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Hypoosmotic Shock Induces Increases in Cytosolic Ca^{2+} in Tobacco Suspension-Culture Cells[1]

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Hypoosmotic shock treatment increased cytosolic Ca^{2+} ion concentration ($[Ca^{2+}]_{cyt}$) in tobacco (*Nicotiana tabacum*) suspension-culture cells. $[Ca^{2+}]_{cyt}$ measurements were made by genetically transforming these cells to express apoaequorin and by reconstituting the Ca^{2+}-dependent photoprotein, aequorin, in the cytosol by incubation with chemically synthesized coelenterazine. Measurement of Ca^{2+}-dependent luminescence output thus allowed the direct monitoring of $[Ca^{2+}]_{cyt}$ changes. When cells were added to a hypoosmotic medium, a biphasic increase in $[Ca^{2+}]_{cyt}$ was observed; an immediate small elevation (phase 1) was observed first, followed by a rapid, large elevation (phase 2). Phase 1 $[Ca^{2+}]_{cyt}$ was stimulated by the V-type ATPase inhibitor bafilomycin A_1. Phase 2 was inhibited by the protein kinase inhibitor K-252a and required the continued presence of the hypoosmotic stimulus to maintain it. Although Ca^{2+} in the medium was needed to produce phase 2, it was not needed to render the cells competent to the hypoosmotic stimulus. If cells were subject to hypoosmotic shock in Ca^{2+}-depleted medium, increases in luminescence could be induced up to 20 min after the shock by adding Ca^{2+} to the medium. These data suggest that hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation results from the activity of a Ca^{2+} channel in the plasma membrane or associated hypoosmotic sensing components that require Ca^{2+}-independent phosphorylation and a continued stimulus to maintain full activity.

The maintenance and regulation of turgor pressure is essential for many aspects of plant growth, development, and movements. Although turgor pressure can be regulated via the synthesis and degradation of osmotic protectants, rapid changes usually occur by modifications of the flux of inorganic ions across the plasma membrane (Schroeder and Hedrich, 1989).

A great deal of research effort has been directed at investigating the responses of higher plants to hyperosmotic signals (Greenway and Munns, 1980), because hyperosmotic stress during salination or drought, for example, is agriculturally important. In contrast, plant responses to hypoosmotic signals have rarely been studied, even though they are directly relevant to understanding both stomatal and leaf movements. Hypoosmotic shock induces increases in both cell volume and turgor pressure, even in walled cells, and the magnitude of the water influx is, in part, dependent on the osmotic potential of the cell. After hypoosmotic shock plant cells can usually recover both their cell volume and their original osmotic potential (Okazaki and Tazawa, 1990), a phenomenon termed "hypoosmotic regulation." To accomplish this, plant cells must have mechanisms both for sensing hypoosmolarity and for transducing this information to affect responses leading to hypoosmotic regulation; the challenge is to identify these components.

Hypoosmotic regulation, which occurs after hypoosmotic shock, involves a reduction in turgor pressure. Substantive evidence in the literature suggests that cytosolic Ca^{2+} may regulate reductions in turgor pressure. Leaf closure in *Mimosa pudica*, which is caused by a decrease in turgor pressure on one side of the pulvinus, can be inhibited by EDTA and La^{3+} (Campbell and Thompson, 1977). Dark-induced stomatal closure in *Commelina communis* is also mediated by a decrease in turgor pressure and can be accelerated by external Ca^{2+} and inhibited by EGTA (Schwartz, 1985). Increases in $[Ca^{2+}]_{cyt}$ in guard cells of *Vicia faba* and *C. communis* precede ABA-induced stomatal closure (McAinsh et al., 1990; Irving et al., 1992). Photolysis of loaded caged Ca^{2+} or caged IP_3 causes stomatal closure in *C. communis* (Gilroy et al., 1990), again indicating the involvement of $[Ca^{2+}]_{cyt}$ and internal IP_3-sensitive Ca^{2+} stores in closure. These reports suggest that $[Ca^{2+}]_{cyt}$ plays a significant role in controlling turgor decrease.

In algal cells, $[Ca^{2+}]_{cyt}$ has been postulated to play a significant role in hypoosmotic responses. Okazaki and Tazawa (1990) reported that hypoosmotic shock induces a transient $[Ca^{2+}]_{cyt}$ elevation and Ca^{2+} acts in turgor regulation in the euryhaline characeaen alga *Lamprothamnium*. A Ca^{2+}-dependent protein kinase has been purified from the halotolerant green alga *Dunaliella tertiolecta* and its involvement in hypoosmotic responses has been suggested (Yuasa and Muto, 1992; Yuasa et al., 1995).

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Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} ion concentration; IP_3, inositol 1,4,5-triphosphate; LS, Linsmaier and Skoog.

