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# CALCIUM AND PLANT DEVELOPMENT

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"Our knowledge about $Ca^{2+}$ is still increasing; the more we learn about $Ca^{2+}$, the more impressed we are by a wide variety of the mode of action of $Ca^{2+}$. It is almost like life itself."

Setsuro Ebashi (55a)

## INTRODUCTION

A revolution is occurring in our thinking about the physiology and development of plants as we realize the importance of calcium ions ($Ca^{2+}$) in mediating many different processes. The function of $Ca^{2+}$ as a "second messenger" in



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animal cells has been acknowledged for years, but it is only recently that botanists have come to appreciate the concept that activities of plant cells are also regulated by $Ca^{2+}$. New $Ca^{2+}$ studies are appearing at a rapid rate, and it seems certain that the impact of these findings will be significant and felt widely. As an emerging area of inquiry, the contribution of $Ca^{2+}$ to plant development brings with it a sense of excitement and expectation. New ways of investigating old problems are made available and this gives us hope that fresh, clarifying insights will appear. The concepts of $Ca^{2+}$ function are being explored in depth by our colleagues studying animal systems, and this provides us with an important reference point. It seems likely that $Ca^{2+}$ activities in all eukaryotic cells will share basic properties. Nevertheless, crucial differences in $Ca^{2+}$ regulation between plant and animal cells may exist, and thus we must not hastily embrace the findings on animal cells until the key experiments on plant cells have been completed.

It is our intention in this review to capture the excitement that has been generated by $Ca^{2+}$ studies on plants, but at the same time to provide a measure of critical judgment concerning $Ca^{2+}$ methodology and interpretation. Modern reviews on the role of $Ca^{2+}$ in plants have appeared (32, 73, 108, 112, 141, 142, 193, 196); certain topics including plant calmodulin (38, 39, 253) and $Ca^{2+}$ ATPases (141, 142) have received authoritative treatment and accordingly will be mentioned only briefly here. There is a vast literature on the role of $Ca^{2+}$ in animal systems to which the reader is directed. Extensive reviews, books, and multivolume treatises have appeared that summarize virtually all known aspects of $Ca^{2+}$ chemistry and metabolism (2–4, 12, 14, 21, 24, 27–30, 62, 100, 101, 120, 182–184, 197, 222, 234, 240, 262).

## GENERAL MECHANISMS OF REGULATION INVOLVING A SECOND MESSENGER

The concept of a second messenger has been successful in explaining the mechanism of hormone and light action in animal cells (182). Cyclic AMP at first was proposed to be the second messenger for all hormone responses, and $Ca^{2+}$ the second messenger in muscle contraction, secretion, and egg activation. Through continued experimentation it became apparent that the participation of $Ca^{2+}$ was more widespread than had been thought originally. A universal $Ca^{2+}$ messenger system emerged with cyclic AMP as an additional system built, in part, upon its interaction with $Ca^{2+}$ (181, 182). Although cAMP, adenylate cyclase, and phosphodiesterase have been found in plants, a cAMP-dependent protein kinase, the only known physiologically important receptor of cAMP, has never been found (16). Furthermore, cAMP has never been shown to be required for any physiological response. Therefore, $Ca^{2+}$ ions alone may contribute to the coupling of stimulus to response in plants.

The general mechanism by which $Ca^{2+}$ modulates a response is through a change in its concentration. At an elevated level $Ca^{2+}$ binds either directly to a protein-response element or it binds to the modulator protein calmodulin (CaM). In the second example the $Ca^{2+}$-CaM complex binds to the response element. In either example a conformational change is induced in the response element that causes activation and allows for the subsequent reactions that comprise the response. The magnitude of the response is proportional to the concentration of the $Ca^{2+}$-(CaM)-response element complex and can be regulated by the $[Ca^{2+}]$ or by changing the affinity of the receptor protein or the response element to $Ca^{2+}$. These have been referred to as "amplitude" and "sensitivity" modulation (183).

Amplitude modulation by the $Ca^{2+}$-messenger system involves the interaction of an agonist with the plasma membrane (PM) and the opening of channels that allow an increase in the rate of $Ca^{2+}$ influx. The external space is the most common source of $Ca^{2+}$ from which the ion may enter the cell through channels (82, 205, 206, 246). However, internal compartments such as the endoplasmic reticulum (ER) and vacuole (203, 220, 250, 265, 275) as well as mitochondria (195) may also provide a store of $Ca^{2+}$ that can be released either through the direct action of the primary stimulus, e.g. light (195), or indirectly through $Ca^{2+}$-induced $Ca^{2+}$ release (61). The result is an increase in the intracellular $[Ca^{2+}]$ from approximately 0.1 μM to 1–10 μM, which is sufficient to activate a response element (169, 241, 272, 273).

Amplitude modulation is an adequate mechanism for controlling cell function when the response is transient. However, because of the cytotoxicity of prolonged exposures to high levels of $Ca^{2+}$, it would not be appropriate for the modulation of sustained responses. Mitochondria, for example, can only accumulate activating levels of $Ca^{2+}$ for a few hours before they are filled to capacity. This problem has been resolved in animal cells through the mechanism of sensitivity modulation where the concentration of the $Ca^{2+}$-receptor-response element increases (thus the response), without a concomitant increase in the intracellular $[Ca^{2+}]$.

Sensitivity modulation is achieved in a number of animal cells through allosteric control. In many cell types, the binding of an agonist results in an increase in the activity of phospholipase *c*, an enzyme that catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate to diacylglycerol and inositol triphosphate. Diacylglycerol binds directly to a $Ca^{2+}$-dependent protein kinase (*c* kinase) and increases its affinity for $Ca^{2+}$ so that the enzyme is essentially fully activated at resting levels of $Ca^{2+}$ (0.1 μM). Experimental studies show, however, that changes in $[Ca^{2+}]$ are also involved, and thus sensitivity modulation is not completely separate from amplitude modulation (183, 184).

Another mode of sensitivity modulation is through the covalent modification

of response elements. Phosphorylation of response elements by protein kinases is a widespread mechanism for the regulation of enzyme reactions (36, 238) and can provide either positive or negative sensitivity modulation. The phosphorylation of phosphorylase *b* kinase, for example, increases its affinity for $Ca^{2+}$ so that the half maximal [$Ca^{2+}$] required for activation is lowered from 3 μM to 0.3 μM. The phosphorylation of myosin light chain kinase (MLCK) is an example of negative sensitivity modulation in which P-MLCK exhibits a lower affinity for $Ca^{2+}$ ($K_{0.5}$ from 0.8 μM to 8.0 μM). When dephosphorylated, MLCK phosphorylates the myosin light chain that allows the interaction of actin with myosin and consequently contraction in many smooth muscle and nonmuscle systems. The protein kinases that are involved with the phosphorylation of MLCK and phosphorylase *b* kinase are cAMP-dependent. A similar pathway in plants, though, would not have to include a cAMP-dependent protein kinase, since a protein kinase that can change the affinity of another response element upon phosphorylation to $Ca^{2+}$ would be sufficient.

$Ca^{2+}$-dependent protein kinases have also been identified in plants. Some are soluble (171–173, 243, 244) and some are associated with membrane fractions (89, 90, 123, 174, 199, 243, 244). However, except for quinate:$NAD^+$ oxidoreductase (67, 180, 186) and $H^+$-ATPase (281), it is not known what proteins these $Ca^{2+}$-dependent protein kinases phosphorylate. The $K_m$s of these enzymes with respect to $Ca^{2+}$ are all in the micromolar and submicromolar ranges expected for cytosolic $Ca^{2+}$ regulation (89, 174, 199). There are at least three different classes of protein kinases found in plants (90), one $Ca^{2+}$-independent, one $Ca^{2+}$-dependent, and one $Ca^{2+}$-CaM-dependent. Since the "CaM-dependent" protein kinases require nonspecific levels of CaM-inhibiting phenothiazines to cause inhibition (89, 90, 123, 174, 199, 243), and the addition of CaM only modestly stimulates protein kinase activity, the involvement of CaM has been questioned. This problem will be resolved when individual protein kinases are isolated and endogenous CaM removed and identified. No $Ca^{2+}$-dependent protein phosphatases have been found as yet. In the future, protein kinases will probably prove to be an extremely common and important response element involved in the amplification cascade in $Ca^{2+}$-mediated cellular responses to external stimuli.

Although cAMP-dependent pathways of regulation may not occur in higher plants, it would seem likely that those pathways involving hydrolysis of inositol phosphates (9, 153) and the subsequent metabolism of the products, e.g. arachadonic acid (106) and prostaglandins (105), do occur, and these could contribute to $Ca^{2+}$-dependent regulation. Plant physiologists generally are testing the amplitude modulation model for transient responses; however, many sustained responses may also exhibit sensitivity modulation. Examination of cAMP-dependent reactions in higher plants has not yielded convincing positive evidence. It would seem that attention to the phospholipase *c*-

dependent hydrolysis of inositol phosphates and diacylglycerol activated *c*-kinase might provide new insight about $Ca^{2+}$-dependent regulation in plants.

## CELLULAR METABOLISM OF $Ca^{2+}$

$Ca^{2+}$ is a cytotoxin (257). At elevated levels it will react with inorganic phosphate forming an insoluble precipitate. It is apparent therefore that phosphate-based energy metabolism would be severely inhibited if the [$Ca^{2+}$] approached the millimolar quantities found outside the cell. Rather than change energy metabolism, it has been suggested that living organisms evolved a method for removing $Ca^{2+}$ from the cell, lowering its concentration to 0.1 μM, at which point its reaction with inorganic phosphate would be insignificant (119).

Maintenance of a low intracellular activity of $Ca^{2+}$ in the presence of millimolar levels in the extracellular milieu requires the active pumping of $Ca^{2+}$ out of the cytoplasm (91, 139). A variety of $Ca^{2+}$ pumps has been characterized from different membrane fractions (208) including PM (46, 47, 50, 51, 70, 224, 242), rough ER (19, 20), tonoplast (69), and mitochondria (47, 86, 87, 95, 195). In animal systems, the plasma membrane $Ca^{2+}$ pump has the highest affinity for $Ca^{2+}$ and thus sets the lower limit for the activity of intracellular $Ca^{2+}$. In plants, ER membranes have the highest affinity, and it would follow that they would be responsible for setting the lower limit. However, $Ca^{2+}$ uptake in the other membrane fractions has not been investigated in the presence of $Ca^{2+}$-EGTA buffers, the medium in which the affinity for $Ca^{2+}$ can be properly established. The PM-enriched fraction and the mitochondrial fraction have been examined under identical conditions (52), and in this instance, the former had a tenfold higher affinity for $Ca^{2+}$ than the latter.

It is evident that plants contain the basic mechanisms needed to ensure $Ca^{2+}$ homeostasis, and therefore a second messenger system can be built upon this foundation. Having achieved a very low internal [$Ca^{2+}$], the cell, in effect, is able to capitalize upon the large concentration gradient tending to force the ion into the cytoplasm. Transport of a relatively small absolute number of $Ca^{2+}$ into the cytoplasm will cause a large change in ion concentration that can act as a signal.

