Yeast Respond to Hypotonic Shock with a Calcium Pulse*

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We have used the transgenic AEOQURIN calcium reporter system to monitor the cytosolic calcium ([Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\)) response of Saccharomyces cerevisiae to hypotonic shock. Such a shock generates an almost immediate and transient rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\) which is eliminated by gadolinium, a blocker of stretch-activated channels. In addition, this transient rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\) is initially insensitive to 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), an extracellular calcium chelator. However, BAPTA abruptly attenuates the maintenance of that transient rise. These data show that hypotonic shock generates a stretch-activated channel-dependent calcium pulse in yeast. They also suggest that the immediate calcium influx is primarily generated from intracellular stores, and that a sustained increase in [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\) depends upon extracellular calcium.

Eukaryotic cells respond to a hypotonic shock with ion or organic solute fluxes in order to restore proper volume and osmotic pressure relationships (1, 2). Aspects of the regulatory volume decrease responses to hypotonic shock including ion flux and organic solute fluxes in order to restore proper volume and osmotic pressure. Changes in cytoplasmic Ca\(^{2+}\) levels in yeast—

pertonic shock stimulates the HOG pathway in yeast (17, 18), hypotonic shock stimulates the PKC1 signal transduction cascade (16, 19). Components of both MAP kinase pathways are functionally conserved in mammalian cells (20–24).

Despite our understanding of the signal transduction pathway involved in transducing an hypotonic shock in yeast and mammalian cells, little is known about the mechanisms involved in the activation of that pathway. We have used transgenic AEOQURIN as an in vivo luminescent cytosolic calcium reporter system (11, 25–27) to investigate hypotonicity-induced changes in cytoplasmic Ca\(^{2+}\) levels in the yeast S. cerevisiae. We discuss our results in view of the possible involvement of Ca\(^{2+}\) as a second messenger in that response.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Transformation, and Culture Conditions—**A2wt20.1 was derived from the S. cerevisiae YPH252 strain (MATa, ura3-52, lys2-801, ade2-101, trpl-1, his3-200, leu2-3,112) (28) by transformation of a 2 μ-type recombinant pEVP11-based, 30 plasmid (pEVP11/AEQ) carrying the APOEQUORIN gene from pMAQ2 (28) under the control of the ADH1 promoter and NOS (nopaline synthase) terminator sequences.

For the luminescence assays shown below, A2wt20.1 or untransformed YPH252 cells were grown in CM-L (pH 4.2) dropout medium (29) at 30 °C to an A\(_{600}\) of 0.92–1.15. These precultures were diluted 1:100, and grown to A\(_{600}\) = 0.92–1.15 before being assayed as described below.

**Measurement of Cytosolic Ca\(^{2+}\): Changes in Response to Hypotonicity—**In order to assess the calcium response of freshly grown cells to hypotonic shock, we analyzed the luminescence of A2wt20.1 or YPH252 cells before, during, and after hypotonic stimulation.

Briefly, 25 or 30 μl of culture (see below) were aliquoted into luminometer tubes at room temperature. Typically, 20 min (min) after removal from the growth chamber, 0.1 volume of 590 μM coelenterazine (C-2944, Molecular Probes) in absolute methanol (Mallinkrodt) was added to the first sample, which was then incubated at room temperature for an additional 20 min in order to reconstitute functional AEOQURIN within the cells. After incubation, the base-line luminescence was recorded every 30 s for about 5 s, using a Monolight 1500 lumimeter (Analytical Luminescence), and reported in relative luminescence units/s (RLU/s). Then the osmotic shock was administered by quickly bathing the sample in 100 μl of the shock solution (described in the figure legends and in the text) by means of a mechanical injector (Analytical Luminescence, Sorente Valley, CA). Luminescence was measured for more than 2 min after the stimulus, as described above. The data were aligned so that the injection point corresponded to the 5-s point on each figure. Samples were treated sequentially, maintaining a constant time of incubation in coelenterazine before the hypotonic shock.

