THE GENERATION OF Ca²⁺ SIGNALS IN PLANTS

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Abstract The calcium ion is firmly established as a ubiquitous intracellular second messenger in plants. At their simplest, Ca²⁺-based signaling systems are composed of a receptor, a system for generating the increase in [Ca²⁺]cyt, downstream components that are capable of reacting to the increase in [Ca²⁺]cyt, and other cellular systems responsible for returning [Ca²⁺]cyt to its prestimulus level. Here we review the various mechanisms responsible for generating the stimulus-induced increases in [Ca²⁺]cyt known as Ca²⁺ signals. We focus particularly on the mechanisms responsible for generating [Ca²⁺]cyt oscillations and transients and use Nod Factor signaling in legume root hairs and stimulus-response coupling in guard cells to assess the physiological significance of these classes of Ca²⁺ signals.

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INTRODUCTION

The calcium ion (Ca$^{2+}$) is firmly established as a ubiquitous intracellular second messenger in plants (114, 116). An increase in the cytosolic concentration of Ca$^{2+}$ ([Ca$^{2+}]_{\text{cyt}}$) couples a diverse array of signals and responses. At their simplest, Ca$^{2+}$-based signaling systems are composed of a receptor, a system for generating the increase in [Ca$^{2+}]_{\text{cyt}}$, downstream components that are capable of reacting to the increase in [Ca$^{2+}]_{\text{cyt}}$, and other cellular systems responsible for returning [Ca$^{2+}]_{\text{cyt}}$ to its prestimulus level. This apparently simple scheme hides considerable complexity. Although it has been argued recently that plant Ca$^{2+}$ elevations may more often act as switches in the signaling process (118) rather than encoding specific information, it is clear that the cell has multiple mechanisms for generating increases in [Ca$^{2+}]_{\text{cyt}}$ (116, 136), which suggests the capacity to produce highly complex spatio-temporal patterns of [Ca$^{2+}]_{\text{cyt}}$ elevation. Downstream of the stimulus-induced [Ca$^{2+}]_{\text{cyt}}$ increase (or Ca$^{2+}$ signal), the cell possesses an array of proteins that can respond to changes in [Ca$^{2+}]_{\text{cyt}}$ such as calmodulin (CaM) (85, 120), Ca$^{2+}$-dependent protein kinases (19, 54), and CaM-binding proteins (113, 143).

Further complexity arises with the involvement of other intracellular messengers and signaling proteins that can modulate Ca$^{2+}$ signaling. In recognition of this increasing complexity, it is perhaps more appropriate to represent intracellular signaling as a network. Within a network-based organization a stimulus-induced increase in [Ca$^{2+}]_{\text{cyt}}$ can be regarded as one of a small number of nodes or hubs responsible for orchestrating the events that comprise the full cellular response. The possibility that guard cell signaling might be organized as and exhibit properties reminiscent of a specific type of network known as a scale-free network has been discussed (57, 58).

In this review we only consider the processes involved in generating Ca$^{2+}$ signals. Currently, our understanding of how complex spatio-temporal patterns of [Ca$^{2+}]_{\text{cyt}}$ elevation contribute to controlling responses in stimulated cells is fragmentary. Nevertheless, recent work has provided strong evidence that information is encoded in [Ca$^{2+}]_{\text{cyt}}$ oscillations and transients, at least in guard cells (2, 3). To assess the physiological importance of [Ca$^{2+}]_{\text{cyt}}$ oscillations and transients we review how they are generated. Although we restrict our discussion to the roles of mitochondria and plasma membrane and endomembrane Ca$^{2+}$-permeable channels in this process, it should not be forgotten that nuclei (106, 107) and chloroplasts...
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(65, 115) also contribute to Ca\textsuperscript{2+} signal generation. The physiological context of our discussions is provided by focusing on the roles of [Ca\textsuperscript{2+}]\textsubscript{cyt} transients and oscillations in Nod Factor (NF) signaling and stimulus-response coupling in guard cells.

DISSECTING THE COMPONENTS OF Ca\textsuperscript{2+} SIGNALS

Entry Points: Ca\textsuperscript{2+}-Permeable Channels

Ca\textsuperscript{2+}-permeable channels are the key entry points for Ca\textsuperscript{2+} into the cytosol (116). Elevation of [Ca\textsuperscript{2+}]\textsubscript{cyt} can arise via increased influx and/or decreased efflux (Figure 1), although generally most Ca\textsuperscript{2+} signals initiate from increased Ca\textsuperscript{2+} channel activity. In animal cells, specific [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations have been ascribed to Ca\textsuperscript{2+} influx through specific Ca\textsuperscript{2+}-selective channels, at both the whole-cell (50) and single-channel level (145). The pharmacological profiles and selectivities of plant plasma membrane Ca\textsuperscript{2+}-permeable channels often differ significantly from their animal counterparts (136). In plants Ca\textsuperscript{2+} permeable, rather than Ca\textsuperscript{2+}-selective, channels predominant and there is a general lack of specificity of pharmacological agents. These differences may reflect signaling strategies of plants that rely largely on adaptive rather than behavioral responses as well as the different modes of energization and more hyperpolarized nature of most plant plasma membranes.

The known properties of the major groups of plant Ca\textsuperscript{2+}-permeable channels have been surveyed in several recent comprehensive reviews (26, 130, 136). At the molecular level these have been broadly classed as nonselective cation channels (NSCCs). They are encoded by 41 genes of the cyclic nucleotide gated channel (CNGC) or glutamate receptor channel (GLR) families (see below) and the two pore calcium channel (TPC) gene. However, in only a few cases have physiologically characterized channels been mapped to their molecular counterparts. Here we focus on those channels that have been implicated in bringing about specific Ca\textsuperscript{2+} elevations.

Plasma Membrane Depolarization-Activated Ca\textsuperscript{2+} Channels (DACCs)

Plasma membrane depolarization is a frequently observed response to various biotic (32, 81) and abiotic (104) stimuli that also give rise to [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations, which suggests the involvement of DACCs (135). Nonselective, cation-permeable DACCs have been described in a number of cell types such as Arabidopsis root cells (128). Their activity appears to be downregulated by the microtubule cytoskeleton. However, their pharmacology is relatively unexplored and there are no reports directly relating DACC activity in situ with elevations in [Ca\textsuperscript{2+}]\textsubscript{cyt}. These are difficult channels to characterize due to small currents and activity rundown, although channel recruitment in whole-cell recordings is possible with strong
depolarization or microtubule disrupters (128). DACCs from cereal root plasma membrane have also been characterized in lipid bilayers (136). The molecular identities of DACCs have not been verified. *AtTPC1*, which has homology with the animal α1 subunit of voltage-dependent Ca$^{2+}$ channels, and the *ScCCH/MID1* gene in yeast (41) has been proposed as a candidate gene (137). However, evidence that *AtTPC1* encodes a depolarization-activated channel is indirect so far. Expression of *AtTPC1* in *SccCH*-deficient yeast enhances Ca$^{2+}$ uptake and growth rate, and in Arabidopsis, *AtTPC1* is required for the [Ca$^{2+}$]$_{cyt}$ elevation in response to sucrose supply (41). The sucrose-induced Ca$^{2+}$ rise depends on the expression of sucrose symporter genes [*AtSUC1* and *AtSUC2*], and it is plausible that depolarization associated with sucrose/H$^{+}$ cotransport underlies channel activation.

**Plasma Membrane Hyperpolarization-Activated Ca$^{2+}$ Channels (HACCs)**

Ca$^{2+}$ channels that underlie hyperpolarization-activated Ca$^{2+}$ currents across the plasma membrane have been relatively well characterized. They were first described in tomato cells (43) and were activated in response to fungal elicitors (44). Significantly, aequorin-transformed parsley cells show a biphasic elevation of [Ca$^{2+}$]$_{cyt}$ in response to a *Phytophara* oligopeptide elicitor (13).

