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A calcium signal is involved in heterocyst differentiation in the cyanobacterium *Anabaena* sp. PCC7120

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The impact of calcium signals in virtually all cells has led to the study of their role in prokaryotic organisms as stress response modulators. Cell differentiation in adverse conditions is a common Ca^{2+}-requiring response. Nitrogen starvation induces the differentiation of N_2-fixing heterocysts in the filamentous cyanobacterium *Anabaena* sp. PCC7120. This paper reports the use of a recombinant strain of this organism expressing the photoprotein aequorin to monitor the intracellular free-calcium concentration during the course of heterocyst differentiation. A specific calcium signature that is triggered exclusively when cells are deprived of combined nitrogen and generated by intracellular calcium stores was identified. The intracellular calcium signal was manipulated by treatment with specific calcium drugs, and the effect of such manipulation on the process of heterocyst differentiation was subsequently assessed. Suppression, magnification or poor regulation of this signal prevented the process of heterocyst differentiation, thereby suggesting that a calcium signal with a defined set of kinetic parameters may be required for differentiation. A *hetR* mutant of *Anabaena* sp. PCC7120 that cannot differentiate into heterocysts retains, however, the capacity to generate the calcium transient in response to nitrogen deprivation, strongly suggesting that Ca^{2+} may be involved in a very early step of the differentiation process.

INTRODUCTION

The prospect of Ca^{2+} having an important role as an intracellular second messenger in prokaryotic, as well as in eukaryotic, organisms is currently supported by a substantial amount of literature. Since Ca^{2+} was first studied in bacteria fifty years ago (Norris & Jensen, 1957) it has been implicated in a broad range of physiological processes in prokaryotes that include chemotaxis and motility (Tisa & Adler, 1992, 1995; Watkins *et al.*, 1995; Pitta *et al.*, 1997), pathogenesis (Rose *et al.*, 1993; Straley *et al.*, 1993), the cell cycle and the control of the initiation of replication (Jiménez-Sanchez *et al.*, 1993; Yu & Margolin, 1997), quorum sensing (Wherten & Lundgren, 2001) and spore and fruiting body formation (Inouye *et al.*, 1983; O'Hara & Hageman, 1990). In many cases, the involvement of Ca^{2+} in the regulation of cellular processes has been roughly described in terms of an influx or efflux of Ca^{2+} from the cytosol. The application in bacteria of the calcium detection method based on the calcium-binding luminescent photoprotein aequorin (Knight *et al.*, 1991) has allowed the monitoring of intracellular free-calcium concentration ($[Ca^{2+}]_i$) in such a way that fluxes of free calcium in prokaryotic cells can be precisely portrayed. In this regard, our group has been the first to report direct evidence of Ca^{2+} signalling in cyanobacteria (Torrecilla *et al.*, 2000, 2001, 2004).

Cyanobacteria are a large group of photosynthetic, oxygen-evolving prokaryotes. Many of them are also capable of fixing atmospheric N_2, a process that requires nitrogenase to be protected from oxygen. The filamentous cyanobacterium *Anabaena* sp. PCC7120 differentiates specialized cells called heterocysts that create a microoxic environment for nitrogen fixation; heterocysts normally form at semi-regular intervals along the filaments, following a developmental pattern, as a response to nitrogen deprivation. Heterocyst differentiation follows a specific scheme and requires global changes in gene expression (reviewed by Wolk *et al.*, 1994; Wolk, 1996, 2000, Golden & Yoon, 1998, 2003) but an overall model of the regulatory networks controlling development remains elusive. In the regulation of early events in the process of differentiation, the expression patterns of several genes have been studied and a few have been placed into an ordered sequence; among

Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester); $[Ca^{2+}]_i$, intracellular free-Ca^{2+} concentration; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; TFP, trifluoperazine.

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these, *hetR* is a key regulator of heterocyst development that starts to be activated within 2 h and is already active in spaced cells in the filament within 3·5 h of nitrogen deprivation. *Anabaena* sp. PCC7120 *hetR* mutants fail to differentiate (Wolk *et al.*, 1994, Black *et al.*, 1993). *hetR* encodes an unusual serine-type protease that has been suggested to have a calcium-modulated activity (Zhou *et al.*, 1998; Dong *et al.*, 2000).

The involvement of a second messenger in the process of heterocyst development is not clear. Hood *et al.* (1979) reported that in *Anabaena variabilis* the cAMP levels increased fourfold after nitrogen removal but more recent studies have cast doubt on the role of cAMP in nitrogen metabolism in cyanobacteria (Cann, 2003). However, growing evidence indicates that calcium might truly be involved in heterocyst development; in early studies Smith *et al.* (1987) noted that the external concentration of Ca^{2+} and the presence of several calcium agonists influenced heterocyst frequency in *Nostoc* PCC 6720. Later studies found a correlation between heterocyst frequency and different treatments that affect the accumulation of Ca^{2+} in cells during the process of differentiation (Smith, 1988; Smith & Wilkins, 1988; Zhao *et al.*, 1991; Onek & Smith, 1992), hence reinforcing the hypothesis that Ca^{2+} might have a regulatory role in the differentiation of heterocysts.

