Use of Recombinant Aequorin to Study Calcium Homeostasis and Monitor Calcium Transients in Response to Heat and Cold Shock in Cyanobacteria[1]

Ignacio Torrecilla, Francisco Leganés, Ildefonso Bonilla, and Francisca Fernández-Piñas*

Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, 28049, Spain

We investigated the possibility of Ca^{2+} signaling in cyanobacteria (blue-green algae) by measuring intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) in a recombinant strain of the nitrogen fixing cyanobacterium *Anabaena* strain sp. PCC7120, which constitutively expresses the Ca^{2+}-binding photoprotein apoaequorin. The homeostasis of intracellular Ca^{2+} in response to increasing external Ca^{2+} has been studied in this strain. The resting level of free Ca^{2+} in *Anabaena* was found to be between 100 and 200 nM. Additions of increasing concentrations of external Ca^{2+} gave a transient burst of $[Ca^{2+}]_i$ followed by a very quick decline, reaching a plateau within seconds that brought the level of $[Ca^{2+}]_i$ back to the resting value. These results indicate that *Anabaena* strain sp. PCC7120 is able to regulate its internal Ca^{2+} levels. We also monitored Ca^{2+} transients in our recombinant strain in response to heat and cold shock. The cell's response to both stresses was dependent on the way they were induced. The use of inhibitors suggests that heat shock mobilizes cytosolic Ca^{2+} from both intracellular and extracellular sources, while the Ca^{2+} source for cold shock signaling is mostly extracellular.

Ca^{2+} is a well-known second messenger in signal transduction of environmental stimuli and hormones in eukaryotic cells (Campbell, 1983). In prokaryotic cells, an equivalent important role for Ca^{2+} has been harder to demonstrate, but is now becoming clearer (Onek and Smith, 1992; Smith, 1995; Norris et al., 1996). However, the role(s) for Ca^{2+} is still not well defined. Dating from the pre-Cambrian era, the cyanobacteria have a long history of adaptation to the earth's environment, and that makes them suitable candidates to study adaptation mechanisms and their regulation. In fact, to survive in extreme or variable environments, cyanobacteria have developed specific regulatory systems (those controlling the differentiation of specialized cells), in addition to more general mechanisms equivalent to those of other prokaryotes or photosynthetic eukaryotes. In this context, Ca^{2+} has attracted the most attention, since it could be implicated in regulatory mechanisms as it is in eukaryotes (in particular, those regulating responses to environmental variables; Smith, 1988, 1995; Tandeau de Marsac and Houmard, 1993; Norris et al., 1996).

To demonstrate a regulatory role for Ca^{2+} in any cell system, it is essential to measure resting intracellular free Ca^{2+} levels as well as those arising in response to stimuli or environmental signals; nevertheless, its accurate quantitation during cellular signaling events has proven very difficult. $^{45}Ca^{2+}$-based methods and especially Ca^{2+}-sensitive fluorescent dyes have been used over the past quite extensively, but not without problems that limit their application. The fluorescent dyes show a general resistance to entry into plant cells (Bush and Jones, 1990), and in bacteria, considerable problems have been encountered with dye loading and autofluorescence (Gangola and Rosen, 1987).

Fortunately, the possibility of transforming animal, plant, and bacterial cells with the Ca^{2+}-binding-sensitive luminescent protein apoaequorin (Knight et al., 1991a, 1991b; Brini et al., 1995) has allowed the quantitation of intracellular Ca^{2+} fluxes accompanying diverse stimuli (Knight et al., 1991a, 1991b; Watkins et al., 1995; Okazaki et al., 1996; Sedbrook et al., 1996; Takahashi et al., 1997; Chandra and Low, 1997; Gong et al., 1998; Volotovski et al., 1998).

The present study was undertaken to investigate whether Ca^{2+} has a regulatory role in cyanobacteria. We report the construction of a recombinant strain of the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120 that constitutively expresses the Ca^{2+}-binding photoprotein apoaequorin. We have used this system to study the homeostasis of intracellular Ca^{2+} levels in this cyanobacterium and to monitor Ca^{2+} transients in response to environmental stresses such as heat and cold shock.

RESULTS

Calibration of the Aequorin Signal

To transform luminescence values into $[Ca^{2+}]_i$ values, we have basically followed the method described by Allen and Blinks (1978), which relies on the relationship between $[Ca^{2+}]_i$ and the ratio L_0/

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* Corresponding author; e-mail Francisca.Pina@uam.es; fax 34–91–3978344.

L_{max}, where L_0, the light intensity at time intervals of 1 s, is obtained by integrating the luminometer output (in millivolts) over these 1-s intervals, and L_{max} is defined as the sum of all L_0 values from that interval to the end of the experiment. As aequorin is being consumed continuously, the value of L_{max} is not constant and decreases steadily during the experiment. The relationship between the ratio (L_0/L_{max}) and $[Ca^{2+}]_i$ has been modeled mathematically (Allen et al., 1976). The model (model B) is based on the assumptions that each of the sites to which Ca^{2+} binds has two states, T and R, which are in equilibrium, and that light is emitted by the molecule only when all sites are in the R state.

The model contains three parameters: K_R, the equilibrium association constant; $K_{TR} = [T]/[R]$; and n, the number of Ca^{2+}-binding sites of the molecule. Figure 1 shows the calibration curves obtained at different temperatures (44°C, 28°C, and 10°C) of recombinant aequorin from cell lysates of *Anabaena* strain sp. PCC7120 calculated according to this model. Experimental data were obtained by mixing a solution containing the recombinant aequorin with solutions containing different $[Ca^{2+}]$ that give defined pCa values in the final solutions, as described in "Materials and Methods." The data plotted in Figure 1 were used to fit a theoretical curve based on the model mentioned above, obtaining the best values for parameters K_R, K_{TR}, and n. Fitting was made using a computer routine designed to use the Marquardt-Levenberg algorithm (Marquardt, 1963). The values obtained in this way for the control curve at 28°C ($K_R = 7 \times 10^6$ M^{-1}; $K_{TR} = 118$; and $n = 3$) for the fitting of the $[Ca^{2+}]/(L_0/L_{max})$ relationship of recombinant aequorin are the same as those found by Allen et al. (1976) with purified aequorin from *Aequorea*, and very similar to those obtained by Brini et al. (1995) ($K_R = 7.23 \times 10^6$ M^{-1}; $K_{TR} = 120$; and $n = 2.99$) using recombinant aequorin from transformed HeLa cells.

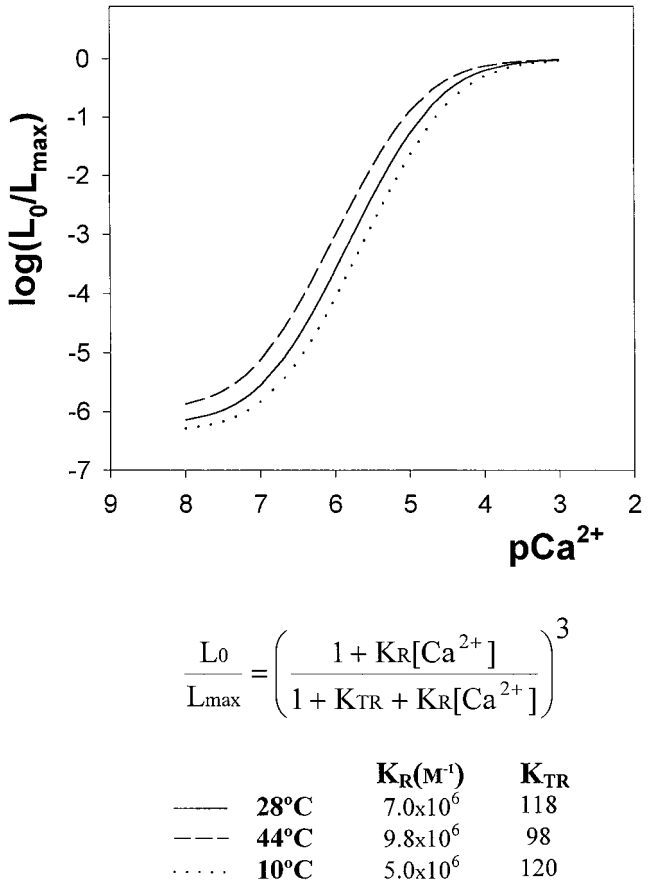

Figure 1. Calibration of recombinant aequorin luminescence at different temperatures (44°C, 28°C, and 10°C). The recombinant aequorin cell lysate (50 μL) at the appropriate temperature was mixed with 950 μL of the Ca^{2+} buffer at the appropriate temperature (see "Materials and Methods" for details). The shown continuous curves correspond to the best fit of the experimental data to model B of Allen et al. (1976) as detailed in "Results."

The influence of temperature on the Ca^{2+} calibration curves, as shown in Figure 1, indicates that warming induces a slight acceleration of the luminescent reaction that is somewhat more pronounced at the lower end of the curve (nanomolar range); as also shown in the figure, cooling has the opposite effect. The calibration curves at different temperatures allowed us to obtain much more accurate data from the heat and cold shock experiments.

