asparagus cells.
Fig. 13. Computer Generated Images of Fluo-3 Loaded Cell During Exogenous GABA Application.

Top left panel - Phase image of the asparagus cell. This cell is loaded with Fluo-3. Fluo-3 was excited at 495 nm and fluorescence intensity was measured at 535 nm (as described in "Methods").

Top right image - initial fluorescence levels at the beginning of the experiment. Fluorescence levels are represented by a pseudo-colour scale, where red and yellow represent low fluorescence intensities and green and blue represents high intensities. These fluorescence intensities indicate free cytosolic Ca$^{2+}$ concentration (low and high, respectively).

Bottom left image - 12 seconds into the experiment. An increase in fluorescence intensity was observed upon perfusion of 10 mM GABA in 1 mM CaSO$_4$ / 5 mM MES pH 6.0. GABA was applied at 5 seconds, and was present for an additional 15 seconds. In discrete regions of the cell, a change in colours from yellow to green occurred.

Bottom right panel - 40 seconds into the experiment. A sustained increase in fluorescence intensity occurred after the removal of 10 mM GABA at 20 seconds, and perfusion of 1 mM CaSO$_4$ / 5 mM MES pH 6.0.

The result shown here is a representative of 5 experiments.
Fig. 14. Continuous Recordings of Fluorescence Levels from Fluo-3 Loaded Cell upon GABA Treatment.
1-Fluorescence levels from whole cell during perfusion of 10 mM GABA in 1 mM CaSO₄/5 mM MES pH 6.0 at 21°C. 2-Control fluorescence level from the same cell. Measurements were performed using the cell shown in Fig 13. GABA was applied at 5 seconds and maintained for an additional 15 seconds as indicated.
5. Discussion.

GABA accumulation in response to many, unrelated, stress conditions has been demonstrated in a variety of plant tissues (Wallace et al. 1984; Crawford et al. 1994; Reggiani et al. 1994). Neither the mechanism(s) nor role(s) of GABA accumulation has been defined. GABA accumulation may result from stimulation of GABA synthesis or inhibition of GABA utilization. There is strong evidence that GABA accumulation follows cytosolic acidification (Crawford et al. 1994, Carrol et al. 1994). Thus GABA accumulation occurs in response to stresses that induce a drop in cytosolic pH. In such cases both induced synthesis and inhibited utilization may contribute to GABA accumulation. GABA synthesis may be stimulated, because of the acidic pH optimum of GAD, and GABA utilization may be inhibited because of the alkaline pH optima of GABA-transaminase and succinic semialdehyde dehydrogenase (Fig. 2 GABA shunt enzymes). However other stresses, touch and cold shock for example, that do not induce cytosolic acidification are known to stimulate GABA synthesis (Wallace et al. 1984, Fig. 5, Table 2). Observations of increases in intracellular Ca\(^{2+}\) levels induced by touch and cold shock (Knight et al. 1991, Fig. 7 and Fig. 8) led to the hypothesis that increased Ca\(^{2+}\) levels may be involved in the mechanisms stimulating GABA synthesis.
The following criteria for evidence of Ca\textsuperscript{2+} involvement in a physiological response have been proposed (Pooviah and Reddy 1993; Hepler and Wayne 1985):

1. Cytosolic Ca\textsuperscript{2+} must change in response to the primary stimulus.
2. This change should precede the physiological response.
3. Artificial induction of Ca\textsuperscript{2+} changes should initiate the physiological response in the absence of the primary stimulus.
4. Blocking changes in cytosolic Ca\textsuperscript{2+} or the Ca\textsuperscript{2+}-sensing system should prevent the response to the primary stimulus.

In this study all four criteria were examined to determine whether or not increased cytosolic Ca\textsuperscript{2+} levels stimulate the synthesis of GABA.

**Increase in cytosolic Ca\textsuperscript{2+} precedes GABA synthesis.**

An increase in cytosolic Ca\textsuperscript{2+} concentration was observed in asparagus cells in response to cold shock. This increase was evident within 2 seconds, lasted for 10 seconds and was followed by a decrease below initial Ca\textsuperscript{2+} levels (Fig.7). Accumulation of GABA during cold shock was observed after 1 minute of cold shock application (Fig.5). Therefore, an increase in cytosolic Ca\textsuperscript{2+} appears to precede detectable GABA synthesis.