We have therefore investigated intracellular free calcium changes following nitrogen deprivation in strain *Anabaena* sp. PCC7120(pBG2001a), which constitutively expresses recombinant aequorin (Torrecilla *et al.*, 2000). We have found a Ca^{2+} transient with a specific calcium signature (spatial and temporal characteristics of stimuli-specific calcium transients) that starts shortly after nitrogen deprivation. We have also shown that a *hetR* mutant retains the calcium transient. Finally, evidence is presented that indicates a correlation between the suppression or alteration of such calcium signals and a subsequent early arrest of heterocyst differentiation.

METHODS

Organisms and growth conditions. The *Anabaena* sp. PCC7120(pBG2001a) strain expressing apoaequorin (Torrecilla *et al.*, 2000) was routinely grown in 100 ml conical flasks containing 50 ml BG11 medium with 25 mM HEPES/NaOH (pH 7·2) and 2·5 μg spectinomycin ml^{-1}, along with the standard calcium concentration (0·25 mM) and standard nitrate concentration (18 mM); for some experiments, $NaNO_3$ was replaced by 5 mM NH_4Cl in the growth medium as the sole source of combined nitrogen. The calcium concentration was also modified when required. The *hetR* mutant strain of *Anabaena* sp. PCC7120 expressing apoaequorin was selected after conjugal transfer of plasmid pBG2001a and was grown as described above with 2·5 μg spectinomycin ml^{-1} and 40 μg neomycin ml^{-1}. Cell cultures were incubated on a rotatory shaker at 28 °C under 65 μE m^{-2} s^{-1} fluorescent white light.

***In vivo* aequorin reconstitution and luminescence measurements.** For aequorin luminescence measurements, *in vivo* reconstitution of aequorin was performed by the addition of 2·5 μM coelenterazine (Molecular Probes) to cell suspensions (15 μg chlorophyll ml^{-1}) and incubation for 4 h in darkness and with shaking, as previously described (Torrecilla *et al.*, 2000). Excess coelenterazine was removed before Ca^{2+} measurements were taken.

Luminescence measurements were made using a digital luminometer with a photomultiplier (BioOrbit 1250). Reconstituted cell suspensions (0·5 ml) in a transparent polystyrene cuvette were placed in the luminometer and luminescence was recorded every 1 s for the duration of the experiment.

Calibration of the $[Ca^{2+}]_i$ changes requires the knowledge of the total available amount of reconstituted aequorin in cell suspensions (L_{max}) at any one point in time during the experiment, as well as the running luminescence (L_0). For estimation of total aequorin luminescence, the remaining aequorin was discharged at the end of the experiment by the addition of 0·5 ml 100 mM $CaCl_2$ and 5 % (v/v) Triton X-100. Rate constants of luminiscence (L_0 $L_{max}{}^{-1}$) were determined for each point along the experiment, and $[Ca^{2+}]_i$ was calculated by using calibration curves obtained for aequorin extracted from the recombinant strain of *Anabaena* sp. PCC7120, according to Torrecilla *et al.* (2000).

Nitrogen deprivation experiments. Before nitrogen deprivation, cell suspensions of *Anabaena* sp. PCC7120(pBG2001a) grown with nitrate or ammonium were reconstituted with coelenterazine. To induce heterocyst formation, reconstituted cell suspensions were washed four times with combined-nitrogen-free medium (medium $BG11_0$, buffered with 25 mM HEPES/NaOH to pH 7·20), followed by incubation in the same combined-nitrogen-free medium. When the experiments required total absence or a lower or higher concentration of Ca^{2+} than the standard, a modified $BG11_0$ with the required calcium concentration was used for nitrogen deprivation. When needed, 200 μM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; Sigma), 5 μM compound A23187 (Sigma) or 5 μM trifluoperazine (TFP; Sigma) was added at this step. To facilitate cell loading of the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxy-methyl ester) (BAPTA-AM; Molecular Probes), it was added at a final concentration of 300 μM 1 h before aequorin reconstitution and then again at this step.

Bright-field and fluorescence micrographs were taken with an Olympus BH-2 microscope equipped with epifluorescence and a digital camera Leica DC 300F. Proheterocysts and mature heterocysts were clearly discernible at 10 and 20 h, respectively, after nitrogen deprivation. Heterocyst frequency was calculated as described by Smith & Wilkins (1988).

Statistical procedures. All tests of statistically significant differences between datasets were performed using Student's *t*-tests or analysis of variance at $P < 0.05$ with the program SigmaStat. All data were obtained from a minimum of three repetitions for each assay situation.

RESULTS

Calcium signals in *Anabaena* in response to nitrogen deprivation

Cell suspensions of *Anabaena* sp. PCC7120(pBG2001a) were first incubated with coelenterazine in order to reconstitute aequorin, and nitrogen deprivation experiments to induce heterocyst differentiation were performed as described in experimental procedures. Immediately after the removal of nitrate from the growth medium, samples were placed in the luminometer chamber in order to monitor the

