An Apoplastic Ca$^{2+}$ Sensor Regulates Internal Ca$^{2+}$ Release in Aequorin-transformed Tobacco Cells*

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Removal of Ca$^{2+}$ from tobacco suspension cell medium has two immediate effects on cytosolic Ca$^{2+}$ fluxes: (i) externally derived Ca$^{2+}$ influx (occurring in response to cold shock or hypo-osmotic shock) is inhibited, and (ii) organellar Ca$^{2+}$ release (induced by a fungaly derived defense elicitor, caffeine, or hypo-osmotic shock) is elevated. We show here that the enhanced release of internal Ca$^{2+}$ is likely due to increased discharge from a caffeine-sensitive store in response to a signal transduced from an extracellular Ca$^{2+}$ sensor. Thus, chelation of extracellular Ca$^{2+}$ in the absence of any other stimulus directly activates release of intracellular Ca$^{2+}$ into the cytosol. Evidence that this chelator-activated Ca$^{2+}$ flux is dependent on a signaling pathway includes its abrogation by prior treatment with caffeine, and its reduction by protein kinase inhibitors (K252a and staurosporine) and anion channel blockers (niflumate and anthracene-9-carboxylate). An unexpected characteristic of tobacco cell adaptation to low external Ca$^{2+}$ was the emergence of a new Ca$^{2+}$ compartment that was inaccessible to external EGTA, yet responsive to the usual stimulants of extracellular Ca$^{2+}$ entry. Thus, cells that are exposed to EGTA for 20 min lose sensitivity to caffeine and defense elicitors, indicating that their intracellular Ca$^{2+}$ pools have been depleted. Surprisingly, these same cells simultaneously regain their ability to respond to stimuli that usually activate extracellular Ca$^{2+}$ influx even though all external Ca$^{2+}$ is chelated. Because this gradual restoration of Ca$^{2+}$ influx can be inhibited by the same kinase inhibitors that block EGTA-activated Ca$^{2+}$ release, we propose that chelator-activated Ca$^{2+}$ release from internal stores leads to deposition of this Ca$^{2+}$ into a novel EGTA- and caffeine-insensitive compartment that can subsequently be activated by stimulants of extracellular Ca$^{2+}$ entry.

A wide variety of stresses stimulate the expression of cytosolic Ca$^{2+}$ transients in plant cells, presumably resulting in the acquisition of tolerance/resistance to the same stresses (1). Examples of such stresses include cold shock, which stimulates an influx primarily of extracellular Ca$^{2+}$ (2, 3); pathogen infection and defense elicitor stimulation, which activate Ca$^{2+}$ pulses largely from internal Ca$^{2+}$ pools (4–6); and hypo-osmotic shock, which activates a biphasic Ca$^{2+}$ transient deriving first from outside and then from inside the cell (7, 8). We have reported previously that, in the case of the hypo-osmotic shock-induced Ca$^{2+}$ pulses, inhibition of either of the two Ca$^{2+}$ pulses generally leads to greater Ca$^{2+}$ entry during the uninhibited pulse (7). We have thus surmised that an internal Ca$^{2+}$ store might receive information regarding the Ca$^{2+}$ status of the external store and vice versa (7). In this way, a deficiency in the Ca$^{2+}$ content of either the intracellular or extracellular Ca$^{2+}$ pool could be compensated by additional release of Ca$^{2+}$ from the undepleted pool.

It has already been established that animal cells can communicate the Ca$^{2+}$ status of their internal compartments to the external Ca$^{2+}$ pool. Thus, after stimulation of internal Ca$^{2+}$ release, the resulting depletion of ER Ca$^{2+}$ has been observed to induce a slow refilling of the ER by influx across the plasma membrane. This phenomenon has been referred to as store-operated calcium entry (9–11). The mechanism of activation of this process is still debated (12), but there is general agreement that some ER protein must monitor luminal Ca$^{2+}$ levels and signal the plasma membrane to initiate Ca$^{2+}$ influx when ER levels fall.