The calibration curves shown in the figure also indicate that the aequorin isoform used for transformation is very sensitive, since the dose-response curve begins below a pCa of 7 (around 100 nM free Ca^{2+}) and is saturated well above pCa 5 (around 10 μM free Ca^{2+}). All in vivo Ca^{2+} measurements were performed at least three times, and the results were highly reproducible. A representative trace from the replicates was taken for each experiment and is represented in the figures. Luminescence values were transformed into calibrated $[Ca^{2+}]_i$ using the appropriate calibration curve according to the temperature needed for the experiment (Fig. 1); as an example, Figure 2B (see below) presents the transformation in free Ca^{2+} (micromolar) of the luminescence data presented in Figure 2A. As shown by Figure 2A, the final consumption of aequorin was never greater than 6% to 10% of total (the area occupied by the Ca^{2+}/Triton discharge at the end of measurements comprised about 90% to 94% of the total signal) in our experiments, except when 10 mM Ca^{2+} and 5 μM A23187 were used (see below) and around 28% of aequorin was consumed (the total Ca^{2+}/Triton discharge at the end of measurement of about 72%). However, in the latter case, the fact that the ionophore at such concentration in the presence of 10 mM Ca^{2+} did not completely discharge the photoprotein is difficult to explain unless, under our experimental conditions, this ionophore concentration does not have a full effect on the cyanobacterial cells.

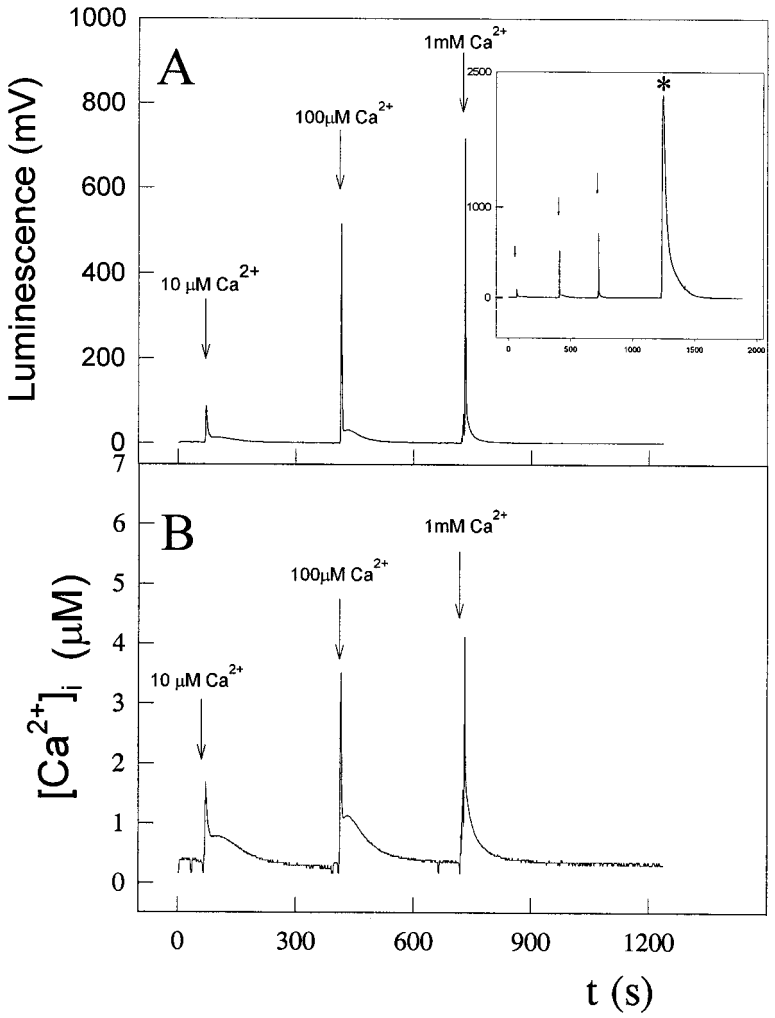

Figure 2. Effect of external Ca^{2+} concentration on the regulation of *Anabaena* $[Ca^{2+}]_i$. Cell suspensions expressing apoaequorin were reconstituted with coelenterazine, as described in "Materials and Methods." Coelenterazine-treated cells (0.5 mL) were placed in a luminometer cuvette, and luminescence was recorded every 1 s. $CaCl_2$ was injected in the sample at the times indicated to give external Ca^{2+} concentrations of 10 μM, 100 μM, and 1 mM. At the end of the experiment, 0.5 mL of 100 mM $CaCl_2$ and 5% (v/v) Triton X-100 was added to discharge the remaining aequorin. A, Light emission of the recombinant aequorin (in mV). In the inset, the asterisk indicates the peak area comprising the remaining aequorin discharge by the addition of an equal volume of 100 mM $CaCl_2$ and 5% Triton X-100. B, Calculated $[Ca^{2+}]_i$ values obtained using the calibration curve depicted in Figure 2. These experiments were repeated 15 times, and the traces represented have been chosen to best represent the average result.

Studies on the Regulation of Intracellular Free Ca^{2+} Levels ($[Ca^{2+}]_i$) by *Anabaena* Strain sp. PCC7120

A fundamental requirement for Ca^{2+}-mediated regulation is the ability of the species in question to regulate intracellular free Ca^{2+} levels. Considering the cytotoxic effects of excess Ca^{2+}, all cells are likely to possess a means of keeping their background free Ca^{2+} levels very low, thus maintaining a Ca^{2+} concentration gradient across the cell membrane. We therefore studied the ability of *Anabaena* cells to regulate internal free Ca^{2+} levels in response to increasing external Ca^{2+} concentrations.

As Figure 2B shows, external additions of 10 μM, 100 μM, and 1 mM $CaCl_2$ gave a transient burst of intracellular free Ca^{2+}, followed by a very quick decline (fast phase of recovery), reaching a plateau within seconds that brought the level of intracellular free Ca^{2+} back to the resting value (slow phase of recovery). To rule out the possibility that the observed spikes could be due to a discharge of aequorin released into the medium by lysed cells or by lysis of cells upon addition of Ca^{2+}, we measured luminescence after the addition of Ca^{2+} to the medium in which reconstituted cells were present after removing cells by centrifugation, and found no luminescence signal. We also measured phycobiliproteins in the supernatant with negative results, indicating that the Ca^{2+} transients were not due to cell lysis. The resting value of $[Ca^{2+}]_i$ was found to be between 100 and 200 nM. These results indicate that *Anabaena* is apparently able to regulate its internal free Ca^{2+} levels.

To confirm that the observed intracellular response was specific to external Ca^{2+} additions, we treated cells with the Ca^{2+} ionophore calcimycin (A23187) and with the Ca^{2+} chelator EGTA. Treatment with calcimycin resulted in larger spikes of intracellular Ca^{2+} compared with controls when cells were challenged with increasing external Ca^{2+}; in addition, the Ca^{2+} transients in the presence of the ionophore were longer lived (Fig. 3A). On the contrary, EGTA inhibited the response and the only elevation of internal free Ca^{2+} levels occurred when the concentration of external Ca^{2+} was higher (1 mM) than the concentration of EGTA used (500 μM) (Fig. 3B). We checked the pH of the EGTA-containing medium throughout

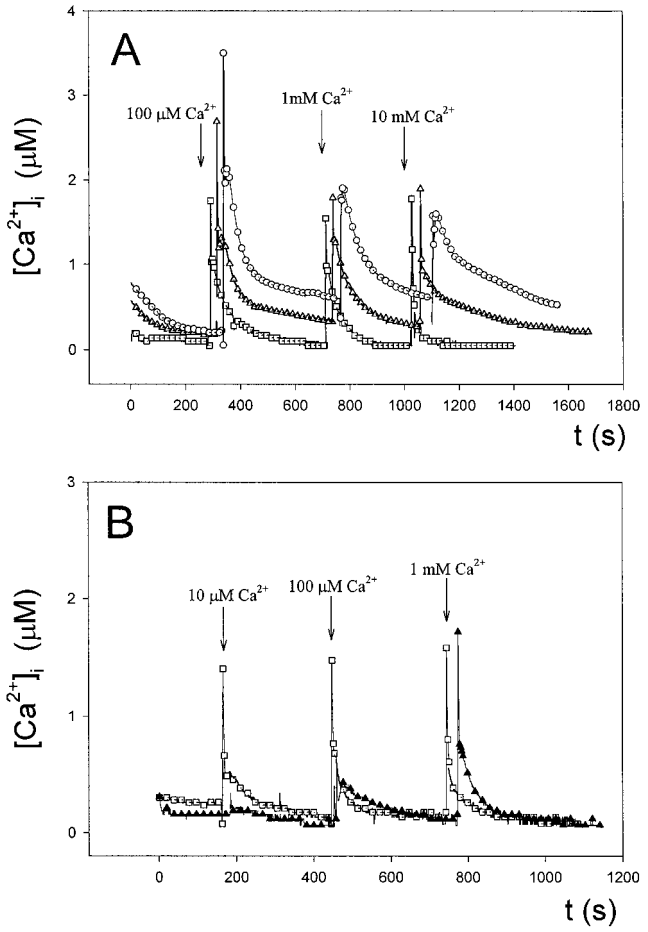

Figure 3. Effect of the Ca^{2+} ionophore calcimycin (compound A23187) and the Ca^{2+} chelator EGTA on the regulation of *Anabaena* $[Ca^{2+}]_i$. A, Effect of preincubation for 15 min in 1 μM (△) or 5 μM (○) calcimycin on intracellular Ca^{2+} transients induced by increasing external Ca^{2+} concentrations compared with the control (□). B, Effect of preincubation for 15 min in 500 μM EGTA (▲) on intracellular Ca^{2+} transients compared with the control (□). $CaCl_2$ was injected in the sample when indicated by the arrows. These experiments were repeated 10 times, and the traces represented have been chosen to best represent the average result.

the experiment and found no significant lowering of the medium pH (already buffered at pH 7.2) when Ca^{2+} was added (not shown). The results with the Ca^{2+} agonist (ionophore) and the Ca^{2+} antagonist (chelator) show that the observed intracellular Ca^{2+} transients are indeed a response to challenge with external Ca^{2+} and that influx of Ca^{2+} from the extracellular space occurs.