The above information suggests that hypoosmotic signals in plant cells might be transduced via $[Ca^{2+}]_{cyt}$. Ca^{2+} functions as a primary second messenger in signal transduction (Muto, 1992; Bush, 1995) and has been shown to modify a number of physiological processes (Hepler and Wayne, 1985; Nagai, 1993; Trewavas and Knight, 1994). To understand the involvement and functions of Ca^{2+} in hypoosmotic signaling, it is necessary to continuously monitor the change in $[Ca^{2+}]_{cyt}$. We have described a novel method for this purpose that uses plants genetically transformed to express apoaequorin, which on incubation with coelenterazine reconstitutes the Ca^{2+}-dependent luminescent protein aequorin. Continuous measurement of luminescence reports the dynamics of $[Ca^{2+}]_{cyt}$ in vivo during signaling (Knight et al., 1991; Knight and Knight, 1995). Using this method we have detailed $[Ca^{2+}]_{cyt}$ changes in response to touch, cold shock, fungal elicitors (Knight et al., 1991), wind (Knight et al., 1992), wounding (Knight et al., 1993), hydrogen peroxide (Price et al., 1994), and circadian rhythms (Johnson et al., 1995). Because of the simplicity and ease of this method, we used it to examine the possible transduction of hypoosmotic signaling. However, hypoosmotic shock is technically difficult to administer to whole plants, so we decided to transform tobacco (*Nicotiana tabacum* BY-2) suspension-culture cells to express apoaequorin and reconstitute aequorin by incubation with chemically synthesized coelenterazine. This approach has the added benefit that biochemical inhibitors can be used more effectively and in the physiological range.

MATERIALS AND METHODS

Chemicals

K-252a (Seikagaku Kogyo Co., Tokyo, Japan) and bafilomycin A_1 (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in DMSO at 2 mM and 1 mM, respectively, and stored at −30°C. Coelenterazine was synthesized as described previously (Isobe et al., 1994), dissolved in ethanol at 1 mM, and stored at −30°C.

Cell Culture and Genetic Transformation

Tobacco (*Nicotiana tabacum* L. cv bright yellow 2) suspension-culture cells (BY-2) were cultured as described previously (Kamada and Muto, 1994). Briefly, the culture was maintained in LS liquid medium (Linsmaier and Skoog, 1965) containing 0.2 $\mu g\ mL^{-1}$ of 2,4-D (LS medium, pH 5.8) at 28°C with shaking at 130 rpm on a gyratory shaker in darkness and subcultured every week with a 4% inoculum.

Transformation of tobacco suspension-culture cells was carried out according to An (1985) as follows: 4 mL of 3-d-old, exponentially growing, suspension-cell culture was transferred to a 90-mm Petri dish and incubated at 28°C with 100 μL of fresh overnight culture of *Agrobacterium tumefaciens* LBA4404 containing the binary vector pMAQ2 (Knight et al., 1991), which contains the apoaequorin-coding region from cDNA clone pAEQ1 (Prasher et al., 1985). After 48 h of co-cultivation, the tobacco cells were washed and plated on the LS agar medium containing 500 $\mu g\ mL^{-1}$ of carbenicillin and 250 $\mu g\ mL^{-1}$ of kanamycin. After 3 to 4 weeks of selection, the transformants were collected and transferred into LS medium.

Hypoosmotic Shock and Luminescence Measurements

Aequorin was reconstituted by incubating 3-d-old transgenic BY-2 cells with 1 μM coelenterazine in the culture medium in darkness for 8 h. The cells were washed with and resuspended in fresh medium and used after 30 min of resting incubation. The cells were subjected to hypoosmotic shock by diluting 200 μL of the cell suspension with 400 μL of the culture medium depleted of Suc in a glass tube for luminescence measurement. Luminescence emitted from the transgenic cells was measured with a Chem-Grow photometer (American Instrument Co., Silver Spring, MD) equipped with a pen recorder (Rikadenki Co., Tokyo, Japan) and expressed as relative light units. The osmotic pressure of solutions was measured with an osmometer (model 3D3, Advanced Instruments, Norwood, MA).

Calibration of $[Ca^{2+}]_{cyt}$

After each experiment all remaining aequorin was discharged with 1 M $CaCl_2$ and 10% ethanol, and the resultant luminescence was measured to estimate the amount of remaining aequorin. Cytosolic Ca^{2+} concentration was calibrated according to Knight et al. (1996) using the calibration equation: $pCa = 0.332588(-\log k) + 5.5593$, where k is a rate constant equal to luminescence counts per second divided by total counts.

Measurement of $[Ca^{2+}]$

The concentration of $[Ca^{2+}]$ in solutions was measured with a Ca^{2+} ion-selective electrode (model IS 561-Ca^{2+}, Philips, Eindhoven, The Netherlands).

Determination of Cell Viability

Cell viability was determined according to Ono et al. (1995) as the percentage of cells that accumulated fluorescein and as the percentage of plasmolyzed cells. Briefly, 5-mL aliquots of the suspension of cells were withdrawn after various treatment. One hundred microliters of 0.5% solution (w/v) of fluorescein diacetate in acetone was added to 5-mL aliquots of the cell suspension and cells were incubated for 20 min at 28°C. The cells were washed twice with fresh medium and cells that accumulated fluorescein were counted using fluorescence microscopy. For monitoring plasmolysis, 5-mL aliquots of the cell suspension were centrifuged (500*g* for 1 min), harvested cells were resuspended in 2 mL of 1 M sorbitol in the culture medium and incubated for 20 min at 28°C, and the plasmolyzed cells were counted under a light microscope.