To test the effect of gadolinium (gadolinium(III) chloride hexahydrate, Aldrich) on the response of freshly grown cells to hypotonic shock, 30 μl of cultured cells were aliquoted into luminometer tubes and maintained at room temperature. Seventeen min after addition of 3 μl of the coelenterazine solution, 3.7 μl of a preaddition aqueous gadolinium solution (pH 4.2 ± 0.1) containing 10 times the final GdCl\(_3\) concentration (see below) was added to that sample, and the treated cells were incubated at room temperature for an additional 3 min. The sample was then shocked with an hypotonic aqueous solution containing the final GdCl\(_3\) concentration (pH 4.2 ± 0.1), and its luminescence was monitored (see below). Subsequent samples were treated sequentially. For all gadolinium concentrations tested, equivalent control sam-

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‡‡‡The abbreviations used are: [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\), cytosolic Ca\(^{2+}\) concentration; BAPTA, 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; MAP, mitogen-activated protein kinase; MeOH, methanol; PKC, protein kinase C; RLU, relative luminescence unit; SAC, stretch-activated channel; TS, Tris-succinate buffer.
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To test the effect of the calcium chelator BAPTA (tetrakis(dimethylamino)ethylenediamine disuccinate) on freshly grown cells, cultured cells were centrifuged for 45 s at 14,000 rpm in a 5415C Eppendorf microcentrifuge at room temperature and resuspended in a 50 mM Tri-succinate buffer (pH 5.9) containing 100 mM glucose (pH 5.9 ± 0.1) to provide a more optimal pH for calcium determination by BAPTA. Then, cells were aliquoted in 25-µl samples into luminometer tubes, and each aliquot was treated with coelenterazine as described above. A twice-concentrated (due to solubility constraints) BAPTA preaddition solution in TS + glucose (pH 5.9 ± 0.1) was added 3 min before shocking the cells with 100 µl of an hypotonic aqueous 1 × BAPTA solution (pH 5.9 ± 0.1). Luminescence was recorded as described above. For all BAPTA concentrations tested, BAPTA-free control samples were treated with normotonic and pH-equivalent preaddition and hypotonic shock solutions, made with either a concentration or dilution of the solution the cells were in. All inhibitor solutions were made in the cold as described above.

For experiments involving cold-pretreated cells, freshly grown yeast were placed in the cold (2–5 °C) for 6 d, 3 h, soaked in a cold 54 µM coelenterazine solution containing 9% methanol for 6 h, centrifuged for 45 s at 14,000 rpm (model 5414 Eppendorf microcentrifuge), and resuspended in cold TS + glucose or CM-L for a minimum of 16.5 h, as described in the text. For experiments testing the effect of a cold pretreatment, freshly grown yeast were placed in the cold for 0–6 d, 3 h, while control cells were maintained at room temperature for 0–6 d and then added in the cold to partially equilibrate coelenterazine uptake. Each sample was then treated with coelenterazine and resuspended in the cold as described above.

At the end of each pretreatment, the samples were aliquoted on ice, sequentially removed from the ice, and incubated at room temperature for 15 min to allow their temperature to equilibrate to that of the room as determined by a thermocouple analysis. They were then sequentially shocked, and their luminescence was recorded as described above.

To determine the effect of gadolinium and BAPTA on the luminescence response to hypotonic shock of cold-pretreated cells, concentrated inhibitor solutions (a 10 times concentrated GdCl₃ solution in water, or a twice-concentrated BAPTA solution in TS + glucose) were added 3 min before the assay to 30- and 25-µl samples of cells resuspended in CM-L or TS + glucose, respectively. The hypotonic shock was performed by diluting the sample with a 1 × solution of the inhibitor (see “Results”). Controls were treated in the same way, using inhibitor-free normotonic preaddition and osmotic shock solutions made with either a concentrate or dilution of the solution the cells were in. All inhibitor and normotonic solutions were pH-adjusted as described above.

In all experiments described in this report, the osmolarities of each preaddition and shock solution were determined with a Wescor Osmometer.

Determination of Cell Viability—To analyze the effect of the tested inhibitors and of the various treatments on cell viability, A2wt20.1 and YPH252 cells were subjected to the treatment regimens described above and plated on CM-L and CM media, respectively. After 3 days of growth at 30 °C, the number of colonies was determined and reported in colony-forming units.

Determination of Plasmid Stability—For some treatments (see below), the percentage of A2wt20.1 cells retaining the plasmid after the treatment was determined by plating the cells on nonselective CM and selective CM-L media, and comparing the number of colony-forming units on each medium.