The presence of a Ca^{2+} homeostat in a cell system implies the existence of mechanisms of Ca^{2+} regulation that control influx and efflux through the membrane. In cyanobacteria, Ca^{2+} influx may involve low passive permeability and/or Ca^{2+}-sensitive channels as those described for eukaryotic cells. To investigate that, we used the plasma membrane Ca^{2+}-channel blockers verapamil and La^{3+} (Fig. 4). The organic blocker verapamil (Fig. 4A) did not inhibit the Ca^{2+} transients induced by increasing the external Ca^{2+} concentration. However, with the higher concentration (500 μM), after the initial rapid decline in $[Ca^{2+}]_i$, basal levels were not approached, and instead remained quite high (between 0.5 and 1 μM). In the lysate assay we found that verapamil at the concentrations indicated in Figure 4A did not affect the response of aequorin (not shown).

La^{3+} (1 mM but not 100 μM) directly stimulated the luminescence of aequorin in our lysate assay (not shown), which should explain why 1 mM La^{3+} evoked a significant elevation of the resting levels of $[Ca^{2+}]_i$ even before $CaCl_2$ was injected into the sample (Fig. 4B). La^{3+} (100 μM) did not stimulate aequorin in the lysate assay; in fact, as shown in Figure 4B, the resting level of Ca^{2+} before the addition of

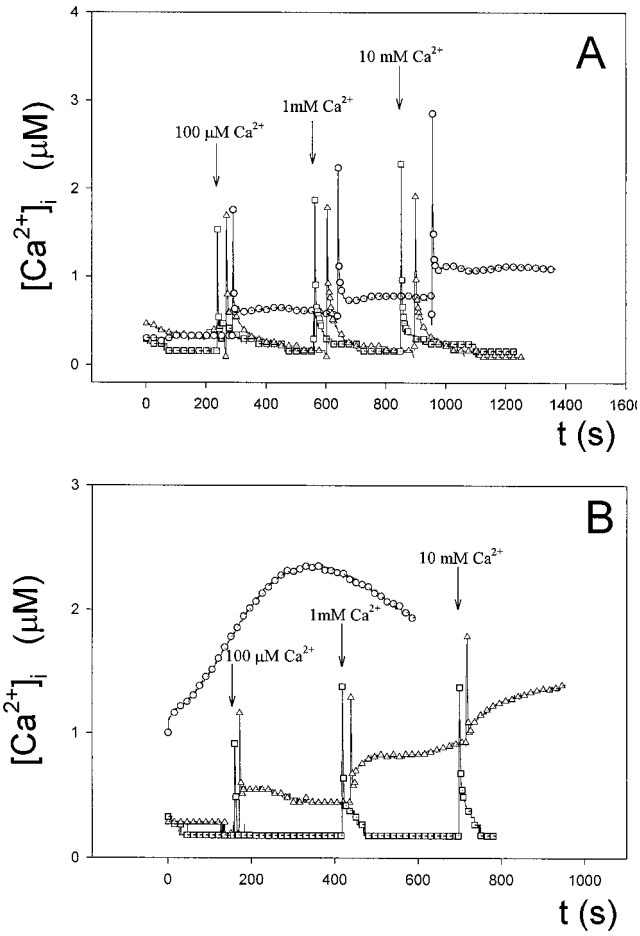

Figure 4. Effect of plasma membrane Ca^{2+} channel blockers verapamil and La^{3+} ($LaCl_3$) on the regulation of *Anabaena* $[Ca^{2+}]_i$. A, Effect of preincubation for 30 min in 200 μM (△) or 500 μM (○) verapamil on intracellular Ca^{2+} transients induced by increasing external Ca^{2+} concentrations compared with the control (□). B, Effect of preincubation for 20 min in 100 μM (△) or 1 mM (○) $LaCl_3$ on intracellular Ca^{2+} transients induced by increasing external Ca^{2+} concentrations compared with the control (□). $CaCl_2$ was injected in the sample when indicated by the arrows. These experiments were repeated 10 times, and the traces represented have been chosen to best represent the average result.

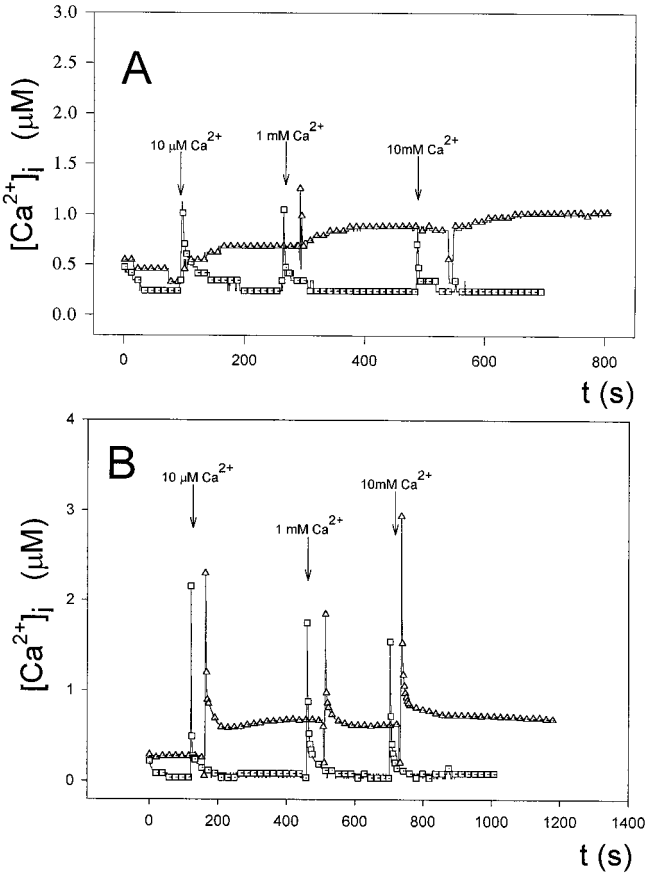

Figure 5. Effect of the calmodulin inhibitor TFP and the Ca^{2+}-exchanger inhibitor diltiazem on the regulation of *Anabaena* $[Ca^{2+}]_i$. A, Effect of preincubation for 1 h in 10 μM TFP (△) on intracellular Ca^{2+} transients induced by increasing external Ca^{2+} concentrations compared with the control (□). B, Effect of preincubation for 45 min in 500 μM diltiazem (△) on intracellular Ca^{2+} transients induced by increasing external Ca^{2+} concentrations compared with the control (□). $CaCl_2$ was injected in the sample when indicated by the arrows. These experiments were repeated eight times, and the traces represented have been chosen to best represent the average result.

$CaCl_2$ was not modified by this concentration of La^{3+}; the addition of $CaCl_2$ to cells treated with 100 μM of La^{3+} provoked a significant and apparently uncontrolled elevation of the levels of $[Ca^{2+}]_i$. In response to increasing external Ca^{2+} concentrations, Ca^{2+} influx apparently does not depend on the activation of verapamil-sensitive channels. Furthermore, La^{3+} at 100 μM provokes an uncontrolled increase in $[Ca^{2+}]_I$, probably due to the fact that La^{3+} may enter the cell and inhibit intracellular Ca^{2+} channels and/or Ca^{2+} pumps (ATPases) needed to regulate the efflux of Ca^{2+} ions. However, when the concentration of La^{3+} used was higher (1 mM), as shown by our lysate assay, the high levels of luminescence encountered in the in vivo assay could be due mostly to direct stimulation of aequorin by the ion.

In connection with this effect of La^{3+}, and as described above (Fig. 2B), after the initial rise of intracellular Ca^{2+}, there was a decline to homeostatic values. As already described, this decline appears to be a two-phase phenomenon and it is important to determine whether the fast or the slow phase of the decrease in $[Ca^{2+}]_i$ is rectified by Ca^{2+} efflux (Ca^{2+} pumps and/or Ca^{2+} exchangers) and/or intracellular binding by Ca^{2+} binding proteins. To address this question, we treated the cells with the calmodulin inhibitor TFP (Fig. 5A) and the Ca^{2+}-exchanger inhibitor diltiazem (Fig. 5B). Treatment with TFP provoked a poor regulation of $[Ca^{2+}]_i$ when cells were challenged with increasing external Ca^{2+}; after the initial rise in $[Ca^{2+}]_i$, the cells maintained throughout the experiment extremely high levels of $[Ca^{2+}]_i$ and no decline was observed, suggesting an important role for Ca^{2+}-binding proteins in Ca^{2+} homeostasis in this cyanobacterium. In this context, one could reasonably expect the observed elevation of the resting $[Ca^{2+}]_i$ values caused by TFP.

These findings are also supported by the fact that, in our lysate assay, TFP at the concentration indicated in Figure 5A did not affect the response of aequorin (not shown). Treatment with diltiazem also elevated the resting level, but had no effect on the initial rise of $[Ca^{2+}]_i$ and the subsequent fast phase of recovery, although it impaired the slow phase of regulation of $[Ca^{2+}]_i$ levels, as they never settled back to basal values. In the lysate assay, diltiazem at the concentration indicated in Figure 5B did not affect the response of aequorin (not shown). Thus, the effect of diltiazem, although not as pronounced as that of TFP, suggests that in vivo the Ca^{2+} electroneutral exchangers also may have a role in allowing the return of $[Ca^{2+}]_i$ to resting values. The combination of both systems, Ca^{2+}-binding proteins and Ca^{2+} exchangers (we cannot rule out the possible role of Ca^{2+} pumps) possibly prevent excessive Ca^{2+} accumulation and, thus, cell damage in cyanobacteria.

Intracellular Free Ca^{2+} Changes in Response to Heat Shock

As indicated in "Materials and Methods," heat shock was applied either by immersing cell suspensions in a water bath at 44°C or by directly irrigating with hot water.

