Molecular and Cellular Aspects of Calcium Action in Plants

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Calcium is known to be a second messenger in many developmental processes in animal systems, but it has only recently become evident that Ca is an important intracellular messenger in plants as well (9, 15, 22, 32–35, 41). The level of free Ca concentration in the cytoplasm is extremely low, and it is influenced by extracellular signals such as light, gravity, and hormones. Investigations from our laboratory indicated that Ca and its binding protein, calmodulin, play an important role in stimulus-response coupling by regulating enzyme activities, especially through protein phosphorylation (33, 34, 48). In vivo and in vitro protein phosphorylation studies have revealed Ca-dependent changes in various plant tissues (18, 37–39, 49, 50). We have also been able to influence various physiological processes such as cell elongation, abscission, senescence, and tuberization by altering extracellular and intracellular Ca levels (33). Other examples of Ca-mediated processes in plants are as follows: a) cell division, b) geotropism, c) protoplasmic streaming, d) stomatal control, e) chloroplast movement, f) secretion, g) hormone-dependent changes, h) enzyme activation, and i) protein phosphorylation (9, 33).

Calcium is essential to maintain structural integrity of membranes and cell walls (8, 11, 14, 20, 26). In plants, the major portion of the Ca is present in the apoplast, primarily complexed with cell wall moieties and the plasma membrane. The importance of Ca in cell-to-cell adhesion is well recognized (8, 11, 23–25, 30, 44). The cementing effect is due primarily to Ca pectate of the middle lamella. Calcium is essential for the maintenance of cell wall structure, especially in fruits and vegetables that are stored for prolonged periods (30). Preharvest Ca treatments are effective in improving overall quality and overcoming physiological disorders such as bitter pit in apples, blossom end rot in tomatoes, tip burn in lettuce, and hollow heart and brown center in potatoes (23, 30). Postharvest dipping or vacuum- or pressure-infiltration with a Ca solution also is effective in improving storage quality in apples (30, 31).

Extracellular calcium

Concentrations of 1 to 5 mM Ca^{2+} are known to occur in the cell wall region. These concentrations are essential to protect the plasma membrane and to maintain the structural integrity of the cell wall. Calcium is known to delay senescence in apples, resulting in firmer fruit (17, 23, 30, 31). During ripening and senescence, dissolution of the middle lamella occurs, resulting in cell separation. Calcium-treated fruits, which remain very firm during storage, have a densely stained middle lamella and increased cell-to-cell adhesion. To study the effect of Ca on fruit firmness and on changes in the ultrastructure of the cell wall, postharvest Ca infiltration of apples was performed. As shown in Fig. 1, Ca protects the middle lamella from normal breakdown associated with senescence. In addition to fruit firmness, Ca-treated apples possess lower membrane permeability and contain more chlorophyll and ascrobic acid than those not treated. Respiratory CO_2 evolution and ethylene production, which are normally high in senescing fruits, are lowered in apples after Ca treatment (17, 31). These results suggest that some of the senescence-related biochemical processes in apples are delayed by Ca (21, 23, 30). The mechanisms involved in these changes are not clearly understood.

Intracellular calcium

The free Ca ion is now considered as a major intracellular regulator of numerous biological and physiological processes in plants (9, 22, 33, 41). Over the past 5 years, a large body of evidence has accumulated that enables us to propose a working hypothesis for the mode of Ca action. This hypothesis states: a) the free cytoplasmic Ca concentration is < 1 μM and under metabolic control; b) the cytoplasmic Ca concentration can be regulated by various extra- or intra-cellular signals such as light, gravity, and hormones; and c) the cytoplasmic Ca binds to calmodulin, thereby activating it. In this activated state, enzymes can bind to the calcium–calmodulin complex leading to the response. The major compartments of the cell where high levels of Ca could be stored include organelles such as endoplasmic reticulum, mitochondria, chloroplasts, and the vacuole (Fig. 2).