HACCs have also been described in root hair apices, and root epidermal, endodermal, and cortical cells from the elongation zone and in guard cells (116). Their activation at hyperpolarized membrane potentials (more negative than $-100$ mV) leads to elevation of Ca$^{2+}$ in guard cells (49, 109), and they play a critical role in the response of stomata to abscisic acid (ABA). In root hairs their preferential activity at the growing apex (129) suggests a role in the sustained tip-localized elevation of [Ca$^{2+}$]$_{cyt}$, and their presence in elongating root cells (68) suggests a role in sustained Ca$^{2+}$ influx. Spontaneous or evoked hyperpolarization leads to inward Ca$^{2+}$ currents and corresponding elevation of [Ca$^{2+}$]$_{cyt}$ (49) in *Vicia* guard cells. The observed [Ca$^{2+}$]$_{cyt}$ elevation is relatively slow, occurring over tens of seconds. Grabov & Blatt (49) suggested that ABA-induced [Ca$^{2+}$]$_{cyt}$ elevation could result from hyperpolarization-induced HACC activity, although it is also likely that Ca$^{2+}$ release from intracellular stores plays a significant role in the ABA response.

In addition to regulation by membrane voltage, Ca$^{2+}$$_{cyt}$ (129) and reactive oxygen species (ROS), specifically OH$^-$, increase HACC activity in *Arabidopsis* root hairs (40). Molecular evidence for upstream activation of HACC activity by ROS, and an essential role in cell growth comes from work with the *Arabidopsis* *rhd2* [*AtatrohC*] nicotinamide adenine dinucleotide phosphate (NADPH) oxidase mutant. These mutants fail to elongate root hairs and are defective in ROS production and the generation of the root hair apical [Ca$^{2+}$]$_{cyt}$ gradient (40, 140). Both mutant and wild-type cells have similar OH$^-$-activated HACC activity. This study suggests that ROS is an essential upstream signal regulating cell growth via HACC regulation. In guard cells, ROS (specifically H$_2$O$_2$) (76, 108) and phosphorylation (73) increase HACC activity (see below), but in contrast to root hairs, elevated Ca$^{2+}$$_{cyt}$ decreases it
This apparent differential regulation by Ca\textsuperscript{2+} and ROS raises the possibility that different isoforms occur in these two cell types.

The molecular identity of HACC is not known. However, two possibilities have been proposed. First, mutation in the \textit{AtCNGC2} gene underlies the defense no death (\textit{dnd}) mutation in \textit{Arabidopsis}. \textit{AtCNGC2} has inward-rectifying properties when expressed in \textit{Xenopus} and is nonselective for cations. This led Demidchik and colleagues (25) to suggest that it may be an HACC. However, the apparent insensitivity to cyclic nucleotides of the \textit{Arabidopsis} root hair HACC (V. Demidchik \& J.M. Davies, unpublished data) argues against the identity of the HACC as a CNGC. Second, White et al. (137) proposed that an annexin may underlie HACC activity based on their Ca\textsuperscript{2+} permeability, localization, and known channel-forming properties of annexins in animal cells (45). Annexins comprise a small family in \textit{Arabidopsis} (21) that have been linked with Ca\textsuperscript{2+} signaling and fluxes (20, 137) and the Ca\textsuperscript{2+}-dependent regulation of membrane cycling (17). Analysis of annexin mutants should shed more light on their roles as Ca\textsuperscript{2+}-permeable channels.

**Plasma Membrane NSCCs Indicate Diverse Functions Including Ca\textsuperscript{2+} Signaling**

NSCCs have been characterized in \textit{Arabidopsis} root epidermal cells and mediate steady-state Ca\textsuperscript{2+} influx at more depolarized membrane potentials than HACCs (25). These rapidly activating channels show little voltage dependence of activation and are active over a wide range of membrane potentials. They also co-occur with HACCs in the same cell type. Such channels may provide a crucial Ca\textsuperscript{2+} influx pathway at moderate membrane potentials as found, for example, in root epidermal cells (86), increasing the range of potentials over which plasma membrane channels may play a role in Ca\textsuperscript{2+} signaling.

CNGCs are represented by 20 members in \textit{Arabidopsis}. In animals their activity is increased by cAMP or cGMP. Members of the \textit{Arabidopsis} CNGC family possess overlapping nucleotide-binding and CaM-binding regions at their C terminal (116, 126, 137). Binding CaM and cyclic nucleotides provides the potential for dual regulation by distinct signaling pathways and possible mechanisms for integrating Ca\textsuperscript{2+} and cyclic nucleotide signals (126). However, direct regulation of Ca\textsuperscript{2+} flux or [Ca\textsuperscript{2+}]	extsubscript{cyt} dynamics by cyclic nucleotides for plant CNGCs remains to be shown. Cyclic nucleotide modulation of a Na\textsuperscript{+}-permeable channel has been reported in \textit{Arabidopsis} root protoplasts, but cGMP or cAMP inhibited rather than enhanced activity (87). CNGCs probably have a wide range of functions not all related to Ca\textsuperscript{2+} signaling. However, human kidney (HEK) cells transformed with \textit{AtCNGC2} showed that [Ca\textsuperscript{2+}]	extsubscript{cyt} increases after treatment with membrane-permeant cAMP and cGMP (80). The demonstration of a role for cAMP in pollen tube growth regulation raises the possibility that CNGC regulation could be involved in the formation of the apical [Ca\textsuperscript{2+}]	extsubscript{cyt} gradient that directs pollen tube growth (97).

The 20 members of the GLR family are also good candidates for nonselective Ca\textsuperscript{2+}-permeable channels (24, 26). GLRs have fundamental roles in animal cells...
in mediating fast chemical transmission and long-term synaptic potentiation (29). Plant GLR ligand binding is suggested by the similarities between the animal and plant genes in the ligand-binding domains (24, 26). Glutamate can induce relatively fast (within a few seconds) increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} in aequorin-transformed whole Arabidopsis seedlings (27). Ca\textsuperscript{2+} influx is implicated in this response by the inhibitory effects of La\textsuperscript{3+}.

**Endomembrane Ca\textsuperscript{2+} Channels: Unidentified Players with Important Roles**

Because many Ca\textsuperscript{2+} responses in plant cells involve Ca\textsuperscript{2+} release from internal stores it is important to understand the nature and regulation of the endomembrane Ca\textsuperscript{2+} release pathways. However, linking the activity of specific endomembrane Ca\textsuperscript{2+} channels to [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations is difficult because they cannot be monitored electrophysiologically in intact cells. None have yet been characterized at the molecular level in plants. Obvious homologues of animal inositol (1, 4, 5) trisphosphate (InsP\textsubscript{3}) or ryanodine receptors that mediate InsP\textsubscript{3} or cyclic ADP ribose (cADPR)-induced Ca\textsuperscript{2+} release, respectively, have not been identified in the Arabidopsis genome. Nevertheless, there is compelling evidence to suggest fundamental roles for endomembrane channels based on direct patch clamp recordings, caged release, pharmacology, imaging, and more recently, genetic studies. A variety of endomembrane channels that could potentially give rise to cytosolic Ca\textsuperscript{2+} elevations have been characterized at the physiological level. These include voltage-dependent calcium channels (VDCCs), slow vacuolar (SV) channels, and InsP\textsubscript{3}–, and cADPR–sensitive Ca\textsuperscript{2+} release channels in the vacuolar membrane (116). Ca\textsuperscript{2+} release from endoplasmic reticulum (ER)-enriched fractions has also been demonstrated in response to InsP\textsubscript{3} (98), cADPR (101), and nicotinic acid adenine dinucleotide phosphate (NAADP) (102), although Ca\textsuperscript{2+}-permeable ER channels have been monitored directly in only two plant systems (Bryonia tendris and Lepidium root tips) by incorporation into lipid bilayers (70, 71).