## $Ca^{2+}$ CHEMISTRY

While intracellular [$Ca^{2+}$] is submicromolar, the concentration of the closely related divalent cation $Mg^{2+}$ is millimolar. Despite the concentration difference that would favor $Mg^{2+}$, cellular processes often display an enormous

selectivity for $Ca^{2+}$. It is reasonable to ask what the physical properties of these ions are that account for $Ca^{2+}$ selectivity.

The group II ions ($Mg^{2+}$ and $Ca^{2+}$) are spherical point charges in which the electrostatic field is proportional to their charge-squared and inversely proportional to their ionic radius (45). For $Ca^{2+}$ the ionic radius is 99 pm while for $Mg^{2+}$ it is 65 pm. Because of its smaller ionic radius, $Mg^{2+}$ binds water more tightly than $Ca^{2+}$, and it follows that it will require more energy to remove bound water from $Mg^{2+}$ than from $Ca^{2+}$ before either can bind to a ligand. The strength of binding of group II ions to spherical ligands, e.g. halides, depends on the energy gained on binding a ligand minus the energy it takes to remove water. With ligands that have a high electric field strength, $Mg^{2+}$ is selected over $Ca^{2+}$ because it can come closer to the ligand, which increases the interaction between the ligand and the cation and thus the energy released upon binding. However, with ligands of low electric field strength, $Ca^{2+}$ binding is selected over $Mg^{2+}$ because there is not enough energy released upon binding to $Mg^{2+}$ to remove water (21, 64, 137, 266–268, 270).

The above model describes the selectivity of cation binding to spherical monodentate ligands. However, within the cell $Ca^{2+}$ and $Mg^{2+}$ do not usually bind to spherical ligands; rather their interaction is with polarized ligands including $R\text{-}COO^-$, $R\text{-}O^-$, $R\text{-}SO_3^-$, R-O-R, and $R_3N$ (where R = H or C). Because of the greater polarizability of nitrogen over oxygen, $Mg^{2+}$ can attract the electronic cloud of nitrogen more closely than it can that of oxygen, and because the energy of this ion-induced dipole interaction is dependent on the inverse of the fourth power of the radius, nitrogen discriminates against $Ca^{2+}$ much more than does oxygen (129). The majority of monodentate ligands that occur in the cell will favor $Mg^{2+}$ binding over $Ca^{2+}$. Because of their low polarizability and low electric field strength, neutral oxygens are the most common biological ligands that will select for $Ca^{2+}$ over $Mg^{2+}$. However, since they are not charged, the strength of the $Ca^{2+}$-neutral oxygen bond will be weak.

Even though neutral oxygens select $Ca^{2+}$ over $Mg^{2+}$, their discrimination is only within one order of magnitude (157). Therefore, in order to obtain a high degree of selectivity ($>10^3$) for a single cation, the number of binding sites on a ligand must be increased. The selectivity of multidentate ligands is best explained by coordination chemistry (40, 143). A coordinate bond requires the donation of a pair of electrons from the ligand to the cation; cations tend to add a sufficient number of electrons by coordination so that the ion in the resulting complex has an effective atomic number of the next noble gas. $Mg^{2+}$ obtains the electronic configuration of argon and $Ca^{2+}$ obtains that of krypton. This rule is only qualitative since a metal can have different coordination numbers depending on the properties of the ligands (7).

When $Mg^{2+}$ forms coordinate bonds it only has 3s and 4p orbitals, whereas

$Ca^{2+}$ has 3d, 4s, and 4p orbitals. The presence of the d orbitals in $Ca^{2+}$ allows it to form a variety of complexes with varying coordination numbers (6–12) and varying bond lengths. $Mg^{2+}$, with only s and p orbitals to fill, generally forms complexes with coordination numbers of six and with highly conservative bond lengths (267). As the number of coordinating ligands increases, their mutual repulsion prevents binding of cations smaller than a critical radius (219). Given the greater ionic radius of $Ca^{2+}$ over $Mg^{2+}$ and the fact that it can form bonds of variable lengths, cavities can be designed that will bind $Ca^{2+}$ greater than 1000-fold more tightly than $Mg^{2+}$. In brief, $Mg^{2+}$ cannot move close enough to bind to all the ligands at once. $Sr^{2+}$ or $Ba^{2+}$ can form coordinate bonds with a high binding strength if the large ions do not mechanically stress the protein, explaining why $Sr^{2+}$ and $Ba^{2+}$, but not $Mg^{2+}$, can often substitute for intracellular $Ca^{2+}$ (166). A rigorous quantitative treatment of cation binding strength with multidentate ligands can be found in Simon et al (219).



## $Ca^{2+}$-BINDING PROTEINS

In the extracellular milieu, the concentrations of $Ca^{2+}$ and $Mg^{2+}$ are both millimolar. Thus extracellular proteins need only bind $Ca^{2+}$ with a log K of about 3. Ligands with two carboxyl groups are sufficient to bind $Ca^{2+}$ at this concentration but they show no selectivity for $Ca^{2+}$ over $Mg^{2+}$. The addition of a neutral oxygen to a ligand with two carboxyl groups is sufficient to provide selectivity for $Ca^{2+}$ (120, 135, 136). The neutral oxygen is usually provided by the carboxyl of the peptide or the hydroxyl of serine or threonine. By replacing the neutral oxygen with a nitrogen as in glutamate, $Mg^{2+}$ binding is favored over $Ca^{2+}$; $Mg^{2+}$ can replace $Ca^{2+}$ in most extracellular processes.

In the cytosol, the concentration of free $Mg^{2+}$ is millimolar while free $Ca^{2+}$ is micromolar. In order to act as a trigger, a protein must be able to bind $Ca^{2+}$ with an affinity of $10^5$ or $10^6$ $M^{-1}$ (but not higher or lower). Three to five carboxyl groups are needed to give this binding strength (four are present in the known $Ca^{2+}$-binding proteins). However, to give a $10^2$ to $10^3$ selectivity of $Ca^{2+}$ binding over $Mg^{2+}$, one to three neutral ether, hydroxyl, or carbonyl oxygens must be added and arranged in such a way that the loss in entropy upon binding $Ca^{2+}$ is not too great. In order to reduce the loss of entropy, the oxygens are not randomly arranged, but are grouped in "EF hands," which are homologous domains found in all intracellular $Ca^{2+}$ binding proteins such as CaM, S-100, troponin *c*, and parvalbumin (10, 120, 135, 138, 190, 269). Each protein with the exception of parvalbumin contains four "EF hands." The carboxylic coordinating oxygens are provided by the glutamic and aspartic acid residues. The hydroxyl oxygens are provided by serine and threonine, while asparagine provides a carbonyl oxygen.

## THE FITNESS OF $Ca^{2+}$

$Ca^{2+}$ has an advantage over the other abundant cations in acting as a carrier of information because of its low intracellular concentration (225). When the concentration rises from 0.1 μM to 1–10 μM there is a resulting increase in the affinity of CaM for $Ca^{2+}$ and a response element that is driven by the change in free energy of the $Ca^{2+}$-CaM complex. There are some interesting consequences from the fact that the energy available to CaM to change its affinity for both $Ca^{2+}$ and the response element upon binding $Ca^{2+}$ depends on the change in concentration of $Ca^{2+}$ and not on its absolute concentration. For $Ca^{2+}$, the intracellular concentration can transiently change 100-fold, from $10^{-7}$ to $10^{-5}$ M without disturbing the ionic milieu of the cell, whereas $Mg^{2+}$ and $Na^{+}$ would have to change from $10^{-3}$ to $10^{-1}$, and $K^{+}$ from $10^{-1}$ to 10 M to provide an equivalent increase in affinity. The indicated increases in $[Mg^{2+}]$, $[Na^{+}]$, or $[K^{+}]$ would seriously disturb the osmotic and charge balance within the cell and would require excessive energy to restore homeostasis.

$Ca^{2+}$ has another property that makes it better suited to be an intracellular trigger than $Mg^{2+}$, namely its low free energy of hydration. Thus $Ca^{2+}$ sheds water at a rate of approximately $10^{9}$ water molecules per second compared with $Mg^{2+}$ which sheds water at a rate of approximately $10^{5}$ per second (137). Since the association constant of a receptor protein is close to the intracellular concentration of the trigger ion, the association constant of a $Ca^{2+}$-binding protein is $10^{5}$ $M^{-1}$. The off rate of $Ca^{2+}$ is $10^{4}$ per second and the half time of the reaction is 0.07 ms (association constant = on rate/off rate; half time = ln 2/off rate). Therefore, $Ca^{2+}$-CaM can control fast reactions although there are some reactions like insect flying muscle contraction that are too fast for $Ca^{2+}$ control (176). In order for $Mg^{2+}$ to control rapid reactions, the association constant of a $Mg^{2+}$-binding protein would have to be $10^{1}$ $M^{-1}$. Increasing the $[Mg^{2+}]$ would be an osmotic, charge and energy burden for the cell, while increasing the affinity of a protein so that it binds $Mg^{2+}$ at resting concentrations ($10^{-3}$ M), would increase the halftime of the reaction to 7 ms. Thus $Mg^{2+}$ is limited to regulating slow reactions.

## $Ca^{2+}$-MEDIATED PROCESSES

An ever increasing number of $Ca^{2+}$-mediated processes in plants are being discovered. In this section we limit our discussion to three cellular processes, i.e. polarized growth, mitosis, and cytoplasmic streaming, that occur widely and that allow us to examine different aspects of $Ca^{2+}$ regulation and to appreciate their complexity. Because of time and space constraints, we will not discuss in detail several processes, including wound-induced cytoplasmic contraction in *Ernodesmis* (122), wound-induced nuclear migration (207),

volume regulation in *Poterioochromonas* (114, 116), circadian leaf movements (201, 237), guard cell swelling (162), protoplast fusion (15), or freezing injury (146, 277, 280) that appear to involve $Ca^{2+}$.

Throughout this section and the one that follows, attention will be given to the evidence showing that $Ca^{2+}$ mediates a response. In establishing proof that $Ca^{2+}$ is involved, we think it is helpful to keep in mind three rules enunciated by Jaffe (101) as follows: 1. the response should be preceded or accompanied by an increase in intracellular [$Ca^{2+}$]; 2. blockage of the natural [$Ca^{2+}$] increase should inhibit the response; and 3. experimental generation of an increase in the intracellular [$Ca^{2+}$] should stimulate the response.



## *Polarized Growth*

The discovery of transcellular ion currents has been an important contribution to our understanding of polarized cell growth (102, 103). In a wide variety of plant cells, including developing algal zygotes, pollen tubes, root hairs, entire roots, and moss and fungal rhizoids, electrical currents enter the growing point and leave at nongrowing zones (260). The currents are known to occur prior to the morphological appearance of a polarized growth zone, and in addition their inhibition blocks the polarization phenomenon. While $K^+$ and $H^+$ are important ions in this process (259), considerable evidence indicates that $Ca^{2+}$ carries a portion of the current (192, 260).

The rhizoid pole of a population of unilaterally illuminated *Pelvetia* zygotes takes up $Ca^{2+}$ more rapidly than the thallus pole (192). Furthermore, the application of an artificial $Ca^{2+}$ gradient, derived from asymmetric administration of the ionophore A 23187, causes *Pelvetia* (191) and *Funaria* rhizoids (26) to grow predominantly toward the high $Ca^{2+}$. It seems likely from these and related experiments that the intracellular [$Ca^{2+}$] becomes elevated in the growth zone, presumably as a result of localized influx.

