Molecular Microbiology (2004) **54**(2), 291–297 doi:10.1111/j.1365-2958.2004.04276.x

MicroReview

Calcium signalling in bacteria

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Summary

Whereas the importance of calcium as a cell regulator is well established in eukaryotes, the role of calcium in prokaryotes is still elusive. Over the past few years, there has been an increased interest in the role of calcium in bacteria. It has been demonstrated that as in eukaryotic organisms, the intracellular calcium concentration in prokaryotes is tightly regulated ranging from 100 to 300 nM. It has been found that calcium ions are involved in the maintenance of cell structure, motility, transport and cell differentiation processes such as sporulation, heterocyst formation and fruiting body development. In addition, a number of calcium-binding proteins have been isolated in several prokaryotic organisms. The characterization of these proteins and the identification of other factors suggest the possibility that calcium signal transduction exists in bacteria. This review presents recent developments of calcium in bacteria as it relates to signal transduction.

Introduction

Among a wide variety of eukaryotic cells, ranging from fungi to mammals, the Ca^{2+} ion serves as a universal messenger, transmitting signals from the cell surface to the interior of the cell. Cells respond to a number of stimuli by transient changes in the intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$), which is tightly regulated. At rest, cells maintain an $[Ca^{2+}]_i$ at very low levels ($\approx 10^{-7}$ M) compared with the extracellular fluid (10^{-3} M). This creates a Ca^{2+} concentration gradient, which is utilized by cells to transmit information (Campbell, 1983). Depending on the signal and cell type, cytosolic free-Ca^{2+} can be increased by an influx of external calcium via calcium channels, a calcium release from intracellular stores or, more speculatively, by ion condensation (Manning, 1969; Ripoll *et al*., 2004). A decrease in cytosolic free calcium occurs through calcium efflux mechanisms such as ATP-requiring calcium pumps and anti-port systems, Ca^{2+}-binding proteins (CaBPs) and ion condensation. Thus, the net effect of all these mechanisms is to keep the $[Ca^{2+}]_i$ in the nanomolar range (Campbell, 1983). The ion condensation hypothesis suggests that because of the physicochemical constitution of the cytosol including the cell cytoskeleton, Ca^{2+} like other counterions condenses onto linear polymers at a critical value of the charge density (Manning, 1969; Ripoll *et al*., 2004). Ion condensation is a dynamic process, which is promoted by linear assemblies of charges. The composition of a population of condensed counterions of the same valence is similar to that of free ions. Because the condensed counterions include Ca^{2+}, condensation may play an important role in determining the free level of Ca^{2+} in the cytosol (Ripoll *et al*., 2004). Changes in $[Ca^{2+}]_i$ have been associated with the regulation of a wide variety of cellular processes as important and disparate as cell differentiation, transport, motility, gene expression, stress signals (cold and heat shock), metabolism, cell cycle and pathogenesis (Mekalanos, 1992; Clapham, 1995; Petterson *et al*., 1996; Sanders *et al*., 1999; Whitaker and Larman, 2001; Ikura *et al*., 2002).

Although a similar role for this divalent cation in prokaryotes is still elusive, there is increasing interest in and evidence for calcium as a regulator in bacteria. Important examples are the demonstration that bacteria keep tight control of their $[Ca^{2+}]_i$ with values very similar to those found in eukaryotes (100–300 nM) (Gangola and Rosen, 1987; Knight *et al*., 1991; Futsaether and Johnsson, 1994; Herbaud *et al*., 1998; Jones *et al*., 1999; Torrecilla *et al*., 2000). Like eukaryotes, bacterial cells have ion channels, primary and secondary transporters, and CaBPs, which may be involved in Ca^{2+} homeostasis (Norris *et al*., 1996; Paulsen *et al*., 2000; Waditee *et al*., 2004). Furthermore, there is evidence that calcium is involved in a number of bacterial processes such as maintenance of cell structure, motility, cell division, gene expression and cell differentiation processes such as sporulation, heterocyst formation and fruiting body development (Smith, 1995; Norris *et al*., 1996). Based on the aforementioned data, the question thus is, do Ca^{2+} ions play a similar role in bacteria as in eukaryotic cells? and if so, what are the molecular mech-

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anisms for calcium regulation in bacteria? Is the eukaryotic paradigm of CaBPs, Ca^{2+} transporters and Ca^{2+} stores applicable to prokaryotes? or perhaps the ion condensation hypothesis explains Ca^{2+} regulation in bacteria?

