

HISTORICAL PERSPECTIVE ESSAY

Calcium: A Central Regulator of Plant Growth and Development

Today no one questions the assertion that Ca^{2+} is a crucial regulator of growth and development in plants. The myriad processes in which this ion participates is large and growing and involves nearly all aspects of plant development (recent reviews in Harper et al., 2004; Hetherington and Brownlee, 2004; Hirschi, 2004; Reddy and Reddy, 2004; Bothwell and Ng, 2005). Despite this wealth of research, the concept of Ca^{2+} as an intracellular regulator is relatively recent and within the professional life span of many people who are still active and working on this topic today. The aim of this essay is to identify those lines of thought and research that led to the idea that Ca^{2+} is a second messenger in plant cell growth and development. This essay thus focuses primarily on work starting in the mid sixties and extending to the mid eighties. I do not provide an exhaustive review of the history of Ca^{2+} research, nor do I attempt to treat modern aspects of Ca^{2+} research. However, I do strive to identify the roots of modern Ca^{2+} research and to chart the origin of the current revolution.

EARLY STUDIES ON PLANT CALCIUM

Ca^{2+} is an essential element; however, its role is elusive. When examining total Ca^{2+} in plants, the concentration is quite large (mM), but its requirement is that of a micronutrient (μM). Ca^{2+} is not usually limiting in field conditions, still there are several defects that can be associated with low levels of this ion, including poor root development, leaf necrosis and curling, blossom end rot, bitter pit, fruit cracking, poor fruit storage, and water soaking (Simon, 1978; White and Broadley, 2003). The underlying causes for these effects are not entirely clear; nevertheless, two areas within the cell have been recognized as being important targets. First is the cell wall, where Ca^{2+} plays a key role in cross-linking acidic pectin residues. The second

is the cellular membrane system, where low $[\text{Ca}^{2+}]_e$ increases the permeability of the plasma membrane. These are briefly discussed below.

Ca^{2+} and the Cell Wall

Since the 19th century, it has been appreciated that Ca^{2+} plays a crucial role in determining the structural rigidity of the cell wall (reviewed in Wyn Jones and Lunt, 1967; Burstrom, 1968). During cell wall formation, the acidic pectin residues (e.g., galacturonic acid) are secreted as methyl esters, and only later deesterified by pectin methylsterase, liberating carboxyl groups, which bind Ca^{2+} . It follows that low $[\text{Ca}^{2+}]_e$ should make the cell wall more pliable and easily ruptured, whereas high concentrations should rigidify the wall and make it less plastic. It had become apparent in the mid to late fifties that modifying the $[\text{Ca}^{2+}]_e$ produced a pronounced effect on cell growth. Thus, elevating the $[\text{Ca}^{2+}]_e$ led to an inhibition in shoot or coleoptile growth, whereas reducing its concentration promoted cell and tissue elongation (Bennet-Clark, 1956; Tagawa and Bonner, 1957). Strong support for the Ca^{2+} /pectate interaction came from a quantitative examination of the cation exchange capacity of the coleoptile cell wall, which was shown to be due to the number of free pectic carboxyl groups (Jansen et al., 1960). Still further support came from studies using the cation chelator EDTA, which had been employed to macerate plant tissues without destroying the cell structure (Letham, 1958). The explanation centered around the idea that EDTA, by chelating Ca^{2+} , led to a marked weakening or loss of pectates in the middle lamella, thus removing the agent that cemented cells together.

The importance of the Ca^{2+} /pectate interaction as a regulator of growth encouraged researchers to include a role for auxin in this scheme, particularly because it was becoming evident that Ca^{2+} and auxin

had antagonistic actions. Thus, auxin promoted shoot growth and inhibited root growth, whereas Ca^{2+} inhibited shoot growth and promoted root growth. Working with oat coleoptiles, Bennet-Clark (1956) proposed that there might be a direct antagonism between indoleacetic acid (IAA) and Ca^{2+} . Noting that Ca^{2+} , and the lanthanide praseodymium, inhibited IAA-induced elongation, whereas EDTA reversed the inhibitory activity of Ca^{2+} , and even promoted growth, Bennet-Clark (1956) suggested that IAA acts as a Ca^{2+} / Mg^{2+} chelator. This model proposed that IAA removes Ca^{2+} and leads to a loss of Ca^{2+} pectates, which are replaced by pectate free acids or methyl esters. The latter, because they are not cross-linked, would render the wall plastic and able to elongate (Bennet-Clark, 1956). This idea was challenged by Cleland (1960), who demonstrated that IAA does not enhance the loss of Ca^{2+} from the cell wall, nor does it cause a redistribution of Ca^{2+} between pectin and proto-pectin. Somewhat later, Burling and Jackson (1965) used atomic absorption spectroscopy to show that Ca^{2+} accumulated in the cell walls of elongating coleoptiles and that this accumulation was unaffected by auxin. Further studies by Baker and Ray (1965) and Ray and Baker (1965) established the separation in action between Ca^{2+} and IAA, providing clear evidence that the inhibition of cell elongation by Ca^{2+} does not prevent IAA from stimulating cell wall synthesis. In the presence of Ca^{2+} , and thus the inhibition of cell enlargement, they demonstrated a general promotion of synthesis of matrix polysaccharides in the presence of IAA (Ray and Baker, 1965).

A compelling interaction between Ca^{2+} , the cell wall, and cell growth was also found in pollen tubes. It was shown in 1963 that Ca^{2+} must be present in the medium to support pollen tube growth in vitro (Brewbaker and Kwack, 1963). Using $^{45}\text{Ca}^{2+}$, Kwack (1967) showed that

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incorporation occurred exclusively in the pollen tube wall; some of the autoradiographic images indicated an enhanced accumulation of Ca^{2+} in the apical region. Because the pollen tube cell wall, especially at the tip, is composed almost entirely of pectin, it is reasonable to assume that a Ca^{2+} /pectate interaction dominates the requirement for this ion.

Despite the attractiveness of the idea that cell wall Ca^{2+} achieves its effects through an interaction with pectates, it must be recognized that not all results can be easily accounted for by this explanation (Cleland and Rayle, 1977; Tepfer and Taylor, 1981). The failure to show a close correspondence between the ability of divalent cations to form a pectic gel with their ability to inhibit growth has led to a consideration of other ideas, for example, a direct effect of Ca^{2+} on cell wall modifying enzymes (Cleland and Rayle, 1977). It is important to keep in mind that within the complex framework of carbohydrates and proteins of the cell wall, there could be interactions between Ca^{2+} and molecules other than pectins that could contribute to cell wall structure and extensibility. Nevertheless, a Ca^{2+} /pectate interaction cannot be ignored and deserves attention today as a factor involved in the control of cell growth.

Ca^{2+} and Membrane Permeability

It has also been known for many years that Ca^{2+} plays an important role in controlling membrane structure and function (Wyn Jones and Lunt, 1967; Burstrom, 1968). A general idea is that Ca^{2+} , by binding to phospholipids, stabilizes lipid bilayers and thus provides structural integrity to cellular membranes. From a physiological point of view, a frequent observation has been that Ca^{2+} controls membrane permeability (Epstein, 1972; Hanson, 1984). Thus, when cells are cultured in solutions of low $[\text{Ca}^{2+}]_e$, especially in the presence of EDTA, there is leakage of ions and metabolites (Hanson, 1984). Using roots of soybean and maize, Hanson (1960) showed that a low $[\text{Ca}^{2+}]_e$ caused a marked decline in the ability of these tissues to absorb and retain solutes. A $[\text{Ca}^{2+}]_e$ between

0.1 to 1.0 mM was found to be necessary to maintain the integrity and selective ion transport of the plasma membrane. Epstein (1961) examined the competition between different monovalent cations and reported that Ca^{2+} (0.1 to 1.0 mM), but not Mg^{2+} , promoted the uptake of potassium in the presence of sodium. Thus, Ca^{2+} by some mechanism, imparts selectivity to the ion transport process. In another example, Van Steveninck (1965) found that low $[\text{Ca}^{2+}]_e$ promoted a release of potassium in cultured beet root tissues, which was completely reversed by adding back Ca^{2+} , but not Mg^{2+} . Pollen tubes also showed changes in permeability in response to low $[\text{Ca}^{2+}]_e$, including a significant release of carbohydrates into the medium (Dickinson, 1967).

In a series of studies on leaf abscission and tissue senescence, Poovaiah and Leopold (1973a, 1973b, 1976) reported that Ca^{2+} inhibited or slowed these processes. Recognizing that Ca^{2+} , through cross-linking pectates and cementing cell walls, will directly retard abscission, they noted that several other processes were also affected. During senescence in maize and rumex leaf disks, they showed that Ca^{2+} retarded the loss of chlorophyll, the loss of protein, and the loss of free space, suggesting that the ion plays a regulatory role in maintaining and controlling membrane structure and function (Poovaiah and Leopold, 1973b).

Early ultrastructural studies echoed this refrain. Thus, marked differences were detected at the electron microscope level in the membranes of barley shoot apices cultured in low $[\text{Ca}^{2+}]_e$ relative to the controls (Marinos, 1962). The low Ca^{2+} -induced effect was apparent as relatively gross discontinuities in the nuclear envelope, plasma membrane, and tonoplast, and later in the mitochondria. It is difficult to imagine that such lesions occur in the intact cell because they would immediately lead to cell death. However, they may indicate reduced membrane stability, which leads to breakage and discontinuities during the permanganate fixation process. For that reason, the details of this report must be treated with caution; nevertheless, the differences observed suggest

that membranes cultured in low Ca^{2+} become structurally weakened.

If low Ca^{2+} makes the membrane more permeable, it should follow that elevated concentrations make the membrane less permeable. Using Ca^{2+} itself as the probe, Robinson (1977) showed this to be true in zygotes of the alga *Pelvetia*. Thus, an increase in the $[\text{Ca}^{2+}]_e$ from only 1 to 3 mM reduced the influx of this ion by >10-fold. These results seem counterintuitive and are not well appreciated. Examples certainly exist in which it is evident that an increase in the $[\text{Ca}^{2+}]_e$ causes a corresponding increase in the $[\text{Ca}^{2+}]_i$ (Gilroy et al., 1986), and an extracellular Ca^{2+} sensor recently has been identified in guard cells (Han et al., 2003). However, this situation does not automatically extend to all cell types, as the study by Robinson (1977) shows. In agreement with the studies on *Pelvetia*, we find that increasing the $[\text{Ca}^{2+}]_e$ to 10 mM inhibits lily pollen tube elongation and causes the tip-focused gradient to drop to basal levels (D.A. Callaham and P.K. Hepler, unpublished data). Thus, in experiments in which the $[\text{Ca}^{2+}]_e$ is modulated, the assumption cannot be made that similar changes occur on the cytosol. Rather, an increase in $[\text{Ca}^{2+}]_e$ may generate a decrease in $[\text{Ca}^{2+}]_i$.