Conversely, vertebrate animal cells are also believed to communicate the availability of external Ca$^{2+}$ across their cytoplasm to internal Ca$^{2+}$ stores by signaling through a Ca$^{2+}$-sensing Receptor (CaR) (13). CaR is a seven-transmembrane G-protein-coupled receptor expressed in the plasma membrane of several different cell types, most notably those involved in Ca$^{2+}$ homeostasis (e.g. parathyroid) (14, 15). CaR is activated in the presence of high levels of extracellular Ca$^{2+}$ and communicates with the cell’s interior by a signal transduction cascade that often involves the release of Ca$^{2+}$ from the ER (16). CaR-mediated signaling pathways modulate the secretion of various Ca$^{2+}$-regulating hormones that maintain Ca$^{2+}$ homeostasis in distal tissues (14, 16). Although it is clear that plants and vertebrates must have very different mechanisms of Ca$^{2+}$ homeostasis, it is also likely that plant cells will likewise monitor both internal and external Ca$^{2+}$ stores and develop some means of compensation when one of these stores becomes depleted.

We report here that plant cells indeed respond to depletion of their extracellular Ca$^{2+}$ reserves by activating the release of Ca$^{2+}$ from internal stores. The route of communication between external and internal Ca$^{2+}$ pools appears to require the function of protein kinases and anion channels. Further, after long term removal of external Ca$^{2+}$ and the consequent depletion of the prominent internal store, plant cells appear to sequester their remaining Ca$^{2+}$ in a caffeine-insensitive, EGTA-inaccessible compartment that can still be activated in

* This work was supported by National Science Foundation Grant MCB-9729534. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ER, endoplasmic reticulum; CaR, Ca$^{2+}$-sensing Receptor; MS, Murashige and Skoog.
response to stimuli that normally promote externally derived Ca\textsuperscript{2+} influx.

**MATERIALS AND METHODS**

*Plant Material—* Tobacco plants (*Nicotiana tabacum* L. var. Wisconsin-38) were transformed with the aequorin-encoding plasmid pMAQ2 (3) by the method of Liu et al. (17). Suspension cultures were then established and maintained as previously reported (5) by continuous shaking in Murashige and Skoog (MS) basal growth medium (Sigma; Ref. 18). MS medium contains the following ionic species in the given concentrations: NH\textsubscript{4}+, 20.61 mM; K\textsuperscript{+}, 20.045 mM; Ca\textsuperscript{2+}, 2.99 mM; Mg\textsuperscript{2+}, 1.05 mM; Na\textsuperscript{+}, 0.101 mM; Mn\textsuperscript{2+}, 0.1 mM; Fe\textsuperscript{2+}, 0.1 mM; Zn\textsuperscript{2+}, 0.029 mM; Cu\textsuperscript{2+}, 0.0001 mM; Co\textsuperscript{2+}, 0.0001 mM; NO\textsubscript{3} \textsuperscript{-}, 39.4 mM; Cl\textsuperscript{-}, 2.99 mM; SO\textsubscript{4} \textsuperscript{2-}, 1.73 mM; PO\textsubscript{4} \textsuperscript{3-}, 1.25 mM; ethylenediaminetetraacetic acid \textsuperscript{2-}, 0.1 mM; BO\textsubscript{3} \textsuperscript{-}, 0.1 mM; I\textsuperscript{-}, 0.005 mM; MnO\textsubscript{2} \textsuperscript{2-}, 0.001 mM; pH 5.7 (18). Nonionic components include: sucrose (30 g/liter), enzymatic casein hydrolysate (1 mg/liter), and traces amount of plant growth regulators, 2,4-dichlorophenoxyacetic acid (3 mg/liter), and kinetin (0.002 mg/liter). The osmolarity of the medium was measured at 180–200 mosM with an osmometer.

Three different aequorin-expressing cell lines from three different transgenic parent plants were studied. Because these different cell lines expressed varying levels of aequorin protein, yet behaved similarly in each of the experiments performed, we conclude that incorporation and expression of the aequorin transgene did not alter plant cell responses.