Determination of the Amount of Reconstituted AEQUORIN—To estimate the total amount of reconstituted AEQUORIN present in yeast cells, total soluble protein was extracted according to a published protocol (27), slightly modified. Briefly, 500 µl of yeast culture (approximately 1 × 10⁷ cells) were centrifuged for 45 s at 14,000 rpm, and the cells were resuspended in 200 µl of a lysis buffer containing 30 mM Tris-HCl (7.6), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, and a protease inhibitor mixture including 2 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, and 0.1 mM benzamidine. The yeast were disrupted by vortexing five times for 30 s each in the presence of glass beads, at 5 °C. Then, 200 µl of cold lysis buffer were added, and the cells were spun at 14,000 rpm for 10 min at 5 °C. The supernatant served as the extract for both protein determination (Bio-Rad Bradford protein microassay) and for luminescence assays.

The total amount of reconstituted AEQUORIN present in cell extracts from A2wt20.1 (minus background luminescence of untrans- 
formed A2wt20.1) was derived by calculating the L₉₀₅₇ / L₀₉₅₇ parameter (25, 31), which was used to estimate the [Ca²⁺]ᵢₑₓₗ prior to (base line) and the peak of the luminescence response to hypotonic shock, using a conversion curve generated by Allen et al. (31).

Specifically for each sample, 10-fold dilutions of extract (from 0.01 to 10 µl in a total extraction buffer volume of 200 µl) were injected with 100 µl of 1 M CaCl₂, 10 mM Tris-HCl (7.6) to saturate AEQUORIN luminescence. Each luminescence over a 115-s period was normalized to a 30-µl cell sample equivalent, taking into account the efficiency of the extraction as determined by the Bio-Rad protein microassay. This integrated value was determined in order to formulate a hypothetical decay curve (25). L₉₀₅₇ is the peak luminescence of an average 30-µl sample equivalent of extract assuming the AEQUORIN decay constant of 9.8 s⁻¹ (25).

Both the average in vivo base-line and peak luminescence responses to an hypotonic shock of 100 µl of double distilled H₂O were determined for 30-µl cell samples from the same cultures as those used to generate extracts. The ratios of these values with regard to L₉₀₅₇ were applied to the curve generated by Allen et al. (31) to estimate the corresponding base-line and peak calcium concentrations.

RESULTS

Yeast Respond to Hypotonic Shock with a Calcium Pulse—In order to determine the effect of hypotonic shock upon [Ca²⁺]ᵢₑₓₗ, we analyzed the luminescence response of freshly grown, APOAEQUORIN-expressing yeast cells to hypotonic treatment. The results shown in Fig. 1A indicate that these cells respond to hypotonic shock by a transient rise in cytoplasmic calcium levels. A similar response was found when A2wt20.1 cells were resuspended in TS + glucose before being assayed.

In contrast, cells treated with an isotonic shock yielded a flat luminescence response (Fig. 1A), indicating no change. It was also found that exposure to the cold over a period of 1–7 days before the hypotonic challenge resulted in an increase in both the base-line (Fig. 1B, blue line) and peak (Fig. 1C, blue line) luminescence responses, allowing for an analysis of both increases and decreases (also see below) in luminescence. These luminescence responses displayed a high level of reproducibility within one assay, as shown by the low S.D.s (Figs. 1, B and C). On the other hand, the base-line and peak luminescence of control cells maintained at room temperature over the same period of time did not increase over time (Figs. 1, B and C, red lines) indicating that the cold pretreatment was indeed responsible for the luminescence increases.

To eliminate the possibility that the increased luminescence of cold pretreated yeast cells was artifactual, we determined the effect of a 7-day cold pretreatment on cell viability and on pEVP11/AEQ plasmid maintenance. Results showed that a cold pretreatment does not decrease the number of viable cells present (3.07 ± 0.17 × 10⁷ and 3.47 ± 0.06 × 10⁷ viable cells per ml for freshly grown and cold-pretreated samples, respectively), the percentage of viable cells that were able to exclude methylene blue when placed in a 0.001% methylene blue solution (96 and 97% of the cells in freshly grown and cold-pretreated samples, respectively), or the percentage of viable cells retaining the pEVP11/AEQ plasmid (86 and 89% of the freshly grown and cold-pretreated cells, respectively).