Intracellular Ca distribution plays a critical role in cell function. Excessive amounts of free Ca^{2+} in the cytoplasm are injurious to the cell (9, 33). At high levels, Ca^{2+} reacts with inorganic phosphate to form an insoluble precipitate. Thus, if cytosolic Ca^{2+} concentrations were allowed to reach the millimolar levels present in the cell wall region, phosphate-based energy metabolism would be seriously inhibited. Plants have evolved a mechanism for removing excess Ca from the cytoplasm and maintaining free Ca^{2+} concentration in the submicromolar range. This mechanism requires active pumping of Ca out of the cytoplasm.

In contrast to the intracellular free Ca level, which is submicromolar, the concentration of the closely related divalent cation, Mg^{2+} is in the millimolar range. Despite this high concentration of Mg^{2+} in the cytoplasm, cellular processes often display a greater selectivity for Ca. It is suggested that Ca acts as a second messenger in the response of plant tissues to external signals (9, 15, 32–35, 41). To understand the precise role of Ca in these processes, one must be able to measure accurately changes in free cytoplasmic Ca concentration. The discovery of fluorescent Ca indicators such as quin-2 and fura-2, have made it possible to monitor free Ca concentrations (45, 47, 51). These materials work very well in animal cells. However, in plant cells there are many obstacles to overcome before the rapid and transient changes in free Ca^{2+} can be determined accurately. It is believed that a 10-fold increase in messenger concentration (Ca) is necessary to alter the state of a target protein such as calmodulin.

Calmodulin

Calmodulin is a Ca-modulated protein that has been isolated and characterized from a large variety of animal and plant tissues and is ubiquitous among eukaryotes (1, 5). The physical and biochemical properties of calmodulins isolated from a variety of sources are similar. This similarity suggests a fundamental role for calmodulin in mediating Ca-dependent processes in plant as well as in animal cells (1, 5, 9, 15, 22, 33, 35, 41). Since the discovery of Ca- and calmodulin-dependent enzymes in plants (1, 15, 18, 19, 41, 48), there is increasing interest in studying the role of Ca as a cellular messenger in plants. Whenever there is a transient increase in the cytoplasmic Ca^{2+} concentration, Ca^{2+} binds to calmodulin, thereby activating it. The calcium–calmodulin complex then binds to the enzyme, ultimately leading to the response (Fig. 3).

Calcium-mediated protein phosphorylation

In recent years, major interest has been directed toward post-translational modification of proteins (46) by Ca-regulated protein

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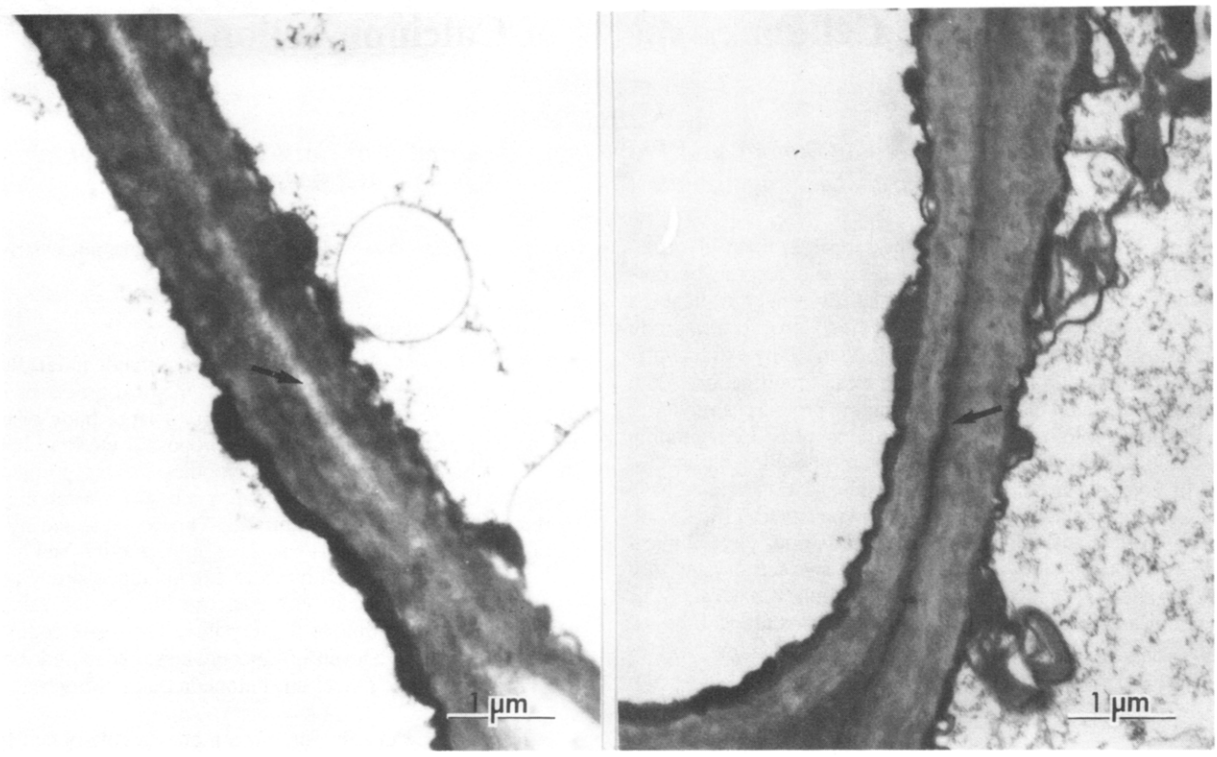