The SV channel has been well studied with respect to its potential role in bringing about Ca\textsuperscript{2+} release from the vacuole during guard cell signaling. There has been considerable discussion about whether this channel can indeed give rise to Ca\textsuperscript{2+} flux into the cytosol under normal physiological conditions (137); at cytosolic Mg\textsuperscript{2+} concentrations the activation threshold for this channel is in the physiological range (−3 to −50 mV, cytosol negative). This channel can potentially give rise to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) from the vacuole because its activity also increases with elevated cytosolic Ca\textsuperscript{2+}. The SV channel is also activated by CaM and the type IIB phosphoprotein phosphatase, calcineurin, which activates at low concentrations but inhibits at high concentrations. The SV channel is also activated significantly by increased cytosolic pH in the physiological range (7.3 to 8.0), potentially providing a link between observed increases in pH in response to ABA (11).
Shaping the Ca^{2+} Signal: Pumping and Binding!

To act as an effective signal [Ca^{2+}]_{cyt} needs to be maintained at sufficiently low levels to allow small influxes through channels to bring about significant changes in [Ca^{2+}]_{cyt}. Adenosine triphosphate (ATP)-fueled pumps (Ca^{2+}-ATPases) and transporters driven by the electrochemical gradients of other ions, particularly H^{+} in higher plants (H^{+}/Ca^{2+} exchangers), play critical housekeeping roles in maintaining low resting [Ca^{2+}]_{cyt} in the unstimulated cell. However, they may also be important in determining the peak amplitudes and duration of Ca^{2+} transients (Figure 1). Evidence that they play active roles in shaping Ca^{2+} signals comes from the knowledge that certain classes of pumps and transporters can be regulated by Ca^{2+}/CaM, among other factors (see below). However, although the properties of transporters and pumps have been elucidated in some detail in various plant cell types, we await concrete evidence to link a specific pump or transporter directly with a specific Ca^{2+} signaling event. It is also becoming clear that disrupting the expression or activity of one type of pump or transporter can lead to compensatory changes in the activity or expression of other pumps or transporters. However, despite these problems, functions for pumps and transporters in stress signaling and Ca^{2+} homeostasis are beginning to emerge.

In general Ca^{2+}-ATPases belong to a group of high-affinity pumps whereas H^{+}/Ca^{2+} exchangers have lower affinity (42, 112, 124). The Ca^{2+}-ATPases can be grouped as type IIA or IIB based on their homologies with animal counterparts. Type IIA ATPases include the ER-type Ca^{2+} ATPases (ECA) whereas type IIB contain the autoinhibited Ca^{2+}-ATPases (ACA) (31). Each group contains multiple members (4 ECA and 10 ACA genes in Arabidopsis). Although there is evidence for an ER location for type IIA Ca^{2+}-ATPases in plants (82, 83), plasma membrane, vacuole, and Golgi locations are also evident (38, 42, 105).

Type IIB Ca^{2+}-ATPases in animal cells are targeted to the plasma membrane whereas plant IIB members are also targeted to endomembranes (42, 60). For example, ACA8 occurs on the plasma membrane (14) but ACA2 has been identified with an ER location (82) and ACA4 occurs on small vacuolar membranes but not the large central vacuole, which suggests functional diversity within the vacuolar membrane system in relation to Ca^{2+} homeostasis or signaling (42). Type IIA and IIB Ca^{2+}-ATPases show distinctly different modes of regulation. Type IIB (ACA) members contain an N-terminal autoinhibitory domain (in contrast to animal counterparts, which contain a C-terminal autoinhibitory domain) and can be activated directly through binding Ca^{2+}/CaM (56). ACA activity can be inhibited by calcium-dependent protein kinase (CDPK) binding and phosphorylation (63). In plants no direct regulation of a type IIA ATPase has been demonstrated, which may suggest a constitutive role in maintaining resting cytosolic Ca^{2+} levels. However, animal type IIB pumps are highly regulated by an inhibitory subunit phospholamban and possibly also ER lumen Ca^{2+} content (31).

Physiological and molecular data indicate vacuolar localization of H^{+}/Ca^{2+} exchangers such as the Arabidopsis cation exchanger CAX1, although there is also
physiological evidence for H\textsuperscript{+}/Ca\textsuperscript{2+} exchange at other locations such as the plasma membrane (67), the chloroplast thylakoid membrane (35), and the nuclear envelope (30). CAX transporter activity can be regulated via an N-terminal autoinhibitory domain. Pittman & Hirschi (111, 112) postulated that this is effected via interaction with a regulatory protein. However, the N terminal does not interact with CaM (112), unlike the ACA Ca\textsuperscript{2+}-ATPases, and the nature of the regulatory protein remains unknown.

**Functional Roles: Evidence from Disruption Studies**

Recent manipulation of expression of both Ca-ATPases and H\textsuperscript{+}/Ca\textsuperscript{2+} exchangers has begun to shed light on their roles in Ca\textsuperscript{2+} homeostasis. T-DNA insertion mutants of \textit{ECA1} (139) showed reduced growth on Ca\textsuperscript{2+}-depleted media. This was the first knockout of a Ca\textsuperscript{2+}-ATPase gene and provided evidence for a role for \textit{ECA1} in ER Ca\textsuperscript{2+} homeostasis. Overexpression of \textit{CAX1} in tobacco plants gave rise to increased vacuolar H\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity and increased plant Ca\textsuperscript{2+} levels. \textit{CAX1} overexpressing plants showed severe Ca\textsuperscript{2+} deficiency symptoms, probably due to reduced [Ca\textsuperscript{2+}]\textsubscript{cyt} levels. These plants also showed increased vacuolar H\textsuperscript{+} pumping. Knockout of \textit{CAX1} (18) led to increased tolerance to both Ca\textsuperscript{2+} deficiency and abiotic stress and also resulted in decreased vacuolar V-ATPase H\textsuperscript{+} pumping activity. Significantly, these knockouts led to increased tonoplast Ca\textsuperscript{2+}-ATPase activity and increased expression of other H\textsuperscript{+}/Ca\textsuperscript{2+} exchangers \textit{CAX3} and \textit{CAX4}. Although evidence for a direct involvement in Ca\textsuperscript{2+} signaling was not provided in these studies, a possible signaling role was suggested by the impaired hormone responses and auxin-regulated gene expression of \textit{cax1} mutants.

These results from overexpression and knockout experiments suggest complex interactions between the activities and expression levels of different pumps and transporters, and the operation of a highly integrated compensatory network that may also include primary H\textsuperscript{+} pumps. Direct monitoring of Ca\textsuperscript{2+} transients in plants where specific pump or transporter activity has been manipulated is eagerly awaited, and their precise cellular localizations need to be cataloged. In frog oocytes, overexpression of a type IIA Ca\textsuperscript{2+}-ATPase led to increased frequency of Ca\textsuperscript{2+} spiking (77). Although there is currently no direct evidence for a role for Ca\textsuperscript{2+} pumps or transporters in shaping Ca\textsuperscript{2+} signals, there is evidence for a role of a V-type H\textsuperscript{+}-ATPase in determining the patterns of Ca\textsuperscript{2+} signals. \textit{Arabidopsis det3} mutants that are defective in a V-type H\textsuperscript{+} ATPase show disrupted Ca\textsuperscript{2+} signaling in stomatal guard cells in response to elevated [Ca\textsuperscript{2+}]\textsubscript{ext} and oxidative stress but not to ABA or cold (3). The simplest mechanistic interpretation of this result may relate to energization of a CAX that is involved in generating stimulus-specific [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations.

**ER Ca\textsuperscript{2+} Binding and Homeostasis**

Ca\textsuperscript{2+}-binding proteins also play important roles in Ca\textsuperscript{2+} homeostasis in plants, although a direct signaling role is not yet evident. Overexpression of maize...
Calreticulin (CRT) in tobacco leads to increased Ca$^{2+}$ storage in the ER (110). Arabidopsis plants overexpressing CRT show increased resistance to Ca$^{2+}$ depletion, and expression of CRT antisense leads to increased sensitivity to low Ca$^{2+}$. This suggests that the ER can provide an exchangeable store of Ca$^{2+}$ for [Ca$^{2+}]_{cyt}$ homeostasis. Vacuolar Ca$^{2+}$-binding proteins have also been identified (141), although their role in Ca$^{2+}$ signaling or homeostasis has not been determined.