In the first case, cells placed in a luminometer cuvette were heat-shocked at 44°C for periods up to 60 min in a thermostated water bath, and cuvettes were removed at specific times to monitor luminescence (Fig. 6A). As shown in the figure, continued heat shock treatment caused a significant increase in $[Ca^{2+}]_i$ that lasted more than 30 min and approached basal levels very slowly. Its magnitude increased from around 1.14 ± 0.17 μM after 2 min, to reach a maximum of 3.10 ± 0.25 μM (n = 15) after 20 min (see Fig. 6A). After that time, no further increases in $[Ca^{2+}]_i$ were observed; in fact, a gradual decrease to about 1.22 ± 0.59 μM occurred after 60 min (n = 15). To monitor the actual temperature of the cell suspen-

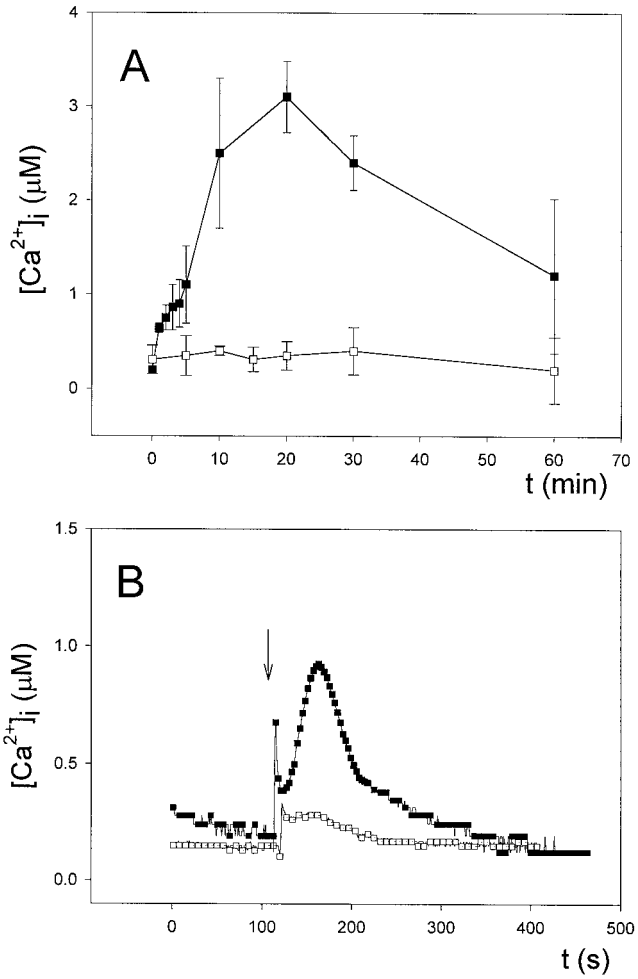

Figure 6. Changes in $[Ca^{2+}]_i$ in response to heat shock. A, Continued heat shock treatment (up to 60 min) in a water bath at 44°C of coelenterazine-treated cells (■). Cuvettes were removed from the water bath at specific times to monitor luminescence as described in "Materials and Methods." Cells maintained in a water bath at 28°C were taken as the control (□). Measurements were made maintaining an external Ca^{2+} concentration of 0.25 mM. B, Coelenterazine-treated cells were heat-shocked by injecting hot water at 65°C to achieve a final temperature of 44°C in the cuvette and luminescence recorded as described in "Materials and Methods" (■). Cells irrigated with water at 28°C were taken as the control (□). Measurements were made maintaining an external Ca^{2+} concentration of 0.25 mM. Experiments were repeated 15 times and the error bars represent ±SE (A). Experiments were repeated 15 times, and the traces represented have been chosen to best represent the average result (B). The vertical arrow in B indicates the injection of hot water.

sions in the water bath throughout the experiment and the temperature fluctuations in the cuvettes during the 15 to 20 s of luminescence measurement, thermocouples were introduced into blank cuvettes containing equal volumes and cell densities as those used for the luminescence assays, and temperatures were recorded continuously.

Using these thermocouples, it took around 5 min for the cell suspension to achieve 44°C, indicating that in a water bath, heating of the sample is a slow process. The temperature fluctuations in the cuvette during the 15 to 20 s of luminescence measurement was only 1°C and lasted around 50 s (by 100 s, the temperature had decreased around 3°C) (not shown). Thus, during the 15 to 20 s measurement in the luminometer, the temperature change is very small (only 1°C). Nonetheless, to determine whether this small decrease in temperature at such a specific rate could be responsible for the observed Ca^{2+} transient, we measured the effect of cooling at that same rate from 28°C (regular growth temperature of *Anabaena* cells) to 27°C, 26°C, and 25°C and found no observable intracellular Ca^{2+} increase (not shown). Therefore, these measurements truly reflect a Ca^{2+} response to heat shock. During the course of the experiment, we did not observe cell lysis.

When heat shock was applied by irrigation of cell suspensions with hot water at temperatures up to 65°C to allow the temperature of the cell suspensions to immediately increase from 28°C to 44°C, the nature of the Ca^{2+} transient was different: injected hot water triggered two contiguous phases of Ca^{2+} release that lasted for a total of about 3 min (Fig. 6B). The peak $[Ca^{2+}]_i$ concentration was approximately 0.80 ± 0.14 μM for the first pulse and 0.98 ± 0.18 μM (n = 15) for the second. The first phase was very short and the second was bell-shaped, accounting for over 90% of the total duration of the transient. The control (the addition of water at 28°C) did not elicit such a response, although a much smaller Ca^{2+} transient in magnitude (peak height of 0.40 ± 0.10 μM [n = 6]) and duration (approximately 20 s) was observed. To rule out that this small transient could be due to a hypoosmotic shock caused by water, we injected growth medium (BG11) both at 65°C and at 28°C and essentially found the same results as those obtained with water (not shown). This small transient could therefore represent a small mechanically induced Ca^{2+} increase.

Thermocouples introduced into blank cuvettes were also used to measure the temperature changes after direct irrigation with hot water or hot growth medium. We found that the actual temperature at the peak of the first phase of the Ca^{2+} transient (observed 2–3 s after irrigation) was already 44°C, so the change in temperature from 28°C to 44°C is almost instantaneous. Also, the actual temperature at the peak of the second, bell-shaped Ca^{2+} transient (observed around 50 s from the irrigation) was 42°C, and only after 100 s did the temperature decrease approximately 4°C to 40°C (not shown). As in the water bath experiment, we also checked whether those small decreases in temperature would account for the observed Ca^{2+} transients and found that cooling *Anabaena* cells from 28°C to 27°C, 26°C, and 25°C at that specific rate did not induce any observable intracellular Ca^{2+} increase (not shown). Thus, the observed biphasic Ca^{2+} transient might correspond to a sudden rise in temperature. Cell lysis was not observed.

The described heat shock experiments were performed with an external Ca^{2+} concentration of 0.25 mM (the standard Ca^{2+} concentration of the medium). An increase in the external Ca^{2+} did not provoke a concomitant increase in $[Ca^{2+}]_i$ in either case (data not shown). Control experiments to test the stability of recombinant aequorin to heat treatment showed that the total luminescence signal remained unchanged at 44°C, although at temperatures of 50°C and over, 40% of the signal was lost. Recombinant aequorin was therefore stable to high temperatures (up to 44°C), and we believe that the changes shown in Figure 6 represent true changes in cytosolic free Ca^{2+}.

Intracellular Free Ca^{2+} Levels in Response to Cold Shock

As indicated in "Materials and Methods," cold shock was applied either by immersing cell suspensions in a water bath at 0°C or by directly irrigating with cold water.

In the first case, cell suspensions placed in luminometer cuvettes were immersed into a water bath at 0°C, removed at specific times, and luminescence recorded. Continued cold shock, applied in this way for up to 60 min, provoked a much smaller increase in $[Ca^{2+}]_i$ than continued heat shock with a maximum magnitude of 1.04 ± 0.23 (n = 8) after 10 min (Fig. 7A). The continued cold-shock-induced Ca^{2+} transient was also shorter in duration than the continued heat-shock-induced Ca^{2+} transient. We found that when cells were incubated in the presence of higher external Ca^{2+} concentrations (5 mM as shown in Fig. 7A), there was a significant (Student's t test, $P < 0.1$) elevation in the Ca^{2+}-induced transient. This result was the opposite of that found with heat shock, after which higher external Ca^{2+} concentrations did not exert any significant effect. Thermocouples introduced into blank cuvettes showed that the cell suspensions reached a temperature of 10°C after 5 min in the water bath at 0°C and that by 10 min, the temperature of the cell suspension decreased even further to 5°C (not shown). No cell lysis was observed at the end of the experiment.