Measurement of cytosolic Ca\textsuperscript{2+} levels using fluorescent probes is assumed to be non perturbing. Plant biologists have
used morphological criteria, such as maintenance of stomatal response, organellar morphology, cell turgor (Gilroy et al. 1990, 1991; McAinsh et al. 1990, 1992), maintenance of growth, and maintenance of cytoplasmic streaming (Miller et al. 1992) as indications that the measurement technique was non-disturbing. We have found that acid loading of asparagus cells with Fluo-3 did not cause any alteration in the cell morphology. Loaded cells did not morphologically differ from control cells (Fig. 4, 6, 13).

Single cell measurements with an imaging system have several advantages over the cuvette-based studies of fluorescence from cell populations. In the spectrophotofluorimeter, the fluorescence signal from living cells may be swamped by the high intensity of fluorescence from dead cells. Unstimulated asparagus cells loaded with Fluo-3 exhibit a low intensity of fluorescence, presumably due to the low level of free Ca$^{2+}$ in the cytosol. However, cells broken during the mechanical isolation procedure (with an average of 20%) exhibit much higher fluorescence intensities. This may mask any signal due to the treatment. In addition the population of cells in the cuvette may not respond synchronously to the treatment. In such cases an average signal may not be indicative of the dynamics of the authentic response.

For example, changes in cytosolic Ca$^{2+}$ levels were observed in monocot protoplasts irradiated with red light using population measurements and single cell imaging. A sustained change in
fluorescence was observed in the population of cells (Chae et al. 1990), whereas the same treatment resulted in transient changes in cytosolic Ca\(^{2+}\) during single cell imaging (Shacklock et al. 1992). Differences in the results obtained in these two experiments result presumably from the two different techniques used.

Fluorescence from Fluo-3 loaded cells is not evenly distributed through the cytosol of the asparagus cells (Fig.4, 6, 13). Image analysis using pseudo colours representing different levels of fluorescence from Fluo-3 allowed observation of discrete cytosolic area “hot spots” of high fluorescence intensity. This may be due to the uneven distribution of the cytosol within the cell, and/or differences in the free Ca\(^{2+}\) concentration in the cytosol. Similar “hot spots” were observed in Commelina communis guard cells loaded with the Ca\(^{2+}\) indicator Indo-1. Confocal microscopy showed that the highest localized increases in Ca\(^{2+}\) concentration were observed in proximity to the endomembrane system adjacent to the nucleus (Gilroy et al. 1991). A Ca\(^{2+}\) gradient was also observed within plant cells. This gradient is believed to be one of the earliest events elicited in the development of cell polarity and subsequent morphogenic processes (Hepler and Calaham 1987).

Cold shock-stimulated increases in the fluorescence intensity of Fluo-3 loaded cells were not evenly distributed
through the cytosol. They were localized in discrete regions of the asparagus cells (Fig. 6). This may be a result of opening of clustered Ca\(^{2+}\) channels in the plasma membrane in these areas, or Ca\(^{2+}\) release from the organelles localized in these regions. To determine whether these "hot spots" of high fluorescence are associated with any organelles, requires confocal microscopy studies.

The largest compartment in the asparagus cell, the vacuole, is surrounded with a thin layer of cytosol and appressed to the PM. The fluorescence signal from this area is extremely weak, although an increase in response to cold shock was detectable (Fig. 7). The observed increase in the fluorescence intensity due to cold shock can be repeated within 5 min, after perfusion of the cells with 21°C buffer.

In this study no attempts were made to estimate intracellular Ca\(^{2+}\) concentrations or magnitude of Ca\(^{2+}\) increases. The resolution of variation in levels of Ca\(^{2+}\) inside the asparagus cells was not adequate to perform calibration. Other factors such as photobleaching or probe leakage from the cell may affect measurements. Therefore, changes in cytosolic Ca\(^{2+}\) were represented as an increases in arbitrary units. Other studies also represented a changes in intracellular Ca\(^{2+}\) as a qualitative increases (Reviewed by Bush 1993)
A possible mechanisms for the transient Ca\(^{2+}\) increase during cold shock may include the following. At the resting stage the Ca\(^{2+}\) concentration in the cytosol is low, in the nM range. At these Ca\(^{2+}\) concentrations, (which are below the CaM dissociation constant), CaM is likely to be inactive. Also, the Ca\(^{2+}\)-ATPase has low affinity for Ca\(^{2+}\) and therefore, may be inactive or may pump Ca\(^{2+}\) at a steady low rate. Cold shock application may cause a change in membrane fluidity and trigger Ca\(^{2+}\) channel opening (Knight et al. 1992). Ca\(^{2+}\) will then passively flow through open channels down its electro-chemical gradient from the apoplast into the cytosol. Rapidly increased Ca\(^{2+}\) concentrations in the cytosol will activate CaM by binding to it and changing its conformation. The activated CaM stimulates Ca\(^{2+}\)-ATPase (Evans 1994) by increasing its affinity for Ca\(^{2+}\). The stimulated Ca\(^{2+}\)-ATPase will pump Ca\(^{2+}\) out of the cytosol until the Ca\(^{2+}\) concentration drops below the CaM dissociation constant. At low nM concentrations, Ca\(^{2+}\) will dissociate from CaM, and there will be no further stimulation of the Ca\(^{2+}\)-ATPase. The Ca\(^{2+}\)-ATPase may again exhibit low affinity for Ca\(^{2+}\). Thus there is a dynamic, negative feedback pathway involving Ca\(^{2+}\) influx and efflux, which is under the control of the cytosolic Ca\(^{2+}\) levels.