To analyze the effect of cold pretreatment on the cytosolic Ca²⁺ levels at base-line and peak luminescence response to hypotonic shock, we quantified the amount of reconstituted AEQUORIN present in samples from fresh cultures and from cells kept in the cold for 1 or 7 days and used the data to determine the corresponding cytosolic Ca²⁺ concentrations. Results indicated an increase in the amount of reconstituted AEQUORIN present in cells treated with coelenterazine after increased periods of cold pretreatment (average L₉₀₅₇ values of 6.7 × 10⁷, 1.9 × 10⁷ and 5.1 × 10⁷ RLUs after 0, 1, and 7 days in the cold, respectively). Furthermore, while no major differences in [Ca²⁺]ᵢₑₓₗ were detected for cells maintained in the cold

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Fig. 1. A, luminescence of freshly grown APOAEQUORIN-transformed A2wt20.1 cells subjected to a hypotonic or an isotonic shock. Each assay was done in triplicate, and the response of each assay is shown in a different color. The hypotonic shock (blue, light blue, and green) was provided after 5 s of recording by injecting 50 μl of cell suspension in CM-L + 9% MeOH medium (osmolality of CM-L = 637 mmol/kg) 100 μl of a 6.7% CM-L solution (pH 4.2 ± 0.1; osmolality = 417 mmol/kg), while the isotonic shock (pink, red, and brown) was provided by injecting 100 μl of an isoosmolar CM-L solution containing 9% MeOH. Overlapping traces obscure replicate samples of each type. B and C, average initial base-line (B) and peak luminescence response (C) to a 100 μl of double distilled H2O hypotonic shock of A2wt20.1 cells in TS + glucose maintained in the cold (blue) or at room temperature (red) for a period of 1–7 days (x axis) (n = 3). The S.D. is shown by horizontal bars at each time point.

Fig. 2. Luminescence response to hypotonic shocks of various intensities of cold-pretreated A2wt20.1 cells resuspended in TS + glucose (pink), TS + sorbitol (green), TS + galactose (light blue), or TS + 2-deoxy-D-glucose (black) treatment. All resuspension solutions contained 50 mm TS and 100 mM carbohydrate, were pH 5.9 ± 0.1, and their osmolalities were 174 ± 4 mmol/kg. Each sample was shocked with the following pH-adjusted dilutions of its resuspension solution: A, 6.7%; B, 33%; C, 67%; or D, 100%. Duplicate samples were subjected to each treatment, and are represented by solid and dotted lines, respectively. E, luminescence response ratio of A2wt20.1 cells resuspended in the various solutions described above and treated with dilutions of the same resuspension medium, as a function of the osmolality of the shocking solution.

for 0 or 1 day (base-line cytosolic Ca2+ concentrations of 195 nM and 150 nM, respectively, and peak Ca2+ levels of 1100 and 1200 nM, respectively), the base-line and peak Ca2+ concentrations increased to 400 and 2500 nM, respectively, after 7 days in the cold.

The [Ca2+]cyt Increase Is Primarily a Response to Hypotonicity but Can Be Altered by the Type of Resuspension and/or Shock Medium—In order to confirm that yeast cells are responding to the hypotonicity of the shock and to eliminate the possibility that the [Ca2+]cyt response to a hypotonic shock is triggered merely by a dilution of glucose, cold-pretreated cells were resuspended in equiosmolar and pH-equivalent succinate buffers containing a variety of carbohydrates (either glucose, galactose, sorbitol, or the nonmetabolizable 2-deoxy-D-glucose) after the coelenterazine treatment, and the base-line and peak luminescence responses to a range of hypotonic shocks were monitored. Fig. 2 shows that the peak luminescence response to hypotonic shock of cold-pretreated cells decreased as the shock solution approached isotonicity. Although the luminescence response ratios (peak RLUs/base-line RLUs) were strikingly similar for cells in TS + glucose, in TS + galactose, and in TS + sorbitol over a range of hypotonic shocks (Fig. 2E), cells in TS + 2-deoxy-D-glucose (TS + 2D) developed a high luminescence response ratio of 56 after a 6.7% hypotonic shock and a high luminescence level was maintained after this peak (Fig. 2E).

Fig. 2 also shows that the initial base-line [Ca2+]cyt was lower for cells maintained in a buffer containing a carbohydrate other than glucose. These differences in base-line levels were not due to changes in cell viability induced by the type of resuspension medium, for the samples contained between 2.74 ± 0.13 × 10^6 and 3.05 ± 0.23 × 10^5 viable cells/ml.