Cold shock applied by irrigation with ice-cold water induced two well-defined Ca^{2+} transients (Fig. 7B). The first Ca^{2+} transient was biphasic, showing two contiguous phases of Ca^{2+} release that lasted for a total of 3 to 4 min, and was very similar to that found after heat shock by irrigation (Fig. 6B). The second Ca^{2+} transient was slower and smaller in magnitude, lasting around 7 to 8 min. This second Ca^{2+} transient after cold shock is a clear difference from that induced by heat shock, since it was never observed under the latter conditions. For the biphasic Ca^{2+} transient, the peak Ca^{2+} concentration was approximately 1.27 ± 0.22 μM for the first phase and approximately 1.36 ± 0.16 μM (n = 8) for the second phase. The first phase was very short and the second bell-shaped phase accounted for over 90% of the total duration of the transient. The peak height concentration for the second, slower transient was around 0.67 ± 0.11 μM (n = 8). The control, the addition of water at 28°C, did not elicit such an increase in $[Ca^{2+}]_i$, although, as already described for heat shock, a much smaller Ca^{2+} transient in magnitude (peak height of approximately 0.40 ± 0.10 μM [n = 6]) and duration (approximately 20 s)

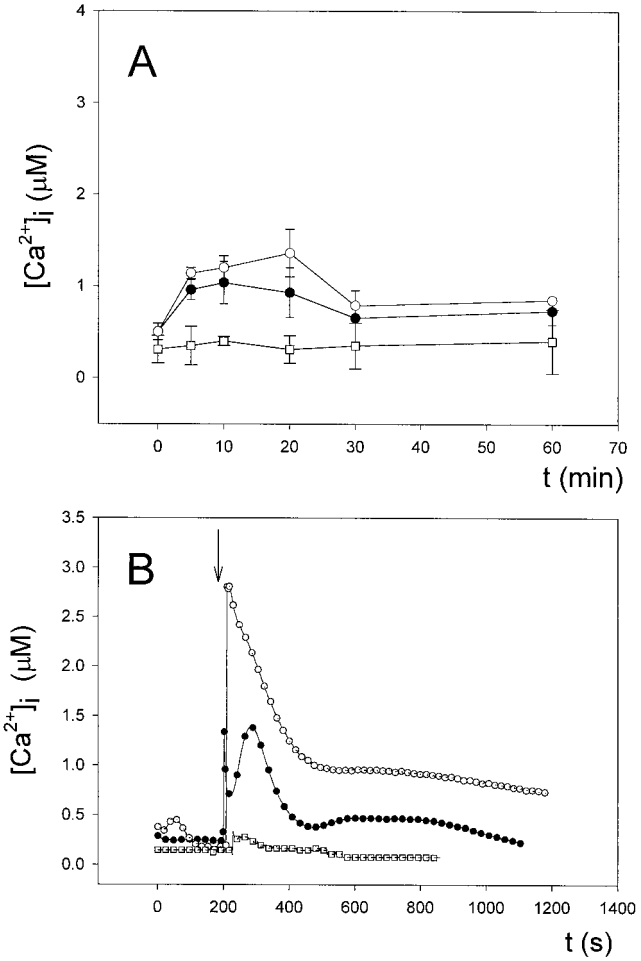

Figure 7. Changes in $[Ca^{2+}]_i$ in response to cold shock. A, Continued cold-shock treatment (up to 60 min) in a water bath at 0°C of coelenterazine-treated cells incubated with increasing external Ca^{2+} concentrations (○, 5 mM Ca^{2+}; ●, 0.25 mM Ca^{2+}). Cuvettes were removed from the water bath at specific times to monitor luminescence as described in "Materials and Methods." Cells maintained in a water bath at 28°C were taken as the control (□). B, Coelenterazine-treated cells incubated with increasing external Ca^{2+} concentrations (○, 1 mM Ca^{2+}; ●, 0.25 mM Ca^{2+}) were cold-shocked by injecting ice-cold water to achieve a final temperature of 10°C in the cuvette and luminescence recorded as described in "Materials and Methods." Cells irrigated with water at 28°C were taken as the control (□). Experiments were repeated eight times and error bars represent ±SE (A). Experiments were repeated eight times, and the traces represented have been chosen to best represent the average result (B). The vertical arrow in B indicates the injection of cold water.

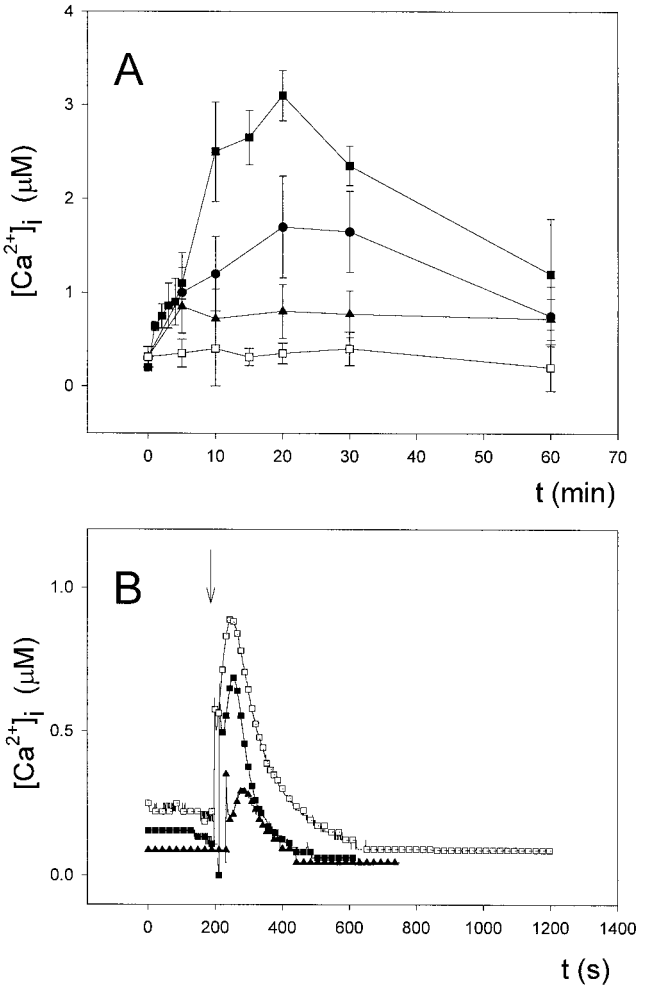

Figure 8. Effect of external Ca^{2+} depletion on the heat-shock-induced changes in $[Ca^{2+}]_i$. A, Heat shock applied in a water bath at 44°C up to 60 min. ■, 0.25 mM Ca^{2+}; ●, 0 mM Ca^{2+}; ▲, 1 mM EGTA; □, control (28°C, 0.25 mM Ca^{2+}). B, Heat shock applied by irrigation with hot water. □, 0.25 mM Ca^{2+} out; ■, 0 mM Ca^{2+} out; ▲, 1 mM EGTA. These experiments were repeated 10 times, and the traces represented have been chosen to best represent the average result. The vertical arrow in B indicates the injection of hot water.

was observed. To rule out that this small transient could be due to a hypoosmotic shock caused by water, we injected growth medium (BG11) both at 0°C and at 28°C and essentially found the same results as those obtained with water (not shown). This small transient could, as indicated before, represent a small, mechanically induced Ca^{2+} increase.

Thermocouples introduced into blank cuvettes showed that the actual temperature at the peak of the first phase of the biphasic Ca^{2+} transient (observed 2–3 s after irrigation) was 12°C, so the change in temperature from 28°C to 12°C was almost instantaneous. Furthermore, the actual temperature at the peak of the second phase (observed around 100 s after the injection of cold water or growth medium) was 16°C. At the beginning of the second, slower transient, the temperature was around 19°C, still 9°C below room temperature (28°C) (not shown). Thus, the observed biphasic Ca^{2+} transient corresponds to a sudden drop in temperature. Cold shock by irrigation also did not provoke cell lysis. As already seen with continued cold shock, cold shock by irrigation after pretreatment with higher external Ca^{2+} concentrations (1 mM, as shown in Fig. 7B), unlike heat shock, induced marked elevations of both the biphasic and the slower Ca^{2+} transient. These results clearly show a strong dependence between $[Ca^{2+}]$ in the external medium and the response of the cell to both types of cold shock (Fig. 7).

Control experiments to test the stability of recombinant aequorin to cold treatment showed that the total luminescence signal remained unchanged (not shown). Thus, we believe that the data reflect genuine changes in cytosolic Ca^{2+} in response to cold shock.

Possible Cellular Origin for the Heat- and Cold-Shock-Induced Increase in $[Ca^{2+}]_i$

To explore the source of the Ca^{2+} for the increased $[Ca^{2+}]_i$ under heat and cold shock, additional experiments were carried out in the absence of added Ca^{2+} in the medium, with EGTA (zero external Ca^{2+}) and with the Ca^{2+} channel blocker verapamil. Inhibitors were added after reconstitution with coelenterazine, and the cultures were incubated in inhibitor for the times indicated in the figures.

When cell suspensions incubated in the absence of added external Ca^{2+} were heat-shocked in a water bath at 44°C for periods up to 60 min, a $[Ca^{2+}]_i$ elevation was observed that, like the control, peaked around 20 min from the beginning of the heat shock; however, the observed Ca^{2+} transient was clearly lower in magnitude compared with the control (after 20 min of heat shock, approximately 1.70 ± 0.54 μM [n = 10] versus 3.10 ± 0.25 [n = 10]). The Ca^{2+} chelator EGTA (zero external Ca^{2+}) induced a significant (Student's t test, $P < 0.05$), although much smaller, $[Ca^{2+}]_i$ transient (maximum value of 0.81 ± 0.29 [n = 10]). These results strongly suggest that both extracellular and intracellular sources contribute to the increase in $[Ca^{2+}]_i$ caused by heat shock applied in a water bath at 44°C.

In the absence of added external Ca^{2+}, heat shock applied by irrigation with hot water (Fig. 8B) induced a biphasic $[Ca^{2+}]_i$ transient with kinetics very similar to those of the control but smaller in magnitude: a first peak height of approximately 0.58 ± 0.13 μM (n = 10) versus approximately 0.80 ± 0.14 μM in the control culture, and a second peak height of approximately 0.71 ± 0.18 μM (n = 10) versus approximately 0.98 ± 0.18 μM in the control culture. The Ca^{2+} chelator EGTA induced a somewhat smaller $[Ca^{2+}]_i$ transient, with peak heights of approximately 0.43 ± 0.09 and 0.35 ± 0.09 μM (n = 10) for the first and second phase, respectively, and were shorter in duration, too. Thus, the response to heat shock by irriga-

tion also involves Ca^{2+} release from intracellular stores.