**Luminometry and Ca\textsuperscript{2+} Quantitation—** Luminometry of aequorin-transformed suspension cultures was performed as previously described, with some minor changes (4). Briefly, 24 h after subculture into fresh MS medium, cell cultures were incubated in 1 mM coelenterazine (Biosynth International, Naperville, IL) for 4–8 h. 1 ml of cell culture was then transferred to a luminometer cuvette that was placed in an LKB-Wallac Bio-Orbit luminometer chamber. Luminescence was recorded 10 times/s using Rainin Dynamax-LC software (Woburn, MA). EGTA and other treatments were added as indicated in the figure legends. A 90 mM stock solution of EGTA buffered to pH 5.7 (osmolality measured at 200 mosm) was used for all experiments. No measurable change in medium pH or osmolality occurred after EGTA additions to cell cultures up to [EGTA] = 45 mM. To quantitate the undischarged aequorin at the end of each experiment, cells were solubilized by injecting 200 \mu l of a solution containing 10% Nonidet P-40 and 500 mM CaCl\textsubscript{2} into the luminometer chamber, and residual luminescence was recorded. Luminescence traces were then transformed by computer program (4) directly into [Ca\textsuperscript{2+}]\textsubscript{cyt} using the equation described by Allen et al. (19).

**Extracellular Ca\textsuperscript{2+} Measurements and Calculations—** Extracellular Ca\textsuperscript{2+} was measured with the fluorescent Ca\textsuperscript{2+}-indicating dye Oregon Green BAPTA 5N (\(\lambda_{\text{ex}} = 494 \text{ nm}; \lambda_{\text{em}} = 523 \text{ nm}; \) Molecular Probes, Eugene, OR). Because of its relatively low affinity for Ca\textsuperscript{2+} (\(K_d \approx 20 \text{ \mu M} \text{Ca}^{2+}\)), at pH = 7), Oregon Green BAPTA 5N accurately reports Ca\textsuperscript{2+} concentrations ranging from <1 \mu M to > 400 \mu M. To quantitate extracellular free Ca\textsuperscript{2+}, 10 \mu g of dye/ml was added to the fluorimeter cuvette together with cell filtrates, and fluorescence was recorded.

Estimates of the free Ca\textsuperscript{2+} content of the medium after addition of EGTA (pK\textsubscript{a}, EGTA-Ca = 7.39) were made using the MINTEQA2 environmental modeling software (United States Environmental Protection Agency, Center for Exposure Assessment Modeling, Athens, GA), assuming all ion concentrations listed in the Murashige and Skoog medium above, a suspension pH of 5.7, and no Ca\textsuperscript{2+} dissociation from the cell wall (20).

**RESULTS**

**Compensation between Internally and Externally derived Ca\textsuperscript{2+} Pulses in Tobacco Cells—** We have previously shown that inhibition of external Ca\textsuperscript{2+} influx can lead to elevation of internally derived Ca\textsuperscript{2+} release (7). Thus, as shown in Fig. 1A, chelation of extracellular Ca\textsuperscript{2+} with EGTA not only inhibits the first phase of hypotonically stimulated Ca\textsuperscript{2+} entry (externally derived influx), but also elevates the second pulse (internally derived influx). OR). Because of its relatively low affinity for Ca\textsuperscript{2+} (\(K_d \approx 20 \text{ \mu M} \text{Ca}^{2+}\)), at pH = 7), Oregon Green BAPTA 5N accurately reports Ca\textsuperscript{2+} concentrations ranging from <1 \mu M to > 400 \mu M. To quantitate extracellular free Ca\textsuperscript{2+}, 10 \mu g of dye/ml was added to the fluorimeter cuvette together with cell filtrates, and fluorescence was recorded.

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The compensatory increase in discharge of intracellular Ca\textsuperscript{2+} following chelation of extracellular Ca\textsuperscript{2+} implies some
type of communication between the two Ca\(^{2+}\) pools. Conceivably, a cytosolic Ca\(^{2+}\) sensor (i.e. a Ca\(^{2+}\) and/or calmodulin-regulated protein kinase; Ref. 21) could detect the absence of the first (externally derived) Ca\(^{2+}\) transient and transmit that information to the intracellular store. Alternatively, the absence of extracellular Ca\(^{2+}\) could be directly detected by an extracellular sensor that signals to the intracellular store independent of any perturbation of the stimulated Ca\(^{2+}\) entry. To distinguish these two mechanisms, we examined the impact of external Ca\(^{2+}\) chelators on the discharge of intracellular Ca\(^{2+}\) following treatment with stimuli that have no effect on external Ca\(^{2+}\) influx. As shown in Fig. 1B, the cytosolic Ca\(^{2+}\) pulse activated by caffeine, a drug that activates the release of Ca\(^{2+}\) only from internal stores (7), is elevated when initiated immediately after EGTA addition. In contrast, cold shock activates a Ca\(^{2+}\) pulse that derives primarily from external stores (2, 3), and EGTA treatment potently inhibits its expression (Fig. 1C).