Briefly summarizing, early studies on the role of Ca^{2+} in plants focused on the cell wall and on membrane permeability. At that time, there was no widespread appreciation that the $[\text{Ca}^{2+}]_e$ might be very low and that this ion might be acting as a regulator of cytoplasmic processes. Botanists exploring Ca^{2+} effects in the concentration range between 0.1 and 100 mM were unlikely to see changes at the submicromolar level. The concept of Ca^{2+} as a regulator initially derives from studies of animal cells and only later in studies of plant cells. To see how this concept arose, I will focus briefly on Ca^{2+} in animal cell physiology, giving attention to the process of muscle contraction.

CALCIUM AND MUSCLE CONTRACTION

More than 120 years ago, Ringer (1883) showed that the repetitive beating of an

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isolated frog heart was sensitive to different $[Ca^{2+}]_i$ (for review, see Carafoli et al., 2001). When cultured in distilled water, the hearts failed to exhibit the proper contraction; however, when cultured in London city tap water, they exhibited repetitive contractions. Using sequential ion addition to the distilled water, Ringer (1883) discovered that Ca^{2+} was the key factor that supported contraction. Despite these early studies, the idea that Ca^{2+} was a regulator of muscle contraction did not expand at this point. Only considerably later through the efforts of Heilbrunn (1940) was the emphasis again focused on Ca^{2+} . Heilbrunn (1940) showed that muscle contraction could be stimulated through the injection of Ca^{2+} into the frog muscle fiber. Of note, the contraction could take place even when the Ca^{2+} solution was highly diluted. Equally important was the observation that muscle contraction was not supported by injection of other important physiological ions, including sodium, potassium, or Mg^{2+} . Because potassium at that time was considered crucial, the additional observation that massive doses of potassium were ineffective further emphasized the primary role of Ca^{2+} in stimulating contraction (Heilbrunn and Wiercinski, 1947).

As insightful and penetrating as these studies were, Heilbrunn and Wiercinski (1947) were not able to establish the actual $[Ca^{2+}]_i$ in the resting muscle fiber. Indeed, determining the $[Ca^{2+}]_i$ has been difficult for any cell type. Hodgkin and Keynes (1957), using $^{45}Ca^{2+}$ to examine the mobility of this ion in squid axoplasm, made two important observations: first, that the mobility of Ca^{2+} is extremely low; second, that the bulk of the Ca^{2+} is bound, with only 10 μM or less being free and ionized. Further work that established the true $[Ca^{2+}]_i$ depended on two technical developments. The first was the application of cation chelators EDTA and EGTA in physiological studies to carefully control the $[Ca^{2+}]_i$ (Bozler, 1954). Before the availability of effective chelators, it was nearly impossible to construct solutions in the submicromolar range because of the presence of Ca^{2+} as a contaminant, or leaching from glassware. Whereas EDTA has a high affinity for Ca^{2+} , it also has a substantial

affinity for Mg^{2+} . With EGTA, the affinity for Ca^{2+} is not as high as with EDTA, but the relative insensitivity of EGTA to Mg^{2+} means that it is a more efficacious chelator for constructing solutions that are specifically buffered for Ca^{2+} . The second important development was the isolation and characterization of the photoprotein aequorin, a Ca^{2+} sensitive, bioluminescent protein from the jelly fish Aequorea, which provided a means for detecting changes in the $[Ca^{2+}]_i$ in the submicromolar range (Shimomura et al., 1963). At resting $[Ca^{2+}]_i$, the protein generates only a faint glow; Shimomura et al. (1963) initially determined that the resting concentration was between 0.1 and 1.0 μM . However, as the $[Ca^{2+}]_i$ increases, there is an exponential (2.3 power) increase in the amount of light generated, making aequorin a suitable reagent for detecting regions of elevated ion concentration or amplitude modulation.

Despite the favorable properties of aequorin as an indicator of the $[Ca^{2+}]_i$ in living cells, there were substantial problems in its use. First was the need to introduce the protein into cells, and second was the difficulty of detecting and imaging a rather weak signal. The first problem was solved using large cells, which are easy to inject. Of course, more recently, using modern molecular biological methods, it is possible to transfect cells with the aequorin gene and express the protein in virtually any cell (Knight et al., 1991), and even within organelles (Rizzuto et al., 1994). The problems associated with the detection and imaging of the aequorin signal remain with us today. Although detection of a signal without imaging can be done effectively with photomultiplier tubes, imaging, especially from single small cells is difficult due to a low number of Ca^{2+} -dependent photons. Progress has been made in the development of extremely sensitive photon imaging equipment, which has permitted the visualization of these weak signals (Gilkey et al., 1978; Knight et al., 1993).

The determination of the $[Ca^{2+}]_i$ in living muscle cells was performed by studies that involve both of these technologies. In 1964, Portzehl and coworkers used EGTA to produce carefully buffered Ca^{2+} solutions and showed that contraction in an isolated

muscle fiber of the crab *Maia squinado* occurred between 0.3 and 1.5 μM . A few years later in 1967, Ridgway and Ashley injected the giant muscle of the acorn barnacle with aequorin. Within 1 ms after electrical stimulation, they recorded a sharp increase in light, indicating that the $[Ca^{2+}]_i$ had risen (Figure 1). This was followed in 5 ms by an increase in muscle tension. Although the results were not strictly quantitative, Ridgway and Ashley (1967) argued, based on the work of Shimomura et al. (1963), that at rest the $[Ca^{2+}]_i$ would be between 0.1 and 1.0 μM ; therefore, upon stimulation it would be substantially higher. These studies are dramatic and compelling; they clearly demonstrate that the stimulated depolarization of the membrane potential is followed almost immediately by an abrupt increase in bioluminescence (i.e., $[Ca^{2+}]_i$) and with only a further slight lag by the generation of tension (Ridgway and Ashley, 1967). These studies were the first direct demonstration of Ca^{2+} amplitude modulation.

Ca^{2+} AMPLITUDE MODULATION IN NONMUSCLE CELLS

During the next decade, in studies of several different nonmuscle systems, both EGTA and aequorin were used to show that the basal $[Ca^{2+}]_i$ was submicromolar and that through stimulation elevations of the $[Ca^{2+}]_i$ could be elicited. For example, activation of the freshwater protozoans, Spirostomum (Ettienne, 1970), cell cleavage in *Xenopus* (Baker and Warner, 1972), response of the photoreceptor of *Limulus* to light (Brown and Blinks, 1974), oscillations in cytoplasmic streaming in the plasmodial slime mold, *Physarum* (Ridgway and Durham, 1976), and egg activation in the medaka fish, *Oryzias latipes* (Ridgway et al., 1977), and sea urchin, *Lytechinus pictus* (Steinhardt et al., 1977) were shown to be anticipated by an increase in the $[Ca^{2+}]_i$. The examples of egg activation are especially efficacious in establishing a primary role for Ca^{2+} amplitude modulation in development. Whereas Ridgway et al. (1977) employed eggs from medaka, a fresh water fish, Steinhardt and coworkers (1977) used eggs from a marine sea urchin.

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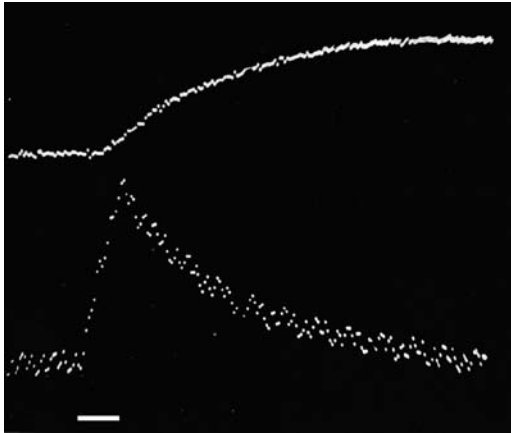


Figure 1. A $[Ca^{2+}]_i$ Increase Precedes Muscle Contraction.

After an electrical stimulus, the giant muscle of the acorn barnacle, which had been injected with aequorin, exhibits an abrupt rise in the $[Ca^{2+}]_i$ (bottom trace). Soon thereafter, an increase in muscle tension begins (top trace), which continues even though the Ca^{2+} quickly returns to basal level. The Ca^{2+} -dependent light emission from aequorin is measured with a photomultiplier tube. Bar = 20 ms. (Figure courtesy of Ridgway and Ashley, 1967, Figure 1a, with permission of Elsevier.)

In both instances, the eggs had been injected with aequorin, and in both examples, clear documentation of a $[Ca^{2+}]_i$ increase was noted after fertilization. In an extension of the studies on medaka eggs, Gilkey et al. (1978), using sensitive imaging equipment, were able to observe the spatial and temporal dynamics of the Ca^{2+} -dependent light emission. Their results reveal that the $[Ca^{2+}]_i$ rises at the point of sperm entry (the micropyle), reaching $\sim 30 \mu M$, and propagates as a wave that travels at the rate of $12 \mu m/s$ through the cortex of the egg. By the late seventies, therefore, it had been established in several cell types that the basal $[Ca^{2+}]_i$ is $\sim 0.1 \mu M$ and, importantly, that a variety of different events can be activated through a change or amplitude modulation of the $[Ca^{2+}]_i$ up to $1 \mu M$ or higher.

Ca^{2+} AMPLITUDE MODULATION IN PLANTS

Although plants do not possess muscles as such, it can be viewed as an interesting example of parallelism that our understanding of Ca^{2+} regulation in plant cells in part originated from studies on the control actomyosin in cytoplasmic stream-

ing. In the sixties, it had been recognized that the action potential in large internode cells of the Characean algae would induce a very rapid but reversible inhibition of cytoplasmic streaming (Barry, 1968; Tazawa and Kishimoto, 1968). Tazawa and Kishimoto (1968) showed that it was not the formation of a gel or the coagulation of the cytoplasm that led to streaming cessation but rather an inhibition of the driving force. Realizing that there were substantial ion changes during the action potential, they focused primarily on chloride and potassium but nevertheless suggested that Ca^{2+} might also be involved. At the same time, Barry (1968), working with *Nitella* and using ion replacements, provided clear evidence that the presence of Ca^{2+} , but not Mg^{2+} , in the extracellular medium caused the cessation of streaming during the action potential. These studies further emphasized that it was not the action potential per se that led to streaming inhibition but rather the presumed influx of Ca^{2+} . Barry (1968) also directed attention to the actomyosin system as the focus for Ca^{2+} activity. Further work, involving the perfusion of the large internode cells of *Nitella* and *Chara*, produced a system that could be readily manipulated experimentally. Williamson (1975) established that

streaming, in addition to requiring ATP, was dependent on a very low $[Ca^{2+}]_i$ ($0.1 \mu M$). If the concentration was elevated to $1.0 \mu M$, there was a decrease in cytoplasmic streaming by 20%, and if the $[Ca^{2+}]_i$ was increased to $10 \mu M$, the streaming would be inhibited by a $>80\%$. Similar results reported by Tazawa et al. (1976) further emphasized the conclusion that elevated $[Ca^{2+}]_i$ inhibited cytoplasmic streaming. At the time these studies were published, they may not have enjoyed widespread acknowledgment because there were questions whether findings from the Characean algae were relevant to equivalent processes in higher plants. The subsequent studies on *Vallisneria* dispelled this concern, showing that cytoplasmic streaming, as in *Nitella* and *Chara*, was regulated by the $[Ca^{2+}]_i$ (Yamaguchi and Nagai, 1981; Takagi and Nagai, 1983). Today, it is widely recognized for non-flowering and flowering plants alike that low $[Ca^{2+}]_i$ ($0.1 \mu M$) permits streaming, whereas elevated $[Ca^{2+}]_i$ ($1.0 \mu M$) inhibits the process.