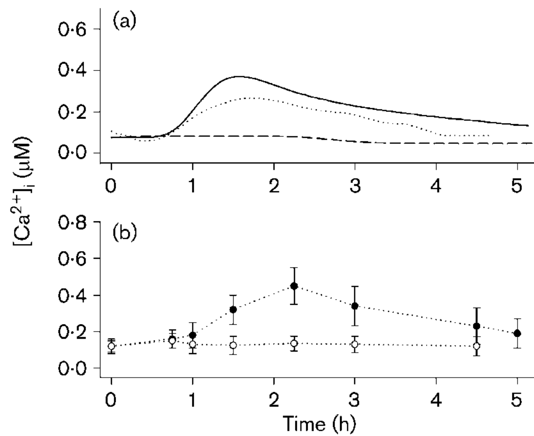

Fig. 1. Effect of combined-nitrogen deprivation on the $[Ca^{2+}]_i$ of *Anabaena* sp. PCC7120(pBG2001a). (a) Cultures of the cyanobacterium were grown in BG11 with 18 mM $NaNO_3$ (solid line) or 5 mM NH_4Cl (dotted line). After loading the cell suspensions with coelenterazine, they were washed four times with $BG11_0$ medium and the luminescence was continuously measured in the luminometer. (b) Cell suspensions were washed four times with $BG11_0$ medium and then were kept under 65 $\mu E\ m^{-2}\ s^{-1}$ fluorescent white light (filled circles). At particular time intervals, punctual measurements of $[Ca^{2+}]_i$ were made. As a control, a cell suspension was washed four times with BG11 medium with nitrate and resuspended in the same medium [dashed line in (a) and open circles in (b)]. The traces have been chosen to best represent the mean results from at least five repetitions.

changes in $[Ca^{2+}]_i$. In this way we could detect a specific and reproducible calcium transient with a particular calcium signature, as represented in Fig. 1(a). This transient began to rise around 45 min after the shift from nitrate to N_2, and gradually increased in magnitude, reaching a maximum $[Ca^{2+}]_i$ value of approximately $0{\cdot}39 \pm 0{\cdot}12$ μM (n = 32) nearly 1 h later; a slow decrease followed afterwards until the $[Ca^{2+}]_i$ reached its original basal level of around 100–200 nM. The total length of the transient was nearly 4 h. Therefore, this calcium transient was relatively low but quite long in comparison with other calcium transients recorded in this cyanobacterium (Torrecilla *et al.*, 2000, 2001, 2004).

As a control, cell suspensions were washed four times with BG11 medium containing the standard nitrate concentration and finally resuspended in the nitrate-containing medium; these cells suspensions did not show any subsequent significant alteration in $[Ca^{2+}]_i$ (Fig. 1a), which indicated that the observed calcium transient was not due to the manipulation of the sample nor to the maintenance of the sample in darkness during the measurements.

To check whether this calcium transient was a specific response to nitrate deprivation or a response to a lack of combined nitrogen, cultures were grown for several days in a modified BG11 medium containing 5 mM ammonium (NH_4Cl) instead of nitrate as the nitrogen source. The subsequent removal of ammonium from the medium gave rise to a calcium transient (Fig. 1a) with very similar features to that described above for removal of nitrate, though the maximum $[Ca^{2+}]_i$ reached was slightly lower, $0{\cdot}31 \pm 0{\cdot}11$ μM (n = 5). Likewise, as a control, cell suspensions washed several times with BG11 with ammonium and finally resuspended in the ammonium-containing medium did not show any change in the levels of $[Ca^{2+}]_i$ (data not shown). Also, a shift in the medium from nitrate to ammonium did not induce the calcium transient (not shown).

The experimental procedures for these measurements, which rely on the continuous detection of the luminescence from the cell suspension, meant that the sample had to be kept in total darkness for several hours. To test the possible effect of such absence of light during the time of measurement on the course of heterocyst differentiation, cell suspensions were transferred after measurements from the luminometer chamber to a thermostatic chamber at 28 °C under white light at a light intensity of 65 $\mu E\ m^{-2}\ s^{-1}$. After 20 h, it was verified that the morphology and the frequency of the heterocysts that had differentiated was not significantly different from those of the sample that had been kept under continuous light (frequencies of 10·2 % and 11·0 %, respectively). This result indicated that exposure to darkness for 4–5 h after nitrogen withdrawal did not affect the process of differentiation. Nonetheless, since light is needed for heterocyst differentiation to take place correctly in the obligate autotroph *Anabaena* sp. PCC7120 (Wolk *et al.*, 1994), we checked whether the observed 'calcium signature' also appeared under light conditions. For this purpose, discontinuous recording of luminescence data at specific intervals was performed. Thus, a cell culture deprived of combined nitrogen was divided into several aliquots and kept under light at a light intensity of 65 $\mu Es\ m^{-2}\ s^{-1}$. At specific time intervals, aliquots were transferred, one by one, to the luminometer chamber and the stable level of $[Ca^{2+}]_i$ was determined. As can be seen in Fig. 1(b), a defined calcium transient also occurred under light conditions, with a magnitude and duration very similar to that described when samples were kept in the luminometer continuously for several hours after nitrogen deprivation. The maximum stable calcium level reached for the assayed times was $0{\cdot}45 \pm 0{\cdot}11$ μM (n = 5), which corresponded to a time of 2 h 35 min. Thus, the observed calcium transient seems to be a physiological response to combined-nitrogen deprivation.