RESULTS

Hypoosmotic Shock Transiently Increases $[Ca^{2+}]_{cyt}$

Transgenic tobacco suspension-culture cells expressing apoaequorin were incubated with 1 μM coelenterazine for

8 h to reconstitute aequorin. The amount of reconstituted aequorin was not sufficient to report resting $[Ca^{2+}]_{cyt}$ in these cells (which we expected to be maintained at a relatively low level of $<10^{-7}$ M), therefore, unstimulated cells did not produce any luminescence above background. When the transgenic tobacco cell suspension in the 200 mosmol medium was diluted with 2 volumes of the culture medium depleted of Suc (90 mosmol), a dramatic increase in the $[Ca^{2+}]_{cyt}$-dependent luminescence was observed (Fig. 1, trace A). An immediate, small increase in $[Ca^{2+}]_{cyt}$ was followed by a rapid, large increase starting 35 s after dilution. Luminescence intensity peaked at 70 s, rapidly decreased for the next 30 s, and then gradually returned to the original level after 4 to 5 min. The peaked $[Ca^{2+}]_{cyt}$ was estimated to be 321.4 ± 1.2 nM (n = 10) using the calibration equation described in "Materials and Methods." Dilution with the ordinary medium (containing 3% Suc, 200 mosmol, trace B) or with a medium containing 120 mM mannitol (320 mosmol, data not shown) did not cause the increase in $[Ca^{2+}]_{cyt}$. When a cell suspension preincubated in culture medium in which mannitol replaced Suc and with a final osmolarity of 200 mosmol was diluted with 2 volumes of culture medium depleted in Suc (90 mosmol), a similar luminescence profile to Figure 1A was observed (data not shown). On the other hand, dilution of the same cell suspension with 200 mosmol mannitol solution did not induce a $[Ca^{2+}]_{cyt}$ elevation and the trace was similar to Figure 1B (data not shown). Taken together, these results indicate that the $[Ca^{2+}]_{cyt}$ elevation observed in Figure 1A was caused by a "hypoosmotic shock" induced by reducing the extracellular osmolarity from 200 to 125 mosmol. This response was not because of the lack of Suc as a nutrient and was specific for hypoosmotic shock, because it could not be mimicked by a hyperosmotic shock.

Increasing the magnitude of the hypoosmotic shock produced a correspondingly increased $[Ca^{2+}]_{cyt}$ response (Fig. 2). The first phase of the response, the small, rapid elevation, became more clearly pronounced with increasing hypoosmotic shock and was clearly detectable at the 96.7 and 126.9 mosmol treatments (Fig. 2, indicated by arrowheads). These results indicate that there are two phases in $[Ca^{2+}]_{cyt}$ elevation in response to hypoosmotic shock in tobacco suspension-culture cells. These two phases, the immediate, small elevation and subsequent large, major $[Ca^{2+}]_{cyt}$ elevations, were termed "phase 1" and "phase 2," respectively. A second correlation with increased hypoosmolarity was a decrease in the lag time before the start of phase 2 (Fig. 2).

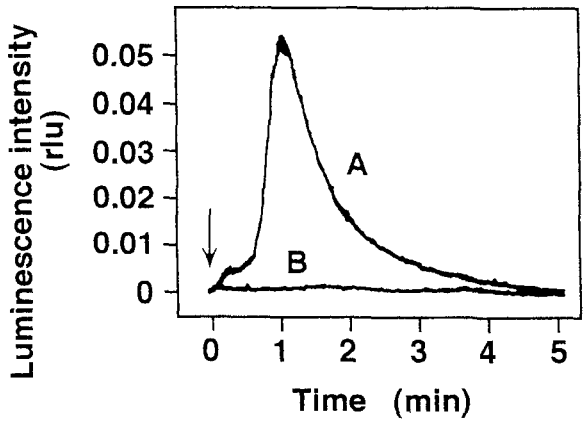

Figure 1. Effect of hypoosmotic shock on the $[Ca^{2+}]_{cyt}$ of cultured tobacco cells. Three-day-old transgenic tobacco suspension-culture cells were incubated with 1 μM coelenterazine to reconstitute aequorin. At the arrow the suspension was diluted (A) with 2 volumes of the medium depleted of Suc (90 mosmol) (B) with the normal medium (200 mosmol) and luminescence was recorded. These experiments were repeated 10 times and the traces represented have been chosen to best represent the average result. rlu, Relative light units.