Direct demonstration of an increased [$Ca^{2+}$] in tip-growing cells has been made using chlorotetracycline (CTC) fluorescence (170, 188, 203), $^{45}Ca^{2+}$ autoradiography (99), and proton-induced X-ray emission analysis (189). CTC fluorescence shows a marked gradient from a high point at the tip backward (170, 188, 203). Since CTC indicates the localization of membrane-associated $Ca^{2+}$, a portion of the observed fluorescence gradient reflects the fact that membranes are generally more concentrated in the tip. Nevertheless, Reiss et al (189), using proton-induced X-ray emission, find that the $Ca^{2+}$ gradient in pollen tubes is steeper than the phosphorus gradient, the latter being assumed to reflect membrane distribution. They conclude that $Ca^{2+}$ is enriched in certain membranes, especially the ER and mitochondria, that are localized a few microns from the tip itself. The $Ca^{2+}$ gradient appears to be closely related to the marked cytoplasmic stratification that can be observed in electron micrographs. High [$Ca^{2+}$] or application of the ionophore A 23187 stops tip growth

and in some instances causes the previously stratified organelles to become dispersed (187, 229), presumably as a result of the dissipation of the $Ca^{2+}$ gradient. The CaM antagonists trifluoperizine (TFP) and chlorpromazine (CPZ) also inhibit germination and tip growth in pollen of pear (170). The ability of these inhibitors to modify normal changes in CTC fluorescence suggests that $Ca^{2+}$ mobilization may be altered in a CaM-dependent process.

The involvement of membrane-associated $Ca^{2+}$ associated with growing points has also been observed in the highly sculptured, multilobed cells of the desmid *Micrasterias* (145). In this example the pattern of CTC fluorescence exactly coincides with those particular regions of the cell surface that are exhibiting rapid growth. The fact that *N*-phenyl-1-naphthylamine, a general membrane marker, shows no particular accumulation indicates that a specific enrichment of $Ca^{2+}$ has occurred at the growing site at the time of active growth (145). Modulation of $Ca^{2+}$ with different agents including EGTA, verapamil, $La^{3+}$, or A 23187 arrests cells of *Micrasterias* from developing their multilobed morphology (130).

$Ca^{2+}$ gradients thus exist in cells exhibiting polarized growth. It seems likely that the $Ca^{2+}$ current is brought about by asymmetric distribution of channels and pumps to opposite sides or regions of the cell (187). The channels at the growing point would allow $Ca^{2+}$ to flow into the cell down its electrochemical gradient while pumps at the distal regions would remove $Ca^{2+}$ against a concentration gradient using ATP.

To establish polarity initially in an unpolarized zygote like *Fucus,* it is likely that the cell is poised, ready to respond to a variety of stimuli such as light, sperm entry, gravity, etc (100). Perception of the stimulus at some place might induce a series of membrane changes that allows for localized entry of $Ca^{2+}$. Having established a point of facilitated $Ca^{2+}$ entry, the cell could respond by amplifying and propagating the signal, thereby creating a polarized current that would govern subsequent morphogenesis (100).

There appear to be two major activities of $Ca^{2+}$ in polarized growth. Initially, $Ca^{2+}$ may act as a trigger; responding to a stimulus-related change in membrane potential (depolarization?), voltage-gated channels might open that would allow $Ca^{2+}$ to flow into the cell. The internal [$Ca^{2+}$] would increase and appropriate response elements would become activated. These early events thus turn on the system and probably contribute to the second phase of development, which might include as a major regulatory component the establishment of a sustained, self-driven current. $Ca^{2+}$ is ideal for regulating polarized growth; because of its low cytosolic concentration, the occurrence of a small leak at one point will quickly create a gradient in the ion (6). Although $K^+$ may carry much more total current than $Ca^{2+}$, its concentration is so high that even a localized introduction of a relatively large number of ions would not

be detected by the cell as a concentration gradient that could be used for polarized activities. In effect, the large concentration changes that $Ca^{2+}$ experiences contain energy that can be transformed into information.

As compelling as the evidence is in favor of $Ca^{2+}$ participation in triggering and maintaining polarized growth, there are important issues that need to be resolved. Most pressing is a direct demonstration that the internal free [$Ca^{2+}$] increases following a stimulus and that a gradient occurs in tip-growing cells. The systems that exhibit tip growth are numerous and many are easy to study. It seems possible that use of a permeant fluorescent $Ca^{2+}$ indicator, quin-2 (240, 241) or fura-2 (169), together with video image intensification, could provide exciting new information about free $Ca^{2+}$ changes and distribution in these cells.



## *Mitosis and Cytokinesis*

It is widely assumed that mitosis and cytokinesis are regulated by $Ca^{2+}$ (83). Early studies on the role of $Ca^{2+}$ in growth noted that low concentrations ($<$ 0.1 mM) led to reduced division rate in roots (108). The targets for low $Ca^{2+}$ action might include the cell plate and/or the mitotic apparatus (83). During cytokinesis, the forming cell plate is known to contain considerable quantities of $Ca^{2+}$ (203, 265), and its inhibition by caffeine has often been attributed to an effect on membrane-bound $Ca^{2+}$ (163). The idea that the mitotic apparatus or the phragmoplast might be the target of $Ca^{2+}$ action grew from the discovery that microtubules are depolymerized by elevated levels of $Ca^{2+}$ (258). It thus seems reasonable that fluctuating levels of $Ca^{2+}$ would control the assembly-disassembly of spindle microtubules and directly regulate both formation and function of the mitotic apparatus and phragmoplast (83, 84).

These ideas are attractive, and evidence supporting a role for $Ca^{2+}$ during mitosis and cytokinesis is beginning to accumulate. Studies on animal cells have shown that elevated [$Ca^{2+}$] causes disassembly of spindle microtubules in vivo (117) and in vitro (200). The calcium regulatory components, including CaM (263) and $Ca^{2+}$-ATPase (167), are present as well as an extensive membrane system (85) that is capable of sequestering $Ca^{2+}$ (217). Plant spindles also contain CaM; in endosperm cells of *Haemanthus*, antibodies to CaM are localized in the spindle pole region and close to kinetochore fibers (124).

Plant mitotic apparatuses often possess an elaborate system of endomembranes (85). In some cell types such as barley leaf cells, the ER shows remarkably close and specific associations with the spindle fibers (81). Elements of reticulate tubular ER invade the spindle along kinetochore tubules and in addition surround the mitotic apparatus, creating a separate spindle compartment within the cytoplasm (81). These membranes contain $Ca^{2+}$ as detected by antimonate staining (265) and CTC fluorescence (275), and it seems reason-

able, given their proximity to the spindle elements, that they contribute to the control of the [$Ca^{2+}$] in the mitotic apparatus.

The dividing plant cell thus contains elements for $Ca^{2+}$ regulation. The question is whether changes in [$Ca^{2+}$] actually occur, and if so what processes are modulated. Indirectly it has been shown that the membrane-associated $Ca^{2+}$, indicated by CTC fluorescence, declines a few minutes before the cells enter anaphase (276). A transient decrease in CTC fluorescence is consistent with an increase in free $Ca^{2+}$, but it does not constitute a proof. Further indirect evidence has been gained from studies with two "voltage-sensitive" dyes showing that their fluorescence sharply increases as the cells enter anaphase (276). It seems clear that ion/charge redistributions occur at the metaphase/anaphase transition as well as at other phases of mitosis and that some of the change may be traceable to $Ca^{2+}$. A direct approach using the fluorescent free $Ca^{2+}$ indicator quin-2 (274) has recently provided tentative data supporting a change in [$Ca^{2+}$] at the onset of anaphase. However, we strongly underscore the need to establish this point firmly.

With a view that $Ca^{2+}$ fluxes do occur, experiments have been performed in which these presumed changes have been modified by pharmacological agents and the effects on mitosis monitored (82). Restricting the passage of $Ca^{2+}$ from the wall into the cell with agents that bind $Ca^{2+}$ (EGTA) or block its transport at the PM ($La^{3+}$ and D 600) either arrest or greatly extend metaphase. With $Ca^{2+}$-EGTA buffers it is found that 0.1 μM $Ca^{2+}$ or less causes a delay in the onset of anaphase while 1 μM and higher supports normal mitotic progression. An additional observation from these studies is that the conditions causing delay in anaphase onset have little or no effect on the subsequent rate of chromosome motion or time of cell plate formation. In short, cells that are undeniably $Ca^{2+}$-stressed exhibit normal anaphase and cytokinesis. It is possible that anaphase motion might be slowed because of an inadequate supply of $Ca^{2+}$ (82). Clarification of the anaphase question in particular and other aspects of mitosis as well may benefit considerably from experiments in which the internal [$Ca^{2+}$] is buffered directly either with a permeant chelator, BAPTA (239), or with $Ca^{2+}$-EGTA solutions that have been microinjected.

Experimental attempts to raise the internal [$Ca^{2+}$] have not produced detectable effects either in *Tradescantia* stamen hair cells (P. K. Hepler, unpublished results) or *Marsilea* microspores (264). Application of the ionophore A 23187 with $Ca^{2+}$ (10 mM) would be expected to raise the internal [$Ca^{2+}$] and possibly prevent assembly or hasten depolymerization of microtubules during anaphase (264). In the studies on *Marsilea*, presence of the ionophore A 23187 plus $Ca^{2+}$ caused marked acidification of the medium. Since A 23187 exchanges $Ca^{2+}$ for $H^+$ it seems evident that $Ca^{2+}$ has indeed entered the cell. What is not known is whether the cellular buffering systems sequestered the influxing $Ca^{2+}$ and protected the mitotic apparatus from excess ion. These few studies indicate that

our knowledge of the contribution of $Ca^{2+}$ in mitosis remains unclear. Although it seems obvious that $Ca^{2+}$ should modulate certain motile events such as anaphase motion, there is only fragmentary supportive evidence at best.

The evidence that $Ca^{2+}$ regulates cytokinesis is also somewhat fragmentary. It has been reported that low [$Ca^{2+}$] (<0.1 μM) blocks mitosis, and while we find that it slows the process, primarily by prolonging metaphase and possibly also prophase, cytokinesis itself is relatively insensitive (82). In the presence of EGTA or $La^{3+}$, if cells enter anaphase, they form a cell plate without further delay and the cell plate grows to completion. It has been argued that caffeine, a well-known inhibitor of cell plate formation, achieves its inhibition through modulation of $Ca^{2+}$ (163). By extrapolation from studies on animal systems, especially muscle cells, caffeine would be expected to cause release of $Ca^{2+}$ from internal stores and thus raise the internal concentration. It seems unlikely that this occurs in plants since if the [$Ca^{2+}$] were raised to 1 μM or higher, cytoplasmic streaming would stop (see below). At concentrations that always cause cell plate failure in *Tradescantia* stamen hair cells, cytoplasmic streaming in neighboring nondividing cells never stops (13). It is possible that caffeine affects a membrane-calcium relationship (163), but that is only speculation.