It would thus appear that removal of external Ca\(^{2+}\) has two immediate effects on Ca\(^{2+}\) fluxes in tobacco cells: (i) it leads to inhibition of any externally derived Ca\(^{2+}\) influx, and (ii) it triggers elevation of internally derived Ca\(^{2+}\) release. Because the elevation of intracellular Ca\(^{2+}\) release proceeds even in the absence of any change in entry of extracellular Ca\(^{2+}\), we suggest that the Ca\(^{2+}\) detection system involves an extracellular sensor.

**Internal Ca\(^{2+}\) Release Is Activated after Removal of External Ca\(^{2+}\)**—To further explore whether extracellular Ca\(^{2+}\) levels might be communicated to intracellular Ca\(^{2+}\) stores, EGTA was added directly to cell suspensions and cytosolic [Ca\(^{2+}\)] was monitored in the luminometer. As shown in Fig. 2A, chelation of external Ca\(^{2+}\) by addition of 9 mM EGTA results in an irregular Ca\(^{2+}\) transient that continues for more than 10 min. A measurable increase in cytosolic Ca\(^{2+}\) could even be detected after adding only 2 mM EGTA, which still leaves the extracellular concentration of free Ca\(^{2+}\) at roughly 1 mM (Fig. 2B). To more quantitatively evaluate the impact of external Ca\(^{2+}\) removal on the magnitude of the stimulated internal Ca\(^{2+}\) transient that continues for more than 10 min. A measurable increase in cytosolic Ca\(^{2+}\) could even be detected after adding only 2 mM EGTA, which still leaves the extracellular concentration of free Ca\(^{2+}\) at roughly 1 mM (Fig. 2B). To more quantitatively evaluate the impact of external Ca\(^{2+}\) removal on the magnitude of the stimulated internal Ca\(^{2+}\) transient that continues for more than 10 min. A measurable increase in cytosolic Ca\(^{2+}\) could even be detected after adding only 2 mM EGTA, which still leaves the extracellular concentration of free Ca\(^{2+}\) at roughly 1 mM (Fig. 2B). To more quantitatively evaluate the impact of external Ca\(^{2+}\) removal on the magnitude of the stimulated internal Ca\(^{2+}\) transient that continues for more than 10 min. A measurable increase in cytosolic Ca\(^{2+}\) could even be detected after adding only 2 mM EGTA, which still leaves the extracellular concentration of free Ca\(^{2+}\) at roughly 1 mM (Fig. 2B).
count the 2.99 mM Ca$^{2+}$ and all other ionic species in the medium, but does not consider the Ca$^{2+}$ stored in the cell wall, which, if substantial, could shift the dotted line to the right. In view of these considerations, the close reciprocal relationship between the two traces is remarkable and suggests some type of functional linkage between log(Ca$^{2+}$) and the amplitude of internal Ca$^{2+}$ release, with half-maximal Ca$^{2+}$ flux occurring when external Ca$^{2+}$ concentrations are reduced to $\sim 10^{-5.5}$ M.

To further demonstrate that the chelator-activated Ca$^{2+}$ flux arises because of removal of external Ca$^{2+}$, and not because of any other effect of EGTA, we conducted analogous studies using BAPTA, an unrelated Ca$^{2+}$ chelator. When BAPTA was added to suspension cultures at a concentration of 5 mM, the cells responded with a cytosolic Ca$^{2+}$ flux that reached an average of 0.083 ± 0.055 mM Ca$^{2+}$ (n = 3). Although the magnitude of the BAPTA-induced Ca$^{2+}$ flux was admittedly more variable than that measured after EGTA addition, the data still support the conclusion that removal of external Ca$^{2+}$ results in a substantial cytosolic Ca$^{2+}$ flux regardless of the method of Ca$^{2+}$ chelation.