The data presented above strongly suggest that the observed calcium transient is triggered by the withdrawal of the source of combined nitrogen from the external medium. Nevertheless, with the aim of further corroborating the specificity of the calcium response, we investigated the possibility that deprivations of other nutrients, such as iron or phosphate, might also induce an analogous early

calcium transient. Reconstituted cultures of *Anabaena* were washed several times with modified BG11 prepared with the omission of the corresponding salts (ferric-ammonium citrate or K_2HPO_4) and finally resuspended in the medium without iron or phosphate. In both cases, the $[Ca^{2+}]_i$ of the cell suspensions remained at basal level (not shown). Nonetheless, although the $[Ca^{2+}]_i$ was monitored for the subsequent 6–7 h after the deprivation of such nutrients, the possibility of the occurrence of calcium transients later on, once the iron or phosphate deficiencies are more pronounced, cannot be rejected.

Determination of the possible cellular origin of the calcium signature induced by combined-nitrogen deprivation

In order to establish the source of calcium responsible for the observed $[Ca^{2+}]_i$ transient, nitrogen deprivation experiments were performed in which cells were finally resuspended in $BG11_0$ medium either without calcium and with 200 μM of the extracellular calcium chelator EGTA or supplemented with calcium up to 5 mM. As shown in Fig. 2, extracellular calcium depletion was not reflected by a diminution in or suppression of the observed calcium transient; the transient had very similar features, in terms of amplitude and kinetics, to those seen with the standard calcium concentration (the highest free-calcium concentration reached was $0{\cdot}32\pm0{\cdot}10$ μM [n=5]). Likewise, the increase in the external calcium concentration up to 5 mM had no significant effect on the magnitude and kinetics of the calcium transient. Therefore, these data suggest that the appearance of the calcium transient is independent of an extracellular source of calcium and thus, the main source of calcium seems to be intracellular.

Effect of artificial alteration of the Ca^{2+} signal on heterocyst differentiation

With the final objective of testing whether the detected calcium transient is involved in the process of heterocyst differentiation, experiments were performed in order to alter this calcium transient and check the effect of such manipulation on the process of differentiation. In eukaryotic cells, alterations in Ca^{2+} responses producing 'altered'

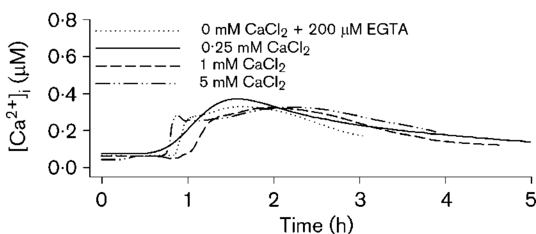

Fig. 2. Effect of EGTA and of increasing calcium concentration on the calcium transient induced by nitrogen deprivation. The traces have been chosen to best represent the mean results from at least three repetitions.

calcium signatures have been shown to have profound effects on the physiological responses mediated by Ca^{2+} (Dolmetsch *et al.*, 1997; Allen & Schroeder, 2001). The calcium transient was altered by adding agents that, according to the literature and to previous experiments in our laboratory, affect calcium homeostasis (Torrecilla *et al.*, 2000, 2001). Calcimycin (compound A23187) is a calcium ionophore that promotes the influx of calcium from the extracellular space; trifluoperazine (TFP) is an inhibitor of eukaryotic calmodulin that affects the regulation of Ca^{2+} signals; and BAPTA-AM is an uncharged calcium-binding molecule that can permeate cell membranes and be cleaved by nonspecific esterases, resulting in a charged form that leaks out of the cell very slowly and therefore acts as an intracellular calcium chelator.

Two sets of contrasting experiments were performed with the Ca^{2+} agents: firstly, agents were added just after nitrogen deprivation so that the agent was present from the beginning of the differentiation process and should, therefore, alter the calcium transient; and secondly, agents were added once the calcium transient was over and the basal intracellular calcium level had recovered (approximately 6 h after nitrogen removal). For both sets of experiments, changes in intracellular calcium levels were continuously measured in the luminometer and, in parallel, the course of differentiation was monitored by bright-field and fluorescence microscopy. Fluorescence microscopy was used to check for patterned loss of red fluorescence which occurs due to the degradation of phycobiliprotein at an early stage of development in cells committed to become heterocysts (Wolk, 2000; Wood & Haselkorn, 1980). As a control, both Ca^{2+} monitoring and microscopy observations were performed in cell suspensions not treated with the calcium agents. Also, the three agents at the tested concentrations did not have any significant effect on vegetative growth in nitrate-containing medium during the experimentation time (not shown).

Effect of the calcium ionophore compound A23187. Previous results in our laboratory have shown that compound A23187 causes a significant elevation of the Ca^{2+} transients induced by environmental shocks in *Anabaena* sp. PCC7120 (Torrecilla *et al.*, 2001). As represented in Fig. 3, when the cell cultures were pretreated with 5 μM A23187 just after nitrogen deprivation, the shape of the induced calcium signature changed dramatically, resulting in a significant rise in the magnitude of the Ca^{2+} elevation ($0{\cdot}74\pm0{\cdot}26$ μM [n=6] versus $0{\cdot}39\pm0{\cdot}12$ μM [n=32] in the control) and in a longer duration of the transient, as 5 μM of the ionophore did not restore $[Ca^{2+}]_i$ basal levels even after 5 h (Fig. 3a). The effect of the addition of 5 μM A23187 immediately after nitrogen removal on heterocyst differentiation was remarkable; no heterocysts or proheterocysts were observed 24 h after nitrogen removal, or even 3 days after nitrogen removal, and no loss of phycobiliprotein fluorescence was observed either (Fig. 3b, c), indicating that heterocyst differentiation

