Hypo-osmotic Shock of Tobacco Cells Stimulates Ca\(^{2+}\) Fluxes Deriving First from External and then Internal Ca\(^{2+}\) Stores*

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Stephen G. Cessna, Sreeganga Chandra‡, and Philip S. Low§

From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393

Hypo-osmotic shock of aequorin-transformed tobacco cells induces a biphasic cytosolic Ca\(^{2+}\) influx. Because both phases of Ca\(^{2+}\) entry are readily blocked by Ca\(^{2+}\) channel inhibitors, we conclude that the Ca\(^{2+}\) transients are mediated by Ca\(^{2+}\) channels. Evidence that the first but not second Ca\(^{2+}\) transient derives from external Ca\(^{2+}\) stores is that the first but not second influx is (i) eliminated by membrane-impermeable Ca\(^{2+}\) chelators, (ii) enlarged by supplementation of the medium with excess Ca\(^{2+}\), and (iii) reduced by the addition of competitive cations such as Mg\(^{2+}\) and Mn\(^{2+}\). Furthermore, entry of \(^{45}\)Ca during osmotic shock is prevented by inhibitors of the first but not second phase of Ca\(^{2+}\) entry. Evidence that the second wave of Ca\(^{2+}\) influx stems from release of intracellular Ca\(^{2+}\) is based on the above data plus observations that probable modulators of intracellular Ca\(^{2+}\) channels selectively block this phase of Ca\(^{2+}\) influx. Finally, a mechanism of communication between the two Ca\(^{2+}\) release pathways has become apparent, since perturbations that elevate or reduce the first Ca\(^{2+}\) transient lead to a compensating diminution/elevation of the second and vice versa. These data thus suggest that osmotic shock leads to the sequential opening of extracellular followed by intracellular Ca\(^{2+}\) stores and that these Ca\(^{2+}\) release pathways are internally compensated.

Ca\(^{2+}\) is well established as a secondary messenger in plant signal transduction pathways, including those initiated by such stimuli as cold shock (1), heat shock, (2) touch (3), anoxia (4, 5), elicitor addition (3, 6–9), pathogen infection (10–12), hormone administration (13–16), oxidative stress (17), far red light (18), drought (19), pollen tube elongation (20, 21), and egg cell fertilization (22) (for recent reviews, see Refs. 23–26). These temporary elevations of cytosolic Ca\(^{2+}\) are believed to participate in signal propagation, resulting in activation/inhibition of such downstream effectors as protein kinases, ion channels, phospholipases, oxidases, hydrolases, and/or calmodulin-dependent enzymes (27). As anticipated, blockade of the above-stimulated Ca\(^{2+}\) influxes either inhibits or retards most, if not all, of the associated pathways. It can be hypothesized that plant cells maintain high Ca\(^{2+}\) concentrations both in their extracellular milieu and in certain intracellular compartments to enable rapid gating of the cation into the low Ca\(^{2+}\) environment of the cytosol (23–29). Indeed, cytosolic Ca\(^{2+}\) transients in hormone/cytokine-treated animal cells have been shown to derive from both internal and external Ca\(^{2+}\) stores (27). Because Ca\(^{2+}\) channels have been identified in both plasma and internal membranes of plant cells (6, 9, 30–38), both Ca\(^{2+}\) pools should be considered as possible sources for mediating plant signaling events.