Although both EGTA and BAPTA prefer Ca$^{2+}$ over many other bivalent cations, it was still conceivable that the Ca$^{2+}$ fluxes might have been induced by removal of other cations. To examine this possibility, tobacco cells were first activated with EGTA (until the usual chelator-induced increase in cytosolic Ca$^{2+}$ was observed), and then external Ca$^{2+}$ was restored by injection of enough CaCl$_2$ to compensate for the presence of EGTA. As shown in Fig. 2C, restoration of external Ca$^{2+}$ to EGTA-treated cells returns the cytosolic Ca$^{2+}$ activity to its basal level. Because addition of MgCl$_2$ does not alter the EGTA-induced Ca$^{2+}$ flux (Fig. 2C), we conclude that the chelator-activated Ca$^{2+}$ flux is mediated by a Ca$^{2+}$ sensor that cannot respond to Mg$^{2+}$.

**Chelator-induced Ca$^{2+}$ Fluxes Derive from a Kinase- and Anion Channel-dependent, Internal Caffeine-sensitive Pool—**To characterize the Ca$^{2+}$ pools involved in the chelator-induced Ca$^{2+}$ transients, we first depleted the internal Ca$^{2+}$ stores by incubating cells in caffeine for an extended period (7) and then tested whether EGTA could still induce an increase in Ca$^{2+}$ levels. As shown in Fig. 3A, caffeine-treated cells no longer respond to EGTA with a transient rise in cytosolic Ca$^{2+}$, indicating that the Ca$^{2+}$ pulses induced by removal of external Ca$^{2+}$ derive from caffeine-sensitive internal stores.

To further confirm that a caffeine-sensitive intracellular Ca$^{2+}$ pool is specifically responsive to external Ca$^{2+}$ removal, the ability of caffeine itself to trigger release of Ca$^{2+}$ was evaluated as a function of time after EGTA addition. As seen in Fig. 3A, caffeine-induced Ca$^{2+}$ release is prominent in tobacco cells immediately prior to Ca$^{2+}$ chelation, but diminishes with time following EGTA addition until its disappearance by 20 min after chelation. *V. dahlia* elicitor-triggered Ca$^{2+}$ pulses, which also derive from a caffeine-sensitive intracellular compartment (5), display the same kinetics of depletion (Fig. 3C). We, therefore, conclude that the EGTA-induced Ca$^{2+}$ pulses derive from the same intracellular compartment that releases Ca$^{2+}$ in response to both caffeine and *Verticillium dahlia* elicitor stimulation.

To begin to assess the mechanism by which a change in extracellular Ca$^{2+}$ might be communicated to caffeine-sensitive intracellular Ca$^{2+}$ stores, we next examined the impact of pharmacological agents that were previously found to regulate intracellular Ca$^{2+}$ fluxes in tobacco cells (5). As shown in Fig. 4, cells that are treated with either the protein kinase inhibitor, K252a (Fig. 4A), or the anion channel blocker, niflumate (Fig. 4B), display little or no response upon subsequent stimulation with 50 mM caffeine or left unmodified (control) 10 min prior to stimulation with 9 mM EGTA (at the time marked by the arrow). Cytosolic Ca$^{2+}$ levels were then measured as described above. Alternatively, tobacco cells were exposed to 9 mM EGTA for the indicated period and then stimulated with 50 mM caffeine (B) or 0.2% (v/v) *V. dahlia* elicitor (C). Both caffeine and the *V. dahlia* elicitor stimulate Ca$^{2+}$ influx from intracellular pools only (5).
concentrations for 10 min prior to EGTA addition (9 mM), and cytosolic 

Because the mobilizable internal Ca\textsuperscript{2+} stores in plants are probably vast (1), it was conceivable that restoration of the externally derived Ca\textsuperscript{2+} pulses following long term removal of external Ca\textsuperscript{2+} was due to the release of enough Ca\textsuperscript{2+} from internal pools to replenish the extracellular medium. To explore this possibility, we monitored Ca\textsuperscript{2+} levels in the extracellular medium after addition of 9 mM EGTA. However, no significant replenishment of external Ca\textsuperscript{2+} could be detected, even following 2 h of EGTA application (data not shown). Furthermore, addition of an extra 9 mM EGTA to cells that had already been incubated in EGTA for 20 min did not prevent activation of the restored cold shock-stimulated Ca\textsuperscript{2+} peak (data not shown). These data demonstrate that the return of responsiveness to stimulants of external Ca\textsuperscript{2+} entry is not due to restoration of external Ca\textsuperscript{2+}. Additionally, the revived Ca\textsuperscript{2+} influx also does not appear to derive from the previously characterized caffeine-sensitive internal Ca\textsuperscript{2+} pool. As shown in Fig. 5E, caffeine injected into the cuvette 20 min after EGTA treatment does not prevent a cold shock-stimulated Ca\textsuperscript{2+} flux. Thus, it appears that long term removal of external Ca\textsuperscript{2+} results in the sequestration of Ca\textsuperscript{2+} into a third compartment that is EGTA-inaccessible and caffeine-insensitive, and which releases Ca\textsuperscript{2+} into the cytosol in response to stimuli that normally activate externally derived influx in Ca\textsuperscript{2+}-bathed cells.