The situation is quite different regarding cold shock (Fig. 9). Continued cold shock in a water bath of cells incubated with EGTA (zero external Ca^{2+}) did not induce a significant (Student's t test, $P < 0.05$) Ca^{2+} transient, indicating that extracellular sources are mostly involved in the observed Ca^{2+} increase. In the absence of added external Ca^{2+}, cold shock applied by irrigation with ice-cold water induced a very small $[Ca^{2+}]_i$ transient compared with the control (peak height of approximately 0.38 ± 0.10 μM [n = 6]). The kinetics were also totally different, since the induced transient was not biphasic and was shorter in duration. The Ca^{2+} chelator EGTA severely limited the capacity of cold shock to increase $[Ca^{2+}]_i$; in fact, its levels remained nearly basal. Thus, after cold shock by irrigation, it appears that the increased $[Ca^{2+}]_i$ observed arises, as in the case of continued cold shock in a water bath, mainly from extracellular sources.

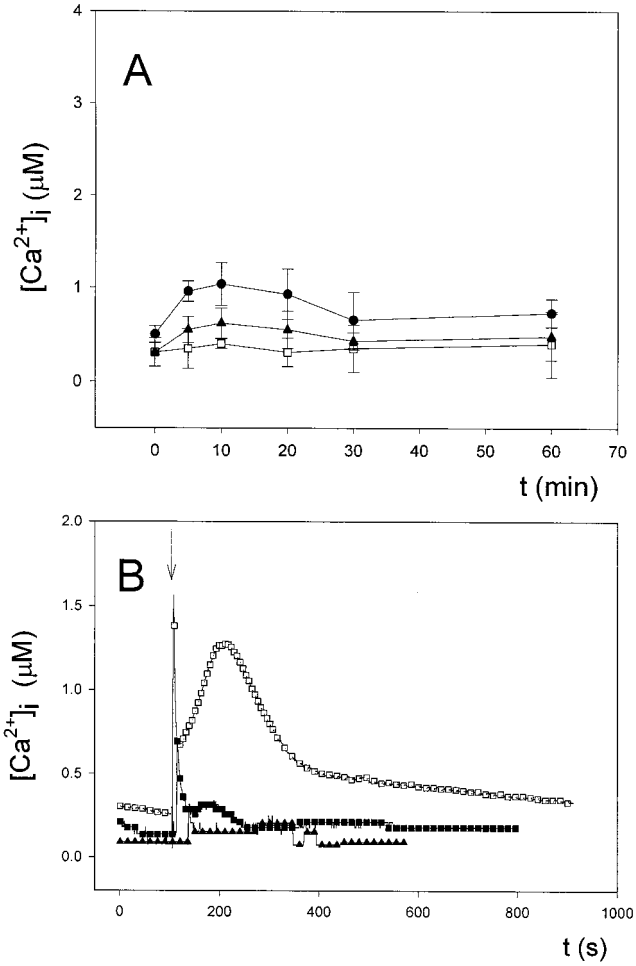

Figure 9. Effect of external Ca^{2+} depletion on the cold-shock-induced changes in $[Ca^{2+}]_i$. A, Cold shock applied in a water bath at 0°C up to 60 min. ●, 0.25 mM Ca^{2+}; ▲, 1 mM EGTA; □, control (28°C, 0.25 mM Ca^{2+}). B, Cold shock applied by irrigation with ice-cold water. □, 0.25 mM Ca^{2+} out; ■, 0 mM Ca^{2+} out; ▲, 1 mM EGTA. These experiments were repeated six times, and the traces represented have been chosen to best represent the average result. The vertical arrow in B indicates the injection of cold water.

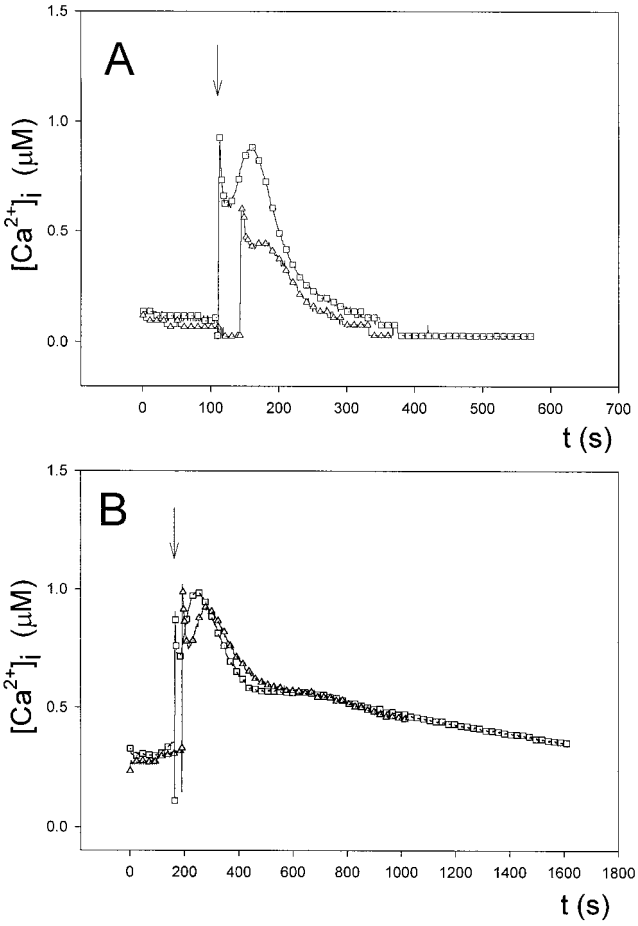

Figure 10. Effect of plasma membrane Ca^{2+} channel blocker verapamil on heat (A) or cold shock (B) induced changes in $[Ca^{2+}]_i$. Cells were preincubated with 200 μM verapamil for 30 min prior to heat or cold shock treatment (△). These experiments were repeated six times and the traces represented have been chosen to best represent the average result. The vertical arrows indicate the injection of hot (A) or cold (B) water. □, Control.

Figure 10 shows the effect of the Ca^{2+}-channel blocker verapamil on heat- and cold-shock-mediated $[Ca^{2+}]_i$ increases, respectively. Verapamil significantly (Student's t test, $P < 0.05$) lowered the observed elevation of $[Ca^{2+}]_i$ under heat shock: a first peak height of 0.55 ± 0.15 μM versus 0.76 ± 0.15 μM [n = 6] of the control, and a second peak height of 0.51 ± 0.18 μM versus 0.84 ± 0.16 μM (n = 6) of the control. However, it did not have the same effect on the Ca^{2+} transient induced by cold shock (Student's t test; $P > 0.50$): a first peak height of the biphasic transient of 1.07 ± 0.18 μM versus 1.02 ± 0.14 μM (n = 6) of the control; a second peak height of the biphasic transient of 1.10 ± 0.11 μM versus 1.04 ± 0.17 μM (n = 6) of the control. The peak height of the second, slower transient was of 0.63 ± 0.10 μM versus

0.62 ± 0.09 μM of the control). These results suggest that the influx of Ca^{2+} needed to induce the Ca^{2+} transient following heat shock may occur through verapamil-sensitive Ca^{2+} channels, while influx of Ca^{2+} due to cold shock probably occurs through a different type of Ca^{2+} channel.

DISCUSSION

We report here, for the first time to our knowledge, the construction of a strain of cyanobacteria that constitutively expresses the apoaequorin gene. Functional recombinant aequorin can be successfully reconstituted upon addition of the hydrophobic luminophore coelenterazine. The Ca^{2+}-sensitive luminescent protein is expressed in cell suspensions of *Anabaena* at sufficient high levels to allow an accurate calibration of the luminescence data into $[Ca^{2+}]_i$ values (Figs. 1 and 2). With regard to the calibration procedure, our results agree with previous observations (Brini et al., 1995) that recombinant aequorin is as sensitive as native aequorin (Allen et al., 1976) to Ca^{2+} changes, since the dose response curve begins at around 100 nM free Ca^{2+} and is saturated well above 10 μM free Ca^{2+} (Fig. 1). Calibration curves should be determined at the temperatures corresponding to that of the experiment, since warming or cooling may have an effect on the luminescent reaction (Fig. 1; Blinks et al., 1982).

The maintenance of a low intracellular free Ca^{2+} concentration is required not only to protect the cell from the toxic effects of Ca^{2+}, but also to permit the use of Ca^{2+} as a second messenger: any increase in the free $[Ca^{2+}]_i$ due to the propagation of a signal must disappear quickly in order for the next signal to occur. Such regulation is accomplished by a complex of processes collectively called the "Ca^{2+} homeostat," which has been mostly studied in eukaryotes (Carafoli, 1987; Bush, 1995).

To assign a regulatory role for Ca^{2+} in cyanobacteria, we thought it necessary to undertake a study of the functioning of the Ca^{2+} homeostat in our recombinant strain. We recorded the response of *Anabaena* cells to increasing external Ca^{2+} concentrations and assayed the effect of several Ca^{2+}-signaling compounds. *Anabaena* sp. strain PCC7120 sensed and responded rapidly to an increase in the external Ca^{2+} concentration (Fig. 2). The induced Ca^{2+} transient was very short, indicating a quick removal of free Ca^{2+} from the cytoplasm to maintain the steady-state concentration very low (between 100 and 200 nM). Thus, *Anabaena* seems to be able to tightly regulate its internal free Ca^{2+} levels. The Ca^{2+} ionophore calcimycin (compound A23187) induced a larger Ca^{2+} transient, while the Ca^{2+} chelator EGTA abolished it (Fig. 3), indicating that the influx of Ca^{2+} from the extracellular space occurred to induce such Ca^{2+} transients.