We and others have observed that elicitation of the oxidative burst in cultured plant cells generally accompanies and requires a rise in cytosolic Ca\(^{2+}\) (7–10). However, it has not been established if the induction of the oxidative burst requires Ca\(^{2+}\) influx from internal or external Ca\(^{2+}\) stores or from both in succession or simultaneously. In the case of the mechanically or osmotically stimulated oxidative burst, the cytoplasmic Ca\(^{2+}\) increase is distinctly biphasic. Thus, using aequorin-transformed tobacco cells to quantify Ca\(^{2+}\) influx into the cytoplasm of the cell (3), Ca\(^{2+}\) peaks have been repeatedly observed ~15 s and 1.5 min after hypotonic stress (8, 39). In addition, other groups have established that both Ca\(^{2+}\) entry across the plasma membrane and Ca\(^{2+}\) release from internal stores may play roles in plant cell volume/turgor regulation (14, 23). Because this unusual pattern of Ca\(^{2+}\) flux offers the opportunity to examine the Ca\(^{2+}\) signal required for both the induction of the oxidative burst and the regulation of cellular turgor pressure, we have decided to identify the Ca\(^{2+}\) stores responsible for both phases of the Ca\(^{2+}\) influx. We report here that the first Ca\(^{2+}\) transient arises from outside the cell, whereas the second derives from intracellular stores. We further identify inhibitors that can selectively block the first, second, or both phases of Ca\(^{2+}\) flow, and we report observations regarding possible communication between the two pathways responsible for Ca\(^{2+}\) influx.

EXPERIMENTAL PROCEDURES

Materials—Ruthenium red, lanthanum chloride, gadolinium chloride, and niflumate were all purchased from Sigma/Aldrich. \(^{45}\)Ca in the form of \(^{45}\)CaCl\(_2\) salt was obtained from ICN (Costa Mesa, CA), and coelenterazine was from Molecular Probes (Eugene, OR). Other chemicals used were of reagent grade or better and were obtained from major chemical suppliers.

Aequorin-transformed Tobacco Cell Suspension Cultures—Aequorin-transformed tobacco cell (Nicotiana plumbaginifolia) suspension cultures were established from transformed seedlings and maintained as described previously (8). Two ml of packed filtered cells were transferred to 100 ml of fresh W-38 liquid medium (40) every 7 days and maintained in suspension by continuous shaking. Cells were used ~12 h later for Ca\(^{2+}\) measurements were transferred at twice their regular cell density. Functional aequorin was reconstituted from the apoenzyme by adding 5 \(\mu\)l of coelenterazine dissolved in ethanol (1 \(\mu\)m final) to 3 ml of suspension-cultured cells at the time of transfer. Functional aequorin capable of reproducible Ca\(^{2+}\) measurements was found to develop 5 to 15 h after transfer of the cells to fresh medium and the addition of coelenterazine.

Aequorin Luminescence Measurements and (Ca\(^{2+}\)\) Quantiitation—Luminescence measurements were carried out in a digital luminometer (LKB Wallac model 1250, Gaithersburg, MD), as described previously.

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‡ Current address: Dept. of Neuroscience, University of California at San Diego, La Jolla, CA 92093.

§ Supported in part by National Science Foundation Grant MCB 97229. To whom correspondence should be addressed: Dept. of Chemistry, 1393 Brown Bldg., Purdue University, West Lafayette, IN 47907-1393. Tel.: 765-494-5273; Fax: 765-494-0239; E-mail: lowps@omni.cc.purdue.edu.
(8). Briefly, 0.5 ml of coelenterazine-treated cells were transferred to the luminometer cuvette and held in suspension by mild stirring during stimulation. Luminescence data was collected 10 times/s. All inhibitors, chelators, and control solvents were added at the times indicated in the figure legends. Plant cells were hypo-osmotically shocked by diluting the suspensions 1:1 with distilled water at the times indicated in the figures. At the end of each experiment, all remaining aequorin was discharged by the addition of 0.5 mM CaCl2 in 10% Nonidet P-40, and luminescence was continually quantitated until recordings returned to basal levels and further addition of CaCl2/detergent solution elicited no further response. Luminescence data were then converted by computer directly to intracellular Ca2
+ concentration using the equation described by Allen et al. (41), [Ca2
+]i = [(LLmax)1/3 + 118(LLmax)2/3]−1\(\times\)\((7 \times 10^{-4} - (7 \times 10^{-6})(L/L_{max})^{1/2})\), where L is the luminescence intensity at any time point, and Lmax is the integrated luminescence intensity from that point to the end of the luminescence recording.