The last review on calcium in bacteria by Michiels *et al*. (2002) had an emphasis on CaBPs. The purpose of this article is to review the recent developments concerning the importance of calcium in bacteria as they relate to Ca^{2+}-signal transduction.

The ability to measure $[Ca^{2+}]_i$ in cells is essential in studying its role as an intracellular messenger. The idea that Ca^{2+} acts as a messenger is based on the concept that environmental signals induce changes in $[Ca^{2+}]_i$. Therefore, determining the cytosolic Ca^{2+} levels in response to stimuli has been crucial in establishing the role of calcium in signal transduction.

Measuring $[Ca^{2+}]_i$ in prokaryotic cells has been a challenge, because of unique structural features of bacteria (size, cell wall, cell membrane), toxic effects of reagents and the difficulty of manipulating live bacteria (Norris *et al*., 1996; Jones *et al*., 1999). In addition, there are a number of problems associated with Ca^{2+} research such as Ca^{2+} contamination of reagents and glassware, unspecificity of chelators such as EGTA and side-effects of inophores to name a few. These problems are raised by Hovi *et al*. (1975), Cobbold and Lee (1991), Dawson *et al*. (1991), Hughes and Poole (1991) and Youatt (1993). Nevertheless, the calcium-binding dye fura-2 and the photoprotein aequorin (calcium indicators widely used in eukaryotes) have been successfully used in prokaryotes (Gangola and Rosen, 1987; Knight *et al*., 1991; Futsaether and Johnsson, 1994; Herbaud *et al*., 1998; Jones *et al*., 1999; Torrecilla *et al*., 2000).

The first measurements of $[Ca^{2+}]_i$ in bacteria were made by Gangola and Rosen (1987). *Escherichia coli* was made permeable to fura-2 pentaacetoxymethyl ester by treatment with EDTA. The resting $[Ca^{2+}]_i$ in energy-replete cells was found to be 90 ± 10 nM independent of external calcium concentrations, which ranged from 10 μM to 10 mM. Similar values were observed when $[Ca^{2+}]_i$ was measured by fura-2 in *Propionebacterium acnes*. However, this study showed that the $[Ca^{2+}]_i$ was transiently influenced by changes in the external Ca^{2+} concentration (Futsaether and Johnsson, 1994).

With the advent of molecular DNA technology, it was possible to constitutively express the aequorin gene in bacterial cells to measure changes in $[Ca^{2+}]_i$ (Knight *et al*., 1991; Jones *et al*., 1999; Torrecilla *et al*., 2000). Using this technique, several investigators were able to continuously monitor cytosolic free-Ca^{2+} in different genera of bacteria. Jones *et al*. (1999) showed that in principle, *E. coli* tightly regulates its $[Ca^{2+}]_i$, ranging from 170 nM to 300 nM. But in contrast to previous studies there were some interesting differences. It was found that the $[Ca^{2+}]_i$ increased slowly as the external Ca^{2+} was increased, returning slowly to the initial level after 80 min. Thus, the internal Ca^{2+} reached ≈ 2 μM, in the face of 1 mM Ca^{2+}, before slowly declining. Cells incubated in medium with a constant level of external Ca^{2+} appeared to display a modest oscillation of the free-Ca^{2+} concentration, 200–600 nM, with similar amplitude, peaking at ≈ 50 min. In addition, when cells were depleted of Ca^{2+} by treatment with EGTA, restoration to normal levels occurred very slowly. All these results suggest that the free-Ca^{2+} pool may be more dynamic than previously thought and that these transient increases in internal free-Ca^{2+} levels could be available as a signalling mechanism. Ca^{2+} oscillations may have many different patterns. Multiple messages can be carried by Ca^{2+} depending on the amplitude and duration of the signal, frequency and location of the Ca^{2+} signal and interaction with other cell components (Clapham, 1995; Sanders *et al*., 1999).