Using transgenic tobacco seedlings with an inserted gene coding for apoaequorin, an increase in Ca\(^{2+}\) concentration in response to various stimuli was detected (Knight et al. 1991). The expression of apoaequorin allows measurement of Ca\(^{2+}\), as its
luminescence is Ca\(^{2+}\) dependent. Rapid and transient increases in luminescence intensity indicating an increase in cytosolic Ca\(^{2+}\) concentration were observed in response to touch, cold shock and fungal elicitors. Each of these responses exhibits different kinetics. Ca\(^{2+}\) increases induced by touch are very short (seconds), intermediate for cold shock (10-20 seconds), and long for fungal elicitors (1-2 minutes). The largest increase in cytosolic Ca\(^{2+}\) level was observed in plants transferred from 20°C to 5°C. After cold treatment, rewarmed plants were sensitive to a further cold shock and exhibited a further Ca\(^{2+}\) spike (Knight et al. 1991).

**Artificial increase in Cytosolic Ca\(^{2+}\) levels stimulates GABA synthesis.**

A23187, a divalent cation ionophore was used to investigate Ca\(^{2+}\) stimulated GABA synthesis. The cytosolic Ca\(^{2+}\) concentration was elevated artificially by incubation of asparagus cells with the Ca\(^{2+}\) ionophore A23187 (Fig.10). This treatment resulted in an increase in GABA levels of 61% as compared to the controls (Table 6).

A23187 is commonly used as a diagnostic tool to identify Ca\(^{2+}\) regulated processes. The presumed mode of action of the ionophore is to facilitate Ca\(^{2+}\) transport across membranes by coupling exchange of protons for Ca\(^{2+}\) (Reed and Lardy 1972). This process is electroneutral, and it has been proposed that the active complex within membranes is composed of two A23187 molecules.
which cycle $2\text{H}^+/\text{Ca}^{2+}$ (Pohl et al. 1990). Under the conditions of the experiments performed with asparagus cells, the possible mechanism of action of A23187 is as follows (Fig 15).

**Fig 15. Possible Mechanism of Action of A23187 in Asparagus Cells.**

A23187 is a lipophilic anion which is capable of binding divalent cations, such as $\text{Ca}^{2+}$, as well as protons. When added to the cell suspension it will penetrate into the PM. In the membrane the anionic form of A23187 will form a complex with $\text{Ca}^{2+}$. The complexed carrier diffuses to the opposite interface of the membrane where $\text{Ca}^{2+}$ is released in the cytosol. The polar ionophore is protonated on the cytosolic side of the PM. The two protons are released on the opposite side of the PM, to the
medium. Under these conditions equilibrium is obtained according to the equation (Kell and Donath 1990):

$$[\text{Ca}^{2+}]_{\text{in}}[\text{H}^+]_{\text{out}}^2 = [\text{Ca}^{2+}]_{\text{out}}[\text{H}^+]_{\text{in}}^2$$

Assuming a cytosolic Ca$^{2+}$ concentration of 100 nM and a pH of 7.0, with 1 mM Ca$^{2+}$ present outside of the cell, the critical pH of equilibrium would be 5.0.