$Ca^{2+}$ is a regulator of mitosis, but its mechanism of action needs to be elucidated. External $Ca^{2+}$ at 0.1 μM or less profoundly prolongs metaphase, but the mechanism of action is unknown. $Ca^{2+}$ is undeniably a major component of the cell wall and also is needed for membrane stability, but low external [$Ca^{2+}$] appears to have no effect on the cell plate in *Tradescantia* stamen hair cells. There are many facets to the $Ca^{2+}$-mitosis story that have to be worked out. These include demonstration that fluxes in free $Ca^{2+}$ actually occur, and if so, when and where in the mitotic apparatus and what the response elements are. A further issue concerns the nature of the stimulus that causes a cell to enter mitosis.

## *Cytoplasmic Streaming*

Remarkable progress has been made during the last decade in our understanding about the role of $Ca^{2+}$ in cytoplasmic streaming (112). Given the fact that $Ca^{2+}$ regulates virtually all motile systems, both muscle and nonmuscle, it seems only natural to expect that the ion would also regulate streaming. Early progress on this problem came from studies of Characean algae in which the internode cells had been opened and the vacuole perfused, allowing one to add media of known composition and to see their effect on streaming (230, 271). From studies on ATP-dependent motion, Williamson (271) noted that the [$Ca^{2+}$] had to be 0.1 μM or lower for streaming to occur. As the concentration increased to 1–10 μM, streaming became progressively inhibited. These initial studies created a certain amount of puzzlement since the implication was that

elevated levels of $Ca^{2+}$ stopped motion, a result that directly contradicted the role of the ion in other known motile systems. Additional support for $Ca^{2+}$ inhibition of streaming came from studies of Tazawa et al (230), also on perfused preparations of *Chara*. Making use of the well-known observation that elicitation of an action potential always caused immediate cessation of streaming, Tazawa et al (230) found that the presence of EGTA would nullify the inhibitory effect of the action potential. They suggested that the action potential caused an increase in intracellular [$Ca^{2+}$] which then blocked streaming; preventing the [$Ca^{2+}$] increase with EGTA permitted streaming to continue. Isolated cytoplasmic droplets of *Chara* that contained rotating chloroplasts were used in further studies to show that $Ca^{2+}$ and $Sr^{2+}$ reversibly blocked motility while $Mg^{2+}$ and $K^+$ had no effect (80).

A concern arising from some of these initial studies was the necessity to use high concentrations of $Ca^{2+}$ (1 mM) in order to mimic the rapid inhibitory effect of an action potential (236). The resolution of this problem came from studies that used cell preparations in which the plasma membrane had been permeabilized rather than the vacuole being perfused (213, 235). With media containing 1 mM ATP, Shimmen & Tazawa (213) found that 1 μM $Ca^{2+}$ rapidly and reversibly inhibited streaming. The implication from these studies is that the vacuolar perfusion technique, while leaving the actin microfilament bundles in place, removes cytoplasmic factors that confer $Ca^{2+}$ sensitivity to the motile process.

A direct demonstration that the action potential causes an increase in intracellular [$Ca^{2+}$] together with an inhibition of streaming has come from the landmark study of Williamson & Ashley (273). Aequorin, a photoprotein that emits light when it binds $Ca^{2+}$, was microinjected into *Chara* or *Nitella* internode cells. The cell was then stimulated, causing a rapid depolarization of the membrane potential and stoppage of streaming. A concomitant increase in light output from aequorin provided direct evidence that the intracellular [$Ca^{2+}$] had risen. Williamson & Ashley (273) find that the [$Ca^{2+}$] in the cell at rest is 0.1–0.4 μM, assuming [$Mg^{2+}$] is 1 mM, and that during the action potential it rises to 6.7 μM for *Chara* and 43 μM for *Nitella*. To the best of our knowledge, these are the only studies on plant cells in which the internal free [$Ca^{2+}$] has been measured.

$Ca^{2+}$ regulation of streaming appears to occur in plants other than the Characean algae. In the plasmodial slime mold *Physarum*, which exhibits a periodic back and forth "shuttle" streaming, it has been found that the [$Ca^{2+}$] oscillates with the same periodicity as that of tension-relaxation (112). Interestingly, the point of high tension correlates with low [$Ca^{2+}$] while relaxation correlates with increased [$Ca^{2+}$]. Again the increasing [$Ca^{2+}$] seems to be related to a slowing or stoppage of streaming. The presence of the $Ca^{2+}$-

sensitive actin-binding protein fragmin that cleaves actin filaments into short lengths may provide a molecular basis for $Ca^{2+}$ action in these organisms (77).

Beyond these two well-studied systems there is evidence that streaming in cells of flowering plants is also inhibited by elevated $Ca^{2+}$. *Vallisneria* leaf cells normally do not stream in the dark or low light, but do when the light intensity is increased (228, 278). If, however, the dark-grown cells are cultured in EGTA (20 mM), streaming begins quickly and reaches rates of 10–20 μm/sec in 10 min (278). Presumably the cellular level of $Ca^{2+}$ or the leakage of the ion into the cell in the dark is too high to permit streaming, but when EGTA is present the $Ca^{2+}$ drops to permissive levels and movement starts. In *Tradescantia* stamen hair cells (53) and cultured tomato cells (277) that normally exhibit streaming, it has been reported that the process can be blocked by application of the ionophore A 23187 plus exogenous $Ca^{2+}$. Woods et al (277) calculate that raising the [$Ca^{2+}$] from 0.1 μM-1.0 μM markedly inhibits motility and simultaneously causes a vesiculation and reticulation of cytoplasmic structure. Finally, microinjection of a Ca-EGTA buffer (10 μM free $Ca^{2+}$) into stamen hair cells of *Tradescantia* blocks streaming, which recovers if the $Ca^{2+}$-EGTA buffer is allowed to diffuse to neighboring cells (P. K. Hepler, unpublished results).

The question of whether CaM is involved in the inhibition of streaming has been approached by Woods et al (277) using several different inhibitors. While 10 μM TFP and CPZ inhibit the expression of $Ca^{2+}$ sensitivity, W-7 does not unless the concentration is very high (1 mM). Since the W series inhibitors are thought to be more specific for CaM activity than either TFP or CPZ, the authors conclude that modulator protein may not be involved in streaming regulation. Emphasizing the nonspecific actions of TFP and CPZ (115, 116), Woods et al (277) attribute the inhibition by these agents to effects other than binding to CaM.

$Ca^{2+}$ inhibition of streaming appears to be a general phenomenon in flowering and nonflowering plants. Of all the $Ca^{2+}$-mediated processes in plants this one is the most completely understood. The rules enunciated by Jaffe (101) have been fully satisfied as follows: 1. the event, namely inhibition of streaming, is accompanied by an increase in intracellular [$Ca^{2+}$]; 2. blockage of the naturally occurring increase in [$Ca^{2+}$] prevents streaming inhibition; and 3. exogenous application of an elevated level of $Ca^{2+}$ causes streaming inhibition. Given the apparent widespread occurrence of this $Ca^{2+}$ regulatory system in plants, it is reasonable to imagine in those circumstances in which the streaming normally stops, for example, when a cell enters mitosis, that the internal [$Ca^{2+}$] has risen. Alternatively, those treatments that cause streaming rates to increase such as light (209) and auxin (233) may be achieving the effect through a reduction in intracellular [$Ca^{2+}$]. Cytoplasmic streaming rates may thus be a

natural indicator of intracellular $Ca^{2+}$ levels that could be used as a reference in cytophysiological investigations.

## STIMULUS-RESPONSE COUPLING

### *Light*

Similarities between the action of red light via phytochrome and the action of $Ca^{2+}$ in coupling many stimulus-response systems in animal cells have been recognized for years (79). Blue light-activated responses also share common features with systems known to employ $Ca^{2+}$ as a signaling agent. In brief, there is a diverse and rapidly growing body of information supporting the assertion that $Ca^{2+}$ acts as a "second messenger" in triggering light-stimulated responses.

Photoreceptor cells of animals provide a model where $Ca^{2+}$ may act as a second messenger in light-response coupling (17, 71). Injections of $Ca^{2+}$-EGTA buffers into the photoreceptor cells demonstrate a micromolar threshold for intracellular $Ca^{2+}$ (18). In addition, light absorbed by rhodopsin causes a release of $Ca^{2+}$ from the membrane discs (65). The presumptive increase in the cytoplasmic [$Ca^{2+}$] inhibits the dark current and thereby the information contained in the light is transferred to the optic nerve.

PHYTOCHROME Red light triggers a large array of physiological and developmental events that require $Ca^{2+}$, including chloroplast rotation in *Mougeotia* (55, 78, 247–249), spore germination (254–256) and cell expansion (37) in *Onoclea,* leaflet closure in *Mimosa* (22, 23, 237), root tip adhesion in *Phaseolus* (229, 279), peroxidase secretion in *Spinacia* (113, 164, 165), membrane depolarization in *Nitella* (261), as well as activation of NAD kinase (1, 218, 232) and inhibition of mitochondrial ATPase (212). Micromolar concentrations of external $Ca^{2+}$ are sufficient to stimulate germination (254), peroxidase secretion (223), activation of NAD kinase (48), and inhibition of mitochondrial ATPase (212); 100 μM concentrations of $Ca^{2+}$ will stimulate membrane depolarization (261), root tip adhesion (229), and chloroplast rotation (248).

In order to modulate the level of intracellular $Ca^{2+}$, phytochrome would have to be associated with membranes either directly or indirectly. Although 95% of phytochrome in an etiolated plant is soluble, approximately 5% of phytochrome pellets with the membrane fraction (177). Furthermore, biochemical (252) and immunological (245) studies show an increase in membrane-associated phytochrome following irradiation and, most convincingly, cells irradiated with polarized red and far-red light show a response that is best explained by assuming that photobiologically relevant phytochrome is localized on the PM (59, 75, 76, 78, 111). Phytochrome can affect many membrane

properties (72, 140, 177). Of particular interest here is the fact that red light induces a small depolarization with a lag time of less than 1 sec (155, 261). A small depolarization may be sufficient to open voltage-dependent $Ca^{2+}$ channels and allow the ion to enter the cell. Phytochrome may also induce a release of $Ca^{2+}$ from internal organelles. The pigment has been localized on mitochondria (195), etioplasts (93), cytoplasmic vesicles (202), and microsomal fractions (177, 252). In plant mitochondria, for example, $Ca^{2+}$ is exchangeably bound (47) and could be available for release into the cytoplasm upon red light irradiation (195).



In contrast to the above examples in which red light initially opens channels allowing the movement of $Ca^{2+}$ into the cell, pretreatment of corn plants with morphogenetically active far-red light inhibits the influx of $Ca^{2+}$ into inside-out PM vesicles by lowering the $V_{max}$ and increasing the $K_m$ of the $Ca^{2+}$-ATPase for $Ca^{2+}$ in the presence of CaM. In essence, far-red irradiation reduces the stimulatory effect of CaM on the $Ca^{2+}$ pump (49, 52). In vivo this would translate to an inhibition of transplasmalemmal calcium efflux with the result that the intracellular [$Ca^{2+}$] would increase since there would be a constant leakage of $Ca^{2+}$ into the cell. However, in the rate of microsomal $Ca^{2+}$ uptake in corn, there is a strict dependence on age (47), and 6 h of far-red light may have the effect of advancing or retarding aging. In animal cells, where $Ca^{2+}$ acts as a second messenger, the rate of calcium influx and efflux is usually increased by the stimulus, but influx is stimulated more than efflux, resulting in a transient increase in the cytosolic [$Ca^{2+}$] (183).