**Mitochondria: Energetic Players in a Dynamic Ca$^{2+}$ Network**

The role(s) of mitochondria in signaling in plants cells are relatively unexplored. There is evidence that they may function to provide a source of Ca$^{2+}$ in maize suspension cells in response to anoxia (122), or respond to elevated [Ca$^{2+}]_{cyt}$ by increasing their Ca$^{2+}$ content in Fucus rhizoid cells (22). Recently, Logan & Knight (84) targeted aequorin to the mitochondrial matrix and recorded average cellular “resting” [Ca$^{2+}]_{mit}$ around 200 nM, approximately double that of [Ca$^{2+}]_{cyt}$. Treatments that caused elevation of [Ca$^{2+}]_{cyt}$ also gave rise to elevations of [Ca$^{2+}]_{mit}$. However, although [Ca$^{2+}]_{mit}$ elevations were comparable in kinetics (though lower in amplitude) to those in the cytosol in response to cold and osmotic treatments, significantly different kinetics were observed in response to touch stimuli, and [Ca$^{2+}]_{cyt}$ recovered to resting levels much more rapidly than [Ca$^{2+}]_{mit}$. In response to H$_2$O$_2$, [Ca$^{2+}]_{mit}$ elevated to a similar amplitude as [Ca$^{2+}]_{cyt}$. These results indicate separate mechanisms for regulating [Ca$^{2+}]_{mit}$ and [Ca$^{2+}]_{cyt}$, and existence of mitochondria-specific Ca$^{2+}$ signals. Logan & Knight (84) proposed that the elevations in [Ca$^{2+}]_{mit}$ might be involved in stimulating dehydrogenases of the TCA cycle, which in turn would stimulate respiration and increased ATP production. Similar mechanisms have been elucidated in animal cells (10, 51).

**Mitochondria as Ca$^{2+}$ Buffers: “Marks and Sparks”**

In animal cells, evidence is emerging for a very tight coordination of ER/SR Ca$^{2+}$ release and uptake by individual mitochondria (51). It is likely that very highly localized [Ca$^{2+}]_{cyt}$ “sparks,” resulting from Ca$^{2+}$ flux although endomembrane InsP$_3$ or ryanodine receptors, permit targeted delivery of Ca$^{2+}$ to neighboring mitochondria, resulting in transient [Ca$^{2+}]_{cyt}$ elevations in the matrix of individual mitochondria (“marks”). This points to a high degree of coordination of cytoplasmic and mitochondrial Ca$^{2+}$ signaling, and highly localized regulation of mitochondrial function that is coordinated with localized signaling activity of the cytosol. However, there is evidence that mitochondria play an important role in regulating the extent of Ca$^{2+}$ signal propagation by highly localized buffering. For example, Malli et al. (89) have shown, using DsRed and yellow chameleon Ca$^{2+}$ indicators targeted to the mitochondria and ER, that localized [Ca$^{2+}]_{cyt}$ elevations in response to histamine were only evident in mitochondrial-free zones and did not occur close to mitochondria. This heterogeneity of Ca$^{2+}$ elevation could allow simultaneous activation of plasma membrane Ca$^{2+}$-activated and Ca$^{2+}$-inhibited channels in the same cell. Thus, by rapidly removing Ca$^{2+}$ from the cytosol, mitochondria can...
play an important role in the spatiotemporal patterning of Ca$^{2+}$ signals. Recently it was shown in HeLa cells that mitochondrial uptake required sustained release of Ca$^{2+}$ from nearby locations on the ER membrane and balanced uptake of Ca$^{2+}$ into the ER via the activity of plasma membrane Ca$^{2+}$ channels (123). Although we can only speculate that similar mechanisms exist in plants (Figure 2), the data of Logan & Knight (84) are broadly consistent with results from mammalian cells. It will be interesting to determine whether the mean increases in [Ca$^{2+}$]$_{mit}$ measured with aequorin reflect a heterogeneity of [Ca$^{2+}$]$_{mit}$ between individual mitochondria in plant cells.

Polarized growing plant cells offer an obvious choice for investigating the role of mitochondria in [Ca$^{2+}$]$_{cyt}$ spatial regulation. Regulating mitochondrial movements in growing pollen tubes and root hairs may be especially significant. The apical [Ca$^{2+}$]$_{cyt}$ gradient in growing lily pollen tubes is associated with large measured fluxes of Ca$^{2+}$ into the tube apex (59). These Ca$^{2+}$ fluxes show oscillations with the same frequency as oscillations in the apical [Ca$^{2+}$]$_{cyt}$ gradient and growth. However, although it is clear that the [Ca$^{2+}$]$_{cyt}$ gradient at the pollen tube apex is required for growth (59), the peak Ca$^{2+}$ elevation at the growing tube apex lags behind the growth peak by approximately 4 s, and the maximum Ca$^{2+}$ influx at the tube tip lags behind growth by approximately 15 s. Studies of mitochondria distribution in pollen tubes show that they are excluded from the extreme apex (28) but they also move with cytoplasmic streaming in a “reverse fountain” pattern (39) that continually delivers and removes mitochondria to the subapical region. Whether these mitochondria play a role in the periodic removal or buffering of [Ca$^{2+}$]$_{cyt}$ in the pollen tube apex remains to be determined.

PROPAGATION OF PLANT Ca$^{2+}$ SIGNALS

Experimental demonstration of the components required for propagated intracellular release provides compelling evidence that plant cells, like animal cells, can generate Ca$^{2+}$ signals that propagate through the cytosol via intracellular Ca$^{2+}$ release. Most recorded Ca$^{2+}$ waves in animal cells fall into the “fast Ca$^{2+}$ wave” category that propagate within a narrow range of velocities that may reflect the highly conserved nature of the intracellular propagation mechanisms (64). However, slow Ca$^{2+}$ waves also occur in animal cells, particularly those that propagate between cells and in developing systems (134), and it has been proposed that both fast and slow waves propagate via reaction-diffusion mechanisms (64, 134). In plants, very few intracellular propagating waves have been visualized directly, although Campbell et al. (15) showed the existence of intercellular waves that propagate slowly through tissue in whole Arabidopsis seedlings. Although [Ca$^{2+}$]$_{cyt}$ elevations that appear to propagate as waves have been observed in guard cells, their velocity is approximately ten times slower than typical fast Ca$^{2+}$ waves (49). So far the only recorded fast Ca$^{2+}$ wave described in detail in a plant or algal cell is that of the osmotically induced wave in the Fucus rhizoid (48). Although this will likely be triggered by activating the mechanosensitive plasma membrane.
channels (127), it propagated by elemental “sparks” through ER-rich regions (48). Fast [Ca^{2+}]_{cyt} responses of higher plants, such as those in response to osmotic stimuli, that are likely to involve intracellular Ca^{2+} release (125) are potentially good candidates for studying plant Ca^{2+} waves. The question remains of how we explain the preponderance of relatively slow increases in cytosolic Ca^{2+} in terms of known mechanisms for Ca^{2+} elevation in plants. The rate of rise of [Ca^{2+}]_{cyt} and velocity of propagation in any cell depends on several factors, including the kinetics of receptor activation, rate of production and diffusion of second messengers such as InsP₃, the rate of permeation of Ca^{2+} through channels (which also depends on their selectivity), the length of time the channels stay open (presumably a sustained elevation of Ca^{2+} requires a sustained increased channel-open probability), and the regulation of efflux systems. The highly convoluted nature of the ER endomembrane system may allow Ca^{2+} release sites to be positioned in close proximity to one another, whereas the reaction-diffusion kinetics along the more linear vacuolar membrane may be significantly different.