The major breakthrough that established the relationship between the action potential, Ca^{2+} , and the inhibition of streaming came from the pioneering studies of Williamson and Ashley (1982). Using internode cells of *Nitella* and *Chara*, into which the photoprotein aequorin had been microinjected, they showed that the action potential elicited an abrupt rise in the $[Ca^{2+}]_i$ (Figure 2) together with a parallel decrease in cytoplasmic streaming. The system also showed impressive recovery with a relatively rapid return to basal $[Ca^{2+}]_i$, followed by a resumption in cytoplasmic streaming. Williamson and Ashley (1982) further established that the basal $[Ca^{2+}]_i$ in *Chara* was $\sim 0.1 \mu M$, whereas in *Nitella*, it was $0.4 \mu M$. When stimulated, the $[Ca^{2+}]_i$ in *Chara* rose to $6.7 \mu M$, whereas in *Nitella*, it rose to $43 \mu M$. A closely following study by Kikuyama and Tazawa (1983) provided results in agreement with Williamson and Ashley (1982), firmly establishing the change of $[Ca^{2+}]_i$ during the action potential in *Nitella* and *Chara*. These studies were the first and for several years remained the most convincing example of Ca^{2+} amplitude modulation in plants.

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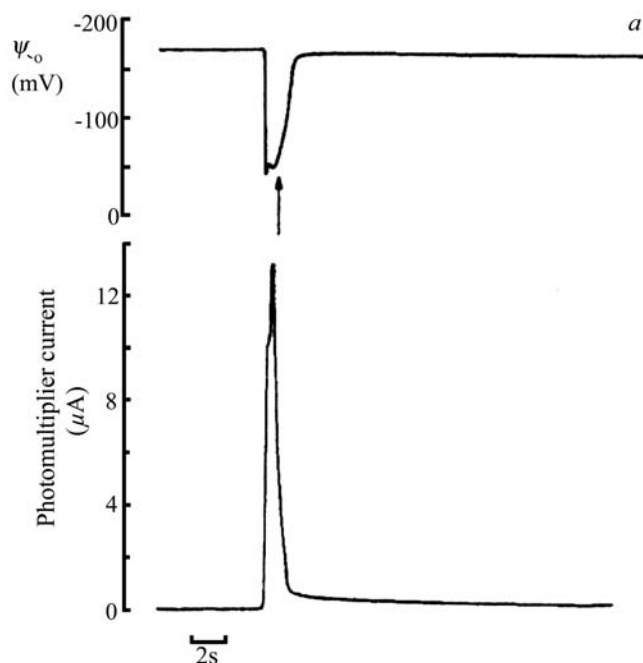


Figure 2. The Action Potential in Chara Elicits a $[Ca^{2+}]_i$ Increase.

A Chara internode cell, which had been injected with aequorin, is stimulated electrically to induce an action potential (top trace). Following closely is a sharp increase in the photomultiplier current indicating Ca^{2+} -dependent light emission from aequorin (bottom trace). Bar = 2 s. (Figure courtesy of Williamson and Ashley, 1982, Figure 2a, with permission of Nature Publishing Group <http://www.nature.com/>).

After these pioneering studies on *Nitella* and *Chara*, there have been additional studies in plants showing that the basal $[Ca^{2+}]_i$ is low and that increases can occur following different stimuli. Gilroy et al. (1986) used the permeant acetoxy methyl-ester of quin2 to show that the $[Ca^{2+}]_i$ in mung bean root protoplasts was 171 nM. This study is important because it was the first to use a fluorescent indicator. Although quin2 is no longer used, the second-generation fluorescent dyes developed by R.Y. Tsien and colleagues, for example fura-2 and indo-1 (Grynkiewicz et al., 1985), and especially in their dextranated forms, have proved extremely effective in allowing us to assay $[Ca^{2+}]_i$ in plants. Other methods have also provided compelling results. For example, Miller and Sanders (1987), using a Ca^{2+} selective intracellular microelectrode, found that the alga *Nitellopsis* had a basal $[Ca^{2+}]_i$ of 400 nM in the dark. However, when cultured in light, the $[Ca^{2+}]_i$ dropped to 150 nM. The interpretation put

forth was that the process of photosynthesis, together with ion uptake by chloroplasts, caused the reduction of the $[Ca^{2+}]_i$. Also using Ca^{2+} selective microelectrodes, Felle (1988) showed that auxin induced Ca^{2+} oscillations in maize coleoptiles. Here, the basal $[Ca^{2+}]_i$ was 119 nM, which in the presence of auxin rose in an oscillatory fashion to 300 nM. Yet another example was the induction of stomatal closure by ABA, which was shown to be accompanied by an increase in the $[Ca^{2+}]_i$ to 600 nM in *Commelina* guard cells that had been injected with the fluorescent indicator dye fura-2 (McAinsh et al., 1990). Note is also made of the dramatic tip-focused Ca^{2+} gradient observed in pollen tubes (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992), a result that was anticipated given the earlier demonstration of $^{45}Ca^{2+}$ influx in these cells (Jaffe et al., 1975). However, the fluorescent dyes allowed direct visualization of free Ca^{2+} . Also, the use of fura-2 covalently

linked to a 10-kD dextran provided a means for avoiding dye sequestration (e.g., into vacuoles) and for permitting long term recording of the $[Ca^{2+}]_i$ (Miller et al., 1992). Finally, in a dramatic development that fused molecular methods to Ca^{2+} cell biology, Knight et al. (1991) introduced the aequorin gene into tobacco plants and were able to show that different agents, including touch, cold shock, and fungal elicitors, induced Ca^{2+} stimulated luminescence. Suffice it to say that by the late eighties and early nineties several studies, using different techniques, had documented a low basal $[Ca^{2+}]_i$ and demonstrated amplitude modulation in plant cells.

CONCEPT OF Ca^{2+} AS A REGULATOR

The studies discussed above make it abundantly clear that the $[Ca^{2+}]_i$ in plant cells, as in animal cells, is low and that plants are able to respond to various stimuli by eliciting a change in the $[Ca^{2+}]_i$. However, just as a professional orchestra does not need the oboist to sound them the appropriate A, neither did the plant biologists need these data to suggest that Ca^{2+} was a potential signal transducer. By the early to mid seventies, the ideas were in the air, and thus well before the actual documentation of the $[Ca^{2+}]_i$, many scientists working on different aspects of plant growth and development were coming to recognize the potential importance of Ca^{2+} as an intracellular signaling agent. Although there were probably several paths that were responsible for focusing attention on the regulatory function of Ca^{2+} , I will mention a few lines of thought and research that I think were important in shaping the ideas of plant biologists.

Ca^{2+} and Cyclic AMP: The Discovery of Calmodulin and Calcium-Dependent Protein Kinases

In the late fifties, Sutherland and Rall (1958) discovered that adenosine 3',5'-monophosphate (cyclic AMP) levels increased in liver tissues in response to epinephrine and furthermore that this small nucleotide was implicated as a second messenger in a wide variety of cellular reactions frequently

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involved in the phosphorylation of proteins (reviewed in Rasmussen, 1970). It soon became apparent that Ca^{2+} was also involved in many of these reactions, where it was recognized that a stimulus that caused an increase in cyclic AMP also generated an increase in Ca^{2+} ion uptake. The confluence of the activities of these two agents led Rasmussen (1970) to speculate that, "The basic elements in this widespread biochemical control mechanism are: calcium ions, adenosine 3', 5'-monophosphate (cyclic AMP), intracellular microtubules, microfilaments, secretory vesicles, and a class of enzymes known as protein kinases which phosphorylate specific proteins with adenosine triphosphate (ATP) as substrate." I can clearly remember reading this article (Rasmussen, 1970) and being struck by its bold vision. At the time it was not possible to make a case for the participation of cyclic AMP in plant development (reviewed in Trewavas, 1976), but all the other components could be recognized as possible contributors, including notably Ca^{2+} , the cytoskeleton, directed secretion, and protein kinases.

The continuing studies in different animal systems on the regulation of cyclic AMP led to discovery of calmodulin. In 1970, it was reported that 3',5' nucleotide phosphodiesterase (PDE), the enzyme that degrades cyclic AMP to 5'-AMP, was in part regulated by a heat stable protein (Cheung, 1970; Kakiuchi and Yamazaki, 1970), which itself did not show enzymatic activity. An important further observation revealed that PDE was controlled by Ca^{2+} with basal rates under 1 μM and maximal activation at 20 μM (Kakiuchi and Yamazaki, 1970). Subsequent biochemical investigations established that the heat stable factor was a protein that was dependent on Ca^{2+} , with half-maximal activation at 2.3 μM (Teo and Wang, 1973). Initially, this was called the "calcium-dependent regulator," a name that was changed to "calmodulin" (Cheung, 1980). It was also soon realized that calmodulin was extremely common, being found in virtually all tissues that were tested, and that it was very similar to troponin C, the Ca^{2+} switch for striated muscle (Cheung, 1980).

The discovery of calmodulin did not escape the attention of the plant biologists. Muto and Miyachi (1977) first showed that NAD kinase isolated from pea seedlings required an activator protein, which was sensitive to acid and alkali conditions, and also heat stable. These properties, together with its relatively low molecular mass (28 kD), led Muto and Miyachi (1977) to draw a tentative connection of the protein they had identified with the PDE activator in animal systems. However, it was Anderson and Cormier (1978), using appropriate metal chelators, who first discovered the Ca^{2+} requirement of the NAD kinase activator protein in plants. Their results firmly established the close relationship between it and the Ca^{2+} -dependent regulator protein or calmodulin. It soon became apparent that calmodulin was widely present in plants (Watterson et al., 1980). These results truly made the case for Ca^{2+} as an intracellular regulator of plant processes.

Continued investigations by many researchers revealed the existence of different kinases, in addition to NAD kinase, that were Ca^{2+} /calmodulin dependent. For example, Ca^{2+} /calmodulin regulation was demonstrated for an unspecified protein kinase (Polya and Davies, 1982) and for plant quinate:NAD⁺ 3-oxidoreductase (Ranjewa et al., 1983). Of particular interest and excitement was the discovery at this time of kinases that were Ca^{2+} dependent but calmodulin independent (Hetherington and Trewavas, 1982). This line of research led eventually to the discovery of calcium-dependent protein kinases (CDPKs) (Putnam-Evans et al., 1986; Harmon et al., 1987), which are now recognized as members of a large family of kinases that does not exist in animals. In animals, there are no kinases that are directly regulated by Ca^{2+} ; rather, their Ca^{2+} -stimulated kinases work through a relay system, often involving calmodulin (for review, see Carafoli et al., 2001). By contrast, plants have the calmodulin pathway (for review, see Snedden and Fromm, 2001; Zhang and Lu, 2003) and a separate system involving the CDPKs (for review, see Harmon et al., 2001; Cheng et al., 2002; Harper et al., 2004); this marks a significant and unique

difference in the mechanism of Ca^{2+} regulation between plant and animal cells.