Among the red light-induced physiological responses there are two, namely chloroplast rotation in *Mougeotia* and spore germination in *Onoclea,* for which a $Ca^{2+}$ participation has been particularly well characterized. Chloroplast rotation in *Mougeotia* depends on the presence of $Ca^{2+}$ in the external medium (248). If $^{45}Ca^{2+}$ is used, red light will stimulate its uptake while far-red reverses this process (55). Although it seems likely that $Ca^{2+}$ enters the cell from the wall, measurement of total flux by murexide absorption reveals that efflux exceeds influx (72, 193, 194). In order to resolve this apparent discrepancy, Roux (193, 194) has suggested that red light induces a small transient influx of $Ca^{2+}$ across the PM and a coincident release from intracellular stores which together increase the intracellular [$Ca^{2+}$]. The net efflux of $Ca^{2+}$ would result from a stimulation of the PM $Ca^{2+}$-ATPase pump. Thus, although more $Ca^{2+}$ appears to be moving out of the cell than in, the prevailing view suggests that the internal [$Ca^{2+}$] initially increases, a conclusion that is supported by the $^{45}Ca^{2+}$ data.

The flux of $Ca^{2+}$ in red light-stimulated *Mougeotia* cells appears to come from different sources. In contrast to hormone-stimulated responses, in which diffusion of a chemical requires its interaction first with the PM and secondly with other compartments, with light (or gravity) the initial stimulus could affect

internal compartments nearly as much as the PM. We noted above, for example, that mitochondria may contain phytochrome and thus be an initial source of $Ca^{2+}$. *Mougeotia* appears to contain a special system of $Ca^{2+}$-containing vesicles that have been implicated in chloroplast rotation (250). If cells are cultured in low $Ca^{2+}$ and are unable to respond to light, these vesicles lose their $Ca^{2+}$ store. In addition, their fluorescence, when labeled with CTC, changes during chloroplast rotation, suggesting a relationship between the membrane-associated $Ca^{2+}$ pool and the response (249).

Further support for the idea that $Ca^{2+}$ mediates red light stimulation of chloroplast rotation comes from the experiments of Serlin & Roux (210, 211) in which they artificially induce an influx of the ion. Using microtips coated with A 23187 that have been placed on opposite sides of a cell near the edges of the chloroplast, they are able to induce rotation in the absence of light. By contrast, no rotation is observed when both A 23187-coated microtips are oriented 90° from the chloroplast edges. In addition, no rotation occurs regardless of the orientation if $Ca^{2+}$ is omitted from the external medium. These results suggest that the chloroplast edges move away from localized zones of $Ca^{2+}$ influx.

CaM may contribute to the phytochrome-mediated signal transduction chain since the $Ca^{2+}$-CaM antagonists TFP (20–50 μM) and W-7 (100 μM) inhibit chloroplast rotation (251). In order to guard against the nonspecific detergent effects of these drugs, Serlin & Roux (211) have used the analogs W-12 / W-13 and have shown that the active analog W-13 is far more effective in inhibiting chloroplast rotation than the inactive analog. Moreover, CaM has been isolated from filaments of *Mougeotia* (251).

An attractive feature of *Mougeotia* chloroplast rotation, which it shares with cytoplasmic streaming, is the likelihood that actomyosin will serve as the intracellular $Ca^{2+}$ target. The chloroplast edge is attached to the PM by actin filaments (118, 248). Although it is much too early to specify a particular mechanism, there are different known $Ca^{2+}$-sensitive steps that could control actin-myosin interactions or actin filament assembly and thus regulate chloroplast movement (249).

Phytochrome controls spore germination in many different organisms (178). Of current interest is the realization that $Ca^{2+}$ mediates the response in the fern *Onoclea* (254) and probably many others as well. A major stumbling block in uncovering the $Ca^{2+}$ requirement for germination has been the presence of large quantities of the ion in the spore wall (156). Even if one sows the spores in $Ca^{2+}$-free media, the response to the ion cannot be detected unless the wall-bound $Ca^{2+}$ has been previously removed through washing in EGTA (255). When the wall-bound $Ca^{2+}$ is removed, the spores display a remarkable sensitivity to the concentration of ion added back. The threshold for $[Ca^{2+}]$ for germination is submicromolar while 3 μM supports a half-maximal response (254). Although $Sr^{2+}$ and $Ba^{2+}$ can substitute for $Ca^{2+}$, $Mg^{2+}$ is completely ineffective.

The relationship between phytochrome and its presumptive second messenger is revealed more pursuasively by the results showing that red light stimulates net $Ca^{2+}$ uptake in *Onoclea* spores while far-red light inhibits this process. Measurements made by atomic absorption spectroscopy indicate that enough $Ca^{2+}$ is taken up during the red light irradiation to raise the internal concentration by 500 μM (256). A large excess may be needed to saturate cellular binding sites so that sufficient $Ca^{2+}$ remains free to act as a stimulus (183, 184). While these data do not directly show that the free [$Ca^{2+}$] has increased, they are entirely consistent with that conclusion.

Further evidence that $Ca^{2+}$ couples the red light stimulus to the response comes from observations showing that $La^{3+}$ blocks $Ca^{2+}$ uptake and also inhibits germination (254, 256). $La^{3+}$ applied prior to irradiation completely inhibits germination whereas it progressively loses its effectiveness if given short times after the onset of irradiation. After 5 minutes of red light, $La^{3+}$ is completely ineffective, indicating that uptake of external $Ca^{2+}$, needed for germination, occurs rapidly following the initiation of irradiation.

One of the most exciting and unexpected results is that in $Ca^{2+}$-free media, the PM remains maximally poised to accept and transport $Ca^{2+}$ for at least 4 hours following red light irradiation (254). Subsequent irradiation with far-red light 0.5–4 hours after the red irradiation fails to prevent germination, indicating that $Ca^{2+}$ transport has been uncoupled from transformed phytochrome (Pfr). These observations provided evidence for the occurrence of intermediary steps in the transduction chain between phytochrome and $Ca^{2+}$ influx. Thus the conversion of Pr to Pfr may alter the conformation of specific $Ca^{2+}$ channel proteins on the PM and lock them into a relatively stable open configuration. Even though Pfr may quickly decay to an inactive state, the channels remain open for several hours during which time $Ca^{2+}$ enters the spore from the extracellular space and stimulates germination.

The final evidence showing that $Ca^{2+}$ couples the stimulus to the response in *Onoclea* comes from studies in which an artificially generated influx of $Ca^{2+}$ causes germination in the absence of red-light irradiation (254). By applying the ionophore A 23187 to dark-sown spores it is possible to partially mimic the effect of red light. Once $Ca^{2+}$ has entered the cell it seems likely that it binds CaM. Compounds that interfere with the function of the $Ca^{2+}$-CaM complex, e.g. CPZ, TFP, and calmidazolium (R 24571), also inhibit germination (254). Although the drug concentrations used are high, raising concern over their specificity, the effectiveness of the drugs corresponds to their affinity in binding to $Ca^{2+}$-CaM (262). Moreover, at high concentrations (300 μM) the effects of CPZ and TFP are reversible.

If all phytochrome-mediated responses are coupled to red light through $Ca^{2+}$, we must also be able to explain responses in which long irradiations are required or long escape times have been demonstrated. Phytochrome control solely by amplitude modulation would be impossible in these long-term re-

sponses because of the cytotoxicity of calcium. However, these responses may be regulated through sensitivity modulation. Regarding the diacylglycerol pathway, one interesting action of morphogenetically active far-red light is the inhibition of lipoxygenase activity (148), the enzyme that catalyzes the breakdown of arachidonic acid. Arachadonic acid (106), a metabolite of diacylglycerol, and its breakdown products, prostaglandins (105), influence cellular $Ca^{2+}$ metabolism in animal cells (153, 156). Interestingly, inhibition of arachadonic acid catabolism with nordihydroguaiaretic acid (10–100 μM) inhibits red light-stimulated germination of *Onoclea* spores (R. O. Wayne and P. K. Hepler, unpublished observations), indicating that the diacylglycerol pathway may have a regulatory function in phytochrome-mediated responses.



BLUE LIGHT Calcium ions contribute to the signal transduction chain in the blue light-induced phototactic response in *Chlamydomonas* (158, 159, 206). In the dark, *Chlamydomonas* swims forward with a ciliary waveform that is independent of external calcium concentration (97, 206). Immediately upon illumination with blue light, the swimming behavior changes from a forward-moving ciliary waveform to a backward-moving flagellar waveform. This transformation is dependent on the concentration of external calcium (97, 206), and can be replaced partially by $Ba^{2+}$ and $Sr^{2+}$ but not by $Mg^{2+}$ (158). Below $10^{-6}$ M $Ca^{2+}$, light has no effect on changing the waveform; above $10^{-6}$ M $Ca^{2+}$, light changes the waveform from ciliary to flagellar. $La^{3+}$ inhibits light-induced phototaxis and this inhibition can be reversed by $Ca^{2+}$ (158). The voltage-dependent $Ca^{2+}$ channel blocker D-600 (100 μM) also inhibits phototaxis (206). It appears that blue light may cause a depolarization of the membrane potential resulting in the opening of voltage-dependent calcium channels and the influx of calcium ions into the cytosol. The elevated $[Ca^{2+}]$ triggers a change in ciliary waveform and causes a reversal in the direction of swimming.

The experiments discussed above provide a firm basis for the conclusion that $Ca^{2+}$ acts as a second messenger in light-stimulated responses. However, we again stress the importance in dircctly measuring a light-induced increase in the intracellular free $[Ca^{2+}]$.

## *Gravity*

During the last few years there has been a new wave of excitement in studies on gravitropism. In part this is the result of an infusion of money from the National Aeronautic and Space Administration (NASA) for basic research on the mechanism of graviperception and response coupling in plant and animal systems. Plants, for several reasons, have provided attractive subjects for the study of gravitropism. Of particular interest to this review is the current attention to the role of $Ca^{2+}$.

A $Ca^{2+}$ involvement in gravitropic curvature derives from studies showing that relatively large amounts of $Ca^{2+}$ move to the upper side of shoots or coleoptiles that have been placed horizontally (5,66,220). These conclusions are based on investigations using different techniques, including $^{45}Ca^{2+}$ movement (5, 66) and antimonate staining (220). The $Ca^{2+}$ that is visualized cytologically is primarily in the wall, although it is recognized that major intracellular sites exist and that these may contribute to the tissue pattern of $Ca^{2+}$ movement. Crucial to a role for $Ca^{2+}$ in a gravitropic response is the observation that the redistribution of the ion precedes tissue curvature (220). Furthermore, removal of the apoplastic $Ca^{2+}$ by culture in the chelator EGTA inhibits $Ca^{2+}$ redistribution and the subsequent curvature, but not growth (41).

Because $Ca^{2+}$ is known to inhibit growth, it is suggested that the asymmetric distribution of the ion causes a similar asymmetry in elongation (196). By reducing growth on the upper side of a shoot or coleoptile while allowing normal rates on the lower side, $Ca^{2+}$ might cause the growing tip to curve upward. Besides $Ca^{2+}$ movement, evidence is accumulating supporting the notion that opposite transport of auxin which is coupled to the redistribution of $Ca^{2+}$ occurs during gravistimulation (see below).