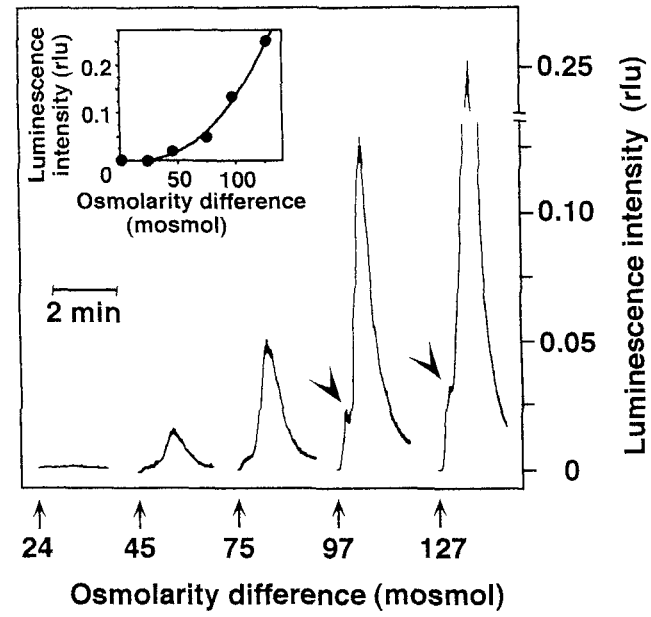

Figure 2. Effect of osmolarity difference on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The suspension of tobacco cells containing reconstituted aequorin was diluted with 2 volumes of various concentration of mannitol solution (175, 150, 90, 50, and 0 mM) as indicated by the arrows. The inset shows the peak values of the luminescence intensity. The values on the abscissa show the osmolarity difference (24, 45, 75, 97, and 127 mosmol) before and after the dilution. The arrowheads indicate the peaks of phase 1 $[Ca^{2+}]_{cyt}$ elevation. These experiments were repeated five times and the traces represented have been chosen to best represent the average result. rlu, Relative light units.

Effect of Various Inhibitors on Hypoosmotic Shock-Induced $[Ca^{2+}]_{cyt}$ Elevation

To characterize the Ca^{2+}-transporting system activated by hypoosmotic shock, the effect of various inhibitors on $[Ca^{2+}]_{cyt}$ elevation was examined. To emphasize the peaks of phases 1 and 2, cells were subjected to a stronger hypoosmotic shock (from 200 to 103 mosmol) in these experiments (Fig. 3). The inhibitors of voltage-dependent Ca^{2+} channels, verapamil, nifedipine, and diltiazem (5 μM each), did not affect $[Ca^{2+}]_{cyt}$ elevation. Bromophenacyl bromide (10 μM) and neomycin (100 μM), respective inhibitors of phosphoinositide metabolism and synthesis of IP_3, and thapsigargin (10 μM), an inhibitor of Ca^{2+} pump on the ER,

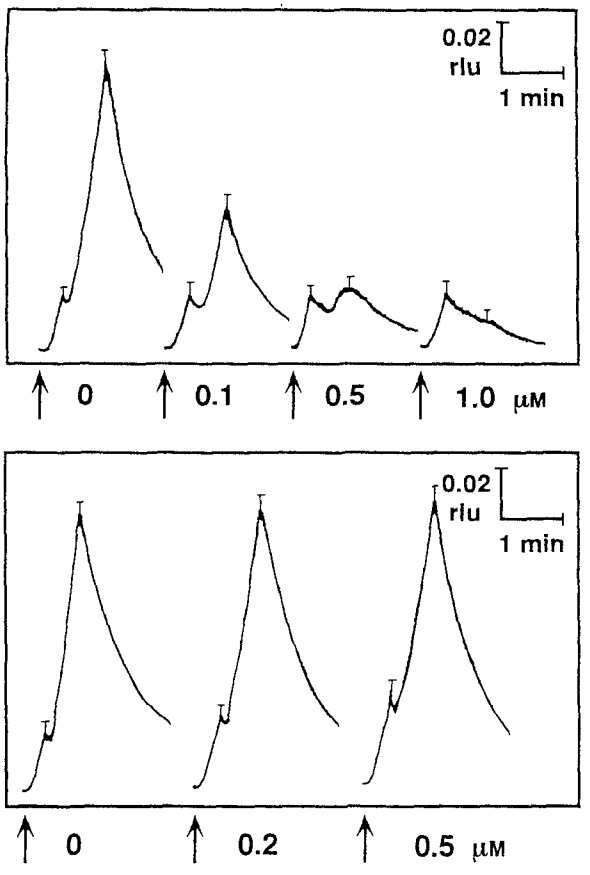

Figure 3. Effect of K-252a and bafilomycin A_1 on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The suspension of tobacco cells containing reconstituted aequorin were treated for 1 min with K-252a at the indicated concentration (top) or for 1 h with bafilomycin A_1 at the indicated concentration (bottom), and were subjected to hypoosmotic shock by diluting with 2 volumes of the 50 mM mannitol solution as indicated by the arrows. The osmolarity difference between the before and the after shock is 97 mosmol. As a solvent control, the cells were treated with 0.05% DMSO. These experiments were repeated four times and the traces represented have been chosen to best represent the average result. Vertical lines at the time of 20 and 70 s represent ± SE. rlu, Relative light units.

also had no effect. Calmodulin inhibitors calmidazolium and trifluoperazine (100 μM each) had no effect. Among the various inhibitors and chemical compounds we tested, only K-252a and bafilomycin A_1 affected the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The protein kinase inhibitor K-252a inhibited phase 2 (Fig. 3, top), and the vacuolar-type H^+-ATPase inhibitor bafilomycin A_1 (Yoshimori et al., 1991) enhanced phase 1 (Fig. 3, bottom). When the cells were treated with 1 μM K-252a 1 min before hypoosmotic shock, phase 2 was completely inhibited, although no effect of K-252a on phase 1 was observed (Fig. 3, top). The inhibitory effect of K-252a on phase 2 was concentration-dependent. This demonstrates that phase 2 is sensitive to K-252a and suggests that phase 2 depends upon one or more phosphorylation events. The other protein kinase inhibitors tested, ML-9, KN-62, M-9, genistein, and geldanamycin (10 μM each), and a phosphoprotein phosphatase inhibitor, calyculin A (5 μM), however, did not affect the $[Ca^{2+}]_{cyt}$ elevation. In cells pretreated with 500 nM bafilomycin A_1 for 60 min, phase 1 was markedly stimulated, but phase 2 was not affected (Fig. 3, bottom). The effect of bafilomycin A_1 on phase 1 was also concentration-dependent. These results show that the sensitivity to inhibitors of phases 1 and 2 is different. The viability of the cells treated with K-252a (1 μM) or bafilomycin A_1 (500 nM) was determined as the percentage of cells that accumulated fluorescein (97.2 and 97.1%, respectively) and as the percentage of plasmolyzed cells (100% each). Both examinations showed that the treatment of cells with K-252a or bafilomycin A_1 did not affect cell viability.