Ca^{2+} and Cell Division

For me, there was a distinct awakening in the early seventies about Ca^{2+} as an intracellular regulator. First, the article by Rasmussen (1970), mentioned above, was enormously stimulating and provided a broad sweep about a role for Ca^{2+} in many different signal transduction processes, perhaps especially including a relationship between Ca^{2+} and the cytoskeleton. But the truly defining moment occurred with the publication by Weisenberg (1972), which showed that a low [Ca^{2+}] (<1.0 μM) was necessary to achieve microtubule polymerization in vitro. Before this, numerous unsuccessful attempts had been made to solve this vexing problem. Weisenberg (1972) had been using phosphate buffers with only limited success. He then switched to the newly introduced family of zwitterionic buffers and found one, *N*-(2-acetamido)-iminodiacetic acid, which dramatically supported microtubule polymerization. Further work indicated that the unique property of this buffer was its ability to chelate Ca^{2+} . Thus, in the presence of 100 mM *N*-(2-acetamido)-iminodiacetic acid and 10 mM calcium, the free [Ca^{2+}] would only be 6 μM , yet this concentration was sufficient to block polymerization of microtubules. Further studies with EDTA, and especially EGTA, clearly established that it was Ca^{2+} and not Mg^{2+} that was responsible for the depolymerization of microtubules (Weisenberg, 1972).

At that time, I was working on mitosis and cytokinesis in plants, focusing on the formation, organization, and function of the mitotic apparatus and phragmoplast. Considerable attention was directed toward microtubules but also to associated structures, including elements of the endoplasmic reticulum, which could be seen to form close structural appositions with microtubules in both the mitotic apparatus and phragmoplast. I was teaching cell biology, which included muscle physiology as a topic, and was aware of the central role that Ca^{2+} played in muscle contraction. By that time, troponin had been discovered,

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and it was well known that contraction was exquisitely attuned to the $[Ca^{2+}]_i$ from resting (0.1 μM) to those that activated contraction (0.3 to 1.5 μM). In addition, studies on the sarcoplasmic reticulum from striated muscle had led to the isolation and partial characterization of the Ca^{2+} pump (MacLennan and Wong, 1971).

Putting these lines of inquiry together switched on the proverbial light in my mind. It seemed plausible that microtubules in the mitotic apparatus might be controlled by the local $[Ca^{2+}]_i$, which in turn would be regulated by the nearby endoplasmic reticulum (Figure 3) (Hepler et al., 1981). This general idea, which Barry Palevitz and I articulated in our review on the cytoskeleton (Hepler and Palevitz, 1974), guided research in my laboratory for several years thereafter. Spindle-associated endoplasmic reticulum was found to contain deposits of Ca^{2+} (Wick and Hepler, 1980; Wolniak et al., 1983), and spindle microtubules were shown to be sensitive to elevations in $[Ca^{2+}]_i$ with depolymerization occurring when the concentration was raised to 1.0 μM or more (Zhang et al., 1992). Restriction of the $[Ca^{2+}]_i$ was seen to affect progress through mitosis (Hepler, 1985), whereas stimulation of Ca^{2+} entry was found to promote bud initial formation in mosses (Saunders and Hepler, 1982) and

red light-stimulated spore development in ferns (Wayne and Hepler, 1984). Nevertheless, evidence for the occurrence of Ca^{2+} amplitude modulation during division was and still is decidedly mixed (Hepler, 1989); indeed, a compelling example of Ca^{2+} amplitude modulation in plant cell division has not been established.

Ca^{2+} and Polarized Cell Growth

An area of endeavor that drew early interest toward Ca^{2+} concerned the regulation of polarized plant cell development. Through the pioneering efforts of Jaffe and coworkers (Jaffe et al., 1974; Weisenseel et al., 1975), it was discovered that polarized cells (e.g., *Fucus* zygotes, pollen tubes, and root hairs) drove substantial ion currents through themselves. The bulk of the ion current appeared to consist of a polarized influx of potassium, focused at the growing point (i.e., the rhizoid of *Fucus* or the growing tip of the pollen tube). However, it was soon appreciated that Ca^{2+} influx constituted a small but potentially important component of the total current (Jaffe et al., 1975; Robinson and Jaffe, 1975; Weisenseel and Jaffe, 1976). Robinson and Jaffe (1975), using *Pelvetia* eggs that were polarized through unilateral illumination, showed that approximately five times more $^{45}Ca^{2+}$ entered the shaded rhizoid pole than

entered the illuminated thallus pole. A subsequent study in which the rhizoids grew toward an experimentally imposed gradient of the Ca^{2+} -ionophore, A-23187, added strong support for the idea of localized Ca^{2+} influx as a regulator of polarized development (Robinson and Cone, 1980).

Studies on growing pollen tubes also provided persuasive support for localized ion fluxes and for a specific role for Ca^{2+} in the regulation of polarized growth. Application of the vibrating electrode revealed strong currents in which an influx of potassium at the apex appeared to be balanced by an outward flux of protons in the region of the grain (Weisenseel et al., 1975; Weisenseel and Jaffe, 1976). Importantly, Ca^{2+} , as noted earlier (Brewbaker and Kwack, 1963), was essential for tube growth (Weisenseel and Jaffe, 1976). Additionally, autoradiography revealed an accumulation of $^{45}Ca^{2+}$ in the apical domain, providing support for the idea that ion flow into the tip created an intracellular gradient (Jaffe et al., 1975). A certain amount of the autoradiographic signal observed by Jaffe et al. (1975) may be due to Ca^{2+} binding in the cell wall as noted by Kwack (1967). Nevertheless, when considered with the other studies on current flow, these data are consistent with a small but significant influx of Ca^{2+} across the plasma membrane.

Ca^{2+} in pollen tubes was subsequently examined for gradients in membrane-associated Ca^{2+} (chlortetracycline) (Reiss and Herth, 1979) and total Ca^{2+} (proton-induced x-ray emission) (Reiss et al., 1983), both of which provide results consistent with the apex being elevated in the ion. However, the demonstration of the steep, tip-focused gradient, using fluorescent dyes (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992), finally provided the necessary proof for the postulated asymmetric distribution of intracellular free Ca^{2+} . Based on the studies on polarized ion currents, the suggestion was made that Ca^{2+} gradients might create an electrical field across the cytoplasm that would be sufficient to segregate cytoplasmic components by electrophoresis (Jaffe et al., 1974; Robinson and Jaffe, 1975). Robinson and Jaffe (1975) also suggested that Ca^{2+} might affect intracellular motility

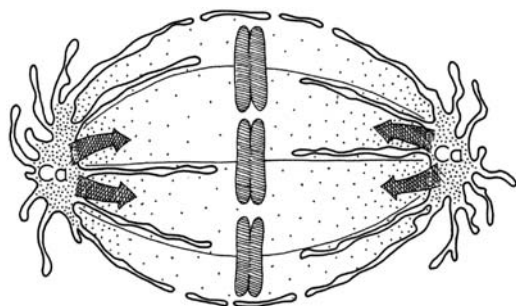


Figure 3. Diagram of a Dividing Plant Cell in Late Metaphase.

This figure depicts a system of Ca^{2+} -containing endoplasmic reticulum that extends from the spindle poles to the chromosomes along kinetochore microtubules. It was suggested that during anaphase, Ca^{2+} release from the endoplasmic reticulum activates motile processes (e.g., microtubule depolymerization) and thus facilitates movement of the chromosomes to the spindle poles. In support of this model, Ca^{2+} -stimulated depolymerization of microtubules and facilitation of chromosome motion have been observed (Zhang et al., 1992). Although an endogenous increase in $[Ca^{2+}]_i$ during anaphase has been reported using the absorbance indicator arsenazo III (Hepler and Callahan, 1987), this has not been repeated with a more efficacious fluorescent dye. (Figure courtesy of Hepler et al., 1981, Figure 7, with kind permission of Springer Science and Business Media.)

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and in particular the formation and function of microtubules. In broad terms, these ideas can be appreciated as antecedents for the view that the Ca^{2+} gradient in polarized cells contributes to the control of secretion (Holdaway-Clarke and Hepler, 2003).

Ca^{2+} and Secretion

The substantial body of work showing that Ca^{2+} affected the permeability properties of the cell membrane, while interesting in itself, did not provide a very compelling or complete understanding of the mechanism of action of the ion. Although usually not explicitly stated, there are different studies where you can almost hear the authors struggling with this rather vague concept and where they are attempting to formulate a more specific model. An example is the induction of α -amylase release in barley aleurone cells by gibberellic acid. Studies on this model system had gained attention because it seemed that the molecular basis for gibberellic acid might emerge. An early and influential observation was that of Chrispeels and Varner (1967), who showed that the presence of Ca^{2+}_e (mM) greatly facilitated the appearance of gibberellic acid-induced α -amylase in the medium. The increase was not trivial; as shown by Jones (1973), 20 mM CaCl_2 stimulated an 18-fold increase over the water control. If an increase in the $[\text{Ca}^{2+}]_e$ renders the membrane less permeable, why then would there be an increased release of α -amylase? It is my supposition that this conundrum puzzled different researchers leading eventually to the realization that Ca^{2+} specifically stimulated the process of enzyme secretion (Jones and Jacobsen, 1983), an idea that stands as a paradigm today in both plant and animal systems (Zorec and Tester, 1992).

Ca^{2+} and Plant Growth Regulators

In addition to a potential interaction between gibberellic acid and Ca^{2+} , connections between this ion and other plant growth regulators were emerging. Thus, Ca^{2+} was seen to enhance the ability of cytokinin to retard senescence (Poovaiah and Leopold, 1973b) and leaf abscission (Poovaiah and Leopold, 1973a) and to

promote cotyledon expansion (Leopold et al., 1974). Ca^{2+} was also found to inhibit cytokinin stimulation of anthocyanin (Elliott, 1977) and betacyanin synthesis (Elliott, 1979). Yet other studies identified a Ca^{2+} /cytokinin/ethylene connection, although there was disagreement between published reports on the nature of the interaction. Lau and Yang (1975), in studies on mung bean hypocotyl segments, reported that kinetin greatly stimulated uptake of $^{45}\text{Ca}^{2+}$, and Ca^{2+} stimulated the uptake and metabolism of kinetin ^{14}C . Of particular note, both Ca^{2+} and kinetin caused a striking increase in ethylene (Lau and Yang, 1975). By contrast, Poovaiah and Leopold (1973a), in studies of leaf abscission in bean petiole explants, found that Ca^{2+} inhibited ethylene production. Quite apart from studies of higher plants, LeJohn and coworkers (1973) identified a Ca^{2+} binding cell surface glycoprotein in the oomycetes, *Achlya* and *Blastocladiella*, which released Ca^{2+} when challenged with cytokinin. They postulated that cytokinin stimulates the availability and uptake of Ca^{2+} , thus promoting metabolism (LeJohn et al., 1973).