In an attempt to define the signal transduction process further, Biro et al (11) have cultured gravistimulated coleoptiles in the calmodulin antagonist CPZ. If the coleoptile tip is removed to allow greater access of the tissue cells to the drug, CPZ at 50 $\mu$M causes a marked inhibition of response to gravity, suggesting that cellular processes involving calmodulin are involved in the stimuli-response coupling. These results, however, are tempered by the realization that CPZ has nonspecific effects, especially on membrane transport properties (115, 116), and that these might contribute to the gravitropic inhibition observed.

Several lines of experimentation support the contention that $Ca^{2+}$ contributes to the downward curvature response of roots to gravity (126–128, 215). Corn roots cultured in the chelators EGTA or EDTA lose their ability to respond to gravity (126). The response can be restored by $CaCl_2$ but not $MgCl_2$, although the amount of $Ca^{2+}$ used (10 mM) is very high (126). If applied asymmetrically to vertically oriented roots, they curve toward the side of high [$Ca^{2+}$] (127). The downward bending of horizontal roots in response to gravity, as in shoots, thus appears to involve the lateral movement of $Ca^{2+}$ to the slower growing side. To demonstrate this point, Lee et al (127) followed the movement of $^{45}Ca^{2+}$ from donor to receiver blocks placed along opposite sides of horizontally growing roots. Large movements of $Ca^{2+}$ from the upper to lower side occurred in the tip region of the root. Removal of the root cap, which is known to contain the gravity-sensing statocytes, inhibited lateral $Ca^{2+}$ transport (127). The metabolic inhibitors DNP and KCN suppressed $Ca^{2+}$ displacements and root curvature.

Of particular interest is the fact that inhibitors of auxin transport blocked the movement of $Ca^{2+}$ to the slower-growing side of roots (128). The concept of the opposite movement of $Ca^{2+}$ and auxin had emerged earlier from studies of gravistimulated shoots, but whether it is the $Ca^{2+}$ that influences auxin or vice versa is not known. Lee et al (128) favor the hypothesis that in roots gravistimulation first causes movement of $Ca^{2+}$. An increased accumulation of the ion on the lower side induces movement of auxin out of the apex into the elongation zone along the lower side. Possibly the accumulation of the hormone may be sufficient to inhibit growth in cells on the lower side and thus cause downward bending by permitting normal growth to continue on the upper side.

In roots the gravity-responding cells, the statocytes, possess $Ca^{2+}$-containing amyloplasts (25). These appear to be the organelles that sense gravity, but how their sedimentation elicits a $Ca^{2+}$ flux or metabolic change causing whole-scale lateral movement of wall-associated $Ca^{2+}$ is unknown. Some pieces of this puzzle are beginning to emerge from studies on the electrophysiological properties of roots and of statocytes in particular. Behrens et al (8) initially showed that the pattern of extracellular currents around roots changed within 30 sec in response to gravity. Whereas currents enter symmetrically around the tip in vertically oriented roots, when the roots are placed horizontally the currents flow acropetally on the upper side and basipetally on the lower side. Intracellular electrical recordings made on the statocytes themselves reveal that the plasma membrane potential in cells on the lower side of the root depolarizes in response to gravity while in those cells on the upper side the PM potential hyperpolarizes (215). The depolarization response is observed within 2 sec of the application of the stimulus and thus constitutes the most rapid gravity effect yet reported.

The significance of these findings to the mechanism of gravity response may be profound. Sievers et al (215) focus their attention on ER-located $Ca^{2+}$ pumps which they think may participate in the rapid changes of membrane potential. We do not deny the importance of the $Ca^{2+}$ pumps, but we think that attention should also be drawn to those channels that might allow $Ca^{2+}$ to enter the cytoplasm in the first instance. The depolarization of the membrane potential in the statocytes on the lower side of the root might be the trigger that opens a voltage-gated $Ca^{2+}$ channel. $Ca^{2+}$ would flow into the cytoplasm and stimulate processes including ATP-dependent $Ca^{2+}$ transport. If the pumps and channels were appropriately positioned, one could imagine that the electrical depolarization event would trigger the release of $Ca^{2+}$ from internal compartments (e.g. ER, mitochondria, plastids, vacuole) into the cytoplasm. Pumps at the PM would then expel $Ca^{2+}$ to the outside, thus raising the concentration of the ion in the cell wall. Alternatively, one could imagine that the PM depolarization induces a small influx of $Ca^{2+}$ from the wall space, which stimulates a more massive release of $Ca^{2+}$ from internal stores via a $Ca^{2+}$-induced $Ca^{2+}$

release mechanism (61). PM pumps would then eject $Ca^{2+}$ to the wall space. In either example the combined electrical-metabolic events result in morc wall-associated $Ca^{2+}$ on the lower side of the root. The statocytes on the upper side of the root that exhibit a slow, transient hyperpolarization (−13 mV) in contrast to the depolarization (+34 mV) (215) would not be expected to participate in $Ca^{2+}$ fluxes. The identification of specific gravity-sensing cells and the discovery of rapid changes in their electrical properties should provide a basis for future experimentation. Questions for which answers might be found include whether the cytoplasmic free [$Ca^{2+}$] increases in response to gravity. Also, using patch clamp recording, $Ca^{2+}$ channels may be identified on statocytes or protoplasts therefrom. To outsiders looking in, gravitropism appears to be an exciting area from which important answers could emerge in the next few years.



## *Cytokinin*

The involvement of cytokinins in the mitotic-cytokinetic processes of plant cells in a general sense alerts one to the possibility that $Ca^{2+}$ might participate in mediating the responses of the hormone. The occurrence in starfish of a hormone, 1-methyladenine, that is chemically related to cytokinin and activates arrested oocytes to continue meiosis in a process involving an increase in intracellular [$Ca^{2+}$] (151) has provided an important model and reference point for analyzing the mode of action of cytokinin.

Several years ago LeJohn and coworkers (131–133) showed that cytokinin stimulates $Ca^{2+}$ release from a glycoprotein on the surface of the water mold *Achlya*. Enhanced uptake of $Ca^{2+}$ subsequently occurred by a transport system that involved $Ca^{2+}$ binding to a cell wall glycopeptide and to the plasma membrane (131). A cytokinin-$Ca^{2+}$ interaction has been noted in several systems where these two factors appear to enhance ethylene production synergistically (68, 125), delay senescence in *Zea* leaf discs (175), and increase cotyledon dry weight in *Xanthium* (134). Furthermore, $Ca^{2+}$ will substitute for cytokinin in leaf disc expansion, and both ion and hormone affect membrane protein phosphorylation (179). That CaM may be involved in some of these events is supported by the findings of Elliott and coworkers (56–58), who have shown that a variety of CaM inhibitors, including TFP, CPZ, dibucaine, and others, blocked betacyanine synthesis in *Amaranthus* cotyledons as well as growth and cell division in soybean callus cultures.

Studies in our laboratory on the regulation of bud formation in the moss *Funaria* have provided additional evidence that $Ca^{2+}$ mediates cytokinin activity (203–205). First, the fluorescent $Ca^{2+}$ chelate probe CTC stains the bud region of the target caulonema cells prior to the first asymmetric division, indicating that the region becomes enriched in $Ca^{2+}$-containing membranes (203). Comparative studies with the general membrane marker *N*-phenyl-1-

naphthylamine show that the amount of membrane increases by 1.5 times. However, the CTC signal in the bud region is elevated fourfold, indicating that the relative amount of $Ca^{2+}$ on the membrane has increased. Microscopic examination of CTC-labeled cells at time intervals following cytokinin induction indicates that the bud site, from the moment it is first recognized as a bulge on the caulonema cell wall, exhibits a marked fluorescence. Thus, cytokinin appears to cause an increase in membrane-associated $Ca^{2+}$, specifically in those regions of the target cell that are undergoing differentiation to become the bud (203). The high degree of polarity exhibited by the budding process indicates an underlying capacity of the target cell to become polarized. It seems likely that self-driven extracellular electrical currents participate in this process.

A second indication that calcium participates in bud induction has been obtained from studies in which the ion has been experimentally increased in the absence of the hormone (204). This has been achieved by culturing the protonemata in the ionophore A 23187 plus $Ca^{2+}$. Under these conditions buds are initiated on every target cell despite the fact that cytokinin has not been added (204). Although A 23187 and $Ca^{2+}$ promote bud initiation, it is important to add that these initials do not develop into complete buds. Cytokinin thus appears to provide stimuli or activate processes in addition to those that can be modulated by $Ca^{2+}$ alone.

Finally, protonemata grown under conditions in which the extracellular $Ca^{2+}$ has been restricted fail to form buds (205). EGTA has been used to chelate $Ca^{2+}$ and markedly lower its extracellular concentration, while $La^{3+}$, verapamil, and D 600 have been used to block $Ca^{2+}$ transport from the wall into the cell. The resulting inhibition of budding, which occurs despite the presence of cytokinin, can be partially overcome by adding back calcium. Blockage of budding by TFP and CPZ provides tentative evidence that $Ca^{2+}$-CaM is involved in the developmental process (205).

Taken together, these studies provide a persuasive argument for the role of $Ca^{2+}$ in mediating cytokinin-stimulated bud development. Based on the results using $Ca^{2+}$ channel blockers and transport inhibitors, we conclude that the hormone, upon binding to the target caulonemata, induces an increase in the influx of $Ca^{2+}$ from the extracellular wall space to the cytoplasm. The increase in $[Ca^{2+}]$ is not thought to be a "trigger" in the usual sense of the word since a continuous supply of cytokinin is needed to complete formation of the buds. If the cytokinin and/or $Ca^{2+}$ is removed, then budding does not occur; if initials have already arisen, they will revert to branches rather than become buds. Given conditions of nonlimiting cytokinin-$Ca^{2+}$, the elevated levels of $Ca^{2+}$ may saturate intracellular $Ca^{2+}$ binding sites such as CaM that in turn activate a variety of processes including notably a marked migration of the nucleus to one end of the cell followed by a highly asymmetrical division.

The evidence from *Funaria* conforms well with Jaffe's (101) rules. What is missing is a direct demonstration that free [$Ca^{2+}$] increases following cytokinin application. However, the CTC data, which show that the membrane-associated $Ca^{2+}$ increases at the bud site and remains elevated during differentiation, provide indirect evidence for an increase in free [$Ca^{2+}$]. It seems plausible that cytokinin enhances $Ca^{2+}$ influx at the PM, and the subsequent uptake of $Ca^{2+}$ by cytoplasmic membrane systems accounts for the increased CTC fluorescence.



## *Gibberellin*

The induction of α-amylase secretion in barley aleurone cells by gibberellic acid (GA) constitutes one of the classic experimental systems in plant physiology. Since protein secretion in several different animal systems is dependent upon $Ca^{2+}$ (197), it is reasonable to assume that the ion will also participate in α-amylase secretion. Chrispeels & Varner (31) first identified the involvement of $Ca^{2+}$ in α-amylase secretion; since that time numerous reports have appeared that address this question (109, 110, 149, 160).