The [Ca2+]cyt Increase in Response to Hypotonic Shock is Inhibited by the Stretch-activated Channel Blocker, Gadolinium—In order to determine if stretch-activated channels are involved in the [Ca2+]cyt response to hypotonic shock, we compared the luminescence responses of gadolinium-pretreated cells (13) with the responses of control cells subjected to the same osmotic stimuli in the absence of gadolinium. Fig. 3 demonstrates that 1 mM gadolinium reduces the luminescence response of freshly grown cells to an hypotonic shock (Fig. 3A), while 10 mM gadolinium completely inhibits that response (Fig. 3B). Interestingly, the response to an hypotonic shock was inhibited by the presence of 10 mM gadolinium in the shock solution independently of whether gadolinium had been preadded to the cells or not.5 The short increase in luminescence
found immediately after the hypotonic shock in both gadolinium-treated and control cells (Fig. 3, A and B) was artifactual, for it was also observed when untransformed YPH252 was gadolinium treated (Fig. 3A).

To verify that the mechanisms responsible for the luminescence response to hypotonic shock of cold-pretreated cells are similar to those of freshly grown cells, we also determined the effect of gadolinium on the luminescence response of cold-pretreated cells. Fig. 3, C and D, shows that the luminescence response of cold-pretreated cells is partially eliminated in the presence of 1 mM gadolinium, and it is completely eliminated in the presence of 10 mM gadolinium. 5 mM gadolinium also completely eliminated the luminescence response, while 0.1 mM gadolinium had almost no effect on the peak response. Viability assays showed that the lack of response of cold-pretreated A2wt20.1 cells exposed to a 10 mM gadolinium solution could not be attributed to a decrease in viability (2.43 ± 0.29 × 10^7 viable cells/ml for control samples, and 2.32 ± 0.26 × 10^7 viable cells/ml for 10 mM GdCl₃-pretreated samples). Finally, the luminescence of gadolinium-treated, cold-pretreated, vectorless cells was similar to that of freshly grown vectorless cells, and was not altered by gadolinium (Fig. 3, A and D).

The [Ca^{2+}]_i Increase in Response to Hypotonic Shock Is Modulated by the Calcium Chelator, BAPTA—To determine if the calcium increase seen upon hypotonic shock was due to the influx of extracellular calcium or to the release of calcium from internal stores, we compared the luminescence response to hypotonicity of BAPTA-treated A2wt20.1 cells with the response of control A2wt20.1 cells treated with BAPTA-less normotonic and pH-equivalent solutions. Fig. 4, A and B, indicates that both 1 and 5 mM BAPTA treatments abruptly attenuate the luminescence response of freshly grown cells, without affecting the kinetics of the initial luminescence rise. A similar 5 mM BAPTA treatment had no effect on the luminescence response to hypotonic shock in the presence of excess calcium. As expected, the background luminescence of untransformed YPH252 control cells was not affected by the presence (Fig. 4A) or absence (Fig. 4B) of BAPTA. Additionally, these treatments did not significantly affect the viability of A2wt20.1 cells (2.67 ± 0.19 × 10^7 and 2.69 ± 0.11 × 10^7 viable cells/ml for 5 mM BAPTA-treated and control A2wt20.1 samples, respectively).

The effect of BAPTA pretreatments on the luminescence response of cold-pretreated cells to hypotonic shocks (Fig. 4, C and D) mimicked that of freshly grown cells (Fig. 4, A and B) in that the presence of BAPTA did not affect the kinetics of the initial hypotonic shock-induced luminescence rise. However, the kinetics of the subsequent calcium decrease relative to the normotonic and pH-equivalent controls was altered for cold-pretreated cells. Additionally for these cells, all BAPTA pretreatments reduced the initial base-line luminescence relative
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to the controls, an effect which would be difficult to detect on freshly grown cells, given their low luminescence levels (Fig. 4). Finally, the luminescence of untransformed YPH252 cells was not modified by BAPTA treatment (compare Fig. 4, C and D); and none of these BAPTA treatments affected the viability of A2wt×201 samples (2.37 ± 0.21 × 105 and 2.11 ± 0.22 × 105 viable cells/ml for untreated control and 5 mM BAPTA-treated samples, respectively).