Either $LaCl_3$ (3 mM) or $GdCl_3$ (3 mM) markedly suppressed both phase 1 and 2 $[Ca^{2+}]_{cyt}$ elevations (data not shown), suggesting that these $[Ca^{2+}]_{cyt}$ elevations may result from an influx of Ca^{2+} into the cytosol through the plasma membrane. However, these two channel blockers may have potential side effects on $[Ca^{2+}]_{cyt}$, because in long-term incubations they seemed to induce cell aggregation. Also, ruthenium red (20 μM) seemed to cause some quenching of aequorin luminescence, and consequently, we have not further examined its effects on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation.

Effect of Extracellular Ca^{2+} Concentration on Hypoosmotic Shock-Induced $[Ca^{2+}]_{cyt}$ Elevation

To ascertain the involvement of extracellular Ca^{2+} in the hypoosmotic $[Ca^{2+}]_{cyt}$ response, we altered the extracellular Ca^{2+} concentration and examined its effect on the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The ordinary culture medium that we used contained 3 mM Ca^{2+}. The transgenic cells containing reconstituted aequorin were washed with and transferred to culture medium depleted of $CaCl_2$ (contaminating free Ca^{2+} concentration, 0.025 mM) and incubated for 30 min. To this suspension 3 mM $CaCl_2$, 5 mM EGTA, or 10 mM mannitol was added. After 1 min the cells were hypoosmotically shocked and the $[Ca^{2+}]_{cyt}$ elevation was monitored (Fig. 4). The biphasic $[Ca^{2+}]_{cyt}$ elevation was observed in the presence of 3.0 mM Ca^{2+} (trace A) and this was greatly reduced at 0.025 mM Ca^{2+} (trace B). No $[Ca^{2+}]_{cyt}$ elevation was detected in cells treated with 5 mM EGTA (trace C). These results suggest that the $[Ca^{2+}]_{cyt}$ elevation mediated by hypoosmotic shock may result from an influx of Ca^{2+} into the cytosol through the plasma membrane.

To examine further the effect of external Ca^{2+} on the $[Ca^{2+}]_{cyt}$ elevation, the cell suspension was subjected to hypoosmotic shock in a Ca^{2+}-depleted medium (free Ca^{2+}, 0.025 mM), and then 3 mM $CaCl_2$ was added back to the cell suspension 2 min after the onset of the osmotic shock. An immediate and sharp luminescence emission was observed by the addition of Ca^{2+} to the hypoosmotically shocked cell suspension (Fig. 5A). After the addition of Ca^{2+}, $[Ca^{2+}]_{cyt}$ increased without a lag period and peaked after only a few seconds before rapidly returning to basal levels after 30 to 40 s. When cells depleted of Ca^{2+} were then treated with 3 mM Ca^{2+} in the absence of any previous hypoosmotic shock, only slight changes in luminescence

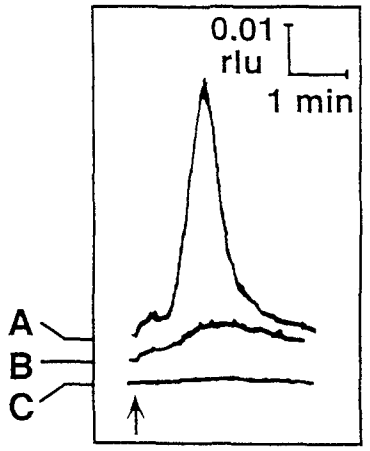

Figure 4. Effect of extracellular Ca^{2+} concentration on hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation. The tobacco cells containing reconstituted aequorin were suspended in a medium depleted of $CaCl_2$. Either 3 mM $CaCl_2$ (A), 10 mM mannitol (B), or 5 mM EGTA (C) was then added to this cell suspension. After 1 additional min (arrow) the cell suspension was diluted with 2 volumes of each of the media depleted of Suc to provide hypoosmotic shock. Free Ca^{2+} concentration in each medium was 3.0 mM (A), 0.025 mM (B), and <0.001 mM (C). These experiments were repeated five times and the traces represented have been chosen to best represent the average result. rlu, Relative light units.

were observed (Fig. 5B). When this experiment was repeated with cells pretreated with K-252a, the $[Ca^{2+}]_{cyt}$ elevation observed was markedly suppressed (Fig. 6C). This K-252a suppression indicated the presence of characteristics of the phase 2 $[Ca^{2+}]_{cyt}$ elevation. In the cells pretreated with bafilomycin A_1 both in the presence and the absence of K-252a, the $[Ca^{2+}]_{cyt}$ elevation was enhanced (Fig. 6, E and G), thereby showing characteristics of phase 1 as well. These results imply that the $[Ca^{2+}]_{cyt}$ elevation shown in Figure 5A, resulting from external Ca^{2+} added back to the medium of cells undergoing hypoosmotic shock, consists of phase 1 and phase 2 merged. Furthermore, this observation would suggest that phase 2 can be induced without phase 1. This transient $[Ca^{2+}]_{cyt}$ elevation could be provoked by the external addition of Ca^{2+} to the cell suspension as late as 20 min after initiation of hypoosmotic shock in the Ca^{2+}-depleted medium (data not shown). This would suggest that the Ca^{2+} channels responsible for hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation can maintain their competence to allow Ca^{2+} influx for at least 20 min, if Ca^{2+} is initially depleted from the medium.