Recently, $[Ca^{2+}]_i$ measurements in the *E. coli* periplasm, determined by targeting the Ca^{2+}-activated photoprotein aequorin to this compartment, showed that the periplasm can accumulate Ca^{2+} ions three- to sixfold with respect to the external medium. These findings are particularly interesting because both the outer membrane and the periplasm could be crucial mechanisms for Ca^{2+} regulation by acting as Ca^{2+} barriers or buffering and sorting Ca^{2+}, thereby regulating the availability of free-Ca^{2+} to Ca^{2+} transporters in the inner membrane (Jones *et al*., 2002). All these findings argue in favour of Ca^{2+} as an intracellular regulator in prokaryotes.

The most direct evidence of Ca^{2+}-mediated stimulus response in bacteria is chemotaxis. Since 1977 it has been shown that the concentration of internal free-Ca^{2+} controls the direction of flagellar rotation in *Bacillus subtilis* (Ordal, 1977). In 1993, Tisa and Adler showed that chemotaxis was also affected by the introduction into *E. coli* of caged campounds, which upon illumination chelate or release Ca^{2+} (Tisa *et al*., 1993). But it was not until 1995 that direct measurements of $[Ca^{2+}]_i$, as measured by fura-2 and aequorin, revealed that addition of a repellant to bacterial cells resulted in tumbling and a transient increase in cytoplasmic free-Ca^{2+} levels, whereas addition of an attractant caused swimming and a transient decrease in cytosolic free-Ca^{2+} levels (Tisa and Adler, 1995; Watkins *et al*., 1995). Furthermore, mutants lacking chemotactic receptors did not show any change in $[Ca^{2+}]_i$ in the presence of attractants or repellants (Tisa and Adler, 1995). Expression of the phosphatase CheZ in these mutants resulted in less tumbling and more smooth swimming. These data support the hypothesis that Ca^{2+} modulates the phosphorylation state of the Che proteins (Tisa and Adler, 1995). Moreover, ω-conotoxin, a Ca^{2+} channel blocker, inhibited chemotaxis by preventing tumbling and promoting swim-

ming (Tisa *et al*., 2000). However, despite the accumulated information on this subject, the role of calcium in chemotaxis is still rarely considered in the literature.

The effects of hydrogen peroxide as an external signal on cytosolic Ca^{2+} homeostasis were investigated in *B. subtilis* using aequorin as a probe to monitor cytosolic Ca^{2+} levels (Herbaud *et al*., 1998). Addition of H_2O_2 caused an increase in aequorin luminescence followed by a slow and steady decrease, as previously observed in chemotaxis (Watkins *et al*., 1995). Data from this study showed that *B. subtilis*, as other bacteria, tightly regulates $[Ca^{2+}]_i$, and that this homeostasis can be changed by an external stimulus such as H_2O_2. In *B. subtilis*, H_2O_2 treatment induces the expression of several proteins. These proteins form a peroxide regulon that is regulated by metals. An interesting finding was that the production of the protein alkyl hydroperoxide reductase (AhpC), a member of this regulon, appears to be modulated by Ca^{2+}. Expression of *ahpC* increased fivefold upon addition of EGTA. Two-dimensional electrophoresis and $^{45}Ca^{2+}$ overlay analyses showed that AhpC is a calcium-binding protein. However, AhpC lacked the typical Ca^{2+}-binding motifs found in eukaryotes. Using ruthenium red, antibody cross-reactivity (anti-calmodulin) and mass spectroscopy, we have also identified AhpC as a CaBP in *Bordetella pertussis* as well as other CaBPs (D.C.D. Dominguez and E.L. Hewlett, unpubl. results). Because bacteria must rapidly adapt to different environments, Ca^{2+} might be used as a signalling system during periods of oxygen stress. This study strongly suggests a link between oxidative stress and Ca^{2+} and the possibility of a Ca^{2+}-dependent regulatory mechanism in cell redox potential (Herbaud *et al*., 1998).