Although an early idea concerning a Ca^{2+} /auxin interaction has already been discussed and largely dismissed, especially to the extent that these agents affect wall structure and expansion, nevertheless, there were physiological processes suggesting a possible coordination in their activity. For example, auxin transport was reduced by low $[\text{Ca}^{2+}]_e$ (EDTA) (DeLa Fuente and Leopold, 1973), whereas auxin-induced proton secretion was stimulated by Ca^{2+} (Rubinstein et al., 1977). The pervasiveness of Ca^{2+} activity led Leopold (1977) to open a summary article with the statement that, "Actions of each of the plant growth hormones can be altered by calcium salts...." Despite these many encouraging leads, the idea of Ca^{2+} as a signaling agent does not directly emerge from these studies. With stimulatory effects being caused by millimolar changes in the $[\text{Ca}^{2+}]_e$, it would be nearly impossible to derive a sense about the Ca^{2+} status in the cytosol. Nevertheless, an awakening was taking place, and one that emphasized the importance of Ca^{2+} over Mg^{2+} , and the

monovalent cations, as a regulator of plant growth and development.

Ca^{2+} and Light

Well before the demonstration that photosynthesis lowers the $[\text{Ca}^{2+}]_i$ in *Nitellopsis* (Miller and Sanders, 1987), a Ca^{2+} /light interaction had been noted in different organisms. The red/far-red reversible phytochrome pigment system was a common focus of attention. Among the early studies, note is made of phytochrome stimulated nyctinastic leaf movement in *Albizia*, which was shown to be inhibited by low $[\text{Ca}^{2+}]$ as generated by culture in EDTA (McEvoy and Koukkari, 1972). Ca^{2+} was found to markedly enhance the photoreversible red light-induced depolarization of the membrane potential in *Nitella* (Weisenseel and Ruppert, 1977). A photoreversible red light-induced increase in Ca^{2+} efflux was noted in oat coleoptiles (Hale and Roux, 1980). Red light, provided as a microbeam to filaments of *Mougeotia*, was also shown to stimulate a 2- to 10-fold increase in the uptake of $^{45}\text{Ca}^{2+}$ (Dreyer and Weisenseel, 1979). Finally, red light-stimulated chloroplast rotation in *Mougeotia* was reduced simply through elimination of Ca^{2+} from the culture medium (Wagner and Klein, 1978). Based on these early studies and also influenced by Rasmussen (1970), Haupt and Weisenseel (1976) suggested that phytochrome molecules located in the plasma membrane function as Ca^{2+} carriers and facilitate an increase in the $[\text{Ca}^{2+}]_i$ when irradiated with red light. Their results supported the idea that Ca^{2+} would control contractile proteins within the cell, and thus the movement of chloroplasts, as demonstrated in *Mougeotia* (Haupt and Weisenseel, 1976). Although most current research on phytochrome is not directed toward Ca^{2+} , a potential role remains likely, given the identification of SUB1, a Ca^{2+} binding protein involved with both cryptochrome and phytochrome (Guo et al., 2001).

Ca^{2+} HOMEOSTASIS: PUMPS AND CHANNELS IN PLANTS

Ca^{2+} and Mitochondria

Given the enormous disparity in $[\text{Ca}^{2+}]$ between the cytosol (0.1 μM) and the

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outside medium or storage compartments (0.1 to 10 mM), it became obvious that the cell must exert extremely close control over the movement of the ion. However, even before the disparity in $[Ca^{2+}]$ was fully appreciated, early studies revealed that certain organelles, in particular mitochondria, exhibited the ability to take up large quantities of Ca^{2+} . Initial studies conducted in the late fifties and early sixties on mitochondria derived from different animal cells (e.g., liver and kidney) revealed that Ca^{2+} sequestration depended on respiration, was sensitive to uncouplers, and required inorganic phosphate (for review, see Carafoli et al., 2001). Also in the sixties, Hodges and Hanson (1965) demonstrated that maize mitochondria, like their animal cell counterparts, actively participate in the uptake and accumulation of Ca^{2+} . These pioneering studies revealed that Ca^{2+} uptake requires respiration or the addition of ATP and is blocked by uncoupling agents. That the process of Ca^{2+} uptake by plant mitochondria was broadly expressed was shown by Chen and Lehninger (1973), who examined and compared the activity of these organelles from 14 different species of higher plants and fungi. Although all mitochondria actively sequestered Ca^{2+} , those from sweet potato and white potato tubers were particularly active and comparable to those from rat liver. Additional work also established that plant mitochondria, like those from animal sources, exhibited greater uptake capacity but lower affinity than the similar process in microsomes (Dieter and Marmé, 1980, 1983). These observations led to the conclusion that mitochondria, while capable of taking up large amounts of ion, would not be the organelle involved in establishing the low basal $[Ca^{2+}]_i$.

A further intriguing observation was the sensitivity of mitochondrial Ca^{2+} uptake to light. Roux et al. (1981) noted that the $[Ca^{2+}]$ in the culture medium surrounding isolated oat mitochondria increased when the preparation was irradiated with red light. Because the change in the $[Ca^{2+}]$ was prevented by subsequent exposure to far-red light, phytochrome was implicated. Additional studies with ruthenium red, which blocks sequestration, led them to

favor the idea that red light, rather than blocking influx, stimulated efflux of Ca^{2+} (Roux et al., 1981). Although further studies at this time (Dieter and Marmé, 1983; Yamaya et al., 1984) failed to agree with the details provided by Roux et al. (1981), they nevertheless supported the basic tenet that Ca^{2+} uptake by plant mitochondria is in part regulated by light.

An important finding concerning basic mitochondrial metabolism was the discovery that at least one associated enzyme, namely NADH dehydrogenase, was regulated by Ca^{2+} . The early studies of Miller et al. (1970) and Coleman and Palmer (1971), using EDTA, directed attention toward Ca^{2+} , but the later study of Møller et al. (1981), using both EDTA and EGTA, definitively established a role for Ca^{2+} and not for Mg^{2+} . It appeared from these studies that plant mitochondria not only were able to sequester Ca^{2+} , but that the ion, through the control of NADH oxidation, participated in regulation of mitochondrial function. In light of these various issues, the mitochondrial/ Ca^{2+} connection in plants deserves renewed attention as emphasized recently by Hetherington and Brownlee (2004). The idea of both plant and animal mitochondria being viewed only as a safety valve capable of responding to a vast overload of Ca^{2+} is being challenged in studies of animal cell mitochondria. Evidence is emerging that mitochondria respond to relatively small changes in the $[Ca^{2+}]_i$ through spatial juxtaposition with the endoplasmic reticulum (Rizzuto et al., 1994; Carafoli et al., 2001). Similarly, plant mitochondria may be playing a more central role in cytosolic Ca^{2+} regulation than has been appreciated heretofore.

Ca^{2+} and Chloroplasts

Chloroplasts also were seen to participate in the uptake of Ca^{2+} . In 1964, Nobel and Packer, drawing parallels with studies on mitochondria, demonstrated that isolated spinach chloroplasts sequestered Ca^{2+} when irradiated with light and supplemented with ATP. Several years later, when it was apparent that plant cells maintained very low $[Ca^{2+}]_i$, the role of the chloroplast in the regulation of this ion

received further attention. Different laboratories using chloroplasts from wheat (Muto et al., 1982) and spinach (Kreimer et al., 1985a, 1985b) confirmed the dramatic light-dependent uptake of Ca^{2+} . Kreimer et al. (1985a, 1985b) further concluded that photosynthetic electron transport was essential and that it was the membrane potential rather than pH that drove Ca^{2+} uptake.

A particular interest in the role of Ca^{2+} in the chloroplast centered on the regulation of NAD kinase. Previous discussion has emphasized the role these studies played in the discovery of calmodulin in plants (Muto and Miyachi, 1977; Anderson and Cormier, 1978). Considerable complexity surrounded this problem because reports arose showing both calmodulin-dependent and -independent forms of the enzyme and both cytoplasmic and organellar location (Moore and Åkerman, 1984). Nevertheless, it seemed apparent that at least some of the NAD kinase, which was associated with chloroplasts, was Ca^{2+} dependent and light activated (Muto et al., 1982). With the further finding that calmodulin was localized in chloroplast stroma, Jarrett et al. (1982) suggested that light stimulated the uptake of Ca^{2+} and that the Ca^{2+} /calmodulin complex activated NAD kinase. The product, NADP, then served its important function as the terminal electron acceptor for photosystem I.

Ca^{2+} and Microsomes

From a historical point of view, our appreciation of a microsomal Ca^{2+} sequestration system is the most recent, although in many ways it emerges as the most important in regulating the basal $[Ca^{2+}]_i$ (Evans, 1998; Sze et al., 2000). Researchers examining animal cells, in particular those working on muscle, made early and important progress on the identification of an ATPase on the sarcoplasmic reticulum that drove the uptake of Ca^{2+} against a concentration gradient (MacLennan and Wong, 1971). Plants may not have such a hypertrophied system for Ca^{2+} uptake, nevertheless early studies provided evidence that microsomal vesicles were capable of Ca^{2+} uptake. Already in their landmark study on

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mitochondria, Hodges and Hanson (1965) made reference to preliminary work showing Ca^{2+} uptake in a microsome preparation from etiolated maize seedlings. However, it was Gross and Marmé (1978) who demonstrated the presence of Mg^{2+} /ATP-dependent Ca^{2+} uptake in microsomes of maize, squash, oats, and mustard, suspension cells of parsley, and the alga *Cryptomonas*. Although the membrane fraction was not fully identified, the studies were consistent with it being derived from the plasma membrane. A more definitive identification of plasma membrane activity derives from studies of *Neurospora*, in which Ca^{2+} accumulation in inverted vesicles depended on a Ca^{2+} / H^{+} antiporter (Stroobant and Scarborough, 1979).

A major problem in these early studies concerned the identification of the source of the membrane vesicles. For example, Dieter and Marmé (1981) provided evidence for a calmodulin-dependent Ca^{2+} -ATPase; however, they were not able to identify the membrane. Rasi-Caldogno et al. (1982) isolated two nonmitochondrial membrane fractions, the heavier of which appeared to be a Ca^{2+} -ATPase and the lighter one a Ca^{2+} / H^{+} -antiporter. Studies in which the source of the membrane system was defined included those of Kubowicz et al. (1982), who isolated a plasma membrane enriched fraction that was active in the sequestration of Ca^{2+} . Of further note here was the observation that auxin promoted Ca^{2+} uptake, whereas cytokinin was inhibitory. Endoplasmic reticulum vesicles were isolated by Buckhout (1984), who demonstrated high affinity Ca^{2+} accumulation that was not dependent on calmodulin. Finally, attention is directed toward the study of Schumaker and Sze (1985), who carefully isolated and identified two membrane fractions from oat roots. The more prominent one derived from the vacuole and the less prominent one from the endoplasmic reticulum. Both fractions sequestered Ca^{2+} , with vacuolar membranes appearing to use a Ca^{2+} / H^{+} -antiporter and the endoplasmic reticulum membranes employing a Ca^{2+} -ATPase. In conclusion, it can be seen that studies conducted between the mid sixties and the mid

eighties and to the present day (Sze et al., 2000) established that plants possess a rich and multifaceted mechanism for Ca^{2+} sequestration.