The Biphasic Ca2
+ Flux in Tobacco Induced by Hypo-osmotic Shock Is the Result of the Activation of Specific Ion Channels—It was conceivable that the previously observed hypo-osmotic shock generated Ca2
+ transients (8, 39) were not due to regulated ion channels but rather to a membrane disturbance resulting in nonspecific Ca2
+ leaks. To evaluate this possibility, we tested the effectiveness of several broad-spectrum Ca2
+ channel inhibitors on the induced cytosolic Ca2
+ fluxes. Ruthe- nium red and the trivalent lanthanum and lanthanum are known to block many types of Ca2
+ channels in both plasma and sarcoplasmic reticular membranes of animal cells (42–44), and all three have also been used for characterization of Ca2
+ channels and Ca2
+-dependent processes in plant cells (5, 8, 30–34, 45, 46). As can be seen in Fig. 1, each of these inhibitors is effective at blocking both phases of Ca2
+ flux into hypo-osmotically stimulated tobacco cells. Inhibition of Ca2
+ influx by ruthenium red at 50 μM (Fig. 1A) is consistent with the pharmacologic data of both Allen et al. (30) and Pinos and Tester (34), who observed blockade of cyclic-ADP-ribose-activated tonoplast Ca2
+ channels and voltage-gated plasma membrane Ca2
+ channels, respectively, at similar concentrations. Although this effect of ruthenium red has been previously published (8), we present these data here to allow direct comparisons of the inhibitory effects of Ca2
+ channel blockers on these Ca2
+ fluxes. Lanthanum and gadolinium ions, which required a 10-min equilibration to achieve maximal channel inhibition, also displayed concentration dependences similar to those observed by others (5, 9, 17). At 10 mM concentration, both lanthanides inhibited greater than 80% total Ca2
+ influx, whereas at 1 mM they primarily suppressed influx during the first Ca2
+ peak (Figs. 1, B and C).

Inhibition by these agents provides little information regarding the location of channels responsible for the cytosolic Ca2
+ transients. Externally provided ruthenium red has been shown to inhibit plasma membrane Ca2
+ channels (34) and to enter cultured plant cells and interrupt internal Ca2
+ release (4). In addition, lanthanum ions, classically used to identify plasma

FIG. 1. Effect of ruthenium red, La3
+, and Gd3
+ on the hypo-osmotic shock induced Ca2
+ fluxes in aequorin-transformed to- bacco cells. Coelenterazine-treated aequorin-transformed tobacco cells (0.5 ml) were transferred to a luminometer cuvette after 15 min of treatment with the following inhibitors: A, ruthenium red (RR); B, LaCl3; and C, GdCl3. Final concentrations of inhibitors are indicated for each trace. Traces marked control represent data from cells not treated with any inhibitor. Luminescence was monitored, and [Ca2
+]i was calculated as outlined under “Experimental Procedures.” The cells were hypo-osmotically shocked in the cuvette by the addition of an equal volume of distilled water at the times indicated by the arrow. The data shown were collected on the same day from the same batch of cells and are representative of three independent experiments conducted on separate days with different batches of cells.

1 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
membrane Ca\(^{2+}\) channel activities, when provided in millimolar concentrations for periods greater than 1 min have also been observed to enter cells and alter internal Ca\(^{2+}\) channels (43, 44). However, the inhibition of Ca\(^{2+}\) influx by these channel blockers does demonstrate that the osmotically induced Ca\(^{2+}\) uptake proceeds through specific Ca\(^{2+}\) channels and not through nonspecific ion leaks.