Fig. 1. Electron micrographs showing the ultrastructure of cell walls of control and Ca-treated apple fruit. Fruit were infiltrated with $CaCl_2$ solution soon after harvest (See ref. 31 for details) and stored for 6 to 8 months. **Left:** Cell wall of a control fruit showing the dissolution of middle lamella (arrow). **Right:** Cell wall of a Ca-treated fruit showing intact, darkly stained middle lamella (arrow) (G.M. Glenn and B.W.P., unpublished data).

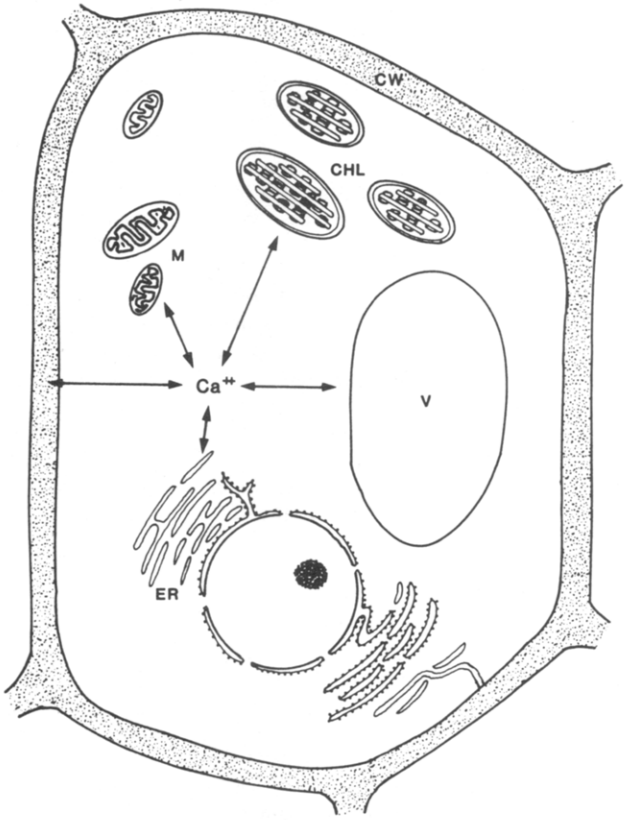

Fig. 2. Major Ca stores in the plant cell. Endoplasmic reticulum (ER), mitochondria (M), vacuole (V), chloroplasts (CHL). The cell wall (CW), which protects the plant cell, also contains high amounts of Ca^{2+} (25).