Barley aleurone cells require a relatively long incubation in GA (6 hrs) before they release α-amylase (149). If $Ca^{2+}$ is added at a concentration of 10 mM, the process can be stimulated 70–80% above GA alone, whereas 0.2 mM is too low to support release. Alternately, if GA and $Ca^{2+}$ (5 mM) are optimal and the [$Ca^{2+}$] is lowered, then α-amylase secretion is reduced by 70–80% (110). The threshold for $Ca^{2+}$ is again 0.1–0.5 mM. Ruthenium red, an inhibitor of enzyme reactions involved in $Ca^{2+}$ uptake into mitochondria (185, 195) and inside-out vesicles made from red blood cells (94), has been used to characterize further the role of $Ca^{2+}$. Moll & Jones (149) report that ruthenium red, when added to aluerone cells that are cultured in $Ca^{2+}$ and GA, has no effect on α-amylase release. However, if the medium [$Ca^{2+}$] is lowered, enzyme release is not inhibited. Ruthenium red thus maintains a stimulated level of α-amylase secretion even under restrictive levels of extracellular $Ca^{2+}$. Moll & Jones (149), noting that ruthenium red blocks $Ca^{2+}$ efflux, argue that the inhibitor, by preventing $Ca^{2+}$ uptake by mitochondria and/or efflux at the PM, causes the intracellular [$Ca^{2+}$] to increase and it is this which permits continued enzyme release. The results obtained with ruthenium red, however, raise concerns that must be addressed before the conclusions can be accepted. Not the least of these is the report of Mitsui et al (147) showing that α-amylase secretion from rice scutellar tissue is completely inhibited by ruthenium red. There is also controversy in the literature about how the inhibitor works. For instance, Dieter & Marmé (47) find that ruthenium red blocks mitochondrial $Ca^{2+}$ uptake but has no effect on microsomal uptake. Can one be certain, in the case of the barley aleurone, that the inhibitor must enter the cell to inhibit mitochondrial $Ca^{2+}$ uptake? On which side of the PM does ruthenium red act?

Future studies that address these and other related questions may provide valuable new information about $Ca^{2+}$ and secretion in plants.

Additional support for $Ca^{2+}$ and for metabolic processes in α-amylase secretion comes from studies using different inhibitors. Low temperature studies indicate that enzyme release is dependent upon metabolic processes, supporting the contention that $Ca^{2+}$ regulates secretion at the PM rather than through an ion exchange process in the wall itself (149). Incubation of aleurone in potent metabolic inhibitors (HCN, DNP, and CCCP) however, causes a rapid burst in α-amylase secretion. Moll & Jones (149) suggest that there may be a release of ions in the presence of the inhibitors which supports α-amylase release. However, to the extent that other metabolic processes are involved it still seems puzzling that such potent inhibitors enhance the process of secretion. Finally, note is made of the findings that synthesis and secretion of α-amylase in scutellar tissue can be enhanced by the ionophore A 23187 together with $Ca^{2+}$ and inhibited by $La^{3+}$, as well as by two CaM antagonists W-7 and CPZ (147).

A recent development has been the observation that the secretion of isozymes of α-amylase are differentially affected by GA and $Ca^{2+}$ (109, 110). α-Amylase isozyme 2, for example, is secreted in the absence of GA and $Ca^{2+}$, whereas isozyme 1 requires only GA (no $Ca^{2+}$) and isozymes 3 and 4 require both GA and $Ca^{2+}$ (109). The differential control suggests that the isozymes are differently compartmentalized or processed and may make it possible in future experimentation to define the $Ca^{2+}$ target.

GA, of course, stimulates much more than α-amylase secretion; its enhancement of cell elongation is well studied. From earlier work showing an inhibition of growth by $Ca^{2+}$ (226), it is not surprising to find that GA and $Ca^{2+}$ are antagonists in this process. In lettuce hypocotyls 5 mM $Ca^{2+}$ reduces growth to zero while 0.5 mM allows rapid growth (150). GA affects the $Ca^{2+}$ growth relationship by stimulating elongation at moderately inhibitory levels of $Ca^{2+}$ (1 mM). Moll & Jones (150) suggest that GA may promote $Ca^{2+}$ uptake and thus remove the ion from the wall and permit enhanced growth. We think that this idea is unlikely since it would require the cell to take up an excessively large amount of $Ca^{2+}$, on the order of half or more of that bound in the wall.

Although $Ca^{2+}$ appears to participate in GA-induced responses, a considerable amount remains to be elucidated. The aluerone system appears to be good, especially with the discovery of α-amylase isozymes that are differentially sensitive to GA and $Ca^{2+}$ (109, 110). We are concerned here about the requirement for very high concentrations of $Ca^{2+}$ (10 mM). We wonder whether it would be possible in a flow-through apparatus to regulate closely the wall-bound $Ca^{2+}$ with EGTA buffers and discover that the levels of $Ca^{2+}$ absolutely needed are much lower than reported heretofore (see concluding section).

## *Auxin*

The multiplicity of auxin actions and the apparent involvement of $Ca^{2+}$ in many of these have made it difficult to derive a consensus about the interaction between these two factors in plant growth and development (60). A general view would suggest that auxin and $Ca^{2+}$ are antagonistic; auxin increases elongation while $Ca^{2+}$ (1–10 mM) inhibits growth (226). There is a complexity to their interaction as can be seen in studies on gravitropism, mentioned earlier, that will require considerable attention before it is elucidated.

The discovery that part of auxin action consists of a stimulated proton release which causes wall loosening and extension has marked an important advancement in our understanding of the mechanism of plant growth regulation (60). Since $Ca^{2+}$ at relatively high concentrations (1–10 mM) inhibits growth (226), it is reasonable to ask by what process it does so and whether it specifically interferes with proton release. The results indicate, however, that $Ca^{2+}$, rather than inhibiting proton release, stimulates the process (33, 35, 198, 231). There is disagreement as to the mechanism or even the need for living tissue. Rubinstein et al (198) find that $Ca^{2+}$ induces $H^+$ release from metabolically inactive tissue, causing them to argue that the process is due in part to ion exchange. Terry & Jones (231), on the other hand, assert from studies on pea internodes that the process requires metabolic activity since it is inhibited at 4°C. Throughout studies on growth (and gravitropic bending) that involve acidification of the wall it is important to keep in mind the effect that $H^+$ release has on $Ca^{2+}$ as well as the reverse. $H^+$ could displace $Ca^{2+}$ from wall-binding sites; in addition, transport enzymes at the PM may exchange $H^+$ for $Ca^{2+}$. Thus, understanding how these two cations interact may help explain wall loosening and cell expansion.

A long held view has been that $Ca^{2+}$, by crosslinking acidic polysaccharides, stiffens the wall thereby retarding the growth process (226, 227). The results showing that $Ca^{2+}$ has no effect on wall extension as measured by the Instron procedure, however, provide persuasive evidence against the pectic-crosslinking theory (33). Cleland & Rayle (33) favor a mechanism in which $Ca^{2+}$ interferes directly with the biochemical process of wall loosening.

There are other possibilities considered in this important study which might deserve a reinvestigation. We refer specifically to the possible role of $Ca^{2+}$ on turgor pressure. We realize that even at relatively high concentrations $Ca^{2+}$ is unlikely to affect turgor alone, but it is timely to recognize the possibility that small changes in intracellular [$Ca^{2+}$] could effect much larger changes in [$K^+$]. Studies on $K^+$ channels in animal cells have identified some that are regulated by $Ca^{2+}$ (92); there is even a pharmacological agent, apamin, a peptide from bee venom, that blocks conduction through the $Ca^{2+}$-regulated $K^+$ channel (96). Thus $K^+$ transport, controlled by changes in intracellular [$Ca^{2+}$], might

contribute to large-scale movement of ions that alter cellular osmotic properties.

A major focus in auxin research has been the elucidation of its polar transport properties. The discovery that EDTA inhibited auxin transport in *Helianthus* hypocotyl sections by 50% and that this inhibition could be overcome by washing in $CaCl_2$ provided an indication that $Ca^{2+}$ might be involved in polar movement of the hormone (43). Yet the effect may be more of a divalent cation action since $Mg^{2+}$ will restore considerable transport activity. However, recent studies performed on tissues that had been grown on low $Ca^{2+}$ without chelator pretreatment showed no stimulation of auxin transport by divalent cations other than $Ca^{2+}$ (44). Nevertheless, the site of cation activity may be the cell wall or the outer surface of the PM since $La^{3+}$, a well-known competitor for $Ca^{2+}$ binding (54, 144) that does not cross the membranes, restores much of the activity lost either through incubation in EDTA (43) or a low $Ca^{2+}$ solution (44).

The relationship of $Ca^{2+}$ to polar auxin transport acquires further complexity and interest in light of the studies showing that $Ca^{2+}$ moves in a direction opposite to that of the hormone (128). De Guzman & DeLa Fuente (42) reported that auxin promotes the acropetal efflux of $Ca^{2+}$ from *Helianthus* hypocotyl segments and further that the process is inhibited by the auxin transport inhibitor TIBA. The suggestion is made that a $Ca^{2+}$ influx regulates IAA efflux through a process of stimulus-secretion coupling. The initial stimulus may be an increase in the cytoplasmic concentration of charged IAA that causes a depolarization of the membrane potential. $Ca^{2+}$ flows into the cell and may activate a hormone carrier mechanism that transports charged IAA to the wall space. Opposite movements of $Ca^{2+}$ and auxin occur in both gravistimulated shoots and roots. Moreover, inhibitors of auxin transport (TIBA, NPA; morphactin) (66, 128) prevent movement of $Ca^{2+}$ to the slower growing side and consequently inhibit gravitropic curvature (see earlier).

Studies at the single cell level (34) and those conducted in vitro (74, 121) may yield insight into the relationship between auxin and $Ca^{2+}$ and provide a basis for understanding the opposite movement of these two growth factors. Cohen & Lilly (34) recently showed that protoplasts from etiolated soybean hypocotyl, when cultured in active auxins but not antiauxin, exhibited a decreased uptake and an increased efflux of $^{45}Ca^{2+}$. They suggest that auxin alters $Ca^{2+}$ flux at the plasma membrane and causes a lowered internal concentration of the ion. The results of Kubowicz et al (121) showing that auxin stimulates $Ca^{2+}$-ATPase activity provides direct evidence for a mechanism for $Ca^{2+}$ efflux. Interestingly, the earlier studies of Thimann & Sweeney (233), reporting that auxin stimulated the rate of cytoplasmic streaming, can be interpreted on similar grounds since streaming is promoted by a lowered $[Ca^{2+}]$. Many factors thus point to the existence of a close interrelationship between auxin and $Ca^{2+}$.

Despite the progress in resolving the auxin-$Ca^{2+}$ relationship, many issues need to be clarified. The data suggest that auxin causes a lowering of intracellular [$Ca^{2+}$] but a direct demonstration of this fact is required. One normally considers the cell to be at a low resting level of $Ca^{2+}$ already and that a stimulus raises that level. Could it be that the [$Ca^{2+}$], following auxin application, first rapidly rises, then subsequently falls, and even undershoots the normal level as a result of $Ca^{2+}$-ATPase activity? In the work to date, few of Jaffe's (101) rules have been satisfied, but perhaps this is an unfair expectation given the apparent reverse nature of the auxin-$Ca^{2+}$ relationship. Certainly an issue that must be resolved concerns the high levels of $Ca^{2+}$ used (see last section). The finding that $Ca^{2+}$ at 0.5 M (sic) induces a thickening of auxin-treated membranes (152) becomes irrelevant in view of the high concentration used.