Finally, to demonstrate that the Ca\textsuperscript{2+} released from internal stores after the depletion of external Ca\textsuperscript{2+} might be responsible for filling this new compartment, signaling modulators that were previously found to block the EGTA-activated release of intracellular Ca\textsuperscript{2+} were again tested for their abilities to prevent the filling of this EGTA-inaccessible Ca\textsuperscript{2+} store. As shown in Fig. 6, K252a (and staurosporine; data not shown) also prevents the restoration of the cold shock-activated Ca\textsuperscript{2+} pulse (Fig. 6). Importantly, these modulators do not affect the cold shock-induced Ca\textsuperscript{2+} pulse either when added to Ca\textsuperscript{2+}-bathed (control) cells, or when administered to Ca\textsuperscript{2+}-depleted cells that have been exposed to EGTA for sufficient time to permit filling of the EGTA-inaccessible compartment (data not shown). These observations suggest that gating of Ca\textsuperscript{2+} from neither the apoplastic space nor the unidentified compartment is altered by the kinase inhibitors. Rather, it would appear that a kinase-dependent pathway must mediate some step in communicating the depletion of external Ca\textsuperscript{2+} to the machinery involved in filling an EGTA-inaccessible Ca\textsuperscript{2+} pool at the expense of the more prominent intracellular Ca\textsuperscript{2+} store.

**DISCUSSION**

We have presented data to suggest that a change in the Ca\textsuperscript{2+} content of an extracellular Ca\textsuperscript{2+} store can be rapidly communicated to an intracellular compartment that responds by releasing Ca\textsuperscript{2+} into the cytosol (Fig. 2). If a stress-related signal to discharge Ca\textsuperscript{2+} is received by the internal organelle soon after removal of the external Ca\textsuperscript{2+}, the magnitude of Ca\textsuperscript{2+} release is heightened (Fig. 1A). If, in contrast, the signal to discharge Ca\textsuperscript{2+} arrives after the store of organellar Ca\textsuperscript{2+} has already been depleted, no further release of the cation is noted (Fig. 3C). Thus, some type of mechanism for sensing extracellular Ca\textsuperscript{2+} appears to modulate the behavior of a major intracellular Ca\textsuperscript{2+} storage compartment. In view of previous data demonstrating communication from the intracellular Ca\textsuperscript{2+} pool to the extracellular Ca\textsuperscript{2+} store (7), we conclude that these two Ca\textsuperscript{2+} compartments are well aware of the Ca\textsuperscript{2+} status of the other.
FIG. 5. Restoration of externally derived Ca\textsuperscript{2+} pulses after long term external Ca\textsuperscript{2+} removal. [Ca\textsuperscript{2+}]\textsubscript{cyt} levels were monitored as described under "Materials and Methods." A, hypo-osmotically activated Ca\textsuperscript{2+} pulses were recorded after treatment with 9 mM EGTA for the indicated periods. The arrow marks the time at which cells were hypo-osmotically shocked by 1:1 dilution with double distilled H\textsubscript{2}O. B, as in A, cells were subjected to varying periods of EGTA treatment (9 mM), and then hypo-osmotically shocked. The % restoration of the first peak of osmotically triggered Ca\textsuperscript{2+} influx is then plotted as a function of the duration of exposure to excess external EGTA (average ± S.D., n = 3). 100% restoration is taken as the magnitude of the Ca\textsuperscript{2+} peak exhibited by Ca\textsuperscript{2+}-bathed control cells. C, cell suspensions were treated with the indicated concentrations of EGTA for 20 min prior to hypo-osmotic shock at the time indicated by the arrow. D, cells were treated with 9 mM EGTA for the indicated periods prior to a cold shock generated by injection of 1 ml of ice-cold cell culture medium into the suspension, at the time indicated by the arrow. E, cells were treated with 9 mM EGTA for 20 min followed by 50 mM caffeine (or buffer as a control) and then subjected to cold shock (as in Fig. 5D) at the indicated time point. Data are representative of three independent experiments in which similar results were obtained.
A question obviously arises regarding the function of the above communication, and more specifically, how the active release of intracellular Ca\(^{2+}\) upon depletion of extracellular Ca\(^{2+}\) might benefit the cell. Although many plausible explanations might be offered, we suggest that different Ca\(^{2+}\) compartments in the cell might have different signaling functions (1, 11). As a consequence, uncompensated emptying of one compartment could conceivably eliminate an entire group of signaling pathways essential for plant survival. Sharing of Ca\(^{2+}\) reserves from a filled compartment to an empty compartment would avoid this potential dysfunction. In this scenario, the release of intracellular Ca\(^{2+}\) upon removal of the external Ca\(^{2+}\) could have evolved to allow replenishing of the depleted extracellular stores or their substitutes.