The First Pulse of Cytosolic Ca\(^{2+}\) Derives from External Ca\(^{2+}\) Pools—To begin to identify the cellular sources of the two pulses of cytosolic Ca\(^{2+}\), we first performed manipulations that would specifically modify the flux of external Ca\(^{2+}\) across the plasma membrane. EGTA, a membrane-impermeable selective chelator of Ca\(^{2+}\) ions, was added to the extracellular medium at a concentration of 1.5 mM, approximately the concentration of free Ca\(^{2+}\) in the medium. As shown in Fig. 2A, chelation of extracellular Ca\(^{2+}\) with EGTA exerts an inhibitory effect selectively on the first Ca\(^{2+}\) spike generated by hypo-osmotic shock, suggesting that this Ca\(^{2+}\) transient, but not the second, derives from an external source. Inhibition of the first peak was also seen using 1.5 mM 1,2-bis-(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) in place of EGTA, indicating that the nature of the Ca\(^{2+}\)-chelating agent is unimportant to the inhibition (data not shown). In contrast to Ca\(^{2+}\) chelation, the addition of extra CaCl\(_2\) to achieve a final medium concentration approximately 2.7 and 4.3 times that normally present in the cell culture was seen to elevate the amount of Ca\(^{2+}\) entering during the first phase of the Ca\(^{2+}\) influx (Fig. 2B). Furthermore, supplementation of the growth medium with excess Mg\(^{2+}\), which can either compete with Ca\(^{2+}\) as a channel substrate or antagonize Ca\(^{2+}\) entry as a weak channel blocker (31, 34), also reduced the magnitude of the first Ca\(^{2+}\) peak (Fig. 2C). Mn\(^{2+}\) and Fe\(^{2+}\) were similarly found to inhibit the first, but not alter the second, of the two Ca\(^{2+}\) peaks (data not shown). Inhibition of the first phase of Ca\(^{2+}\) influx was also observed after replacement of the chloride with the sulfate salts of Mg\(^{2+}\) and Fe\(^{2+}\) (data not shown), indicating that this initial phase of Ca\(^{2+}\) entry into the cytosol is not controlled by the nature of the counter ion. Based on the selective suppression of the first Ca\(^{2+}\) transient by either EGTA or nonsubstrate cations and the selective enhancement of this peak by elevated extracellular Ca\(^{2+}\), we propose that the first peak of Ca\(^{2+}\) influx during osmotic shock originates from an apoplastic pool.

To further confirm the above channel assignment, measurement of \(^{45}\)Ca uptake by hypo-osmotically shocked cells was also performed. Dilution of the suspension-cultured tobacco cells with water caused an increase in the uptake of \(^{45}\)Ca to \(5 \times\) the basal level measured in iso-osmotically treated cells. As expected, 10 mM La\(^{3+}\) was seen to largely inhibit this influx (Fig. 3), confirming that the \(^{45}\)Ca uptake is mediated by a Ca\(^{2+}\) channel. More importantly, 10 mM MgCl\(_2\) was observed to reduce the uptake by \(-60\%\), a value not inconsistent with the data shown in Fig. 2C. Since the added \(^{45}\)Ca was unequivocally apoplastic and since Mg\(^{2+}\) only reduces the first Ca\(^{2+}\) transient measured by aequorin luminescence, we conclude that this first pulse of osmotically stimulated Ca\(^{2+}\) entry indeed derives from extracellular Ca\(^{2+}\).

The Second Peak of Ca\(^{2+}\) Derives from Intracellular Ca\(^{2+}\) Stores—Although the results shown in Fig. 2 also agree that the second peak of cytosolic Ca\(^{2+}\) must stem from opening an internal store, we set out to confirm this hypothesis with selective modulators of internal Ca\(^{2+}\) release. For this purpose, caffeine, a Ca\(^{2+}\) channel regulator believed to activate Ca\(^{2+}\) release from cyclic-ADP-ribose and ryanodine-sensitive stores in many eukaryotic systems (4, 47, 48), was added to the tobacco suspensions, and the osmotically induced Ca\(^{2+}\) transients were again examined. As anticipated, the addition of

![Fig. 2. Effect of extracellular Ca\(^{2+}\) modulators on Ca\(^{2+}\) fluxes.](Image)
caffeine in the absence of any other stimulus induced substan-

tial entry of Ca\(^{2+}\) into the cytosol of the tobacco cell (Fig. 4A).