## AN EVALUATION OF $Ca^{2+}$ IN PLANT DEVELOPMENT

Studies on the role of $Ca^{2+}$ in plants are accelerating at a rapid rate. Many exciting new results have emerged and for a whole variety of processes, mainly in the realm of stimulus-response coupling, it seems evident that $Ca^{2+}$ will be found to participate as a second messenger. The central importance of $Ca^{2+}$ to growth and development acquires new dimensions in light of its regulatory function and provides the experimenter with new ways of thinking and approaching old problems. Despite our general enthusiasm about the $Ca^{2+}$-plant biology field, we think that there are some major problems concerning the emerging results that warrant critical attention. In particular we are concerned and perplexed about the large apparent disparities that exist between the concentration of $Ca^{2+}$ used and that required by the cell to perform certain functions. The problem to which we refer below is widespread in studies on plant systems. It is our purpose not only to draw attention to the issue but to provide a framework within which to perform future $Ca^{2+}$ studies. We hope that by establishing guidelines for future investigations it will be possible to create a sound basis for the interpretation of new results.

We have emphasized the fact that the intracellular [$Ca^{2+}$] is submicromolar and that processes are activated by raising the concentration to 1–10 $\mu$M. What, then, is the meaning of the numerous studies that show a requirement for $Ca^{2+}$ at the millimolar level? Virtually all the studies on auxin, gibberellin, cytokinin, and gravity and many others as well have been performed using [$Ca^{2+}$] between 0.1–10 mM, a full thousandfold higher than would seem to be required by the cell. We do not have an answer for this problem but think that it is important and deserves attention. One cannot continue to show "$Ca^{2+}$ effects" disregarding the concentration of ion used and assume that the results obtained fit neatly into the "$Ca^{2+}$ story."

If all studies showed a requirement for millimolar amounts of exogenous $Ca^{2+}$, then the problem would not be so perplexing, but they do not; there are

several different examples in which the process under investigation is modulated at the micromolar level. We hasten to add in making our argument that we are not including results from studies on cell-free preparations or opened cells. A growing list including flagellar beat reversal in *Chlamydomonas* (206), spore germination in *Onoclea* (254), the metaphase/anaphase transition in *Tradescantia* (82), cytoplasmic streaming inhibition in cultured cells of *Lycopersicon* (277), volume regulation in *Poterioochromonas* (116), peroxidase secretion from *Spinacia* (223), and pollen tube growth in *Lillium* (168) reveals that many processes are stimulated at micromolar concentrations of $Ca^{2+}$. There are other systems including serine uptake in culture cells of *Nicotiana* (221), bud formation in *Funaria* (205), membrane depolarization in *Nitella* (261), root tip adhesion in *Phaseolus* (229), and chloroplast rotation in *Mougeotia* (248) that are markedly stimulated at 100 μM $Ca^{2+}$ and might well show need for less.

A feature that characterizes the examples cited above and that may explain their response to low levels of $Ca^{2+}$ is the fact that they are single cells, single files of cells, or cultured cell preparations. Regulation of the external [$Ca^{2+}$] is made easier in these systems than in multicellular tissues probably because of the accessibility of the cell wall compartment. Nevertheless, the results show that response coupling of widely different processes in intact cells from both higher and lower plants is modulated by micromolar quantities of $Ca^{2+}$. Since these levels of $Ca^{2+}$ conform to those known to be required for intracellular reactions, the issue becomes one of explaining why millimolar $Ca^{2+}$ is found to be needed in the bulk of the processes that have been examined.

Resolution of the $Ca^{2+}$ concentration problem will probably benefit from a careful examination of the cell wall requirements for $Ca^{2+}$ and a separation of those from the requirements of the cytoplast itself. The cell wall is loaded with $Ca^{2+}$ (73), and since its inner layer is appressed to the cytoplast, the PM experiences a high concentration of ion on its outside and a low concentration on its inside. One can argue that these asymmetric conditions of $Ca^{2+}$ distribution must be met in order for the PM transport properties to operate normally. However, we have just pointed out that there are several different examples in which the cell, usually bounded by a wall, can be depleted of $Ca^{2+}$ and show a response at the micromolar level.

One approach for overcoming the problem may be to select simple systems. As heretical as it sounds, perhaps the classical objects, e.g. coleoptiles, aluerone layers, hypocotyl segments, and entire roots, are inappropriate for physiological studies on $Ca^{2+}$. The cell wall content, and thus the residual $Ca^{2+}$ quantity, may be too large and/or too inaccessible for easy removal. Ways for selectively isolating the responding tissues or cells should be developed. Alternatively, it may prove useful to explore other systems which by their normal development consist of only one or a few cells. Among the lower,

nonflowering plants, for example, there are numerous examples of developmental processes that are confined to a few cells in which a $Ca^{2+}$ contribution to stimulus-response coupling could be examined; we have already discussed light-stimulated spore germination in *Onoclea* and cytokinin activated bud formation in *Funaria,* but in addition there are many others, including gravitropism in rhizoids of *Nitella* (216), GA-induced antheridia initiation in fern gametophytes (154), auxin-induced caulonemata formation in mosses (107), and rhizoid formation in *Bryopsis* (98).

A second consideration in developing experiments designed to overcome the confusion that arises from wall-bound $Ca^{2+}$ is to remove the ion with chelators. Both EGTA and EDTA are being used; EGTA is recommended because of its greater selectivity than EDTA for $Ca^{2+}$ over $Mg^{2+}$. We further emphasize the desirability of using $Ca^{2+}$-EGTA buffers that contain nonlimiting amounts of $Mg^{2+}$. If the process under examination conforms to one that is $Ca^{2+}$-dependent, then it will not be stimulated by $Mg^{2+}$ at 1 mM. Chelators can cause serious damage, and thus it may be necessary to use low concentrations ($<$ 1 mM) for long times (hours up to 24) and to use solutions that contain a small amount of $Ca^{2+}$ ($10^{-8}$ M) as an initial incubation medium. In addition, entire experiments should be performed in a $Ca^{2+}$-EGTA buffer. Rather than soaking a tissue in EGTA and then replacing that solution with one containing $CaCl_2$, a preferred method is to culture the tissue continuously in a $Ca^{2+}$-EGTA buffer. By keeping the EGTA and $Mg^{2+}$ concentrations constant one can change only the [$Ca^{2+}$] and in this way define more precisely the limiting amount of $Ca^{2+}$ needed for a given event. Further regulation over the internal $Ca^{2+}$ milieu may be gained by using ionophores (A 23187, ionomycin) in conjunction with the $Ca^{2+}$-EGTA buffer. A final note of procedural detail is the necessity in all of these studies to use plastic vessels rather than glass; micromolar quantities of $Ca^{2+}$ leach out of glass, even after careful washing, and confound one's most carefully executed experiments (214).

An additional factor that must be considered in attempting to decipher the contribution of $Ca^{2+}$ is the potential complexity of the process that is under investigation. For example, tissue elongation or gravitropic bending involve many processes, of which more than one may depend on $Ca^{2+}$. Indeed the confusion about $Ca^{2+}$ participation in growth may revolve around the interaction of the ion with both cytoplast function and cell wall behavior. Attempts to analyze less complex events and also those that occur rapidly may offer dividends to the experimenter and allow him/her to come closer to the primary action in stimulus-response coupling.

The above comments have been aimed at the concentration problem, but there are other aspects of $Ca^{2+}$ experimentation in plant systems that deserve attention. One in particular concerns the mechanism of $Ca^{2+}$ transport both into and out of cells. Considerable effort has been directed toward elucidating the

character and function of $Ca^{2+}$-ATPase pumps (141, 142). We applaud this effort and hope that studies will continue and expand since understanding the mechanisms about how $Ca^{2+}$ is removed from the cytoplasm is central to the regulation of ion concentration. Although there has been focus on the $Ca^{2+}$-ATPase, there has been surprisingly little attention given to the $Ca^{2+}$ channels, the pores that allow $Ca^{2+}$ to move rapidly into the cells In a broad scheme of stimulus-response coupling the $[Ca^{2+}]$ must first rise, and thus an understanding of how these channels open and close is crucial.

The properties of $Ca^{2+}$ channels in animal systems are under intense investigation using pharmacological and electrophysiological procedures (88, 92). There are numerous drugs that fit the general description of a $Ca^{2+}$ entry blocker (verapamil, D 600, nifedipine, diltiazem) (63, 104); they appear to block movement of the ion through voltage-gated $Ca^{2+}$ channels. The general concept has emerged that these channels open in response to a depolarization of the membrane potential and $Ca^{2+}$ flows into the cell, raising the internal free $[Ca^{2+}]$. In the dihydropyridine (nifedipine) class of blockers there are some that are agonists (BAY K8644) and stimulate channel opening (88). In brief, there is a huge arsenal of drugs to which we can turn for $Ca^{2+}$ channel studies. Some of the drugs may, of course, not work on plant systems, but studies with verapamil (205, 246) and D 600 (82, 205, 206) indicate that these at least work well. Together with electrophysiological studies on the membrane potential and on the properties of the channels themselves, it may be possible to define more precisely some of the early and rapid changes that occur following a stimulus.

Finally, in discussing some of the goals in $Ca^{2+}$ research on plant cells, we again emphasize the importance of directly determining the free $[Ca^{2+}]$ in living cells. If $Ca^{2+}$ mediates stimulus-response coupling, then an increase in its cytoplasmic free concentration should occur. To our knowledge the only report on plants is that of Williamson & Ashley (273), who microinjected the photoprotein aequorin into Characean algal internode cells and measured changes in $[Ca^{2+}]$ following an action potential. One reason for the paucity of reports on plant cells is the difficulty of performing the experiments. Nevertheless, the importance of knowing the internal $[Ca^{2+}]$ and whether it changes following a stimulus is absolutely central to understanding the role of $Ca^{2+}$. There are a variety of agents besides the photoprotein aequorin that can be used, including metallochromic dyes, e.g. arsenazo III (12), fluorescent chelators, quin-2 (240, 241), and fura-2 (169), and $Ca^{2+}$ electrodes (12). The fluorescent indicators, especially the newly introduced fura-2 (169) in its acetoxymethyl ester form, could prove to be extremely valuable in permitting us to measure free $Ca^{2+}$ in cells that could not otherwise be loaded by microinjection with arsenazo III or aequorin.

The determination that $Ca^{2+}$ mediates many different processes constitutes

an important advancement in our knowledge, but it is only the beginning in our effort to decipher the entire transduction chain from the initial stimulus to the response. What are the response elements to which $Ca^{2+}$ or $Ca^{2+}$-CaM bind? Which reactions within the cell do the response elements control? How is the $Ca^{2+}$ signal modified so that it can be sustained? What determines how one cell responds to one stimulus and neighboring cells to another? How can two nearby cells respond differently to the same stimulus? These are but a few of the many exciting questions that should occupy our attention in the years ahead. Through $Ca^{2+}$ we have a means of probing basic aspects of growth and development in plants from which unifying fundamental concepts and mechanisms may emerge.



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