Examples of significant deviation from the typical animal reaction-diffusion model for Ca^{2+} waves do exist in plants. For example, a slowly propagating Ca^{2+} signal occurs in the maize egg during in vitro fertilization (8). In this system, elevation of Ca^{2+} is brought about by a slow wave of Ca^{2+} influx across the plasma membrane, which propagates around the egg. Another example is provided by cytoplasmic droplets from the giant alga *Nitella* that display Ca^{2+} spikes in response to hypo-osmotic treatment. The most likely explanation for this phenomenon is the activation of mechanosensitive Ca^{2+}-permeable endomembrane channels (69).

**[Ca^{2+}]_{cyt} OSCILLATIONS AND TRANSIENTS**

In animal cells, stimulus-induced [Ca^{2+}]_{cyt} oscillations are well documented and their mechanisms of generation and the potential for the information to be encoded in the frequency of [Ca^{2+}]_{cyt} oscillation was recently reviewed (10). [Ca^{2+}]_{cyt} oscillations and transients also occur in plant cells with, to the best of our knowledge, the first report being IAA-stimulated [Ca^{2+}]_{cyt} oscillations in maize coleoptiles (37). The frequency, period, and amplitude of [Ca^{2+}]_{cyt} oscillations vary among different cell types and in response to different stimuli (36), and the possibility that [Ca^{2+}]_{cyt} oscillations might encode information has been discussed (36, 93, 114, 116, 117), as have mechanisms for generating [Ca^{2+}]_{cyt} oscillations (9, 55). To discuss how [Ca^{2+}]_{cyt} oscillations and transients are generated, and their possible roles in plant cell signaling, we focus on NF signaling in legume root hair cells and stimulus-response coupling in guard cells.

**Calcium Oscillations in Legume Root Hair NF Signaling**

NFs are bacterial lipochitooligosaccharide signals that play an important role in the early stages of nodule development. They stimulate root hair cells of susceptible plants to undergo morphological changes that lead to the entrapment and invasion
of the bacteria. The role of NFs in this process has been comprehensively reviewed (16, 46, 81). Among the NFs’ induced changes in susceptible root hair are increases in \([\text{Ca}^{2+}]_{\text{cyt}}\), which include oscillations. There are two phases to NF-induced root hair \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations. The first phase is rapid. In *Medicago truncatula* it consists of a rapid spike followed by a sustained \([\text{Ca}^{2+}]_{\text{cyt}}\) increase or plateau that lasts for 3–4 min. Approximately 10 mins after NF application, the second phase occurs, which consists of “calcium spiking” in the nuclear region (7, 119).

A typical spike in *M. sativa* or *M. truncatula* consists of a rapid increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) (approximately 500 nM) followed by a more gradual return to resting levels. This produces an asymmetric peak with a sharp rising phase and a slower recovery. The duration of the whole spike from onset to return to resting \([\text{Ca}^{2+}]_{\text{cyt}}\) is between 30–120 s, and the frequency of occurrence is typically approximately 1 min (7, 33). Because \(\text{Ca}^{2+}\) spiking is an example of an oscillatory phenomenon we refer to it as \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillation.

**Generation of NF-Induced \([\text{Ca}^{2+}]_{\text{cyt}}\) Oscillations in Root Hair Cells**

In *M. truncatula*, (34) NF-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations were inhibited separately by 50 \(\mu\)M 2-APB and 50 mM caffeine, which are putative antagonists of intracellular store-mediated \(\text{Ca}^{2+}\) release. 5 \(\mu\)M cyclopiazonic acid (CPA), an inhibitor of type IIA calcium ATPases in plants, 10 \(\mu\)M 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ), an inhibitor of mammalian sarco(endo)plasmic reticulum calcium ATPases (SERCA), and the phosphatidyl inositol specific phospholipase C (PI-PLC) inhibitor U73122 (20 \(\mu\)M) also inhibited NF-induced root hair \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations. Because both U73122 (121) and CPA (83) inhibit plant enzymes the data obtained by Engstrom and colleagues support a role for PI-PLC and Type IIA Calcium ATPases in NF generation of \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations. No effects on \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations were observed when the root hairs were treated with the putative plasma membrane calcium channel blockers verapamil (100 \(\mu\)M) and LaCl\(_3\) (1 mM) (34). However, it might be unwise to rule out the involvement of plasma-membrane \(\text{Ca}^{2+}\) channels because it is possible that plants possess \(\text{Ca}^{2+}\) channels in the plasma membrane that are La\(^{3+}\)- and verapamil-insensitive.

Genetic approaches have also been used to identify components involved in the NF signaling pathway. Walker and colleagues (132) investigated the occurrence of NF-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations in the pea nodulation defective mutants *sym8*, *sym10*, and *sym19*. In all three of these mutants NF-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations were blocked, suggesting that the products of all three genes are upstream of and required for the generation of NF-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations. Long and colleagues (131) reached similar results and conclusions using the *dmi1* and *dmi2* nodulation mutants of *M. truncatula*.

Shaw & Long (119) recently investigated whether the first rapid NF-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase is required for generating NF-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations. When they challenged *M. truncatula* with 10 nM NF they observed the familiar
two-phase Ca$^{2+}$ response. However, when they applied 1 nM NF they did not observe any rapid NF-induced [Ca$^{2+}$]$_{cyt}$ increase, although they saw the NF-induced [Ca$^{2+}$]$_{cyt}$ oscillations. These results suggest that generating [Ca$^{2+}$]$_{cyt}$ oscillations does not require the first NF-induced [Ca$^{2+}$]$_{cyt}$ elevation.

Stimulus-Induced Oscillations and Transients of Guard Cell [Ca$^{2+}$]$_{cyt}$

Stimulus-induced increases in guard cell [Ca$^{2+}$]$_{cyt}$ are part of signal transduction network(s) responsible for coupling ABA and other external signals to alterations in stomatal aperture (11, 12, 57, 93, 117). Stimulus-induced oscillations in guard cell [Ca$^{2+}$]$_{cyt}$ were observed when *Commelina communis*. Fura-2-loaded guard cells were bathed in 0.1 mM [Ca$^{2+}$]$_{ext}$ (symmetrical oscillations with an average amplitude of approximately 430 nM and a mean period of 8.3 min) or 1.0 mM [Ca$^{2+}$]$_{ext}$ (asymmetrical oscillations with an amplitude of 625–850 nM and a mean period of 13.6 min) (94). Oscillations in guard cell [Ca$^{2+}$]$_{cyt}$ were more extensively investigated in *Arabidopsis* using cameleon [Ca$^{2+}$]$_{cyt}$ technology (5). Using this approach (3), 1 mM [Ca$^{2+}$]$_{ext}$ induced oscillations with an average amplitude of approximately 160 nM and an average period of 2.6 min, whereas 10 mM [Ca$^{2+}$]$_{ext}$ induced oscillations with an average amplitude of 1020 nM and a period of 6.6 min. In a separate study, 10 mM [Ca$^{2+}$]$_{ext}$ induced [Ca$^{2+}$]$_{cyt}$ oscillations with a period of approximately 8.2 min (2). The periods of the [Ca$^{2+}$]$_{ext}$-induced oscillations reported using the cameleon technique correlate well with data obtained in the same species using the fluorescent ratiometric [Ca$^{2+}$]$_{cyt}$ indicator Fura-2 (4). ABA can also induce [Ca$^{2+}$]$_{cyt}$ oscillations in *C. communis* (121) and *Arabidopsis*. ABA-induced [Ca$^{2+}$]$_{cyt}$ oscillations have been reported in different *Arabidopsis* ecotypes (Col, Ler, WS), and when they occur they are either in the form of regular oscillations with a magnitude of about 500 nM and a period of approximately 7.8 min or have no obvious periodicity. Schroeder and colleagues refer to these irregular increases as “transients” and we adopt this terminology here (2, 6, 61, 66, 72, 75, 76). Similar to earlier work (47, 90, 91), not all guard cells responded to ABA with detectable increases in [Ca$^{2+}$]$_{cyt}$. Transients were also observed when guard cells were challenged with a yeast elicitor (72), 100 µM H$_2$O$_2$, and cold (3).