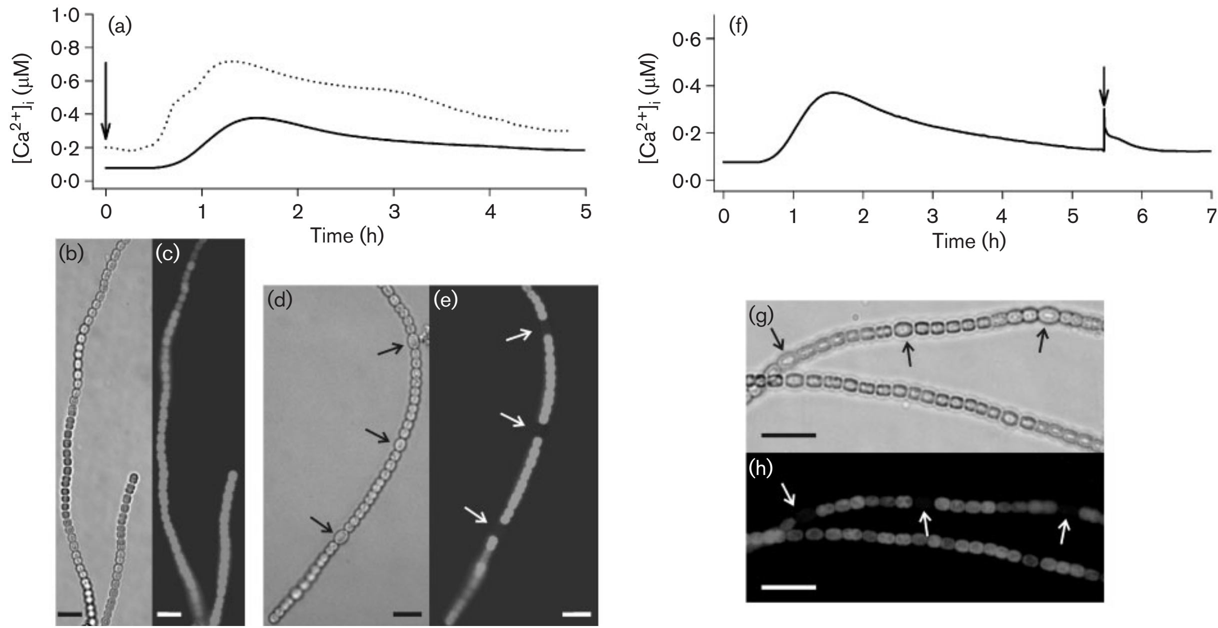

Fig. 3. Effect of the addition of 5 μM of the calcium ionophore compound A23187 on the calcium transient induced by nitrogen deprivation (a, f) and on the process of heterocyst differentiation (b, c, g, h). The ionophore was added either immediately after nitrogen deprivation (a–c) or after approximately 6 h (f–h), at the timepoints indicated by an arrow in (a) and (f). The traces shown in (a) and (f) have been chosen to best represent the mean results from at least three repetitions. In (a) the dotted line represents the suspension with A23187 added and the solid line represents the suspension with no A23187 added. Micrographs of *Anabaena* filaments after 24 h of nitrogen deprivation are shown (b–e, g, h). Control cells not treated with the ionophore are shown in (d) and (e). Bright-field and fluorescence images of the same filament are shown. Arrows indicate mature heterocysts. Bars, 20 μm.

had been arrested at an early stage of development. Control cultures without A23187 showed a normal pattern of heterocyst differentiation (Fig. 3d, e). However, when the ionophore was added after the Ca^{2+} transient was over and $[Ca^{2+}]_i$ basal levels had been restored, it did not modify calcium basal levels except for a short spike that represented a small, mechanically induced Ca^{2+} increase upon ionophore injection (Torrecilla *et al.*, 2000) and did not have any effect on the process of heterocyst differentiation (Fig. 3f–h). The data obtained with the calcium ionophore A23187 indicated that a significant alteration of the specific calcium signature, by means of a change in signal amplitude and duration, is correlated with an early inhibition of heterocyst differentiation. In addition, these results rule out the possibility that the ionophore itself (Fig. 3g, h) could impair differentiation.

Effect of the calmodulin inhibitor TFP. As shown in Fig. 4(a), the addition of 5 μM TFP immediately after nitrogen deprivation yielded a prolonged calcium transient that affected the homeostasis of the signal as no return to $[Ca^{2+}]_i$ basal levels occurred during the time of measurement. Thus, after nitrogen removal $[Ca^{2+}]_i$ rose to $0{\cdot}72 \pm 0{\cdot}27$ μM (n = 8) in the presence of 5 μM TFP. TFP treatment just after nitrogen deprivation suppressed heterocyst differentiation at an early stage since no loss of fluorescence along the filament could be observed (Fig. 4b, c). Control cultures without TFP showed a normal pattern of heterocyst differentiation (not shown). On the other hand, when TFP was added once the calcium transient induced by nitrate removal was over (6 h after nitrogen deprivation), it did not modify Ca^{2+} basal levels, except for the short spike upon addition, and did not have any remarkable effect on heterocyst differentiation (Fig. 4d, e). Therefore, the results with TFP suggest that prolonged elevated $[Ca^{2+}]_i$ levels correlate with an early arrest of heterocyst differentiation. This effect is comparable to that of A23187, suggesting that a specific calcium signature is involved in the process of differentiation. The results also indicate (Fig. 4e, f) that TFP itself does not impair heterocyst differentiation.