Effect of Osmotic Restoration and K-252a Added during $[Ca^{2+}]_{cyt}$ Elevation in Response to Hypoosmotic Shock

To look at the effect of restoring osmolarity of the medium on $[Ca^{2+}]_{cyt}$, the extracellular osmolarity was returned to the original level (200 mosmol) at various times after the hypoosmotic shock (Fig. 7). When the hypoosmotic condition was canceled 30 s after the shock, i.e. at the time when the phase 1 elevation had already peaked, the peak height of the phase 2 was lowered and $[Ca^{2+}]_{cyt}$ rapidly returned to the original resting level (Fig. 7B). When 60 s were allowed to elapse before cancellation of the shock, no change in the peak height of phase 2 was seen; however, the decrease in $[Ca^{2+}]_{cyt}$ was more rapid (Fig. 7C) than that of the control (Fig. 7A). These results indicate that osmotic restoration in some way either suppresses the activation of the Ca^{2+} channel involved in phase 2 or stimulates Ca^{2+} pumping activity. If K-252a was added after 30 s, instead of osmotic restoration being carried out, the peak luminescence was lowered but the rate of decline of the luminescence intensity was similar to the control (Fig. 7D). The addition of K-252a 60 s after the shock had no effect on the elevation and also did not change the rate of decline (Fig. 7E). These results indicate that K-252a added after the shock can suppress the Ca^{2+} channel activity responsible for phase 2 but has little effect on Ca^{2+} pumping activity.

DISCUSSION

In the present study we have genetically transformed *N. tabacum* suspension cells in culture to investigate the effect of hypoosmotic shock on $[Ca^{2+}]_{cyt}$. Hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation consists of two phases (Fig. 1A), an immediate, small $[Ca^{2+}]_{cyt}$ elevation (phase 1), followed by a rapid and more prolonged elevation (phase 2). Hypoosmotic shock causes water influx due to the osmotic potential of plant cells, and as a result increases

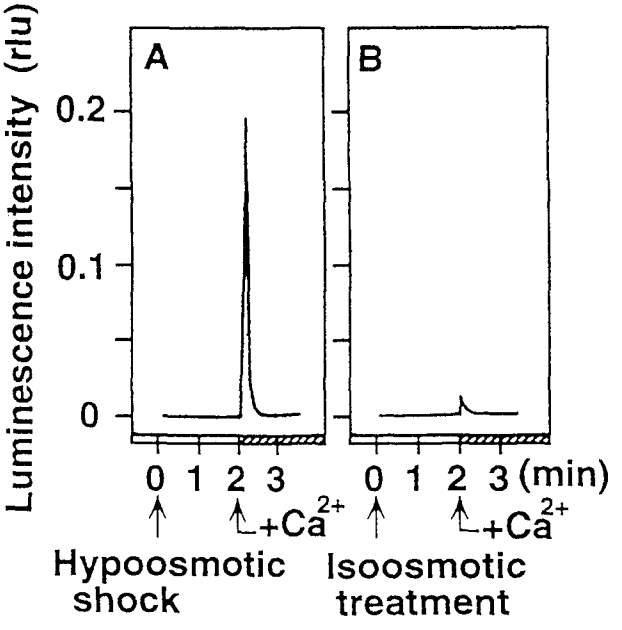

Figure 5. Effect of delay in adding externally added Ca^{2+} on $[Ca^{2+}]_{cyt}$ elevation in cells subjected to hypoosmotic shock. Tobacco cells containing reconstituted aequorin were transferred to the Ca^{2+}-depleted medium for 60 min. A, The cell suspension was subjected to hypoosmotic shock by adding 2 volumes of the Ca^{2+}- and Suc-depleted medium. B, Isoosmotic treatment with the Ca^{2+}-depleted medium. Then, 3 mM $CaCl_2$ was added to the cell suspension 2 min after the shock. These experiments were repeated five times and the traces represented have been chosen to best represent the average result. rlu, Relative light units.