phosphorylation in plants and animals (6, 37, 48–50). The role of protein kinases in phosphorylation and phosphatases in dephosphorylation has been demonstrated in both plants and animals (6, 33). Reversible protein phosphorylation offers a unique advantage in cellular regulation, since an enzyme activated after phosphorylation by a protein kinase can be inactivated as a result of dephosphorylation by a phosphatase. When intracellular Ca concentration increases in response to an external stimulus, Ca- and calmodulin-dependent protein kinases are activated, resulting in protein phosphorylation. Extracts from various plant tissues have demonstrated Ca- and calmodulin-dependent phosphorylation of proteins (1, 18, 19, 35, 38, 48, 50). An example of Ca-dependent phosporylation in oat coleoptiles at micromolar levels of free Ca is shown in Fig. 4.

Role of calcium in hormone action in plants

Previous studies by Poovaiah and Leopold (26–29) and Leopold et al. (12) have shown that Ca could have strong modifying effects on the functions of each of the five known plant hormones, in some instances amplifying the hormonal response and in others suppressing it. Recently, the role of Ca in mediating the cytokinin effect was studied by first depleting Ca from corn leaf disks by an EGTA pretreatment and then transferring the disks to a medium containing cytokinin with or without Ca. After the EGTA pretreatment, cytokinin is no longer effective in delaying the loss of protein, a key parameter of senescence (Fig. 5). The cytokinin effect is restored, however, by the addition of Ca. This effect suggests that the response to cytokinin is mediated by Ca. Previous investigations by Saunders and Hepler (42) have shown that the Ca ionophore A 23187 could mimic the effect of cytokinin in the bud formation of *Funaria*. Their results indicate that stimulus-response coupling involves an influx of Ca into the cytoplasm. Calmodulin antagonists inhibit auxin-induced elongation in corn and oat coleoptiles, suggesting the involvement of Ca and calmodulin in auxin-induced elongation (36). Recent evidence indicates auxin-regulated and calmodulin-dependent changes in protein phosphorylation (39), which further emphasizes the overall significance of Ca and calmodulin in hormonal control in plants. Tuberization in potato has also been shown to be influenced by Ca (2).

Abscisic acid (ABA) is known to induce stomatal closure (13). Recent studies show a synergistic interaction between Ca^{2+} and ABA in preventing stomatal opening (10). These results are consistent with the hypothesis that ABA increases the permeability of