Since these caffeine-activated Ca\(^{2+}\) transients were found to be

insensitive to membrane-permeant Ca\(^{2+}\) chelators and to

competition with extracellular Mn\(^{2+}\) or Mg\(^{2+}\) (data not shown),

we reason that this Ca\(^{2+}\) signal in tobacco cells is derived from

the caffeine-induced emptying of an intracellular Ca\(^{2+}\) pool.

Based on its impact in animal cells, the ability of caffeine to

autologously discharge certain internal Ca\(^{2+}\) stores should also

lead to an insensitivity of the treated cells to stimuli that

discharge the same stores. Therefore, we evaluated the impact

of caffeine pretreatment on the subsequent ability of hypo-

osmotic shock to activate the second phase of cytosolic Ca\(^{2+}\) release.

As shown in Fig. 4B, prior exposure to caffeine eliminates

the second Ca\(^{2+}\) transient without affecting or perhaps

even enhancing the first. This loss of sensitivity to osmotic

stimulation confirms that the latter Ca\(^{2+}\) influx is indeed derived from caffeine-sensitive internal stores. Unfortunately,

other modulators of intracellular Ca\(^{2+}\) release commonly used in

mammalian systems such as ryanodine (20 \(\mu M\), the inositol

1,4,5-trisphosphate receptor Ca\(^{2+}\) channel modulator TMB-8

(200 \(\mu M\), or the sarcoplasmic reticular Ca\(^{2+}\) ATPase in-

hibitor thapsigargin (up to 100 \(\mu M\)) had little or no effect on

either of the osmotically activated transients, suggesting that

the similarity in pharmacology of internal Ca\(^{2+}\) channels in

animals and plants may extend no further than caffeine sensi-

tivity. Nevertheless, the fact that moderate caffeine concen-

trations were able to specifically inhibit expression of the second

phase of Ca\(^{2+}\) entry suggests that this phase of influx is mech-

anistically distinct from the first and is likely gated through an

intracellular channel.

Niflumic acid, an inhibitor of anion channels in plant and

animal cells, was also found to serve as a selective inhibitor of

the second peak of Ca\(^{2+}\) entry (Fig. 4B). Other groups have

observed that anion channel blockers such as niflumate and anthracene-9-carboxylate readily inhibit various anion channel

activities in plant cells (14, 49–52), and they have suggested

that release of Ca\(^{2+}\) from vacuolar stores may depend on con-

FIG. 3. Effect of Ca\(^{2+}\) modulators on 45Ca influx. Aequorin-

transformed tobacco cells (3 ml) were exposed to 0.1 \(\mu M\) Cl\(^{-}\) 45Ca accord-

ing to the protocol outlined under “Experimental Procedures” and sub-

jected to the following treatments: control, hypo-osmotic shock induced

by 1:1 dilution with distilled water; La\(^{3+}\), 10 mM LaCl\(_3\) was added and

followed immediately by a 1:1 dilution with distilled water; Mg\(^{2+}\), 10

mM MgCl\(_2\) was added and followed immediately by a 1:1 dilution with

distilled water; and niflumate, 500 \(\mu M\) niflumate dissolved in Me\(_2\)SO

was added 10 min before 45Ca exposure and dilution with distilled

water. Data are presented as relative nuclide uptake/mg of cells and

Error bars represent mean (S.D.) for three independent experiments.