DISCUSSION

Using the transgenic AEQUORIN calcium reporter system in S. cerevisiae, we have shown that hypotonic shock promotes a transient increase in cytosolic Ca2+ levels from a base line of 195 nM to a peak [Ca2+]cyt of 1100 nM. We have also shown that a 7-day cold pretreatment results in a dramatic increase in base-line and peak luminescence response to hypotonicity, reflecting a doubling of both base-line and peak cytosolic calcium concentrations. In both cases the response ratios of 5.6 for freshly grown cells and 6.3 for cells after 7 days in the cold are quite similar. In contrast, an isostonic shock of either freshly grown (Fig. 1A) or cold pretreated cells (Fig. 2D) results in essentially no change in luminescence.

Using cold-pretreated cells we have demonstrated that the intensity of the shock directly determines the peak calcium response (Fig. 2). These results parallel the hypotonic induction of phosphorylation of the PKC1 pathway MAP kinase Mpk1p (Ref. 19, and see also below).

Cells resuspended in media containing a carbohydrate different from glucose showed a reduced base-line luminescence and demonstrated a lag in their luminescence response to an hypotonic shock (Fig. 2A–D). However, with one exception, the response ratios of cells maintained in these media were strikingly similar to those of cells maintained in TS+glucose (Fig. 2E). For cells resuspended in a medium containing 2-deoxy-d-glucose, a nonmetabolizable analogue of glucose, the response ratio was dramatically increased and the cells were less able to bring the luminescence back to base-line levels after stimulation, suggesting that these cells are unable to efficiently pump calcium out of their cytoplasm, or to regulate the entry of calcium into their cytosol, or both. Therefore, the calcium response to an hypotonic shock can be altered by the physiological state of the cells.

10 mM gadolinium completely eliminates the hypotonic shock response of both freshly grown and cold-pretreated cells, suggesting that the calcium rise upon hypotonic shock is dependent upon SACs (Fig. 3). At lower concentrations gadolinium decreases the kinetics of the hypotonically induced calcium flux in a concentration-dependent fashion without affecting the time of the maximum increase2 (Fig. 3). This hypothesis can be responsible for this inhibition. If SACs mediate the perception of an hypotonic shock with various ion fluxes in yeast as is suggested for eukaryotes (7, 12, 14, 32, 33), gadolinium could inhibit the complete physiological response to an hypotonic shock by inhibiting both cation and anion flux through SACs (13). On the other hand, gadolinium could also inhibit the entrance of a “trigger” amount of calcium (13, 34), which when present in the cell, might activate the release of calcium from internal stores, initiating a signal transduction cascade. As discussed above, hypotonically induced membrane stress precipitating calcium depletion from intracellular stores has been shown to capacitate the influx of extracellular calcium in mammalian cells (6). One or both mechanisms would be expected to affect interevents in the response to hypotonic shock.

The inhibitory effect of GdCl3 cannot be attributed to a nonspecific inhibitory effect of chloride ions on the luminescence response, since treatment with osmotic- and pH-equivalent 10 mM MgCl2 generates an hypotonic shock response which is indistinguishable from that of a normotonic and pH-equivalent control lacking MgCl2 or GdCl3. Additionally, because gadolinium has been shown to activate in vitro AEQUORIN luminescence as efficiently as Ca2+ (while Mg2+ inhibits it (25, 35)), the luminescence response must be intracellular, and the gadolinium effects on that response must derive from an inactivation of the SACs, rather than from its penetrateing the cells.

Very low Gd3+ concentrations (10 μM) were sufficient to block the opening of SACs in patch-clamped yeast protoplasts and Xenopus oocytes (13, 36). The lower sensitivity to gadolinium reflected in our data (Fig. 3, B and D) may simply result from the fact that we have been using yeast cells with their cell wall intact, rather than patch-clamped spheroplasts.

BAPTA, an extracellular Ca2+ chelator, affects later stages of the luminescence response of yeast cells to the hypotonic treatments described here, without affecting the kinetics of the initial rise (Fig. 4). This result implies that the calcium influx upon hypotonic shock is initially derived from intracellular stores, or from an extraplasmamembranous source which is inaccessible to BAPTA. It also confirms that the luminescence response to an hypotonic shock reports the level of intracellular calcium, rather than reporting AEQUORIN secretion upon hypotonic shock. Because BAPTA has no effect on the luminescence response to hypotonic shock in the presence of excess calcium (see above), it seems likely that the BAPTA effect derives from extracellular calcium chelation, although we cannot completely eliminate the possibility that at least some of that effect derives from the chelation of some other ion(s).