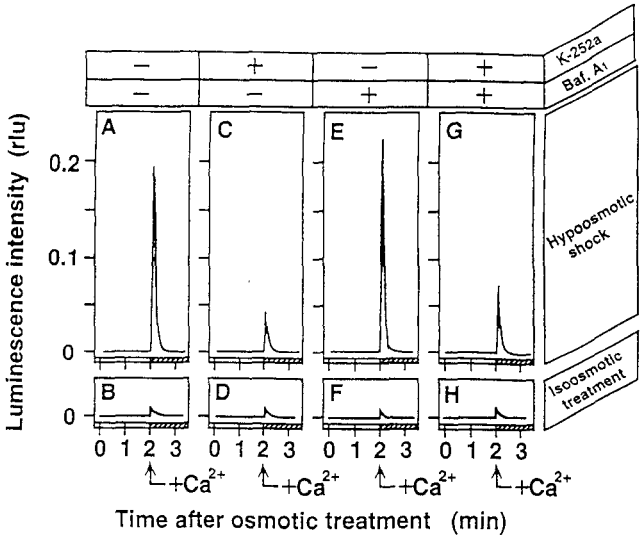

Figure 6. Effect of K-252a and bafilomycin A_1 on $[Ca^{2+}]_{cyt}$ elevation by adding externally added Ca^{2+} to cells subjected to hypoosmotic shock. Tobacco cells containing reconstituted aequorin were transferred to the Ca^{2+}-depleted medium for 60 min. The cell suspension was treated with 1 μM K-252a (C, D, G, and H) or DMSO as a solvent control (A, B, E, and F) for 1 min, or with 500 nM bafilomycin A_1 (Baf. A_1, E, F, G, and H) or DMSO (A, B, C, and D) for 60 min. Then, the cell suspension was subjected to hypoosmotic shock by adding 2 volumes of the Ca^{2+}- and Suc-depleted medium (A, C, E, and G), or isoosmotic treatment with the Ca^{2+}-depleted medium (B, D, F, and H). These experiments were repeated five times and the traces represented have been chosen to best represent the average result. rlu, Relative light units.

turgor pressure, thus causing extension of the plasma membrane. In a brackish water charaphyte *Lamprothamnium succinctum*, increases in turgor pressure were shown to be caused by hypoosmotic shock (Okazaki et al., 1984). Microinjection of aequorin into the cytosol of *L. succinctum* cells was used to demonstrate that hypoosmotic shock induced a transient $[Ca^{2+}]_{cyt}$ elevation (Okazaki et al., 1987) and this elevation was shown to be a prerequisite for turgor regulation of the cells (Okazaki and Tazawa, 1990). However, the change in turgor pressure caused by hypoosmotic shock was not measured in tobacco suspension cells because it is technically difficult to do. Hypoosmotic shock may then result in an increase in the turgor pressure of tobacco suspension cells and thus could act as a mechanical stress. Plant cells are known to be very sensitive to mechanical stress (Trewavas and Knight, 1994). We have previously reported the use of tobacco plants genetically transformed with aequorin to detect instantaneous touch- and wind-induced $[Ca^{2+}]_{cyt}$ transient elevations (Knight et al., 1991, 1992). Touch and wind stimuli cause tension and compression in plant cells because of bending of the tissue. However, the kinetics of hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation are different from those described for touch and wind (Knight et al., 1991, 1992), where the increases in luminescence were observed without obvious lag periods.

The phase 2 $[Ca^{2+}]_{cyt}$ elevation occurred 30 s after imposition of stress and required the continued presence of hypoosmotic stress. This biphasic $[Ca^{2+}]_{cyt}$ elevation observed in the tobacco cells shows features similar to Ca^{2+}-induced Ca^{2+} release in animal cells in which intracellular Ca^{2+} pools are mobilized. However, the results obtained in the present study suggest that elevated $[Ca^{2+}]_{cyt}$ originated instead from extracellular sources. The magnitudes of both the phase 1 and 2 elevations were definitely dependent on the extracellular Ca^{2+} concentration. This again is in contrast to the situation with touch- and wind-induced $[Ca^{2+}]_{cyt}$ elevation in whole tobacco seedlings, epidermal strips and protoplasts (Knight et al., 1992; Haley et al., 1995), and *Bryonia dioica* (Klüsener et al., 1995), where the Ca^{2+} source is thought to be predominantly intracellular. This contrast provides an argument for a separate mechanism for hypoosmotic sensing/signaling. However, in mosses extracellular Ca^{2+} is thought to be involved in the touch-induced $[Ca^{2+}]_{cyt}$ response (Haley et al., 1995), so the situation is not yet clear. With regard to the Ca^{2+}-induced Ca^{2+} release, it is unlikely that phase 1 stimulates the appearance of phase 2, because the two phases occurred independently and phase 1 was not a prerequisite for phase 2 (Fig. 6).

K-252a inhibited $[Ca^{2+}]_{cyt}$ elevation in phase 2 but not in phase 1 (Fig. 3A), suggesting that a protein phosphorylation step is involved in the control of phase 2. The implication of this result is that hypoosmotically induced $[Ca^{2+}]_{cyt}$ elevation involves the activation of either a Ca^{2+}