Generating Stimulus-Induced Oscillations and Transients in Guard Cell [Ca$^{2+}$]$_{cyt}$

Ca$^{2+}$ influx across the plasma membrane and release from internal stores both contribute to the generation of [Ca$^{2+}$]$_{cyt}$ oscillations. In *C. communis* the results of manganese quench experiments and application of verapamil suggests that plasma membrane calcium-permeable channels participate in the generation of [Ca$^{2+}$]$_{ext}$-induced [Ca$^{2+}$]$_{cyt}$ oscillations (94). A similar conclusion was reached from experiments in which *Arabidopsis* guard cells bathed in Ca$^{2+}$-free media failed to exhibit [Ca$^{2+}$]$_{cyt}$ transients when stimulated by a yeast elicitor or ABA (72).
Although it is likely that more than one guard cell plasma membrane calcium channel is involved in the generation of \([Ca^{2+}]_{cyt}\) oscillations (53, 72), most attention has focused on an HACC in *Vicia faba* (53) and *Arabidopsis* (where it is known as \(I_{Ca}\)) (108). In *Vicia* the open probability of the HACC is increased by ABA, the concentration of extracellular divalent cations (52, 53), and inhibitors of Type 1 and 2A phosphoprotein phosphatases (73). This latter result, coupled with the observation that ATP hydrolysis was required for channel activity, led Kohler & Blatt (73) to propose that phosphorylation of sites on or close to the channel facilitates opening. Increasing \([Ca^{2+}]_{cyt}\) from 200 nM to 2 \(\mu\)M reduces the open probability of the channel and provides a mechanism for feedback inhibition of calcium influx (53). In *Arabidopsis* the HACCs are permeable to several cations, including \(Mg^{2+}\), require cytosolic NAD(P)H, and are involved in the generation of \([Ca^{2+}]_{cyt}\) increases by \(H_2O_2\), ABA (108), and pathogenic elicitors (72). Schroeder and colleagues proposed a model for ABA action on *Arabidopsis* guard cells that involves ABA stimulating \(H_2O_2\) production and the resulting \(H_2O_2\) increasing the open probability of HACC (108). The suggestion that ABA action involves \(H_2O_2\) is corroborated by investigations carried out in *Vicia* guard cells (144), and Schroeder and colleagues further suggest that ABA and a fungal elicitor signaling pathways converge on HACC (72). They also place the Type 2C phosphoprotein phosphatases encoded by the *ABI1-1* and *ABI2-1* genes upstream of \(I_{Ca}\) in ABA signaling (4, 99). The most recent work shows that although ABA-induced \([Ca^{2+}]_{cyt}\) transients were not abolished, they were significantly reduced in *Arabidopsis* NADPH oxidase catalytic subunit mutants (atrbohD/F double mutant), supporting a role for HACCs and ROS in *Arabidopsis* guard cell ABA signaling (76). However, the Blatt laboratory’s (74) recent work in *Vicia* questions whether HACCs are critical second messenger in guard cell ABA signaling. Kohler and colleagues (74) found that in *Vicia* although \(H_2O_2\) regulated two key cellular components involved in guard cell turgor control (HACC and inward rectifying \(K^+\) channels) in a manner reminiscent of ABA, and \(H_2O_2\) stimulates \([Ca^{2+}]_{cyt}\) increases in *C. communis* (92) and *Arabidopsis* (108) it also depresses guard cell outward-rectifying \(K^+\) channels (74). This latter action is the direct opposite of what happens in guard cell ABA signaling. Ignoring possible species-specific differences as an explanation for these apparently conflicting sets of data, it seems likely that, as guard cell ABA signaling is partially disrupted in the *Arabidopsis* AtrbohD/F double mutant (76), \(H_2O_2\) and possibly other ROS are involved in some but not all aspects of guard cell ABA signaling. Finally, there is evidence that Type 2A phosphoprotein phosphatase (PP2A) activity is involved in the regulation of ABA-induced \([Ca^{2+}]_{cyt}\) transients. In the loss of function *ren1* mutant, which encodes the regulatory subunit of a guard cell–expressed PP2A, stomatal responsiveness to ABA was greatly reduced, as was the probability of ABA-induced increases in \([Ca^{2+}]_{cyt}\) (75).

There are also data indicating that release of \(Ca^{2+}\) from internal stores contributes to the generation of stimulus induced \([Ca^{2+}]_{cyt}\) transients and oscillations. There is evidence pointing to a role for the PI-PLC–InsP3 system in generating
[Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations and transients in guard cell ABA signaling. In both *C. communis* and *Arabidopsis* guard cells the pharmacological PI-PLC inhibitor U73122 interferes with ABA-induced alterations in stomatal aperture and ABA-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations (121) and transients (72). U73122 also interferes with ABA-induced K\(^{+}\) efflux from guard cells (88).

Is it possible to place PI-PLC in the sequence of events leading to the generation of ABA-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations and transients? Hunt and colleagues (62) present a model in which a primary ABA-generated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) activates guard cell PI-PLC, which then generates InsP\(_3\), leading to Ca\(^{2+}\) release from internal stores. In this model PI-PLC–InsP\(_3\) amplifies the primary ABA-induced increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) and contributes to the rising phase of the oscillation. For this system to generate a series of oscillations, a separate mechanism for repriming [Ca\(^{2+}\)]\(_{\text{cyt}}\) to a level whereby it could activate PI-PLC would be required after the completion of each oscillation and the return of [Ca\(^{2+}\)]\(_{\text{cyt}}\) to resting levels. ABA-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) influx through the plasma membrane HACC (discussed above) might be a prime candidate to fulfill such a role. Although PI-PLC plays a role in the generation of guard cell ABA-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations it is not apparently involved in [Ca\(^{2+}\)]\(_{\text{ext}}\)-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations in *C. communis* (121).

Just as MacRobbie (88) showed that the mechanism used to generate ABA-induced increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) depends on the concentration of applied ABA, it is possible that other Ca\(^{2+}\)-mobilizing messengers might be involved in ABA signaling. In guard (78) and other plant cells (1) cADPR induces Ca\(^{2+}\) release from vacuoles. There is evidence that cADPR might be involved in the generation of ABA-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations and transients. Microinjecting guard cells with cADPR induces increases in guard cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) that include oscillations (78) and nicotinamide, which blocks cADPR synthesis and interferes with ABA-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) calcium transients (72). However, in contrast to the situation with the PI-PLC–InsP\(_3\) system, it is unlikely that cADPR is involved in Ca\(^{2+}\) signal amplification or the phenomenon of CICR because cADPR-gated currents downregulate over the range of guard cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevated by cADPR microinjection (78). This latter finding suggests that cADPR might be involved in the initial priming phase required in the generation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations and transients. Understanding the contribution of cADPR to stimulus-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations and transients will be greatly facilitated with the identification of the enzyme systems responsible for its production and its intracellular receptors.

Another molecule capable of generating increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in animal and plant cells is sphingosine-1-phosphate (S1P) (138). When applied to *C. communis* guard cells 6 \(\mu\)M S1P induced asymmetrical [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations with a period of 3.8 min and peaks of up to 50 nM above resting levels of [Ca\(^{2+}\)]\(_{\text{cyt}}\). 50 nM S1P induced symmetrical [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations with a shorter period (2.8 min) but greater peak height (100 nM above resting levels of [Ca\(^{2+}\)]\(_{\text{cyt}}\)) (103). Recent work from the Assmann lab (23) resulted in the construction of a model for the involvement of S1P in guard cell ABA signaling, in which ABA activates sphingosine kinase in *Arabidopsis* guard cells resulting in the production of elevated
SIP. The SIP then interacts with an unidentified receptor followed by interactions with the product of the GPA1 gene and generation of \([\text{Ca}^{2+}]_{\text{cyt}}\) increases (23). Currently, we do not know whether other \(\text{Ca}^{2+}\)-mobilizing messengers such as inositol hexakisphosphate (InsP\(_6\)) (79) and NAADP (101) are involved in generating \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations ortransients.