In our experiments, an increase in fluorescence intensities in asparagus cells loaded with Fluo-3 and incubated in 1 mM Ca$^{2+}$, at pH 5.0, was observed following application of 25 µM A23187. A sustained increase in fluorescence was observed under fluorescence microscopy within 1 second. This indicates that the cytosolic Ca$^{2+}$ concentration was elevated. The magnitude of this increase was not determined because no calibration for Ca$^{2+}$ levels was performed. However observed increases were consistent and occurred in every experiment performed. This increase was confirmed spectrofluorometrically in the Perkin-Elmer LS-50 (Fig.10). The increase in fluorescence was quenched by addition of the Ca$^{2+}$ chelator EDTA. Chelation of external Ca$^{2+}$ by EDTA will favour Ca$^{2+}$ efflux from the cell, which is facilitated by A23187. In addition EDTA penetrates the cell and chelates intracellular Ca$^{2+}$ (Mechra and Deiman 1983). These results indicate that the increase in fluorescence is due to an influx of Ca$^{2+}$. Influx of $^{45}\text{Ca}^{2+}$ increased 10-20 fold when internodal cells of Chara corallina were treated with 24-40 µM A23187. This increase was
similar when the cells were treated at an external pH of 5.0 and 8.0 (Reid and Smith 1993).

Incubation with 25 μM A23187 resulted in an increase in GABA levels ranging from 31% to 117% with an average of 61% from 6 trials (Table 6). Stimulation of GABA synthesis in response to treatment with A23187 is presumably mediated by elevated cytosolic Ca$^{2+}$. A23187 is a weak acid with a pK of 6. At the concentration used in these experiments it would not accumulate in the cytosol to the levels that would significantly shift the pH. However, a progressive acidification of the cytoplasm of Chara cells was reported by Reid and Smith (1993), who observed a drop of 0.2 pH units in the first hour of their experiment, reaching 1 pH unit after 6 hours (Reid and Smith 1993). Asparagus cells were incubated with A23187 for 15 min prior to GABA extraction. Therefore, prolonged exposure of the cells to the ionophore was eliminated. In addition A23187 did not change the cytosolic pH of BCECF-loaded asparagus cells (Henry BSc thesis 1993).

However A23187 cannot be viewed as simply catalysing Ca$^{2+}$ fluxes across the PM. A23187 is not specific for Ca$^{2+}$ ions, but also forms complexes with Mg$^{2+}$ of almost the same affinity (Pfeiffer et al. 1974). Because the concentration of Mg$^{2+}$ in plant cells is higher than Ca$^{2+}$, loss of Mg$^{2+}$ has been proposed to be involved in the process of protoplast lysis. It was shown that incubation of isolated Avena sativa protoplasts with A23187 disrupted PM integrity and caused cell lysis (Kell and Donath
The percentage of the disturbed protoplasts increased strongly if the external pH was decreased from 6.5 to 5.6. This was thought to be due to the depletion of cytoplasmic Ca$^{2+}$ (Kell and Donath 1990). In contrast to these results, external pH had little effect on A23187-stimulated Ca$^{2+}$ influx in Chara, and a similar rate of Ca$^{2+}$ influx was observed at pH 5 and 8 (Reid and Smith 1993). Treatment of the Chara cells with A23187 resulted in reduction of the ATP concentration and reduction of protoplasmic streaming, which is known to be sensitive to the concentration of the free Ca$^{2+}$ in the cytoplasm (Williamson and Ashley 1982). This was observed also in the absence of external Ca$^{2+}$. This suggests that A23187 releases Ca$^{2+}$ from intracellular stores.

Prolonged elevation of the cytosolic free Ca$^{2+}$ concentration may be toxic. Free Ca$^{2+}$ will form insoluble precipitates with inorganic phosphate and interfere with ATP-based metabolism. Observed increases in cytosolic Ca$^{2+}$ in response to environmental stresses are usually transient and last for a very short time (e.g., 10 seconds, Fig. 7; Knight et al. 1991). High Ca$^{2+}$ concentrations may also stimulate enzymes with proteolytic activity. In this case, GABA accumulation may be the result of protein hydrolysis and subsequent amino acid degradation. The stimulation of GABA synthesis is most probably mediated by increased Ca$^{2+}$ influx and Ca$^{2+}$/CaM stimulated GAD activity.
However, nonspecific effects of A23187 can not be ruled out at this time.

**Inhibition of GABA synthesis by blocking Ca\(^{2+}\) entry and inactivating CaM.**

Treatment of the asparagus cells with lanthanum, a known Ca\(^{2+}\) channel blocker, resulted in limitation of GABA accumulation during cold shock. A 45% inhibition of cold shock-stimulated GABA synthesis was observed in the samples preincubated with lanthanum (Table 4). This result suggests that external Ca\(^{2+}\) plays a role in cold shock-stimulated GABA synthesis.