Although we cannot completely eliminate the possibility that some trace extracellular calcium remains unchelated in the presence of 5 mM BAPTA, perhaps protected by the cell wall, and is able to enter the cell and stimulate the release from intracellular stores (34), the abrupt alteration of the luminescence rise in the presence of BAPTA suggests that extracellular calcium is involved in the maintenance of a transiently high level of [Ca2+]cyt after the initial influx from intracellular stores. This hypothesis is consistent with the fact that the luminescence response to hypotonic shock of control A2wt×201 cells resuspended in TS + glucose is clearly biphasic when the cells are stimulated early after being removed from the incubator. Under these conditions, BAPTA eliminates only the second peak of the response (Fig. 4, A and B). The role of extracellular calcium in less pronounced in the sustained calcium increase of cold-pretreated cells (Fig. 4, C and D).

The pattern of luminescence response of freshly grown cells to an hypotonic shock in the presence of BAPTA, mimics, albeit on a larger scale, the effect of preaddition of a calcium chelator upon the cytosolic Ca2+ rise in hypotonically stimulated mammalian cells (9) and is consistent with the findings of Oike et al. (6) discussed above.

In conclusion, the data presented in this study suggest that hypotonic shock induces a transient rise in cytosolic Ca2+ levels in yeast, mediated initially by an opening of stretch-activated channels whose gating promotes the release of calcium from intracellular stores. A continued [Ca2+]cyt increase is dependent upon extracellular calcium.

Hypotonic stimuli activate the PKC1 MAP kinase pathway in S. cerevisiae (16, 19). Interestingly, the shock intensity positively affects phosphorylation of the Mpk1p MAP kinase in the PKC1 pathway (19) and the amplitude of the peak [Ca2+]cyt response demonstrated here (Fig. 2). In addition, mutants in the PKC1 pathway in S. cerevisiae have either an absolute (37, 38) or temperature-sensitive (ts) (37–41) lysis phenotype. Our data suggest a possible role for an initial transient calcium influx in the activation of that pathway. Although the in vitro
Pkc1p activity is calcium-independent (42), the pckΔ phenotype is suppressed by growth in high levels of calcium (37), and the yeast Pkc1p is related to the Ca\(^{2+}\)-activated family of PKC isoforms (42, 43).

This work has allowed the characterization of a temporal cytosolic response to hypotonic shock in *S. cerevisiae*. The molecular and genetic analysis of coelenterazine-treated, AEQUORIN-expressing yeast mutants\(^2\) will allow us to better understand the mechanisms regulating the [Ca\(^{2+}\)]\(_{cyt}\) response to hypotonic shock as well as the role of [Ca\(^{2+}\)]\(_{cyt}\) in the response of yeast cells to hypotonicity.

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REFERENCES

1. Hoffmann, E. K., and Simonsen, L. O. (1989) Physiol. Rev. 69, 315–382
2. Pierce, S. K., and Politis, A. D. (1990) J. Biol. Chem. 265, 2940–2944
3. Rothstein, A., and Mack, E. (1990) Mol. Cell. Biol. 10, 3967–3971
4. Nakajima-Shimada, J., Iida, H., Tsuji, F. I., and Anraku, Y. (1991) J. Biol. Chem. 266, 1760–1763
5. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) J. Biol. Chem. 268, 19019–19022
6. Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., and Thorner, J. (1996) Cell 86, 621–624
7. Kamada, Y., Jung, U. S., Piotrowski, J., and Levin, D. E. (1995) Genes Dev. 9, 1559–1571
8. Brezinski, L. J., de Valoir, T., Dryer, N. D., Winter, E., and Gustin, M. C. (1993) Science 259, 1760–1763
9. Maeda, T., Takekawa, M., and Saito, H. (1995) Science 269, 554–557
10. Davenport, K. R., Somanath, M., Kamada, Y., Levin, D. E., and Gustin, M. C. (1995) J. Biol. Chem. 270, 30157–30161
11. Tilly B. C., van den Berge, N., Tervooren, L. G. J., Edizhoven, M. J., and de Jonge, H. R. (1994) J. Biol. Chem. 269, 19019–19022