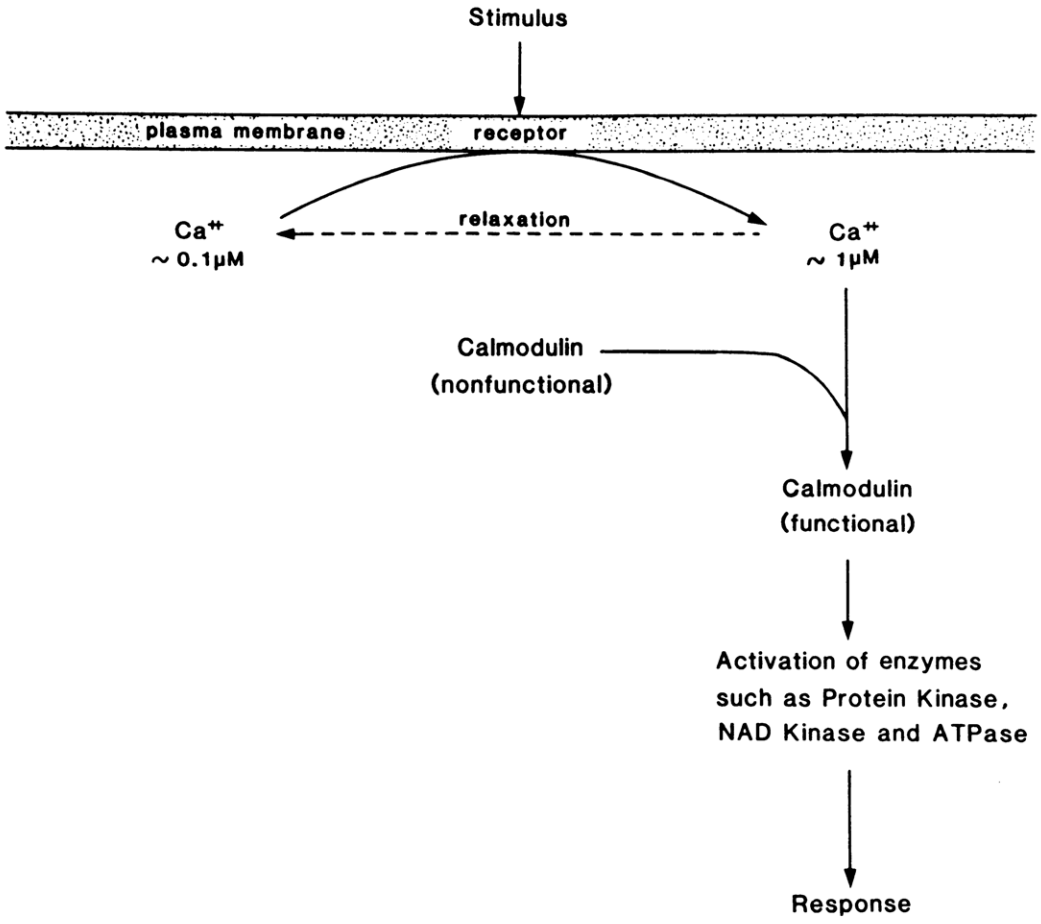

Fig. 3. Calcium- and calmodulin-dependent regulation in plant cells. In the unstimulated cell, free Ca concentration in the cytoplasm remains low, submicromolar range. Following stimulation, the Ca concentration in the cytoplasm increases and Ca binds to calmodulin, making it functional. The calcium–calmodulin complex binds to the enzyme, forming the functional calmodulin–Ca–enzyme complex and induction of the response (25).

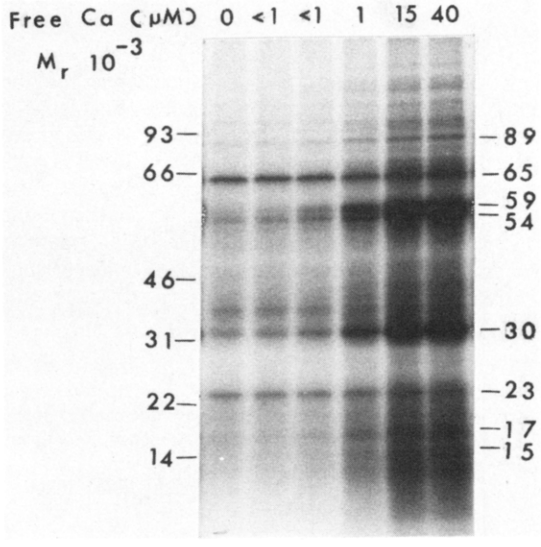

Fig. 4. Effect of micromolar concentrations of free Ca^{2+} on in vitro protein phosphorylation. All reaction mixtures contained 0.2 mM EGTA and total Ca was varied from 0 to 0.25 mM. Free Ca^{2+} concentrations as determined using a Ca^{2+}-sensitive electrode are indicated on the top. M_r values of some representative polypeptides and standards are indicated on the sides (50).

the guard cell to Ca^{2+}. Calcium then might operate as a second messenger to regulate the ionic fluxes that determine guard cell turgor.