The results with the plasma membrane Ca^{2+} channel blocker verapamil showed, however, that influx was not due to the opening of verapamil-sensitive Ca^{2+} channels (Fig. 4A). The other Ca^{2+} channel blocker, La^{3+} provoked an uncontrolled rise of $[Ca^{2+}]_i$ when cells were challenged with increasing external Ca^{2+} concentrations (Fig. 4B). However, the data with La^{3+} should be taken with caution since, in our lysate assay, we found that La^{3+} at 1 mM (although not at 100 μM) largely stimulated aequorin luminescence. This effect has already been described by Blinks et al. (1982). Nevertheless, we believe that our data with 100 μM La^{3+} truly reflects an effect of the ion on Ca^{2+} levels. The other clear evidence that we get from the lysate assay and the in vivo assay (Fig. 4B) is that La^{3+} may be entering the cyanobacterial cells and exert its effect intracellularly. In fact, there is evidence that lanthanides can enter eukaryotic cells (Quiquampoix et al., 1990).

In cyanobacteria, it has been found, using $^{45}Ca^{2+}$, that La^{3+} increases the concentration of intracellular Ca^{2+} between 2- and 3-fold, and this was attributed to increased Ca^{2+} influx (Smith, 1988). That author suggested that La^{3+} may enter cells and, by disrupting Ca^{2+}-protein interactions, may lead to the observed effect on Ca^{2+} uptake. We believe that, rather than affecting Ca^{2+} uptake, La^{3+}, once inside the cell, may inhibit Ca^{2+} efflux systems such as Ca^{2+}/nH^+ antiporters and Ca^{2+} ATPases, impairing the homeostasis of Ca^{2+} and thus giving rise to the observed high levels of intracellular Ca^{2+}. In fact, La^{3+} is not an specific inhibitor of voltage-operated Ca^{2+} channels, and in a variety of environmental systems has been found to block Ca^{2+} pumps, Ca^{2+} exchangers, and K^+ channels (Bush, 1995; Lewis and Spalding, 1998).

The results with the calmodulin inhibitor TFP (Fig. 5A) suggest that Ca^{2+} binding proteins may play an important role in the rapid reestablishment of the resting levels of $[Ca^{2+}]_i$. Ca^{2+} efflux by Ca^{2+} exchangers also appear to contribute to restoration of the steady-state free Ca^{2+} concentrations, as evidenced by the effect of the Ca^{2+}-exchanger-inhibitor diltiazem (Vaghy et al., 1982; Rizzuto et al., 1987; Brini et al., 1995).

Most of the studies on the regulation of intracellular free Ca^{2+} levels have been in eukaryotes, and very little is as yet known about real levels of intracellular free Ca^{2+} in bacteria and how these levels change in response to external stimuli. In fact, in prokaryotes, most if not all of the available data comes from *Escherichia coli*. Using Fura-2 fluorescence and $^{45}Ca^{2+}$, Gangola and Rosen (1987) found that *E. coli* is able to maintain resting levels of free $[Ca^{2+}]_i$ comparable to that of eukaryotic cells. Using recombinant aequorin for the first time in a prokaryotic cell, Knight et al. (1991b) reported that a variety of conditions promote a brief increase in the free $[Ca^{2+}]_i$ in *E. coli* and confirmed the low levels of intracellular free Ca^{2+} in a steady-state situation (around 100 nM) found by Gangola and Rosen (1987). The fact that a photosyn-

thetic bacterium such as *Anabaena* also maintains a low level of $[Ca^{2+}]_i$ and that this concentration is tightly regulated even when cells are challenged with high external Ca^{2+} concentrations favors the idea that Ca^{2+}-mediated regulation may be a general feature of prokaryotic organisms.

Ca^{2+} has been shown to respond to environmental variables in plant cells (Knight et al., 1991a, 1992, 1996; Chandra and Low, 1997; Takahashi et al., 1997; Gong et al., 1998), and there is increasing evidence that the same might be true for cyanobacteria (Smith, 1995; Norris et al., 1996; Giraldez-Ruiz et al., 1997, 1999). In this context, we were interested in determining whether Ca^{2+} was involved in signaling of heat and cold shock, because little is known about the initial perception of both environmental stresses in cyanobacteria since most of the studies have dealt with environmental-stress-induced modifications of protein synthesis (Borbely et al., 1985; Nicholson et al., 1987; Bhagwat and Apte, 1989). To study Ca^{2+} involvement and to elucidate whether the cell response varies according to the specific way of inducing the shock, we applied heat and cold shock in two different ways: (a) cells were placed in cuvettes and immersed in water baths at the appropriate temperature, allowing for continuous heat or cold shock (in this method, cells did not come into contact with hot or cold water); (b) water was injected directly into the sample, resulting in an almost instantaneous change of temperature (in this method, cells came into contact with hot or cold water).

We found that continuous heat shock induced $[Ca^{2+}]_i$ transients with a maximum magnitude after 20 min of heat shock; after this time, $[Ca^{2+}]_i$ gradually returned to resting levels even when heat shock continued to 60 min (Fig. 6B). Surprisingly, our results are almost the same as those found by Gong et al. (1998) using young tobacco seedlings. These authors suggested that refractory periods when a shock is continuously applied (Knight et al., 1991a, 1992, 1996) prevent cells from damage that would be caused by a prolonged increase in $[Ca^{2+}]_i$, which is known to be cytotoxic. Unlike the results of Knight et al. (1991b) and Gong et al. (1998), who reported that irrigation of tobacco seedlings with hot water did not induce detectable changes in luminescence, we found that a biphasic Ca^{2+} transient was originated when the shock was applied by irrigation (Fig. 6C).

Continued cold shock in a cold water bath elicited a Ca^{2+} transient that was significantly smaller in magnitude and duration than the one obtained under continued heat shock (Fig. 7A), indicating that Ca^{2+} changes after a slow decrease in temperature are not so strong as those following a slow increase in temperature. Irrigation of the cell suspension with cold water induced two Ca^{2+} transients: a biphasic Ca^{2+} transient with similar kinetics to that obtained with irrigation with hot water, and a second, slower Ca^{2+} transient (Fig. 7B) that never appeared under heat shock by irrigation. These data suggest that cyanobacterial cells distinguish between these two different ways of inducing heat or cold shock. Also, the fact that irrigation with cold or hot water induces a similar Ca^{2+} transient might indicate that the cells are sensing a sudden change in temperature, and may discriminate between cold and heat shock by the appearance of that second Ca^{2+} transient.

A clear difference between both shocks comes from the source of Ca^{2+} involved in the induction of the Ca^{2+} transient. The results in the absence of added external Ca^{2+} and those using the Ca^{2+} chelator EGTA (zero external Ca^{2+}) indicate that the increased $[Ca^{2+}]_i$ observed during heat shock arises from both extracellular and intracellular spaces (Fig. 8), while that of cold shock arises mainly from the extracellular space (Fig. 9). The fact that an increase in extracellular Ca^{2+} increases the Ca^{2+} transients induced by cold shock, while after heat shock, an increase of extracellular Ca^{2+} does not result in a concomitant increase in $[Ca^{2+}]_i$ supports the above. The data with the plasma membrane Ca^{2+} channel blocker verapamil (Fig. 10) indicate that the influx of Ca^{2+} from the extracellular space after heat shock may occur through a different type of Ca^{2+} channel than that after cold shock.

A major question remains regarding the intracellular stores of Ca^{2+} in cyanobacteria. Cytoplasmic Ca^{2+} chelation by Ca^{2+}-binding proteins could probably complex a significant portion of the total cell Ca^{2+}. Polyphosphate bodies contain significant amounts of K^+, Mg^{2+}, and Ca^{2+} (Jensen et al., 1982) that would meet the needs of cells for these essential metals; however, it is not clear whether Ca^{2+} can easily be mobilized from such bodies when the propagation of a Ca^{2+} signal is needed. We present direct evidence that Ca^{2+} signaling exists in cyanobacteria, but we believe that further research is required to determine the extent of Ca^{2+}-mediated regulation in this relevant group of prokaryotes that, according to the endosymbiotic theory, are phylogenetically and physiologically related to the chloroplast and thus may serve as a model for the photosynthetic eukaryotes.

MATERIALS AND METHODS

Organism and Growth Conditions

The recombinant strain of *Anabaena* strain sp. PCC7120 expressing apoaequorin was routinely grown in BG11 medium buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2 (Rippka et al., 1979), at 28°C on a rotary shaker under a constant irradiance of 100 μmol m^{-2} s^{-1}. The strain was supplemented when grown in liquid cultures with 2.5 μg spectinomycin dihydrochloride mL^{-1}.

Construction of the Apoaequorin Expression Vector for *Anabaena* Strain sp. PCC7120

A fragment of 0.78 Kb containing the apoaequorin (*aaeq*) cDNA was cut with *Pst*I and *Kpn*I from plasmid pSV0AQ (Tanahashi et al., 1990) and ligated into the *Pst*I and *Kpn*I sites of plasmid pBluescript SK+ (Stratagene Cloning Systems, San Diego), generating pBG2000. From pBG2000, the 0.78-Kb fragment with the apoaequorin cDNA could be easily excised with *Asp*718 and *Sma*I; after Klenow treatment, the fragment was ligated to pRL1404 (Fernandez-Piñas et al., 1994) cut with *Sma*I, creating the expression vector pBG2001 (Fig. 11) that can replicate in *Anabaena* strain sp. PCC7120. The apoaequorin gene is oriented to be transcribed from a promoter within the *Nostoc* replicon pDU1 (Walton et al., 1992). This promoter has been widely used in filamentous cyanobacteria to promote expression of several genes (Fernandez-Piñas and Wolk, 1994; Fernandez-Piñas et al., 1994; Maldener et al., 1994). Plasmid pBG2001 was introduced into *Anabaena* strain sp. PCC7120 by conjugation (Wolk et al., 1984; Elhai and Wolk, 1988).