As with NF signaling in root hair cells, genetic approaches have also resulted in significant insights into the generation of \([\text{Ca}^{2+}]_{\text{cyt}}\) transients and oscillations. Schroeder and coworkers investigated whether the products of genes involved in ABA signaling are involved in the generation of \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations and transients by examining ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations in the corresponding Arabidopsis mutants. Ten \(\mu\text{M ABA induced } [\text{Ca}^{2+}]_{\text{cyt}}\) transients in 86% of wild-type guard cells but failed to induce \([\text{Ca}^{2+}]_{\text{cyt}}\) increases in any abi2-1 guard cells (6). The result suggests that the product of the ABI2-1 gene (a type 2C phosphoprotein phosphatases) is required for the generating ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) transients. Stomata of the Arabidopsis rcn1 mutant are less sensitive to ABA. 14% of rcn1 guard cells showed two or more \([\text{Ca}^{2+}]_{\text{cyt}}\) transients when treated with 5 \(\mu\text{M ABA, in contrast to the 44% of wild-type stomata that exhibited two or more } [\text{Ca}^{2+}]_{\text{cyt}}\) transients. The conclusion from this work is that the type 2A phosphoprotein phosphatase encoded by the RCN1 gene is also involved in the generation of ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) transients. Similar experimental approaches have shown that the ERA1 gene product (encoding a farnesyltransferase) (6), the catalytic subunits of NADPH oxidase encoded by AtRBOHD and AtRBOHF genes (76), the product of the GCA2 gene (2), and the mRNA cap binding protein encoded by ABH1 (61) can influence ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) transients.

Significance of \([\text{Ca}^{2+}]_{\text{cyt}}\) Oscillations and Transients in Plant Cell Signaling

In animal cells experimental evidence supports the hypothesis that information is encoded in \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations (10). In plant cells it has been suggested that \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations and transients might encode information that helps to dictate the nature of the downstream response (36, 55, 93, 114, 116, 117). We focus on three experimental systems, pollen tube growth, NF signaling in root hair cells, and stimulus-response coupling in guard cells, to assess the physiological significance of \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations and transients in plant cell signaling.

The role of \([\text{Ca}^{2+}]_{\text{cyt}}\) in the control of pollen tube growth was the subject of a recent comprehensive review (59). However, in brief, the growing pollen tube is an endogenous oscillator and accompanying growth oscillations are oscillations in \([\text{Ca}^{2+}]_{\text{cyt}}, \text{pH}_{\text{cyt}}, \text{membrane potential, exo and endocytosis, cell wall thickness, and the fluxes of extracellular Ca}^{2+}, \text{H}^+, \text{K}^+, \text{and Cl}^-. Because it has long been recognized that } \text{Ca}^{2+}\text{ is essential for pollen tube growth, an attractive proposition was that } [\text{Ca}^{2+}]_{\text{cyt}}\text{ oscillations might be the coordinator or pacemaker controlling growth. Current evidence suggests that this is unlikely for two reasons. First, } [\text{Ca}^{2+}]_{\text{cyt}}\text{ oscillations follow rather than anticipate changes in pollen tube growth rate (95), and second, pollen tube } [\text{Ca}^{2+}]_{\text{cyt}}\text{ oscillations have been observed in}
the absence of growth (96). Accordingly, the current situation in this system is that there are well-characterized \([\text{Ca}^{2+}]_{cyt}\) oscillations but their physiological role remains to be established.

Currently, a similar situation holds for NF-induced \([\text{Ca}^{2+}]_{cyt}\) oscillations in legume root hairs, although this may change in the near future as a result of work by Shaw & Long (119). They showed that by judicial choice of NF concentration it is possible to induce \([\text{Ca}^{2+}]_{cyt}\) oscillations in the absence of the first NF-induced \([\text{Ca}^{2+}]_{cyt}\) elevation. This seems to provide the ideal experimental system to address the question of the physiological role of \([\text{Ca}^{2+}]_{cyt}\) oscillations. Given the perinuclear location of the \([\text{Ca}^{2+}]_{cyt}\) oscillations, an obvious experiment to conduct would be a comparative analysis of 1 nM NF- (induces only \([\text{Ca}^{2+}]_{cyt}\) oscillations) and 10 nM NF- (induces first-phase \([\text{Ca}^{2+}]_{cyt}\) elevations and second-phase oscillations) induced gene expression.

Currently, the most compelling evidence that \([\text{Ca}^{2+}]_{cyt}\) oscillations and transients might be responsible for encoding specific signaling information comes from guard cells. McAinsh and coworkers showed that that it is possible to correlate the pattern of \([\text{Ca}^{2+}]_{cyt}\) oscillation with both the strength of the external stimulus and the extent of the final response (stomatal closure) in \([\text{Ca}^{2+}]_{ext}\) signaling (94). However, these data revealed a correlation rather than provided evidence of a causal relationship between \([\text{Ca}^{2+}]_{cyt}\) oscillations and the control of stomatal aperture. Allen and colleagues (3) established a much stronger link between \([\text{Ca}^{2+}]_{cyt}\) oscillations and the control of stomatal aperture using the Arabidopsis det3 mutant that exhibits a 60% reduction in expression of the C-subunit of the V-type H+-ATPase. The authors hypothesized that a reduction in V-type H+-ATPase activity might result in reduced \([\text{Ca}^{2+}]_{cyt}\) sequestration into internal stores, and this in turn might affect stimulus-induced \([\text{Ca}^{2+}]_{cyt}\) oscillations. Whatever the precise effects of the reduction in expression of the DET3 gene the authors observed, in contrast to wild-type guard cells, that det3 guard cells failed to exhibit \([\text{Ca}^{2+}]_{cyt}\) oscillations and reductions in guard cell turgor in response to 1 and 10 mM \([\text{Ca}^{2+}]_{ext}\). When total guard cell \([\text{Ca}^{2+}]_{ext}\)-stimulated \([\text{Ca}^{2+}]_{cyt}\) was integrated over a 30-minute period it was significantly higher in the det3 mutant than in wild type. This result clearly shows that the reason the det3 mutant failed to close its stomata in response to \([\text{Ca}^{2+}]_{ext}\) was not due to an inability to increase \([\text{Ca}^{2+}]_{cyt}\), but rather due to the failure to generate \([\text{Ca}^{2+}]_{cyt}\) oscillations that seemed to underlie the phenotype. Allen and colleagues also showed that experimentally induced \([\text{Ca}^{2+}]_{cyt}\) oscillations brought about (rescued) closure in the det3 mutant. These data provide strong evidence that \([\text{Ca}^{2+}]_{cyt}\) oscillations are required in the signaling pathway leading to stomatal closure.

With evidence for the importance of \([\text{Ca}^{2+}]_{cyt}\) oscillations in guard cell signaling the next question to address is what their specific role is in the control of stomatal aperture. One possibility is that \([\text{Ca}^{2+}]_{cyt}\) oscillations are responsible for holding the guard cell in a steady low-turgor state while protecting it from the presumed deleterious effects of long-term exposure to elevated \([\text{Ca}^{2+}]_{cyt}\) (94). However, although this might be beneficial to the guard cell it is not a requirement in guard cell signaling because there are examples of closure-inducing,
[Ca$^{2+}$]$_{cyt}$-elevating stimuli that do not bring about [Ca$^{2+}$]$_{cyt}$ oscillations such as ABA (47, 91, 99), CO$_2$ (133), and H$_2$O$_2$ (92). Instead, Schroeder and colleagues propose that [Ca$^{2+}$]$_{cyt}$ controls stomatal aperture by two mechanisms, based on experiments carried out on the Arabidopsis gca2 mutant. They distinguish between short-term stomatal closure that occurs in response to elevated [Ca$^{2+}$]$_{cyt}$ and long-term (steady-state) closure, the degree of which is programmed or controlled by [Ca$^{2+}$]$_{cyt}$ oscillations within a defined range of frequency, transient number, duration, and amplitude (2).