Effect of the intracellular calcium chelator BAPTA-AM. As shown in Fig. 5(a), when cell cultures were pretreated with 300 μM BAPTA-AM and thereafter subjected to nitrogen deprivation no calcium transient was induced since $[Ca^{2+}]_i$ remained at basal levels with no apparent significant increase. The effect of the early addition of BAPTA-AM was also reflected in the inhibition of heterocyst differentiation. The observation that all cells in the filament preserved fluorescence 24 h after nitrate removal suggested that heterocyst differentiation was inhibited at

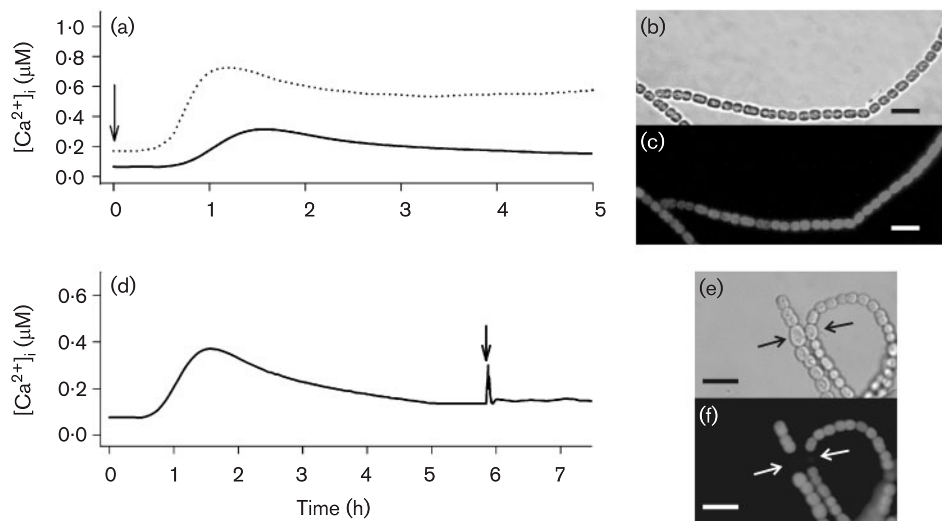

Fig. 4. Effect of the addition of 5 μM of the calmodulin inhibitor trifluoperazine (TFP) on the calcium transient induced by nitrogen deprivation (a, d) and on the process of heterocyst differentiation (b, c, e, f). TFP was added either immediately after nitrogen deprivation (a–c), or after approximately 6 h (d–f), at the timepoints indicated by an arrow in (a) and (d). The traces shown in (a) and (d) have been chosen to best represent the mean results from at least three repetitions. In (a) the dotted line represents the suspension with TFP added and the solid line represents the suspension with no TFP added. Micrographs of *Anabaena* filaments after 24 h of nitrogen deprivation are shown (b, c, e, f). Bright-field and fluorescence images of the same filaments are shown. Arrows indicate mature heterocysts. Bars, 20 μm.

an early stage of differentiation (Fig. 5b, c). Control cultures not treated with BAPTA-AM showed a normal pattern of heterocyst differentiation (not shown). Likewise, as in the case of A23187 and TFP, when BAPTA-AM was added after the $[Ca^{2+}]_i$ had returned to the basal level (approximately 6 h after nitrogen deprivation) no