The inhibitory effect of lanthanum was observed in cell suspension cultures of alfalfa (Monroy et al. 1993). Lanthanum inhibited a cold-dependent increase in protein phosphorylation. The phosphoprotein profile of cold treated cells in the presence of lanthanum was similar to that of nonacclimated cells (Monroy et al. 1993). Preincubation of alfalfa cultured protoplast suspensions with lanthanum caused a reduction in \(^{45}\text{Ca}^{2+}\) influx, which was induced by transferring protoplasts from 25°C to 4°C and incubating for 20 minutes. Ca\(^{2+}\) influx decreased from 3.3 in controls to 0.6 nmoles/cm\(^2\) in the presence of lanthanum (Monroy and Dhindsa 1995). Similar results were obtained in experiments with transgenic *Nicotiana* seedlings (Knight et al. 1992). Ca\(^{2+}\) channels were blocked with lanthanum or gadolinium. This completely abolished cold shock-induced Ca\(^{2+}\) increases. Ruthenium
red, which is a putative mitochondrial and endoplasmic reticulum Ca\(^{2+}\) channel blocker, did not affect this response (Knight et al. 1992). These findings suggest that during cold shock, increased cytosolic Ca\(^{2+}\) concentration is due to the opening of Ca\(^{2+}\) channels at the plasma membrane.

CaM plays a fundamental role in modulating various cellular processes. The following approaches have been taken in studies of CaM function: the identification of CaM-stimulated enzymes, the incubation of cells with pharmacological agents that inhibit CaM, and the genetic manipulation of the CaM system (Roberts and Harmon 1992). Compounds that inhibit the activity of CaM are useful tools in research into CaM function. There are several proposed mechanisms by which such compounds may antagonize CaM action (Asano and Hidaka 1984):

1. By decreasing the concentration of intracellular Ca\(^{2+}\) and preventing the formation of the active Ca\(^{2+}\)-CaM complex.
2. By binding to CaM and altering its affinity for Ca\(^{2+}\).
3. By binding to the active Ca\(^{2+}\)-CaM complex and reducing its affinity for target proteins.
4. By binding to the CaM-binding site of the target enzyme and preventing interaction with Ca\(^{2+}\)-CaM complex.
5. By interacting with the catalytic site of the nonactivated or activated CaM-dependent enzymes.
6. By interacting with the ternary Ca\(^{2+}\)-CaM-enzyme complex and altering its activity.

The most frequently used CaM antagonists interact with the active Ca\(^{2+}\)-CaM complex. These are naphtalenesulfoamides (W-series), phenothiazine antipsychotics (eg trifluoperazine), and calmidazolium. In plants this group of CaM antagonists at concentrations of 10\(^{-3}\) to 10\(^{-6}\) M have been shown to affect processes such as cytokinin-induced pigment accumulation (Elliot 1983), secretion (Elliot et al. 1983), mitotic progression (Lambert and Vantard 1986), protein phosphorylation (Veluthambi and Poovaiah 1986), growth of cucumber cotyledons (Szweikowska and Ganczarska 1993), and cold-induced changes in protein phosphorylation (Monroy et al. 1993).

W-7 binds to CaM in a Ca\(^{2+}\)-dependent manner. In the absence of Ca\(^{2+}\) \([^3]H\)W-7 does not bind to CaM. The binding is increased significantly with 1 µM Ca\(^{2+}\) and is almost maximal with 10 mM Ca\(^{2+}\). Two classes of W-7 binding sites on CaM were revealed. One with a \(K_d = 11\) µM and three molecules of W-7 per molecule of CaM, the other with a \(K_d = 200\) µM and seven molecules of W-7 per CaM. (Asano and Hidaka 1984). The mechanism of binding of W-7 to CaM is poorly understood, but it seems possible that hydrophobic regions of CaM which are exposed upon binding Ca\(^{2+}\) bind W7.
In the present study the possible involvement of CaM in GABA synthesis was investigated by preincubation of asparagus cell suspensions with the CaM antagonists W5 and W7. Cold shock stimulated GABA synthesis was inhibited 70% by W7 (Table 3). ABA induced GABA synthesis was inhibited 55% by W7 (Table 11). Observed inhibition of GAD by W7 in vivo is in agreement with an in vitro inhibition of GAD from asparagus cladophylls. A partially purified asparagus GAD exhibited an activity of 39.8±1.8 nmol CO₂/min/mg protein in the presence of Ca²⁺/CaM. Upon addition of W7 to the assay medium this activity decreased to 22±1.14 nmol CO₂/min/mg protein (Personal communication Dr A. Snedden). In vitro experiments with partially purified GAD from soybeans resulted in a 75% inhibition of Ca²⁺/CaM stimulated GAD activity when W7 was present in the medium (Snedden et al. 1995). These observations strongly suggest that CaM plays a role in GAD regulation. However, CaM antagonists did not have any effect on butyrate or glutamate stimulated GABA synthesis (Table 9 and Table 7). At a pH of 5.8 (which is optimal for in vitro GAD activity) no stimulation of GAD by Ca²⁺ and CaM addition was observed (Snedden et al. 1995). These results suggest that stimulation of GAD activity involves increases in cytosolic Ca²⁺ levels or H⁺ levels.