Ca^{2+} Influx

In parallel with the concept of uptake and sequestration, which lowers $[\text{Ca}^{2+}]_i$, is the equally important matter of entry or release, which raises the $[\text{Ca}^{2+}]_i$. Given the huge concentration gradient, which may be in the order of 1000- to 10,000-fold, together with a substantial charge gradient, which may be -100 mV or more, there is an enormous combined force that will drive Ca^{2+} into the cell. It follows that only a few Ca^{2+} channels may be required to impart a rapid increase in the $[\text{Ca}^{2+}]_i$. The activity of Ca^{2+} channels at the whole cell level is shown directly in the previously cited work by Williamson and Ashley (1982), who recorded a sharp $[\text{Ca}^{2+}]_i$ rise immediately after the action potential in *Nitella* and *Chara* (Figure 2). The Characean algae were also used to demonstrate the activity of single Ca^{2+} channels (Berestovsky et al., 1976); however, this is a topic that has exploded in more recent years (for review, see Tester, 1990; Hetherington and Brownlee, 2004).

CONCLUSIONS

Beginning in the sixties and extending through the seventies to the early eighties, several lines of investigation were being followed by plant biologists that were consistent with the notion that Ca^{2+} is a crucial cellular regulator. The stage was set, and as a result of these pioneering efforts there was a virtual explosion of work in the eighties (Hepler and Wayne, 1985; Trewavas, 1986; Kauss, 1987) (Figure 4) and to the present day that continues to define and characterize the enormous role that Ca^{2+} plays in the regulation of plant growth and development. To some degree, studies on plants were impeded by the presence of a cell wall, which provides an enormous reservoir for Ca^{2+} . With its own requirements being very high ($10 \mu\text{M}$ to 10mM), the $[\text{Ca}^{2+}]_e$ in the wall would easily swamp the relatively trivial amounts seen in the cytosol (0.1 to $10 \mu\text{M}$). But as small as the concentration is, Ca^{2+}_i clearly has a powerful impact on a host of growth and developmental processes.

The extensive involvement of Ca^{2+} frequently leads to the vexing question: how can one ion control so many events? The answer is not entirely known, but the broad framework for its solution can be drawn. In

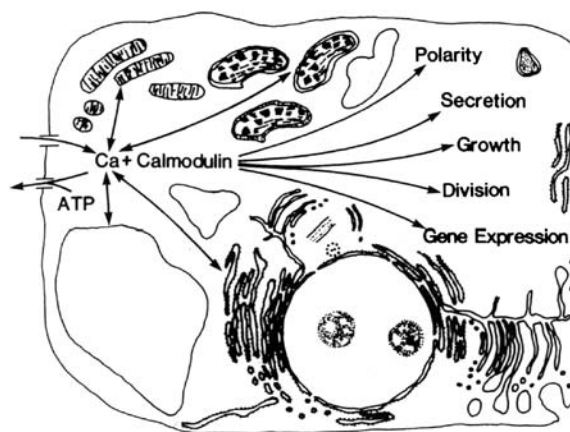


Figure 4. Diagram Depicting the Role of Ca^{2+} as a Signaling Agent in Plant Cells Published in 1985/86 (Trewavas, 1986).

Although some important features of Ca^{2+} signaling were not known at this time (e.g., the existence of CDPKs), the figure nevertheless represents and anticipates the central role that Ca^{2+} plays in many aspects of plant growth and development. (Figure courtesy of Trewavas, 1986, frontispiece figure, with permission of Springer Science and Business Media.)

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brief, Ca^{2+} regulation in plants is richly endowed with many components that can define and adjust responses in both time and space (Reddy and Reddy, 2004). Influx channels on the plasma membrane and release channels from internal stores (endoplasmic reticulum, vacuole, and mitochondria) provide several ways to generate rapid ion elevations or create local gradients. Once the $[\text{Ca}^{2+}]_i$ has risen, there are then a wide variety of response factors, including both calmodulin-dependent kinases and notably the CDPKs, which will phosphorylate a response element and thus stimulate or inhibit an event or process. Finally, plants exhibit frequency as well as amplitude modulation, providing yet another means of generating signals that have unique properties (Evans et al., 2001; Holdaway-Clarke and Hepler, 2003). Because these different pathways can interact, the number of individual signatures multiplies. As noted by Reddy and Reddy (2004) from their analysis of the Arabidopsis proteome, there are ~700 known protein components that function at various stages of Ca^{2+} signaling. Plants thus possess myriad ways in which Ca^{2+} can operate as the intermediary in transducing the stimulus into the appropriate response. The challenge for the future lies in identifying and characterizing these many different events and processes.

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REFERENCES

- Anderson, J.M., and Cormier, M.J.** (1978). Calcium-dependent regulator of NAD kinase. *Biochem. Biophys. Res. Commun.* **84**, 595–602.
- Baker, D.B., and Ray, P.M.** (1965). Direct and indirect effects of auxin on cell wall synthesis in oat coleoptile tissue. *Plant Physiol.* **40**, 345–352.
- Baker, P.F., and Warner, A.E.** (1972). Intracellular calcium and cell cleavage in early embryos of *Xenopus laevis*. *J. Cell Biol.* **53**, 579–581.
- Barry, W.H.** (1968). Coupling of excitation and cessation of cyclosis in *Nitella*: Role of divalent cations. *J. Cell. Physiol.* **72**, 153–159.
- Bennet-Clark, T.A.** (1956). Salt accumulation and mode of action of auxin. A preliminary hypothesis. In *Chemistry and Mode of Action of Plant Growth Substances*, R.L. Wain and F. Wightman, eds (London: Butterworths), pp. 284–291.
- Berestovsky, G.N., Vostrikov, I.Y., and Lunevsky, V.Z.** (1976). Ionic channels of the tonoplast of the cells of Charophyte algae. Role of calcium ions in excitation. *Biofizika* **21**, 829–833.
- Bothwell, J.H.F., and Ng, C.K.-Y.** (2005). The evolution of Ca^{2+} signalling in photosynthetic eukaryotes. *New Phytol.* **166**, 21–38.
- Bozler, E.** (1954). Relaxation in extracted muscle fibers. *J. Gen. Physiol.* **38**, 149–159.
- Brewbaker, J.L., and Kwack, B.H.** (1963). The essential role of calcium ion in pollen germination and pollen tube growth. *Am. J. Bot.* **50**, 859–865.
- Brown, J.E., and Blinks, J.R.** (1974). Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. Detection with aequorin. *J. Gen. Physiol.* **64**, 643–665.
- Buckhout, T.J.** (1984). Characterization of Ca^{2+} transport in purified endoplasmic reticulum membrane vesicles from *Lepidium sativum* L. roots. *Plant Physiol.* **76**, 962–967.
- Burling, E., and Jackson, W.T.** (1965). Changes in calcium levels in cell walls during elongation of oat coleoptile sections. *Plant Physiol.* **40**, 138–141.
- Burstrom, H.G.** (1968). Calcium and plant growth. *Biol. Rev. (Camb.)* **43**, 287–316.
- Carafoli, E., Santella, L., Branca, D., and Brini, M.** (2001). Generation, control, and processing of cellular calcium signals. *Crit. Rev. Biochem. Mol. Biol.* **36**, 107–206.
- Chen, C.-H., and Lehninger, A.L.** (1973). Ca^{2+} transport activity in mitochondria from some plant tissues. *Arch. Biochem. Biophys.* **157**, 183–196.
- Cheng, S.H., Willmann, M.R., Chen, H.C., and Sheen, J.** (2002). Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. *Plant Physiol.* **129**, 469–485.
- Cheung, W.Y.** (1970). Cyclic 3',5'-nucleotide phosphodiesterase. Demonstration of an activator. *Biochem. Biophys. Res. Commun.* **38**, 533–538.
- Cheung, W.Y.** (1980). Calmodulin plays a pivotal role in cellular-regulation. *Science* **207**, 19–27.
- Chrispeels, M.J., and Varner, J.E.** (1967). Gibberellic acid-enhanced synthesis and release of α -amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiol.* **42**, 398–406.
- Cleland, R.** (1960). Effect of auxin upon loss of calcium from cell walls. *Plant Physiol.* **35**, 581–584.
- Cleland, R., and Rayle, D.L.** (1977). Reevaluation of the effect of calcium ions on auxin-induced elongation. *Plant Physiol.* **60**, 709–712.
- Coleman, J.O.D., and Palmer, J.M.** (1971). Role of Ca^{2+} in the oxidation of exogenous NADH by plant mitochondria. *FEBS Lett.* **17**, 203–208.
- DeLa Fuente, R.K., and Leopold, A.C.** (1973). A role for calcium in auxin transport. *Plant Physiol.* **51**, 845–847.
- Dickinson, D.B.** (1967). Permeability and respiration properties of germinating pollen. *Physiol. Plant.* **20**, 118–127.
- Dieter, P., and Marmé, D.** (1980). Ca^{2+} transport in mitochondria and microsome fractions from higher plants. *Planta* **150**, 1–8.
- Dieter, P., and Marmé, D.** (1981). Far-red light irradiation of intact corn seedlings affects mitochondrial and calmodulin dependent Ca^{2+} transport. *Biochem. Biophys. Res. Commun.* **101**, 749–755.
- Dieter, P., and Marmé, D.** (1983). The effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and microsomal calcium-ion transport system from corn. *Planta* **159**, 277–281.
- Dreyer, E.M., and Weisenseel, M.H.** (1979). Phytochrome-mediated uptake of calcium in *Mougeotia* cells. *Planta* **146**, 31–39.