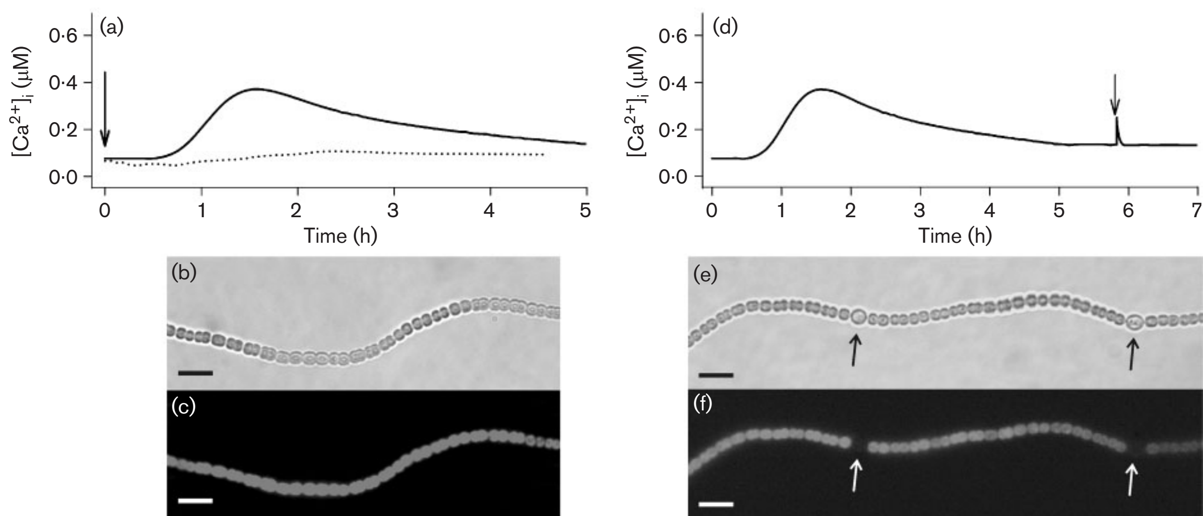

Fig. 5. Effect of the addition of 300 μM of the intracellular calcium chelator BAPTA-AM on the calcium transient induced by nitrogen deprivation (a, d) and on the process of heterocyst differentiation (b, c, e, f). BAPTA-AM was added 1 h before reconstitution of apoaequorin with coelenterazine and again either immediately after nitrogen deprivation (a–c), or after approximately 6 h (d–f), at the timepoints indicated by an arrow in (a) and (d). The traces shown in (a) and (d) have been chosen to best represent the mean results from at least three repetitions. In (a) the dotted line represents the suspension with BAPTA-AM added and the solid line represents the suspension with no BAPTA-AM added. Micrographs of *Anabaena* filaments after 24 h of nitrogen deprivation are shown (b, c, e, f). Bright-field and fluorescence images of the same filaments are shown. Arrows indicate mature heterocysts. Bars, 20 μm.

significant changes in $[Ca^{2+}]_i$ were detected, except for the short spike upon addition of the chelator, and heterocyst differentiation took place normally (Fig. 5d–f). These data suggest that the suppression of the signal arrests heterocyst differentiation and that there is no negative effect of BAPTA-AM itself on differentiation (Fig. 5e, f).

The results obtained independently by using A23187, TFP and BAPTA-AM strongly suggest that a calcium transient with a defined set of kinetic parameters (i.e. a specific calcium signature) is needed for the process of heterocyst differentiation. The suppression or alteration of such a calcium signature leads to an early arrest of differentiation.

Calcium signals in a *hetR* mutant of *Anabaena* sp. PCC7120

A recombinant strain of an *Anabaena* sp. PCC7120 *hetR* mutant expressing apoaequorin was constructed. Due to the critical and early involvement of *hetR* in heterocyst differentiation, we wanted to know whether the observed calcium transient following nitrogen deprivation was induced in the *hetR* mutant. As Fig. 6 shows, a calcium transient with quite similar features regarding amplitude and kinetics to those found in the wild-type strain could be detected, suggesting that the calcium transient may be an upstream event to *hetR* expression, being involved in an earlier step of the differentiation process.

DISCUSSION

There are several examples in the literature where a requirement for calcium in bacterial (Norris *et al.*, 1996) and eukaryotic (Renzel *et al.*, 2000) differentiation has been demonstrated. In addition, protein degradation during sporulation of *Bacillus subtilis* has been reported to be a calcium-dependent process (O'Hara & Hageman, 1990), and in cyanobacteria it has long been recognized that there is a correlation between extracellular calcium concentration and heterocyst frequency (Smith & Wilkins, 1988; Zhao *et al.*, 1991; Onek & Smith, 1992). However, our work represents probably the first example of continuous monitoring of $[Ca^{2+}]_i$ during a process of cell differentiation in a prokaryotic organism.

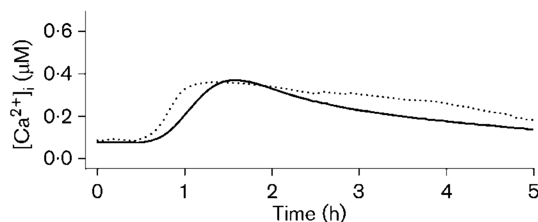

Fig. 6. Effect of combined-nitrogen deprivation on the $[Ca^{2+}]_i$ of the *hetR* mutant of *Anabaena* sp. PCC7120(pBG2001a) expressing apoaequorin. The wild-type strain calcium trace is shown (solid line) for comparison. The traces have been chosen to best represent the mean results from at least five repetitions.

We have observed that upon nitrogen deprivation, resulting from either removing nitrate or ammonium, an early calcium transient is triggered that constitutes a specific calcium signature that differs from the calcium signatures observed upon exposure of the cyanobacteria to other environmental stresses such as heat and cold shock, acid shock, osmotic and salt stress and light-to-dark transitions (Torrecilla *et al.*, 2000, 2001, 2004). Compared with the previously tested environmental stimuli, the observed monophasic and bell-shaped Ca^{2+} signal reaches a modest magnitude (0·39 μM versus 3·10 μM after cold shock or 2·30 μM after a hyperosmotic shock) but lasts nearly 4 h (the transient duration of other environmental stimuli ranges from 3–4 min to over 1 h). Another main difference is that the calcium source responsible for the calcium elevation induced after nitrogen deprivation seems to be entirely intracellular, while for most of the other tested stimuli the calcium source appears to be mainly or entirely extracellular.