Because our Ca\(^{2+}\) depletion studies were conducted in the presence of sufficient EGTA to prevent resupply of extracellular stores, we were unable to evaluate whether the external Ca\(^{2+}\) pool might normally be replenished by the above mechanism. Sharing of Ca\(^{2+}\) reserves from a filled compartment to an empty compartment would avoid this potential dysfunction. In this scenario, the release of intracellular Ca\(^{2+}\) upon removal of the external Ca\(^{2+}\) could have evolved to allow replenishing of the depleted extracellular stores or their substitutes.

Because our Ca\(^{2+}\) depletion studies were conducted in the presence of sufficient EGTA to prevent resupply of extracellular stores, we were unable to evaluate whether the external Ca\(^{2+}\) pool might normally be replenished by the above mechanism. We did observe, however, that in the presence of excess apoplastic chelating capacity, a new Ca\(^{2+}\) compartment was formed that could substitute for extracellular Ca\(^{2+}\) in transducing cold shock and hypo-osmotic stress-induced signals. This new Ca\(^{2+}\) pool was not in the culture medium, since it could neither be detected by Ca\(^{2+}\) indicator dyes nor depleted by treatment with additional EGTA. It was also not likely associated with the primary intracellular store, since it was still functional after this caffeine- and elicitor-responsive store had been emptied. Although we currently have no information on the identity of this new compartment, we hypothesize that it may reside in an internal organelle located near the signaling machinery that triggers entry of extracellular Ca\(^{2+}\). In this way, it could readily substitute for the depleted extracellular Ca\(^{2+}\) in mediating cold shock and hypo-osmotic stress signals to the nucleus.

Because release of intracellular Ca\(^{2+}\) in response to chelation of extracellular Ca\(^{2+}\) is so immediate, we suspect that the putative Ca\(^{2+}\) sensor is extracellular. As such, it is conceivable that the sensor might be homologous to animal CaRs, which are G-protein-coupled receptors that signal in response to elevated serum [Ca\(^{2+}\)] via phospholipase C and inositol trisphosphate (14). In fact, the Arabidopsis genome contains several open reading frames with substantial homology to the animal CaRs (e.g. emb CAB63012.1). The function of these proteins in plants has, unfortunately, not yet been elucidated. However, because the EGTA-induced Ca\(^{2+}\) fluxes in tobacco cells are insensitive to inhibitors of phospholipase C (i.e. neomycin sulfate and U73122; data not shown), but are inhibited by modulators of anion channels and kinases (Fig. 4), it appears that the Ca\(^{2+}\) sensor in our studies must differ at least in its downstream effector components from the animal CaRs (14). An alternative possibility is that the plant extracellular Ca\(^{2+}\) sensor involves the cell wall itself. He and colleagues (22) have recently identified a family of transmembrane cell wall-integrated receptor-like protein kinases, the extracellular domains of which interact with the pectic portion of the cell wall in tobacco. Because pectin is known to bind large amounts of Ca\(^{2+}\) and to dramatically change conformation upon Ca\(^{2+}\) removal (23, 24), it is possible that Ca\(^{2+}\)-regulated changes in cell wall-kinase interactions could also initiate the signaling cascade leading to release of organellar Ca\(^{2+}\).

Acknowledgments—We thank Dr. Darrell Schulze of the Purdue University Department of Agronomy for providing excellent technical assistance.
REFERENCES