However, some additional factors need to be considered before any generalizations can be made about the role of [Ca$^{2+}$]$_{cyt}$ oscillations in guard cells. Perhaps the most obvious issue to consider is that ABA-, CO$_2$-, and H$_2$O$_2$-induced stomatal closure can occur in the absence of [Ca$^{2+}$]$_{cyt}$ oscillations (47, 90–92, 133). It is possible that the experiments in these studies were not long enough for the stomata to reach steady state and thus failed to exhibit [Ca$^{2+}$]$_{cyt}$ oscillations. A further complication concerns the occurrence of spontaneous [Ca$^{2+}$]$_{cyt}$ transients in guard cells (72). The lack of closure in the stomata exhibiting spontaneous [Ca$^{2+}$]$_{cyt}$ transients is presumably due to the fact that the period of the spontaneous [Ca$^{2+}$]$_{cyt}$ transients was shorter than ABA-induced [Ca$^{2+}$]$_{cyt}$ oscillations and transients associated with closure. However, there are some difficulties with this explanation because 10 mM [Ca$^{2+}$]$_{ext}$ induces [Ca$^{2+}$]$_{cyt}$ oscillations with a similar period to the spontaneous [Ca$^{2+}$]$_{cyt}$ oscillations and 10 mM [Ca$^{2+}$]$_{ext}$ closes stomata (3, 4).

In summary, [Ca$^{2+}$]$_{cyt}$ oscillations and transients are found in different plant cells, and their mechanism of generation and possible physiological significance have been the subjects of considerable scrutiny in three well-studied plant signaling systems. Although we are learning much about the origin of [Ca$^{2+}$]$_{cyt}$ oscillations and transients, pinning down their physiological role has proved more difficult. To date, the clearest evidence that stimulus-induced [Ca$^{2+}$]$_{cyt}$ oscillations (as opposed to stimulus-induced nonoscillatory [Ca$^{2+}$]$_{cyt}$ elevations) are required during signaling comes from the work performed on the det3 mutant (3). However, with the identification of new signaling mutants and ways of manipulating Ca$^{2+}$ homeostasis it is likely that we are poised to learn much more about the possible functions of [Ca$^{2+}$]$_{cyt}$ oscillations and transients in plant cell signaling.

CONCLUSIONS AND FUTURE PERSPECTIVES

It was recently proposed, on the basis of a well-argued case, that many (but not all) Ca$^{2+}$ signals act as simple on-off binary switches and thus the Ca$^{2+}$ signal is an unlikely mechanism for ensuring fidelity in plant signaling systems (118). However, as Scrase-Field & Knight (118) acknowledge and we discuss here, the guard cell provides examples of where Ca$^{2+}$ signals do appear to encode the information for defining the outcome of responses (2, 3). The question that we now need to address is whether the guard cell is unique in using the Ca$^{2+}$ signal
to help provide fidelity in stimulus-response coupling, or whether it exists in other cells but we have failed to detect it so far. Unless the symplastic isolation of mature guard cells is a factor, there is no obvious reason why stomata should have evolved a unique method of signaling.

Guard cells have provided important insights into signaling because it is possible to investigate the effects of different stimuli on Ca$^{2+}$ dynamics at the single-cell level while monitoring a robust readout (changes in stomatal aperture). When the Schroeder lab combined the experimental advantages of the guard cell with the use of mutants the definitive evidence that Ca$^{2+}$ signals can encode information was obtained. Before we conclude that information-encoding Ca$^{2+}$ signals are unique to guard cells we need to thoroughly investigate whether they occur in other cell types. In this respect, NF signaling holds great promise, especially if a robust readout, such as an alteration in gene expression, can be identified similar to the way Shaw & Long (119) were able to experimentally isolate NF-induced [Ca$^{2+}$]$_{cyt}$ oscillations from other NF-induced [Ca$^{2+}$]$_{cyt}$ elevations.

The technology for addressing whether the binary switch or the Ca$^{2+}$ signal model predominates in plant cell signaling is advancing on several fronts. In this review we identify a range of targets for potential disruption and modification. The availability of mutants displaying lesions in these targets is increasing and should enable investigation of the effect of manipulating Ca$^{2+}$ signals on stimulus-induced responses. Significant advances are also being made in the use of FRET-based Ca$^{2+}$ indicators such as cameleons and pericams (100, 142) that can be expressed in a targeted manner. These advances in reporter technology are being matched by fast improving camera– and scanning-based imaging technology, and several options are now available for monitoring Ca$^{2+}$ from total internal reflection fluorescence (TIRF) for near-membrane fast events in wall-free cells to multiphoton microscopy for deeper penetration and improved signal-to-noise ratio (39) in whole tissues and plants. Combining these techniques with robust cellular or tissue responses will allow the global significance of Ca$^{2+}$ signals to be assessed. Overall, the field is poised for exciting developments and insights over the next few years.

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NOTE ADDED IN PROOF

Recently, a specific receptor for apoplastic Ca$^{2+}$ that is expressed in guard cells and is required for oscillatory elevations in guard cell Ca$^{2+}$ and stomatal closure in response to elevated [Ca$^{2+}$]$_{ext}$ was cloned from Arabidopsis (53a). This suggests a specific external messenger role for [Ca$^{2+}$]$_{ext}$. It will be interesting to determine the downstream components of this signal transduction pathway.
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Figure 1  Schematic representation of complex interactions between channels, pumps, transporters, and Ca\(^{2+}\)-binding proteins in bringing about \([Ca^{2+}]_{\text{cyt}}\) homeostasis and stimulus-specific elevations (as represented by the solid black trace) or oscillations of \([Ca^{2+}]_{\text{cyt}}\). Light arrows indicate hypothetical actions in raising or lowering \([Ca^{2+}]_{\text{cyt}}\) at particular points in a Ca\(^{2+}\) transient. The assignment of Ca\(^{2+}\) pumps and transporters to different membranes is indicative rather than exhaustive. The combined activity of channels and pumps contributes to resting \([Ca^{2+}]_{\text{cyt}}\). NSCCs and HACCs may be particularly involved in maintaining steady Ca\(^{2+}\) influx. The chloroplast and nucleus are not shown. ACA, ECA, Ca\(^{2+}\)-ATPases; CAX, H\(^{+}\)/Ca\(^{2+}\) exchangers; NSCC, nonselective cation channels; DACC, depolarization-activated Ca\(^{2+}\) channels; HACC, hyperpolarization-activated Ca\(^{2+}\) channels; GLR, glutamate receptor channels; CNGC, cyclic nucleotide gated channels; PM, plasma membrane; mit, mitochondria; ER, endoplasmic reticulum; CRT, calreticulin; VCaB, vacuolar Ca\(^{2+}\)-binding protein.
Figure 2  Hypothetical scheme for Ca\textsuperscript{2+} cycling between the cytosol, the ER, and mitochondria. [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations arising via influx through plasma membrane (PM) or endoplasmic reticulum (ER) channels (e.g., in response to touch, osmotic, or cold) are offset by mitochondrial and ER uptake or reuptake. Ca\textsuperscript{2+} efflux from mitochondria is removed from the cytosol by adjacent ER. [Ca\textsuperscript{2+}]\textsubscript{mit} elevations stimulate ATP production, which may be used to fuel Ca\textsuperscript{2+} efflux via Ca\textsuperscript{2+} ATPase activity. Green arrows represent the proposed Ca\textsuperscript{2+} cycling pathway.
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