To rule out possible pharmacological effects of W7, cells were incubated with W-5, a relatively inactive analog of W7. The
presence of W5 during stimulation of GABA synthesis with various
treatments did not have any effect on GABA accumulation (Tables
3, 7, 9, 11, 12). W-5 serves as a “control compound” of the CaM
antagonist W-7. These compounds are structurally related. W-5 is a
dechlorinated analog of W7 and it is seven times less potent in
its ability to displace bound $[^3]H$W-7 from CaM, and nine times
less potent in the inhibition of CaM-induced activation of
phosphodiesterase (Asano and Hidaka 1984).

**Changes in cytosolic pH during cold shock.**

The response of the intracellular pH of plant cells to
temperature variations is uncertain and not well documented.
Perfusion of BCECF-loaded asparagus cells with ice-cold 1 mM
CaSO$_4$/ 5 mM MES pH 6.0 did not cause a drop in the fluorescence
ratio $I_{501}/I_{435}$ (as described in “Methods”) indicating that no
acidification of the cytosol occurred. In contrast, a slight
increase in the fluorescence ratio indicates that alkalanization
of the cytosol may be occurring (Fig. 9).

This result agrees with the finding that transfer of maize
root tips from 4°C to 28°C resulted in a pH decline of about 0.5
pH units (Aducci et al. 1882). Similar results were also observed
in Chara corallina (Raven and Smith 1978). However, low
temperature-induced acidosis was observed in cultured mung bean
cells. The change in cytoplasmic pH in these cells during cold
incubation depended on the growth stage and their chilling sensitivity. At an early stage of exponential growth, cells are most sensitive to chilling, and exhibited a pH drop of 0.6 pH units after 4 hours, and 1.1 pH units after 18 hours. No significant acidification of the cytoplasm occurred in cells at the late stage of exponential growth (Yoshida 1994).

Results obtained with mung bean cells cannot readily be compared to those with asparagus cells (Fig. 9), because of the duration of the experiment (45 seconds for asparagus cells and 4 hours for mung bean cells). Asparagus cells experienced a rapid cold shock, whereas mung bean cells were preincubated at 0°C for various periods, then loaded with the pH indicator for 30 min at 0°C and maintained at 2±0.2°C during the experiment. The observed acidification may be a completely different response from that observed with asparagus cells. It may reflect a long term change in metabolism at low temperature.

The lack of cytosolic acidification when asparagus cells are exposed to a cold shock suggests that increased H⁺ levels are not responsible for the activation of GAD and subsequent GABA synthesis. In contrast, the data presented support the hypothesis that GABA synthesis is mediated by increased cytosolic Ca²⁺ levels when cold shock stimulates Ca²⁺ influx.
The preceding discussion indicates that all four criteria required to establish the role of Ca\(^{2+}\) in cold shock stimulated GABA synthesis have been satisfied.

**GABA synthesis induced by ABA.**

ABA is a growth regulating phytohormone found ubiquitously in vascular plants. Its synthesis has been implicated in stress tolerance. ABA seems to play a role in induced cold and drought tolerance. Endogenous ABA content has been shown to rise in cold-treated tomato tissue (Daie and Campbell 1981), potato leaves (Chenet al. 1983), and in the above-ground parts of winter wheat (Taylor et al. 1990). The link between ABA and induced cold resistance was strengthened by the observation that an ABA-deficient mutant of *Arabidopsis thaliana* is unable to undergo cold-acclimation. The addition of exogenous ABA restored the ability of these plants to cold-acclimate (Heino et al. 1990).