FIG. 4. The effect of intracellular Ca\(^{2+}\) modulators on Ca\(^{2+}\)

fluxes. Aequorin luminescence recordings and Ca\(^{2+}\) calculations were

performed as outlined under “Experimental Procedures.” A, 0.5 ml of

suspension-cultured cells were treated with either 100 or 40 \(mM\) caf-

feine at the time indicated by the arrow. Aequorin luminescence record-

ings of control cells treated with isotonic sucrose did not exceed 0.15

mCi 45Ca accord-

FIG. 2. A, B, C. Results of experiments consistent with a model of

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ion uptake: control, 

Mg\(^{2+}\), niflumate, and 

La\(^{3+}\). C, treatments of control cells with either 500 or 100 \(\mu M\) niflumate or with a corresponding

volume of Me\(_2\)SO alone (control) 10 min before hypo-osmotic stimulation

in the luminometer. Cells were diluted 1:1 with distilled water at

the time indicated by the arrow.
current anion fluxes to depolarize the membrane (14). Consistent with these interpretations, anthracene-9-carboxylate was also observed to block the second phase of Ca$^{2+}$ uptake at concentrations (100–500 μM) similar to those found effective in the studies mentioned above (data not shown). Importantly and in contrast to the effect of Mg$^{2+}$ on 45Ca influx, the addition of 500 μM niflumate to the plant cell culture failed to inhibit 45Ca uptake upon hypo-osmotic shock (Fig. 3). Thus, since 45Ca influx only measures the externally derived portion of the biphasic Ca$^{2+}$ transients and since niflumate has no effect on this 45Ca influx, we conclude that the second Ca$^{2+}$ peak is generated by release of the cation from internal stores.

The Two Channels Regulating Ca$^{2+}$ Influx during Hypo-osmotic Shock Communicate—It did not escape our attention that inhibition of the first Ca$^{2+}$ transient generally resulted in enlargement of the second (Fig. 2A). Only in the case of Mg$^{2+}$ addition was this compensating increase in the truing Ca$^{2+}$ pulse not consistently observed. Conversely, enhancement of the initial phase of Ca$^{2+}$ influx by supplementation of the medium with extra Ca$^{2+}$ invariably reduced the amplitude of the latter (Fig. 2B). Furthermore, inhibition of the second phase of Ca$^{2+}$ uptake by niflumic acid consistently enhanced the first (Fig. 4B). Similar but somewhat reduced effects were also seen with moderate concentrations of caffeine (Fig. 4A).

Taken together, these data argue that some type of communication must occur between the two phases of osmotically stimulated Ca$^{2+}$-gating and that inhibition or enhancement of one phase of influx somehow leads to a compensating Ca$^{2+}$ flux during the other. It will be interesting to explore the pathways along which such communication travels.

**DISCUSSION**

We have provided evidence that the two phases of Ca$^{2+}$ entry into the cytoplasm of osmotically shocked tobacco cells sequentially involve (i) the influx of extracellular Ca$^{2+}$ and (ii) the release of intracellular (compartmentalized) Ca$^{2+}$. Thus, prevention of external Ca$^{2+}$ entry by the addition of EGTA or competing cations (i.e. Mg$^{2+}$, Mn$^{2+}$, Fe$^{2+}$) diminished the first phase of Ca$^{2+}$ uptake, whereas augmentation of the extracellular Ca$^{2+}$ pool specifically enhanced this same wave of Ca$^{2+}$ uptake. Taken together with data on the blockade of the second phase of Ca$^{2+}$ influx by caffeine and niflumate, i.e. probable modulators of intracellular Ca$^{2+}$ release, a strong case for the above Ca$^{2+}$-gating assignments can now be made. This contention is also bolstered by observations that Mg$^{2+}$, which blocks only the initial phase of Ca$^{2+}$ influx, prevents externally added 45Ca uptake, and niflumic acid, which eliminates only the second phase of Ca$^{2+}$ influx, does not.