Role of phosphoinositides in calcium messenger system

In recent years, a great deal of attention has been given to the turnover of phosphoinositides in the membrane. A schematic dia-

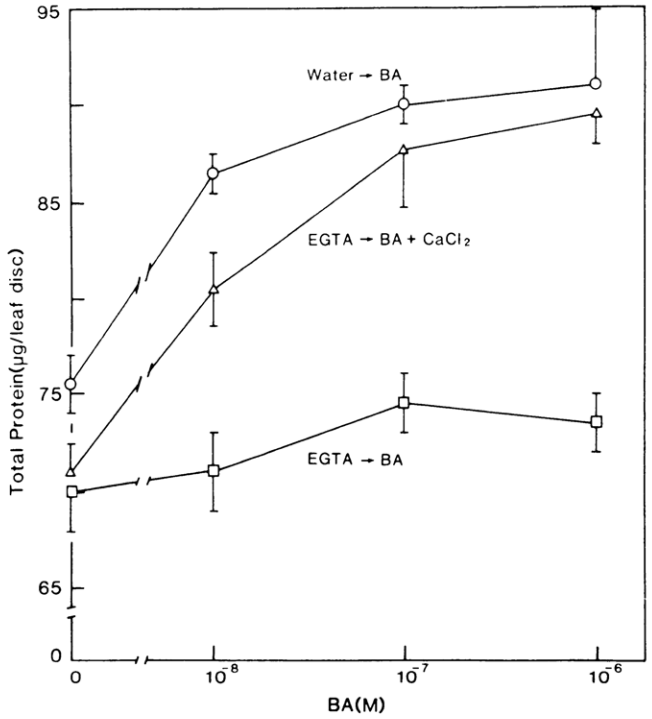

Fig. 5. Effect of pretreatment of 1 mM EGTA on the protein content of corn leaf disks. After pretreatment for 5 hr, leaf disks were transferred to 10^{-8} to 10^{-6} M cytokinin (BA) with or without 1 mM $CaCl_2$ and incubated in the dark for 4 days. Initial value of total protein 120.2 ± 6.2 (24).