The first visible sign of water stress is the closure of stomata. This process has been shown to be ABA-induced. The proposed mechanism which transduces the ABA signal into a physiological response is through elevated Ca\(^{2+}\) levels. This was demonstrated by ABA stimulated increases in cytosolic Ca\(^{2+}\) levels in *Commelina communis* L. guard cells, which preceded stomatal closure (McAinsh et al. 1990). Corn coleoptiles and parsley
hypocotyls and their roots responded to ABA application by increases in cytosolic Ca\textsuperscript{2+} (Gehring et al. 1990). In other studies, however, ABA treatment caused inconsistent results. A variable effect of ABA on Commelina communis guard cell Ca\textsuperscript{2+} levels was reported (Gilroy et al. 1991). No effect of ABA on cytosolic Ca\textsuperscript{2+} levels was observed in root hair of Lycopersicium esculentum and Brassica napus (Clarkson et al. 1988), and 37\% of Vicia faba guard cell protoplasts showed a 2.5 to 25 fold increase in cytosolic Ca\textsuperscript{2+} level (Schroeder and Hagiwara 1990). The link between ABA and Ca\textsuperscript{2+} in a signal transduction pathway is supported by the finding that IP\textsubscript{3} levels increased two fold 10 seconds after application of ABA to isolated Vicia faba guard cells (Lee et al. 1993). In other studies ABA-stimulated turnover of inositol phospholipids and inositol phosphates was also reported (MacRobbie 1992).

Inconsistent observations of increases in Ca\textsuperscript{2+} response to ABA treatment raise the possibility of Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent mechanisms in the ABA sensory system. Other studies concluded that low temperature and ABA can induce the freezing tolerance through separate signal transduction pathways (Nordin et al. 1991, Monroy et al. 1993). More than one mechanism of action could reflect the vital role of ABA in response to diverse stress conditions.
In the present study ABA induced GABA synthesis was investigated. Concentrations of 1, 10, and 100 μM ABA stimulated GABA synthesis by 58% or more as compared to the controls (Fig 12). ABA induced GABA synthesis was inhibited 55% by the CaM antagonist W7 (Table 11). This observation supports the hypothesis that ABA-stimulated GABA synthesis involves activation of GAD by the active Ca\textsuperscript{2+}-CaM complex. The time course for ABA induced GABA synthesis revealed a maximal increase of 83% after 5 minutes of treatment with 100 μM ABA. GABA levels decreased after 10 or 20 minutes of incubation with ABA (Fig.11). The significance, if any, of this decrease is not understood.

A similar accumulation of GABA in the presence of ABA was observed in wheat roots, where values increased from 1.15 to 1.7 μM GABA g\textsuperscript{-1} fresh wt in 2 hours. Also GAD activity was assayed by feeding wheat seedlings \textsuperscript{14}C-glutamate, and measuring the production of \textsuperscript{14}C-GABA. GAD activity was reported to be increased upon ABA treatment (Reggiani et al. 1993). This study suggested that increased GABA production on ABA addition was mediated by increased cytosolic H\textsuperscript{+} levels. However, no measurements of changes in cytosolic H\textsuperscript{+} or Ca\textsuperscript{2+} levels were made. The data presented in our study suggest that elevated Ca\textsuperscript{2+} may be responsible for ABA-stimulated GABA synthesis.
The possible mechanism(s) and role(s) of stimulated GABA synthesis.

Decarboxylation of glutamate is the first reaction in the GABA shunt pathway (Glutamate $\rightarrow$ GABA $\leftrightarrow$ Succinic Semialdehyde $\rightarrow$ Succinate) (Fig.2). GAD catalyses the first irreversible reaction in this pathway. Regulation of GAD may represent a key regulatory point in the GABA shunt pathway in plants. The fact that GAD is a CaM-regulated enzyme strongly supports the suggestion that GABA synthesis is tightly regulated.

The stimulation of GABA synthesis during cytosolic acidosis is mediated through elevated cytosolic H$^+$ concentration. Therefore, GABA synthesis is a proton consuming reaction which appears to have a role in a metabolic pH-stat process during stresses that result in a drop in cytosolic pH (Crawford et al. 1994). However, the possibility of GAD activation by CaM despite the changes in cytosolic pH can not be excluded. GAD activity was assayed in carrot cell suspensions exposed to a mildly hypoxic conditions where cytosolic pH decreased by 0.1. An increase in GAD activity correlated with the drop in cytosolic pH was observed, followed by a decrease when cytosolic pH recovered. Extracted GAD from these cells was assayed at a fixed 5.8 pH, therefore direct effects of pH on GAD activity was excluded (Carroll et al. 1994). The authors of this study concluded that
GABA synthesis is controlled by cytosolic pH, but they did not considered the possibility of CaM-mediated activation of GAD.