We have also observed that communication may occur between the two waves of Ca$^{2+}$ entry into the cytoplasm of a stimulated tobacco cell. Unfortunately, there are currently few clues in the literature regarding the mechanism through which this communication might occur. Although hypo-osmotic stress has been frequently observed in eukaryotic cells to activate release of both intracellular and extracellular Ca$^{2+}$ stores, little data regarding cross-talk between these two Ca$^{2+}$ depots has yet been presented (53–58). Furthermore, the gating and signaling pathways activated by hypo-osmotic stimulus in non-plant systems may be very different from those in tobacco, since, opposite to the order of Ca$^{2+}$-gating in tobacco cells, internal release of Ca$^{2+}$ appears to precede influx of externally derived Ca$^{2+}$ in mammalian cells (55–58). This order of Ca$^{2+}$ channel activation in animal cells is reminiscent of the phenomenon of capacitative Ca$^{2+}$ entry, in which intracellular release of Ca$^{2+}$ leads to the activation of plasma membrane Ca$^{2+}$ channels and the refilling of internal stores (27, 59–61). Although the mechanisms of communication between internal and external Ca$^{2+}$ stores during capacitative Ca$^{2+}$ entry are currently an area of intense research, no consensus mechanistic theory has been put forth (61). In osmotically stressed tobacco cells, a Ca$^{2+}$-sensing mechanism that would adjust the Ca$^{2+}$ efflux from the second (intracellular) Ca$^{2+}$ store depending on the quantity of Ca$^{2+}$ delivered during the first (extracellular) phase of Ca$^{2+}$ entry can be easily envisioned. The intracellular Ca$^{2+}$ gate would simply need to be negatively regulated by Ca$^{2+}$ or a Ca$^{2+}$-activated enzyme (e.g. a kinase) (62, 63). It will be important in the future to look for such a signaling mediator following treatment of stimulated cells with excess Ca$^{2+}$ or EGTA (Figs. 2, A and B).

In contrast to the feed-forward signaling mechanism hypothesized above, identification of a communication pathway that responds to the readiness state of the second phase of Ca$^{2+}$ entry and pre-emptively modulates the first is more difficult to imagine. Nevertheless, pretreatment of osmotically stimulated tobacco cells with moderate concentrations of caffeine or niflumic acid invariably augments the initial Ca$^{2+}$ transient before eliminating the latter (Fig. 4, A and B). However, Takahashi et al. (39) have presented evidence that the general serine/threonine protein kinase inhibitors K252a and staurosporine selectively inhibit the second phase of Ca$^{2+}$ influx without a compensating increase in the first phase. Thus, this modification of extracellular Ca$^{2+}$ influx by internal Ca$^{2+}$ transport capability may not be a general phenomenon and may depend on the type inhibitors used. There are clearly complexities in these Ca$^{2+}$ signaling pathways that will require considerable research to resolve. It will be interesting to explore the area of Ca$^{2+}$ channel communication in plant cells and to possibly identify at both the protein and DNA level the Ca$^{2+}$ channels and other molecules involved.

It was encouraging to observe that several inhibitors could be identified that selectively block cytosolic influx of Ca$^{2+}$ from either intracellular or extracellular Ca$^{2+}$ stores. Although lanthanides and ruthenium red were found to inhibit both pathways of Ca$^{2+}$ influx, the other modulators detailed above were highly selective for just one pathway. Based on these observations, it should now be possible to resolve which downstream consequences of hypo-osmotic shock rely on which phases of Ca$^{2+}$ entry for signal propagation. It is conceivable, for example, that pathways designed to adjust cellular volume/turgor pressure during osmotic stress (14, 23, 64, 65) might depend on a different Ca$^{2+}$ signal than pathways evolved to initiate the osmotically induced oxidative burst (8, 66). Indeed, preliminary studies indicate that only the second of the two phases of Ca$^{2+}$ entry is required to stimulate reactive oxygen biosynthesis following hypo-osmotic stimulation.2

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