Although it has been proposed that calcium may merely act as a chemical switch in signal transduction (Scrase-Field & Knight, 2003), it is generally assumed that the spatio-temporal components of the increase in $[Ca^{2+}]_i$ or Ca^{2+} signature dictates the outcome of the cellular end response (Ng & McAinsh, 2003). There are a few examples in the literature where modulation or alteration of specific calcium signatures in eukaryotic cells correlates with a concomitant modulation of physiological responses (Dolmetsch *et al.*, 1997, 1998; Allen & Schroeder, 2001; Gu & Spitzer, 1995; Allen *et al.*, 2000, 2001). We have also manipulated the Ca^{2+} signal recorded upon nitrogen deprivation by using Ca^{2+} agents that affect calcium homeostasis (the ionophore A23187, the calmodulin inhibitor TFP and the intracellular calcium chelator BAPTA-AM) and have monitored the subsequent effect on the process of differentiation. We have found that suppression, magnification or poor regulation of the signal is correlated with an inhibition of heterocyst differentiation. However, addition of the calcium agents once the signal is over does not have any significant effect on Ca^{2+} basal levels, and heterocyst formation is not affected.

Zhao *et al.* (1991) also reported that the ionophore A23187 in the presence of external calcium inhibited heterocyst differentiation. However, if they added the ionophore 6–7 h after nitrogen deprivation, a time that noticeably matches with the end of the calcium signal that we report here, nitrogen differentiation was not arrested. They did not monitor intracellular free-calcium levels, nor could they detect the specific calcium signature. Subsequently they concluded that calcium may have a role in suppressing an early stage of heterocyst differentiation. However, our results suggest that a specific calcium signal with a defined set of temporal and kinetic parameters may be required for the process of heterocyst differentiation.

The observed effect of the eukaryotic calmodulin inhibitor TFP on the regulation of the Ca^{2+} signal and on heterocyst differentiation suggests the presence of calmodulin or calmodulin-like activities in *Anabaena* sp. PCC7120. Since 1984 there has been immunological as well as biochemical evidence for the presence of cyanobacterial calmodulins (Kerson *et al*., 1984); Pettersson & Bergman (1989) previously found a 17 kDa polypeptide with calmodulin-like activity in *Anabaena* sp. PCC7120 and in *Anabaena cylindrica*. Recent genomic analyses also revealed the existence of cyanobacterial proteins with Ca^{2+}-binding domains (Michiels *et al*., 2002; http://smart.embl-heidelberg.de).

The fact that a *hetR* mutant of *Anabaena* sp. PCC7120 also displays the calcium signature upon nitrogen deprivation suggests that the Ca^{2+} signal may be an upstream event that occurs before the induction of the expression of *hetR*, which is already active in spaced cells within 3·5 h of nitrogen deprivation. During the early phases of differentiation, intracellular protein degradation increases severalfold (Wood & Haselkorn, 1980); proteolysis appears to supply amino acids for heterocyst-specific protein synthesis and must play an important role in the differentiation process. Two proteolytic activities have been related to this process: a Ca^{2+}-stimulated protease degrading numerous proteins of vegetative cells *in vitro*, and a protease apparently specific for phycobiliproteins (Wood & Haselkorn, 1980).

Under our experimental conditions, alteration of the observed Ca^{2+} signal correlated with an early arrest of heterocyst differentiation as evidenced by the absence of patterned loss of phycobiliprotein fluorescence. Therefore it is tempting to correlate the calcium transient with the signalling for proteolysis of phycobiliproteins. In fact, in *B. subtilis* a Ca^{2+} influx has been shown to be essential for the early proteolysis that takes place during sporulation (O'Hara & Hageman, 1990; Shyu & Foegeding, 1991; Dominguez *et al*., 1999). However, the relevance of the calcium-dependent protease in the process of heterocyst differentiation remains unclear since Lockau *et al.* (1988) and Maldener *et al.* (1991) demonstrated that this protease is not essential for the development of functional heterocysts. Also, the non-differentiating *hetR* mutant of *Anabaena* sp. PCC7120 shows no loss of phycobiliprotein fluorescence but retains the Ca^{2+} signal, implying that the observed signal may not be directly related to phycobiliprotein degradation.

As a final point, it should be noted that the calcium transients presented in this work reflect the luminescence emerging from the whole population of filaments in the cell suspension, which raises two key questions to be taken into account. Firstly, the data can only be interpreted in terms of the mean $[Ca^{2+}]_i$ in the totality of cells in the sample, with no indication of the distribution of Ca^{2+} along the filaments. The method used to detect aequorin luminescence does not provide information on whether the increase in $[Ca^{2+}]_i$ is confined to a fraction of cells within the filament or whether the whole filament contributes evenly to the signal. In fact, in order to detect which cells in the filament were undergoing the increase in intracellular calcium, we tried to image, at the single cell level, calcium dynamics in our recombinant *Anabaena* strain, but we failed, probably due to the low quantum yield of aequorin luminescence. At present, we are trying to express in *Anabaena* a GFP cameleon construct (yellow cameleon 2.1) that has allowed successful calcium single-cell imaging in plant cells (Allen *et al*., 1999). Secondly, the observed calcium transient may be covering more complex individual dynamics such as calcium oscillations, which would be hidden by cells that are not in phase with each other. Also, further research is needed to identify the initial sensor or mechanism that triggers the Ca^{2+} signal upon nitrogen deprivation as well as the downstream responses, in order to better define the role of calcium in heterocyst differentiation.

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