HISTORICAL PERSPECTIVE ESSAY

- Elliott, D.C.** (1977). Induction by EDTA of anthocyanin synthesis in *Spirodela oligorrhiza*. *Aust. J. Plant Physiol.* **4**, 39–49.
- Elliott, D.C.** (1979). Ionic regulation for cytokinin-dependent betacyanine synthesis in *Amaranthus* seedlings. *Plant Physiol.* **63**, 264–268.
- Epstein, E.** (1961). The essential role of calcium in selective cation transport by plant cells. *Plant Physiol.* **36**, 437–444.
- Epstein, E.** (1972). *Mineral Nutrition of Plants: Principles and Perspectives.* (New York: John Wiley & Sons).
- Ettienne, E.M.** (1970). Control of contractility in *Spirostomum* by dissociated calcium ions. *J. Gen. Physiol.* **56**, 168–179.
- Evans, D.E.** (1998). Calcium in plants. In *Calcium as a Cellular Regulator*, E. Carafoli and C. Klee, eds (Oxford: Oxford University Press), pp. 417–440.
- Evans, N.H., McAinsh, M.R., and Hetherington, A.M.** (2001). Calcium oscillations in higher plants. *Curr. Opin. Plant Biol.* **4**, 415–420.
- Felle, H.** (1988). Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. *Planta* **174**, 495–499.
- Gilkey, J.C., Jaffe, L.F., Ridgway, E.B., and Reynolds, G.T.** (1978). A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. *J. Cell Biol.* **76**, 448–466.
- Gilroy, A., Hughes, W.A., and Trewavas, A.J.** (1986). The measurement of intracellular calcium levels in protoplasts from higher plant cells. *FEBS Lett.* **199**, 217–221.
- Gross, J., and Marmé, D.** (1978). ATP-dependent Ca^{2+} uptake into plant membrane vesicles. *Proc. Natl. Acad. Sci. USA* **75**, 1232–1236.
- Gryniewicz, G., Poenie, M., and Tsien, R.Y.** (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450.
- Guo, H., Mockler, T., Duong, H., and Lin, C.** (2001). SUB1, an *Arabidopsis* Ca^{2+} -binding protein involved in cryptochrome and phytochrome coaction. *Science* **291**, 487–490.
- Hale, C.C., and Roux, S.J.** (1980). Photoreversible calcium fluxes induced by phytochrome in oat coleoptile cells. *Plant Physiol.* **65**, 658–662.
- Han, S., Tang, R., Anderson, L.K., Woerner, T.E., and Pei, Z.-M.** (2003). A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells. *Nature* **425**, 196–200.
- Hanson, J.B.** (1960). Impairment of respiration, ion accumulation, and ion retention in root tissue treated with ribonuclease and ethylenediamine tetraacetic acid. *Plant Physiol.* **35**, 372–379.
- Hanson, J.B.** (1984). The functions of calcium in plant nutrition. In *Advances in Plant Nutrition*, P.B. Tinker and A. Lauchli, eds (New York: Praeger Publishers), pp. 149–208.
- Harmon, A.C., Gribskov, M., Gubrium, E., and Harper, J.F.** (2001). The CDPK superfamily of protein kinases. *New Phytol.* **151**, 175–183.
- Harmon, A.C., Putnam-Evans, C., and Cormier, M.J.** (1987). A calcium-dependent but calmodulin-independent protein kinase from soybean. *Plant Physiol.* **83**, 830–837.
- Harper, J.F., Breton, G., and Harmon, A.** (2004). Decoding Ca^{2+} signals through plant protein kinases. *Annu. Rev. Plant Biol.* **55**, 263–288.
- Haupt, W., and Weisenseel, M.H.** (1976). Physiological evidence and some thoughts on localized responses, intracellular localization and action of phytochrome. In *Light and Plant Development*, H. Smith, ed (London: Butterworths), pp. 63–74.
- Heilbrunn, L.V.** (1940). The action of calcium on muscle protoplasm. *Physiol. Zool.* **13**, 88–94.
- Heilbrunn, L.V., and Wiercinski, F.J.** (1947). The action of various cations on muscle protoplasm. *J. Cell Comp. Physiol.* **29**, 15–32.
- Hepler, P.K.** (1985). Calcium restriction prolongs metaphase in dividing *Tradescantia* stamen hair cells. *J. Cell Biol.* **100**, 1363–1368.
- Hepler, P.K.** (1989). Calcium transients during mitosis: Observations in flux. *J. Cell Biol.* **109**, 2567–2573.
- Hepler, P.K., and Callahan, D.A.** (1987). Free calcium increases during anaphase in stamen hair cells of *Tradescantia*. *J. Cell Biol.* **105**, 2137–2143.
- Hepler, P.K., and Palevitz, B.A.** (1974). Microtubules and microfilaments. *Annu. Rev. Plant Physiol.* **25**, 309–362.
- Hepler, P.K., and Wayne, R.O.** (1985). Calcium and plant development. *Annu. Rev. Plant Physiol.* **36**, 379–439.
- Hepler, P.K., Wick, S.M., and Wolniak, S.M.** (1981). The structure and role of membranes in the mitotic apparatus. In *International Cell Biology 1980–1981*, H.G. Schweiger, ed (Berlin: Springer-Verlag), pp. 673–686.
- Hetherington, A., and Trewavas, A.** (1982). Calcium-dependent protein kinase in pea shoot membrane. *FEBS Lett.* **145**, 67–71.
- Hetherington, A.M., and Brownlee, C.** (2004). The generation of Ca^{2+} signals in plants. *Annu. Rev. Plant Biol.* **55**, 401–427.
- Hirschi, K.D.** (2004). The calcium conundrum. Both versatile nutrient and specific signal. *Plant Physiol.* **136**, 2438–2442.
- Hodges, T.K., and Hanson, J.B.** (1965). Calcium accumulation in maize mitochondria. *Plant Physiol.* **40**, 101–109.
- Hodgkin, A.L., and Keynes, R.D.** (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.
- Holdaway-Clarke, T.L., and Hepler, P.K.** (2003). Control of pollen tube growth: Role of ion gradients and fluxes. *New Phytol.* **159**, 539–563.
- Jaffe, L.A., Weisenseel, M.H., and Jaffe, L.F.** (1975). Calcium accumulations within the growing tips of pollen tubes. *J. Cell Biol.* **67**, 488–492.
- Jaffe, L.F., Robinson, K.R., and Nuccitelli, R.** (1974). Local cation entry and self-electrophoresis as an intracellular localization mechanism. *Ann. N. Y. Acad. Sci.* **238**, 372–389.
- Jansen, E.F., Jang, R., Albersheim, P., and Bonner, J.** (1960). Pectic metabolism of growing cell walls. *Plant Physiol.* **35**, 87–97.
- Jarrett, H.W., Brown, C.J., Black, C.C., and Cormier, M.J.** (1982). Evidence that calmodulin is in the chloroplast of peas and serves a regulatory role in photosynthesis. *J. Biol. Chem.* **257**, 13795–13804.
- Jones, R.L.** (1973). Gibberellic acid and ion release from barley aleurone tissue. Evidence for hormone-dependent ion transport capacity. *Plant Physiol.* **52**, 303–308.
- Jones, R.L., and Jacobsen, J.V.** (1983). Calcium regulation of the secretion of α -amylase isoenzymes and other proteins from barley aleurone layers. *Planta* **158**, 1–9.
- Kakiuchi, S., and Yamazaki, R.** (1970). Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. Studies on cyclic 3',5'-nucleotide phosphodiesterase III. *Biochem. Biophys. Res. Commun.* **41**, 1104–1110.
- Kauss, H.** (1987). Some aspects of calcium-dependent regulation in plant metabolism. *Annu. Rev. Plant Physiol.* **38**, 47–72.
- Kikuyama, M., and Tazawa, M.** (1983). Transient increase of intracellular Ca^{2+} during excitation on tonoplast-free *Chara* cells. *Protoplasma* **117**, 62–67.
- Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J.** (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**, 524–526.
- Knight, M.R., Read, N.D., Campbell, A.K., and Trewavas, A.J.** (1993). Imaging calcium dynamics in living plants using semi-synthetic recombinant aequorins. *J. Cell Biol.* **121**, 83–90.
- Kreimer, G., Melkonian, M., Holtrum, J.A.M., and Latzko, E.** (1985b). Characterization of calcium fluxes across the envelope of intact spinach chloroplasts. *Planta* **166**, 515–523.

HISTORICAL PERSPECTIVE ESSAY

- Kreimer, G., Melkonian, M., and Lutzko, E.** (1985a). An electrogenic uniport mediates light-dependent Ca^{2+} influx into intact spinach chloroplasts. *FEBS Lett.* **180**, 253–258.
- Kubowicz, B.D., Vanderhoef, L.N., and Hanson, J.B.** (1982). ATP-dependent calcium transport in plasmalemma preparations from soybean hypocotyls. *Plant Physiol.* **69**, 187–191.
- Kwack, B.H.** (1967). Studies on cellular site of calcium action in promoting pollen growth. *Physiol. Plant.* **20**, 825–833.
- Lau, O.L., and Yang, S.F.** (1975). Interaction of kinetin and calcium in relation to their effect on stimulation of ethylene production. *Plant Physiol.* **55**, 738–740.
- LeJohn, H.B., Stevenson, R.M., and Meuser, R.** (1973). Cytokinins and magnesium ions may control the flow of metabolites and calcium ions through fungal cell membranes. *Biochem. Biophys. Res. Commun.* **54**, 1061–1066.
- Leopold, A.C.** (1977). Modification of growth regulatory action with inorganic solutes. In *Plant Growth Regulators. Chemical Activity, Plant Responses, and Economic Potential*, C.A. Stutte, ed (Washington, D.C.: American Chemical Society), pp. 33–41.
- Leopold, A.C., Poovaiah, B.W., DeLa Fuente, R.K., and Williams, R.J.** (1974). Regulation of growth with inorganic solutes. In *Plant Growth Substances*, Y. Sumiki, ed (Tokyo: Hirokawa), pp. 780–788.
- Letham, D.S.** (1958). Maceration of plant tissues with ethylene-diamine-tetra-acetic acid. *Nature* **181**, 135–136.
- MacLennan, D.H., and Wong, P.T.S.** (1971). Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **68**, 1231–1235.
- Marinos, N.G.** (1962). Studies on submicroscopic aspects of mineral deficiencies. 1. Calcium deficiency in shoot apex of barley. *Am. J. Bot.* **49**, 834–841.
- McAinch, M.R., Brownlee, C., and Hetherington, A.M.** (1990). Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* **343**, 186–188.
- McEvoy, R.C., and Koukkari, W.L.** (1972). Effects of ethylenediaminetetraacetic acid, auxin and gibberellic acid on phytochrome controlled nyctinasty in *Albizia julibrissin*. *Physiol. Plant.* **26**, 143–147.
- Miller, A.J., and Sanders, D.** (1987). Depletion of cytosolic free calcium induced by photosynthesis. *Nature* **326**, 397–400.
- Miller, D.D., Callaham, D.A., Gross, D.J., and Hepler, P.K.** (1992). Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. *J. Cell Sci.* **101**, 7–12.
- Miller, R.J., Dumford, S.W., Koeppe, D.E., and Hanson, J.B.** (1970). Divalent cation stimulation of substrate oxidation by corn mitochondria. *Plant Physiol.* **45**, 649–653.
- Møller, I.M., Johnston, S.P., and Palmer, J.M.** (1981). A specific role for Ca^{2+} in the oxidation of exogenous NADH by Jerusalem-artichoke (*Helianthus tuberosus*) mitochondria. *Biochemistry* **194**, 487–495.
- Moore, A.L., and Åkerman, K.E.O.** (1984). Calcium and plant organelles. *Plant Cell Environ.* **7**, 423–429.
- Muto, S., Izawa, S., and Miyachi, S.** (1982). Light-induced Ca^{2+} uptake by intact chloroplasts. *FEBS Lett.* **139**, 250–254.
- Muto, S., and Miyachi, S.** (1977). Properties of a protein activator of NAD kinase from plants. *Plant Physiol.* **59**, 55–60.
- Nobel, P.S., and Packer, L.** (1964). Energy-dependent ion uptake in spinach chloroplasts. *Biochim. Biophys. Acta* **88**, 453–455.
- Obermeyer, G., and Weisenseel, M.H.** (1991). Calcium channel blocker and calmodulin antagonists affect the gradient of free calcium ions in lily pollen tubes. *Eur. J. Cell Biol.* **56**, 319–327.
- Polya, G.M., and Davies, J.R.** (1982). Resolution of Ca^{2+} -calmodulin-activated protein-kinase from wheat-germ. *FEBS Lett.* **150**, 167–171.
- Poovaiah, B.W., and Leopold, A.C.** (1973a). Inhibition of abscission by calcium. *Plant Physiol.* **51**, 848–851.
- Poovaiah, B.W., and Leopold, A.C.** (1973b). Deferral of leaf senescence with calcium. *Plant Physiol.* **52**, 236–239.
- Poovaiah, B.W., and Leopold, A.C.** (1976). Effects of inorganic salts on tissue permeability. *Plant Physiol.* **58**, 182–185.
- Portzehl, H., Ruegg, J.C., and Caldwell, P.C.** (1964). The dependence of contraction and relaxation of muscle fibers from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta* **79**, 581–591.
- Putnam-Evans, C., Harmon, A.C., and Cormier, M.J.** (1986). Calcium-dependent protein phosphorylation in suspension-cultured soybean cells. In *Molecular and Cellular Aspects Calcium in Plant Development*, A.J. Trewavas, ed (New York: Plenum), pp. 99–106.
- Ranjeva, R., Refeno, G., Boudet, A.M., and Marmé, D.** (1983). Activation of plant quinate NAD^+ 3-oxidoreductase by Ca^{2+} and calmodulin. *Proc. Natl. Acad. Sci. USA* **80**, 5222–5224.
- Rasi-Caldogno, F., de Michelis, M.I., and Pagliarello, M.C.** (1982). Active transport of Ca^{2+} in membrane vesicles from pea. Evidence for a $\text{H}^+/\text{Ca}^{2+}$ antiport. *Biochim. Biophys. Acta* **693**, 287–295.
- Rasmussen, H.** (1970). Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* **170**, 404–412.
- Rathore, K.S., Cork, R.J., and Robinson, K.R.** (1991). A cytoplasmic gradient of Ca^{2+} is correlated with the growth of lily pollen tubes. *Dev. Biol.* **148**, 612–619.
- Ray, P.M., and Baker, D.B.** (1965). The effect of auxin on synthesis of oat coleoptile cell wall constituents. *Plant Physiol.* **40**, 353–360.
- Reddy, V.S., and Reddy, A.S.N.** (2004). Proteomics of calcium-signaling components in plants. *Phytochemistry* **65**, 1745–1776.
- Reiss, H.-D., and Herth, W.** (1979). Calcium gradients in tip growing plant cells visualized by chlorotetracycline fluorescence. *Planta* **146**, 615–621.
- Reiss, H.-D., Herth, W., and Schnepf, E.** (1983). The tip-to-base calcium gradient in pollen tubes of *Lilium longiflorum* measured by proton-induced X-ray emission (PIXE). *Protoplasma* **115**, 153–159.
- Ridgway, E.B., and Ashley, C.C.** (1967). Calcium transients in single muscle fibers. *Biochem. Biophys. Res. Commun.* **29**, 229–234.
- Ridgway, E.B., and Durham, A.C.H.** (1976). Oscillations of calcium ion concentration in *Physarum polycephalum*. *J. Cell Biol.* **69**, 223–226.
- Ridgway, E.B., Gilkey, J.C., and Jaffe, L.F.** (1977). Free calcium increases explosively in activating medaka eggs. *Proc. Natl. Acad. Sci. USA* **74**, 623–627.
- Ringer, S.** (1883). A further contribution regarding the influence of different constituents of the blood on the contraction of the heart. *J. Physiol.* **4**, 29–43.
- Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M., and Pozzan, T.** (1994). Mitochondrial Ca^{2+} homeostasis in intact cells. *J. Cell Biol.* **126**, 1183–1194.
- Robinson, K.R.** (1977). Reduced external calcium and sodium stimulates calcium influx in *Pelvetia* eggs. *Planta* **136**, 153–158.
- Robinson, K.R., and Cone, R.** (1980). Polarization of Fucooid eggs by a calcium ionophore gradient. *Science* **207**, 77–78.
- Robinson, K.R., and Jaffe, L.F.** (1975). Polarizing Fucooid eggs drive a calcium current through themselves. *Science* **187**, 70–72.
- Roux, S.J., McEntire, K., Slocum, R.D., Cedel, T.E., and Hale, C.C.** (1981). Phytochrome induces photoreversible calcium fluxes in