Evidence of Ca^{2+}-signal transduction in cyanobacteria was presented by Torrecilla *et al*. (2000). Calcium transients in response to environmental stimuli (heat and cold shock) were studied in the blue green alga *Anabena* strain PCC7120 using aequorin. Heat and cold shock were applied by two different methods: (i) by immersion of bacterial cell suspensions in a hot/cold water bath and (ii) by direct irrigation of cell suspensions with hot or cold water. Results from these studies suggest that cyanobacteria are capable of sensing and distinguishing cold versus heat shock as evidenced by the responses shown in terms of the Ca^{2+} transients. Heat shock treatment of cells in a water bath (44°C) resulted in a threefold increase in cytosolic free-Ca^{2+} (1.14 ± 0.17 μM after 2 min to 3.10 ± 0.25 μM after 20 min) gradually returning to basal levels whereas cold shock treatment under the same conditions evoked a smaller Ca^{2+} transient of shorter duration (1.04 ± 0.23 after 10 min) (Figs 1A and 2 A). Interestingly, heat treatment by irrigation showed a different response triggering a biphasic Ca^{2+} transient with similar kinetics for both heat- and cold-shocked cells (Figs 1B and 2B). Furthermore, when cold-shocked cells were incubated in the presence of higher external Ca^{2+} (5 mM), there was a significant increase (Student's *t*-test, $P < 0.1$) in the Ca^{2+} transient whereas heat-shocked cells incubated under the same conditions did not show an increase in $[Ca^{2+}]_i$. Studies to determine the source of the cold–heat Ca^{2+} transient using Ca^{2+} channel blockers, such as verapamil, the Ca^{2+}-chelator EGTA and the calmodulin-inhibitor trifluoperazine, suggest that the increased calcium associated with heat shock derives from both extracellular and intracellular spaces while that with cold shock arises from extracellular space. Results obtained from experiments using verapamil should be interpreted with caution because studies in *E. coli* have shown that verapamil modestly increases Ca^{2+} levels rather than the expected decrease and it apparently has major effects on the lipid bilayer altering the activity of some protein membranes (I.B. Holland, pers. comm.). In 2001, using the same aequorin-expressing cyanobacterium strain, Torrecilla *et al*., 2001) showed that salt and osmotic stress evoked $[Ca^{2+}]_i$ transients in *Anabaena* cells. These findings provide further evidence that Ca^{2+} signalling may be involved in early sensing of temperature, salt and osmotic stress in cyanobacteria.

Over the years, a number of bacterial proteins have been identified to be involved in calcium transport mechanisms. Primary and secondary transporters as well as ion channels (Ca^2, K^+, Na^+) have been documented in several genera of bacteria (Norris *et al*., 1996; Paulsen *et al*., 2000; Waditee *et al*., 2004). Recent data showed that at the structural level, prokaryotic Ca^{2+}-ATPases and ion channels have striking similarities with their eukaryotic counterparts (Ren *et al*., 2001; Raeymaekers *et al*., 2002). The discovery that polyhydroxybutyrate-polyphosphate (PHB-P) complexes, which function as Ca^{2+} channels in *E. coli*, are present in *Streptomyces lividans* in association with K^+ channels has been a surprising and interesting finding (Reusch, 1999). Moreover, these PHB-P complexes are modulated by pH (Das and Reusch, 2001). At physiological pH, PHB-P channels have a strong preference for Ca^{2+} over Na^+. Lowering the pH to 6.5–6.8 not only decreases the preference for Ca^{2+}, but abolishes it. Thus, Das and Reusch (2001) hypothesized that basic amino acids associate with the channel, inhibit the binding of divalent cations and increase the permeability for monovalent ions.