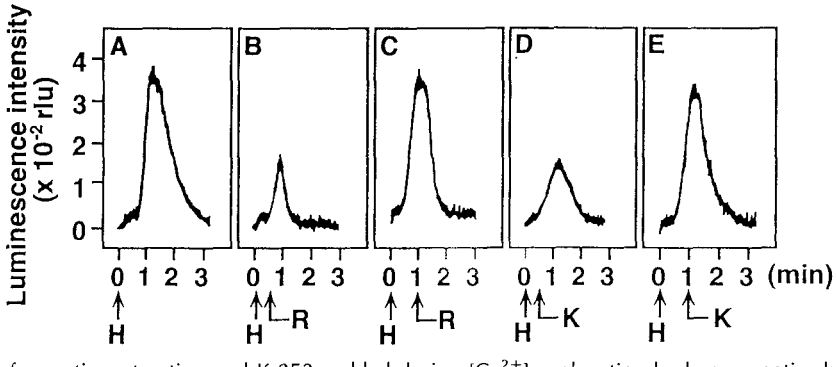

Figure 7. Effect of osmotic restoration and K-252a added during $[Ca^{2+}]_{cyt}$ elevation by hypoosmotic shock. The tobacco cells containing reconstituted aequorin were subjected to hypoosmotic shock by adding 2 volumes of the Suc-depleted medium (A) and then the hypoosmotic condition was canceled by adding 0.275 volume of a medium containing 400 mM mannitol 30 s (B) or 60 s (C) after the shock, or 1 μM K-252a was added 30 s (D) or 60 s (E) after the shock. H, R, and K indicate the time of hypoosmotic shock, osmotic restoration, and addition of K-252a, respectively. These experiments were repeated five times and the traces represented have been chosen to best represent the average result.

channel(s), which requires phosphorylation for activity, or a calcium pump(s), which is inactivated by phosphorylation or associated hypoosmotic sensing component(s).

Bafilomycin A_1, a vacuolar-type H^+-ATPase inhibitor, is thought to negate the H^+ gradient between the vacuole and the cytosol. Consequently, it suppresses Ca^{2+} sequestration into the vacuole, which is facilitated by a Ca^{2+}/H^+ antiporter(s) on the vacuolar membrane. Thus, the presumed effect of bafilomycin A_1 is a reduction of storage Ca^{2+} in the vacuole. If hypoosmotic shock induces intracellular Ca^{2+} release, the cells treated with bafilomycin A_1 might then show a reduced response in $[Ca^{2+}]_{cyt}$ elevation. However, the inhibitor stimulated phase 1 $[Ca^{2+}]_{cyt}$ elevation (Fig. 3B). This indicates that the effect does not result from the presumed mechanism, and therefore the stimulatory effect of bafilomycin A_1 on phase 1 $[Ca^{2+}]_{cyt}$ remains unknown.

When Ca^{2+} was added to the cell suspension subjected to hypoosmotic shock in the low-Ca^{2+} medium, the K-252a-sensitive (phase 2) and -insensitive (phase 1) $[Ca^{2+}]_{cyt}$ elevations were observed immediately without a lag period (Fig. 6). This suggests that the Ca^{2+} channel(s)/pump(s) or associated hypoosmotic sensing component(s) involved in phase 2 are controlled through protein phosphorylation without Ca^{2+} elevation and that the phase 1 $[Ca^{2+}]_{cyt}$ elevation is not required for the activation of phase 2.

None of the inhibitors of voltage-dependent Ca^{2+} channels had any effect on the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation, suggesting that these types of Ca^{2+} channels are not a component of this system. Mechanical stimuli probably induce physical changes in the plasma membrane and it has been suggested that these signals are perceived by mechanosensory Ca^{2+}-sensitive channels (Pickard and Ding, 1993). Although the sources of $[Ca^{2+}]_{cyt}$ elevated by wind and touch are likely to be intracellular (Knight et al., 1992; Haley et al., 1995; Klüsener et al., 1995), the data presented here strongly suggest that hypoosmotic shock induced Ca^{2+} influx through the plasma membrane. Mechanosensory Ca^{2+} permeable channels on the plasma membrane have been reported in tobacco protoplasts (Falke et al., 1988), guard cells of *Vicia faba* (Cosgrove and Hedrich, 1991), and epidermal cells of sweet red onion (Ding and Pickard, 1993). The Ca^{2+} channels involved in phases 1 and 2 may then be of this type, with the possibility of Ca^{2+}-independent phosphorylation-sensitive channel(s) in phase 2. It is also possible that the associated hypoosmotic sensing system is regulated by protein phosphorylation.

The physiological role of $[Ca^{2+}]_{cyt}$ increased by hypoosmotic shock is unclear. However, elevated $[Ca^{2+}]_{cyt}$ has been reported to result in a decrease in cell volume (Pierce and Politis, 1990) and turgor pressure (Okazaki et al., 1987; Okazaki and Tazawa, 1990) by activating outward-rectifying ion channels. This is also the case for guard cell closure (Gilroy et al., 1990). The implication is that the hypoosmotic shock-induced $[Ca^{2+}]_{cyt}$ elevation we have observed in tobacco subsequently induces hypoosmotic regulation, leading to a reduction in cell volume and turgor pressure. However, in wheat leaf protoplasts $[Ca^{2+}]_{cyt}$ elevation can cause increases in cell volume presumably resulting from increased turgor (Shacklock et al., 1992). Specific cell types, therefore, may interpret the same basic $[Ca^{2+}]_{cyt}$ signal in entirely different ways. The implication is that differentiation and development provide specific aspects to the interpretation of signals—that $[Ca^{2+}]_{cyt}$ alone cannot specify these responses and some form of signal discrimination must occur. The challenge now will be to identify how this discrimination occurs and to identify the signaling pathways and components other than $[Ca^{2+}]_{cyt}$ that are involved specifically in plant responses to hypoosmotic shock. Our unpublished observation that hypoosmotic shock induces activation of protein kinases following $[Ca^{2+}]_{cyt}$ elevation and that the activation is regulated by cytosolic Ca^{2+} may help explain some aspects of the specificity of the hypoosmotic shock-signaling pathway.

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