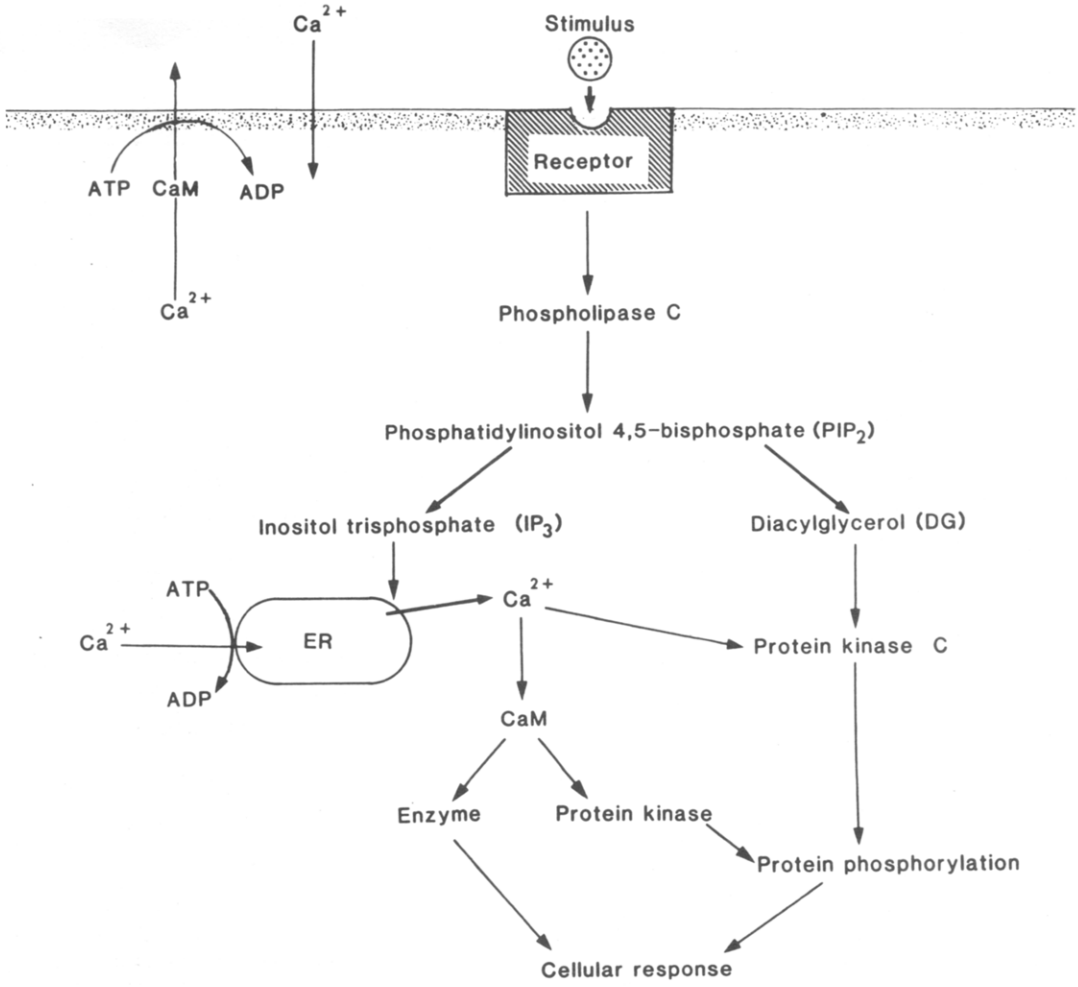

Fig. 6. Diagram illustrating the turnover of phosphoinositides, Ca signal pathways, and the induction of cellular response. CaM, calmodulin, ER, endoplasmic reticulum (34).

gram illustrating the turnover of phosphoinositides and Ca mobilization leading to cellular response is shown in Fig. 6. As indicated in this model, the primary stimuli such as hormones, light, and gravity interact with the receptor. This interaction results in the activation of phospholipase C and the cleavage of the membrane lipid phosphatidylinositol-4,5-bisphosphate into diacylglycerol and inositol trisphosphate (3, 33, 34). Inositol trisphosphate then releases Ca from the endoplasmic reticulum (7, 40). This released Ca can activate calmodulin-dependent enzymes, such as protein kinases. Together with diacylglycerol, Ca can also activate protein kinase C, ultimately leading to protein phosphorylation and cellular response (3, 16, 33, 34). The presence of phosphoinositides has been shown in plant tissues (4), and recent evidence suggests that some similarities exist in the phosphoinositide pathway in plants and animals (33, 34). Inositol trisphosphate-induced Ca mobilization from microsomal fraction has been observed in our laboratory (40) and elsewhere (7). Protein kinase C has recently been reported in plants (43). We also have evidence to show the involvement of phosphoinositides in promoting phosphorylation of soluble proteins (33). More information is needed to clarify the significance of this pathway in plants.

Conclusion

The mechanism of Ca action is just beginning to be understood at the molecular level. A dramatic unfolding of information during the past 5 years suggests that Ca is not only a micronutrient, but it also has major metabolic and developmental control in plants. A detailed understanding of the biochemical processes mediated by Ca and calmodulin would help our understanding of various processes involved in plant growth and development. Although Ca- and calmodulin-mediated biochemical processes as well as the turnover of phosphoinositides have been well-studied in animal systems, such studies are in their infancy in plants. However, the regulation of plant growth processes by Ca points to a second messenger role for Ca similar to that already known for animal systems. What is lacking in plant research is information at the molecular level on how Ca transport from membranes, organelles, and the cell wall is controlled and what are the initial events that trigger the biochemical processes that depend on changes in cytoplasmic Ca concentration. We need to perfect techniques for precisely measuring transient changes in free cytoplasmic Ca levels. Accumulating evidence suggests a major role for Ca in cell function and signal transduction in plants.

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Intracellular Calcium Dynamics, Cell Development, and Stress Tolerance

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Abstract. Intracellular Ca^{2+} and regulation of cell Ca^{2+} play important roles in cell development and in the maintenance and modulation of various cell functions. This report will describe research on the role of membrane and free cytoplasmic Ca^{2+} in cell development and stress tolerance. Results to be presented include microscopic fluorometric data obtained using fluorescent probes for Ca^{2+} and cytoskeletal proteins within individual cells to investigate the role of CA^{2+} in membrane organization, establishment of cell polarity, and maintenance of cytoplasmic streaming under stress conditions.