During cold shock no cytosolic acidification was observed (Fig.9), excluding a role for increased H⁺ levels in stimulated GABA synthesis. The cold shock-stimulated increase in cytosolic Ca²⁺ levels (Fig.7; Knight et al. 1991), together with the findings of a GAD CaM-binding domain (Baum et al. 1993), and in vitro GAD stimulation by the Ca²⁺/CaM complex (Snedden et al. 1995), suggest that some environmental stress conditions stimulate GABA synthesis via a Ca²⁺/CaM signal transduction pathway (Fig. 16). The involvement of intracellular Ca²⁺ in GAD stimulation is supported by the findings of increased GABA levels upon treatment with the Ca²⁺ ionophore A23187 (Table 6), and the CaM-stimulation of GABA synthesis is supported by observed inhibition of GABA accumulation during CaM inactivation with W7 (Table 3).

Evidence for the involvement of a Ca²⁺/CaM complex in stimulating GABA synthesis in response to environmental stimuli includes the following:

1. Increased cytosolic Ca²⁺ in response to cold shock (Knight et al. 1991, Fig. 7)

2. Increased ⁴⁵Ca²⁺ influx in response to cold shock (Monroy and Dhindsa 1995)
3. Increased GABA synthesis in response to cold shock (Wallace et al. 1984, Table 2, Fig. 5).

4. A CaM binding domain of GAD (Baum et al. 1993).

5. *In vitro* stimulation of GAD by Ca$^{2+}$/CaM (Snedden et al. 1995).

6. *In vitro* inhibition of Ca$^{2+}$/CaM dependent GAD by W7 (Snedden et al. 1995).

7. *In vivo* inhibition of stimulated GABA synthesis by W7 (Table 3, Table 11).

8. *In vivo* stimulation of GABA synthesis by A23187 (Table 6).

The role of cold shock-stimulated GABA accumulation remains unclear. It is possible that elevated GABA concentrations in the plant cells may change the ionic strength of the cell sap and decrease the freezing point of plants. There is a possibility that newly synthesized GABA in one cell acts as an extracellular signal to adjacent cells. This hypothesis is supported by the findings of GABA efflux from asparagus cells (Snedden et al. 1992, Crawford et al. 1994) and GABA stimulated cytosolic Ca$^{2+}$ increase (Fig. 14). The role of GABA as an extracellular signal in plants was not examined in this study, and no reference to this hypothesis has been found.
Fig. 16. The Mechanism of Cold Shock Stimulated GABA Synthesis

APOPLAST + - CYTOSOL (Ca\(^{2+}\))

COLD SHOCK Ca\(^{2+}\)

La\(^{3+}\) ATP Pi + ADP

Ca\(^{2+}\) ATP Pi + ADP

2H\(^+\) CALMODULIN W7

H\(^+\) L-GLU GABA + CO\(_2\)

VACUOLE Ca\(^{2+}\)

A23187
Conclusions.

The hypothesis that GABA synthesis and accumulation involves elevated cytosolic Ca\(^{2+}\) concentrations was investigated in this study using mechanically isolated *Asparagus sprengeri* Regel mesophyll cells.

1. GABA accumulation was observed in response to a variety of treatments including cold shock, A23187, ABA, butyrate and glutamate.

2. Cold shock treatment resulted in increases in cytosolic Ca\(^{2+}\) levels.

3. No cytosolic acidification was observed during cold shock.

4. GABA accumulation during cold shock was limited by lanthanum, a PM Ca\(^{2+}\) channel blocker.

5. A CaM antagonist, W7, inhibited GABA synthesis during cold shock application.

6. An artificially induced increase in intracellular Ca\(^{2+}\) stimulated GABA accumulation.

The evidence presented in this study supports the hypothesis that GABA synthesis is mediated via increases in cytosolic Ca\(^{2+}\). To my knowledge it represents the first demonstration that increased cytosolic Ca\(^{2+}\) mediates a signal transduction pathway leading to GABA synthesis.
7. References.


aminobutyric acid in pH homeostasis in carrot cell suspensions. Plant Physiol. 106:513-520


Cleland, R.E., Virk, S.S., Tylor, D. and Bjorkman, T. (1990) Calcium, cell walls and growth. In Calcium, Plant Growth and


Evans D.E. (1994) PM-type calcium pumps are associated with higher plant cell intracellular membranes. Cell Calcium 15:241-246


Russ V., Grolig F., Wagner G. (1991) Changes in cytoplasmic free Ca²⁺ in the green alga Molgeotia scalaris as monitored with Indo-1, and
their effect on the velocity of chloroplast movements. *Planta* 184:105-112


**Satya Narayan V., Nair P.M.** (1990) Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochemistry* 29(2):367-375


Wickremasinghe L.R., Swain T. (1965) The accumulation of y-amino butyric acid in bean callus tissue. Phytochemistry 4:687