In Vivo Reconstitution of Apoaequorin

In vivo reconstitution of apoaequorin was carried out by adding coelenterazine in methanol to a cell suspension (at a cell concentration of 15 μg chlorophyll mL^{-1}) to reach a final concentration of 2.5 μM, and incubating the cells for 4 h in the dark. Before Ca^{2+} measurements were made, cells were washed twice in BG11 medium buffered with 25 mM HEPES, pH 7.2, containing 0.5 mM EGTA to remove the excess coelenterazine.

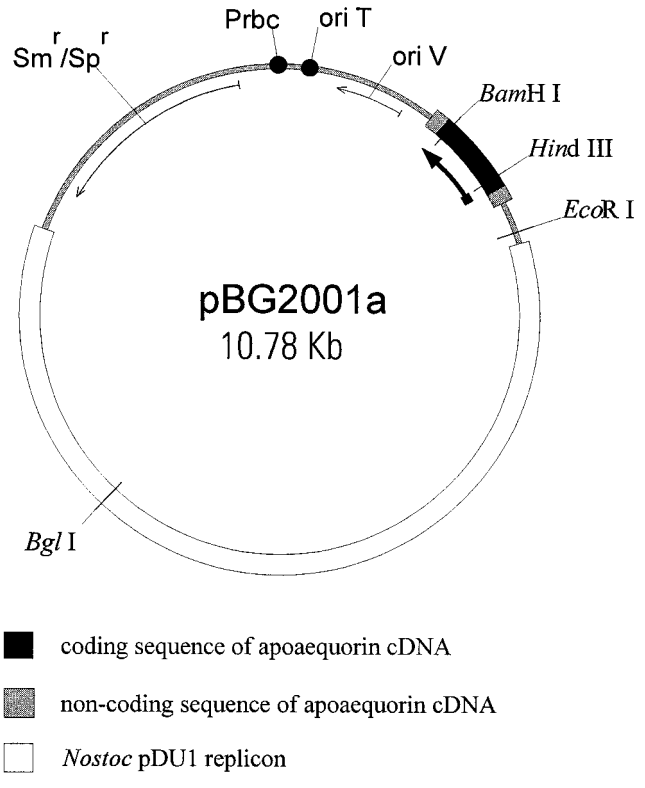

Figure 11. Physical map of the apoaequorin expression vector pBG2001a. Only positions of relevant restriction sites are indicated. The thick black arrow indicates the direction of transcription of the apoaequorin gene.

Aequorin Luminescence Measurements

Aequorin light emission was measured using a digital luminometer (Bio Orbit 1250, Turku, Finland). The luminometer was calibrated by setting the background counts to 0 and a 0.26 μCi of ^{14}C internal standard to 10 mV. Coelenterazine-treated cultures (0.5 mL) were transferred to a luminometer cuvette and luminescence was recorded every 1 s for the duration of the experiment. At the end of the experiment, the remaining reconstituted aequorin was estimated by discharging with the addition of an equal volume of 100 mM $CaCl_2$ and 5% (v/v) Triton X-100.

In Vitro Reconstitution of Apoaequorin and Quantitation of Intracellular Ca^{2+} Concentrations

In vitro calibration of recombinant apoaequorin was made by exposing cell lysates of apoaequorin-expressing cells to solutions with known Ca^{2+} concentrations. For this purpose, the apoaequorin-expressing *Anabaena* strain sp. PCC7120 strain was grown in BG11 medium plus 2.5 μg spectinomycin dihydrochloride mL^{-1}, washed, and resuspended in a buffer solution containing 0.5 mM EGTA, 10 mM HEPES/NaOH (pH 7.2), and 0.8 mM paramethylsulfoxide, and lysed in a French press (140 MPa). After centrifuging at 23,000*g* for 10 min at 4°C, the cell pellet was discarded. The supernatant (cell lysate) was utilized for the experiments after reconstituting apoaequorin with 5 μM coelenterazine for 4 h in the presence of 5 mM β-mercaptoethanol, as described previously (Shimomura et al., 1990). The Ca^{2+} buffers used were the Ca^{2+} calibration kits no. 2 and no. 3 (C-3009 and C-6775, respectively, Molecular Probes Europe, Leiden, The Netherlands). Kit no. 2 provides the following free Ca^{2+} concentrations: 0, 0.017, 0.038, 0.065, 0.100, 0.150, 0.225, 0.351, 0.602, 1.35, and 39.8 μM. Kit no. 3 provides the following free Ca^{2+} concentrations: 0, 1.35, 2.85, 5, 10, 20, 30, 50, 75, 100, and 1,000 μM. In the experiment, 50 μL of the cell lysate at 28°C was added to a luminometer cuvette and transferred to the sample chamber of the luminometer, light emission recording was started, and 950 μL of the Ca^{2+} buffer at 28°C was injected in the chamber. After approximately 1 min, 100 μL of a 100 mM $CaCl_2$ solution was injected and recording was continued until all aequorin was consumed; i.e. until light emission returned to basal values. Based on the experimental data, the recombinant aequorin calibration curve at 28°C was obtained as explained in the text. The in vitro calibration of recombinant apoaequorin was also made at 44°C and at 10°C; both the cell lysate and the Ca^{2+}-EGTA buffers were maintained at such temperatures before and during luminescence measurements.

Heat Shock Treatments of Apoaequorin-Expressing *Anabaena* Cells

Heat shock was induced in two different ways. In the first, reconstituted cell suspensions were placed in luminometer cuvettes that were immersed in a water bath at 44°C and removed at specific times to monitor luminescence. At the end of the experiment, aequorin was completely discharged by cell lysis and the addition of a saturating Ca^{2+} concentration in the luminometer. In the second method of inducing heat shock, reconstituted cell suspensions were placed in a cuvette in the luminometer chamber and directly irrigated at temperatures up to 65°C to achieve a final temperature of 44°C by injecting 0.5 mL of hot water or hot BG11 medium in the cuvette via a light-tight 1-mL syringe inserted into a light-tight port in the luminometer sample housing. Changes in luminescence were then instantaneously recorded. At the end of the experiment, the remaining aequorin was completely discharged as already described.

Intracellular free Ca^{2+} concentrations were calculated from the luminescence data according to our calibration curve at 44°C, as explained in the text.

Cold Shock Treatments of Apoaequorin-Expressing *Anabaena* Cells

Cold shock was induced in two different ways. In the first, reconstituted cell suspensions were placed in luminometer cuvettes immersed into a water bath at 0°C, and removed at specific times to monitor luminescence. At the end of the experiments, aequorin was completely discharged as already described. In the second method, reconstituted cell suspensions were placed in a cuvette in the luminometer chamber and directly irrigated at 0°C to achieve a final temperature of around 10°C by injecting 0.5 mL of ice-cold water or ice-cold BG11 medium in the cuvette via a light-tight 1-mL syringe inserted into a light-tight port in the luminometer sample housing. Changes in the luminescence were then instantaneously recorded. At the end of the experiment, the remaining aequorin was completely discharged as already described.

Intracellular free Ca^{2+} concentrations were calculated from the luminescence data according to our calibration curve at 10°C, as explained in the text.

Extracellular Ca^{2+}, Ca^{2+} Chelator, Ca^{2+} Agonist, and Inhibitor Treatment

When $CaCl_2$, EGTA, $LaCl_3$, verapamil, trifluoperazine (TFP), calcimycin (compound A23187), and diltiazem were used, aequorin reconstitution was performed as described above, followed by the incubation with the above-mentioned chemicals at the concentrations and times indicated in the figure legends. After the incubation time, treated cells were challenged with increasing external Ca^{2+} concentrations or were heat or cold shocked and used for luminescence measurements. For these treatments, stock solutions of $CaCl_2$, EGTA, $LaCl_3$, TFP, and diltiazem were made by dissolving these compounds in water at 10, 10, 10, 1, and 50 mM, respectively. Stock solutions of verapamil and calcimycin (compound A23187) were made by dissolving these compounds in ethanol at 200 and 10 mM, respectively; in the latter case, the amount of ethanol that was present in the assay at the highest concentration of inhibitor added was never above 1‰ to 2‰.

To determine the response of aequorin to each of the inhibitors and to the solvent used for verapamil and calcimycin (ethanol at final concentrations of 1‰-2‰), cell lysates of the apoaequorin-expressing *Anabaena* strain sp. PCC7120 were reconstituted with coelenterazine, as already described, and aliquots of 0.5 mL were treated with the inhibitors at the same concentrations and times as those used for the in vivo treatments. After the treatments, the aliquots were taken to the luminometer chamber, and aequorin was completely discharged by adding an equal volume of 100 mM $CaCl_2$ to determine the total light output. From all of the tested compounds (including the solvent), only 1 mM La^{3+} directly stimulated the luminescence in the lysate assay (see "Results").

Stability Test of Recombinant Aequorin to Heat and Cold Treatment

To test the stability of recombinant aequorin to heat and cold treatment, cell lysates of apoaequorin-expressing *Anabaena* strain sp. PCC7120 were reconstituted with coelenterazine as described above, and aliquots of 1 mL were cold or heat shocked for different times. After the treatment, the aliquots were taken to the luminometer chamber and aequorin was completely discharged by adding an equal volume of 100 mM $CaCl_2$ to determine the total light output.

Cell Lysis Check

For each of the treatments used in the present work, the occurrence of cell lysis was checked by the following methods: (a) examination by optical microscopy; (b) measurements of luminescence after addition of Ca^{2+} to the medium in which reconstituted cells were present after removing the cells by centrifugation; (c) measurement of phycobiliproteins in the medium in which reconstituted cells were present after removing the cells by centrifugation.

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