HISTORICAL PERSPECTIVE ESSAY

- a purified mitochondrial-fraction from oats. Proc. Natl. Acad. Sci. USA **78**, 283–287.
- Rubinstein, B., Johnson, K.D., and Rayle, D.L.** (1977). Calcium-enhanced acidification in oat coleoptiles. In Regulation of Cell Membrane Activities in Plants, E. Marre and O. Ciferri, eds (Amsterdam: Elsevier/North Holland Biomedical Press), pp. 307–316.
- Saunders, M.J., and Hepler, P.K.** (1982). Calcium ionophore A23187 stimulates cytokinin-like mitosis in *Funaria*. Science **217**, 943–945.
- Schumaker, K.S., and Sze, H.** (1985). A $\text{Ca}^{2+}/\text{H}^{+}$ antiport system driven by the proton electrochemical gradient of a tonoplast H^{+} -ATPase from oat roots. Plant Physiol. **79**, 1111–1117.
- Shimomura, O., Johnson, F.H., and Saiga, Y.** (1963). Further data on the bioluminescent protein, aequorin. J. Cell. Comp. Physiol. **62**, 1–8.
- Simon, E.W.** (1978). Symptoms of calcium deficiency in plants. New Phytol. **80**, 1–15.
- Snedden, W.A., and Fromm, H.** (2001). Calmodulin as a versatile calcium signal transducer in plants. New Phytol. **151**, 35–66.
- Steinhardt, R., Zuker, R., and Schatten, G.** (1977). Intracellular calcium release at fertilization in the sea urchin egg. Dev. Biol. **58**, 185–196.
- Stroobant, P., and Scarborough, G.A.** (1979). Active transport of calcium in *Neurospora* plasma membrane vesicles. Proc. Natl. Acad. Sci. USA **76**, 3102–3106.
- Sutherland, E.W., and Rall, T.W.** (1958). Fractionation and characterization of a cyclin adenine ribonucleotide formed by tissue particles. J. Biol. Chem. **232**, 1077–1091.
- Sze, H., Liang, F., Hwang, I., Curran, A.C., and Harper, J.F.** (2000). Diversity and regulation of plant Ca^{2+} pumps: Insights from expression in yeast. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51**, 433–462.
- Tagawa, T., and Bonner, J.** (1957). Mechanical properties of the *Avena* coleoptile as related to auxin and to ionic interactions. Plant Physiol. **32**, 207–212.
- Takagi, S., and Nagai, R.** (1983). Regulation of cytoplasmic streaming in *Vallisneria* mesophyll cells. J. Cell Sci. **62**, 385–405.
- Tazawa, M., Kikuyama, M., and Shimmen, T.** (1976). Electrical characteristics and cytoplasmic streaming of *Characeae* cells lacking tonoplast. Cell Struct. Funct. **1**, 165–176.
- Tazawa, M., and Kishimoto, U.** (1968). Cessation of cytoplasmic streaming in *Chara* internodes during action potential. Plant Cell Physiol. **9**, 361–368.
- Teo, T.S., and Wang, J.H.** (1973). Mechanism of activation of a cyclic adenosine 3'-5'-monophosphate phosphodiesterase from bovine heart by calcium-ions: Identification of protein activator as a Ca^{2+} binding protein. J. Biol. Chem. **248**, 5950–5955.
- Tepfer, M., and Taylor, I.E.P.** (1981). The interaction of divalent cations with pectic substances and their influence on acid-induced cell wall loosening. Can. J. Bot. **59**, 1522–1525.
- Tester, M.** (1990). Plant ion channels: Whole-cell and single-channel studies. New Phytol. **114**, 305–334.
- Trewavas, A.J.** (1976). Post-translational modification of proteins by phosphorylation. Annu. Rev. Plant Physiol. **27**, 349–374.
- Trewavas, A.J.** (1986). Molecular and Cellular Aspects of Calcium in Plant Development. (New York: Plenum Press).
- Van Steveninck, R.F.M.** (1965). The significance of calcium on the apparent permeability of cell membranes and the effects of substitution with other divalent cations. Physiol. Plant. **18**, 54–69.
- Wagner, G., and Klein, K.** (1978). Differential effects of calcium on chloroplast movement in *Mougeotia*. Photochem. Photobiol. **27**, 137–140.
- Watterson, D.M., Iverson, D.B., and Van Eldik, L.J.** (1980). Spinach calmodulin: Isolation, characterization and comparison with vertebrate calmodulins. Biochemistry **19**, 5762–5768.
- Wayne, R., and Hepler, P.K.** (1984). The role of calcium ions in phytochrome-mediated germination of spores of *Onoclea sensibilis* L. Planta **160**, 12–20.
- Weisenberg, R.C.** (1972). Microtubule formation in vitro in solutions containing low calcium concentration. Science **177**, 1104–1105.
- Weisenseel, M.H., and Jaffe, L.F.** (1976). The major growth current through lily pollen tubes enters as K^{+} and leaves as H^{+} . Planta **133**, 1–7.
- Weisenseel, M.H., Nuccitelli, R., and Jaffe, L.F.** (1975). Large electrical currents traverse growing pollen tubes. J. Cell Biol. **66**, 556–567.
- Weisenseel, M.H., and Ruppert, H.K.** (1977). Phytochrome and calcium ions are involved in light-induced membrane depolarization in *Nitella*. Planta **137**, 225–229.
- White, P.J., and Broadley, M.R.** (2003). Calcium in plants. Ann. Bot. (Lond.) **92**, 487–511.
- Wick, S.M., and Hepler, P.K.** (1980). Localization of Ca^{++} -containing antimonate precipitates during mitosis. J. Cell Biol. **86**, 500–513.
- Williamson, R.E.** (1975). Cytoplasmic streaming in *Chara*: A cell model activated by ATP and inhibited by cytochalasin B. J. Cell Sci. **17**, 655–688.
- Williamson, R.E., and Ashley, C.C.** (1982). Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature **296**, 647–651.
- Wolniak, S.M., Hepler, P.K., and Jackson, W.T.** (1983). Ionic changes in the mitotic apparatus at the metaphase/anaphase transition. J. Cell Biol. **96**, 598–605.
- Wyn Jones, R.G., and Lunt, O.R.** (1967). The function of calcium in plants. Bot. Rev. **33**, 407–426.
- Yamaguchi, Y., and Nagai, R.** (1981). Motile apparatus in *Vallisneria* leaf cells. I. Organization of microfilaments. J. Cell Sci. **48**, 193–205.
- Yamaya, T., Oaks, A., and Matsumoto, H.** (1984). Stimulation of mitochondrial calcium uptake by light during growth in corn roots. Plant Physiol. **75**, 773–777.
- Zhang, D.H., Wadsworth, P., and Hepler, P.K.** (1992). Modulation of anaphase spindle microtubule structure in stamen hair cells of *Tradescantia* by calcium and related agents. J. Cell Sci. **102**, 79–89.
- Zhang, L., and Lu, Y.T.** (2003). Calmodulin-binding protein kinases in plants. Trends Plant Sci. **8**, 123–127.
- Zorec, R., and Tester, M.** (1992). Cytoplasmic calcium stimulates exocytosis in a plant secretory cell. Biophys. J. **63**, 864–867.