The report of a Na^+ channel in *Bacillus halodurans* (Catterall, 2001; Ren *et al*., 2001) has been an exciting development because it was believed that this ion channel family did not exist in prokaryotes. The channel shows a high degree of conservation, but is simpler in structure than eukaryotic channels. Furthermore, in relation to this review, an interesting and intriguing finding is that the channel's pore region sequence is very similar to that of a calcium-gated channel, in fact this Na channel is

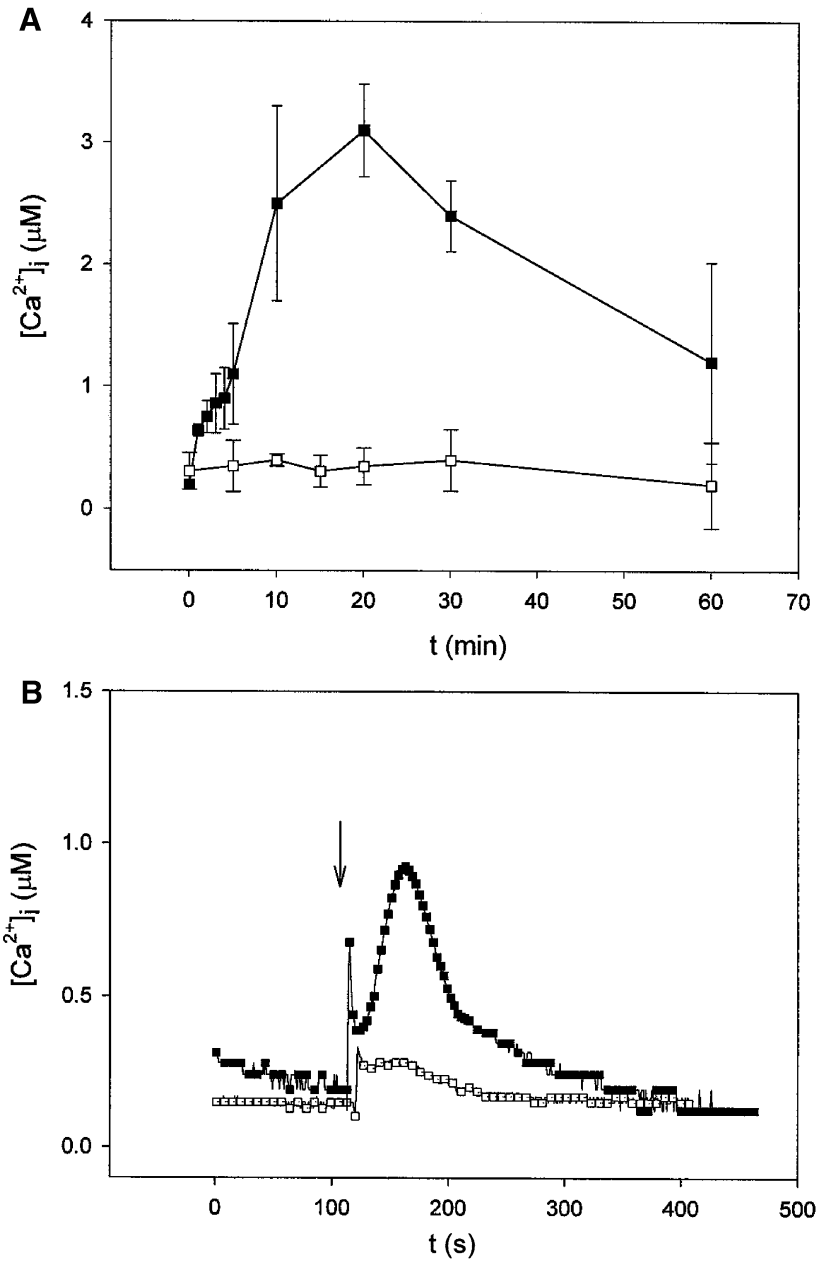

Fig. 1. Changes in $[Ca^{2+}]_i$ response to heat shock in *Anabaena* strain PCC7120.
A. Continued heat shock treatment (up to 60 min) in a water bath at 44°C of coelenterazine-treated cells (■). Cuvettes were removed from the water bath at specific times to monitor luminescence. Cells maintained in 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 (■). 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. This material is copyrighted by the American Society of Plant Biologists and is reprinted from Torrecilla *et al*. (2000) with permission.

activated by voltage and blocked by calcium channel blockers. It would be very interesting to know whether this channel contains PBH-P complexes and behaves similarly.

A transporter that shows striking similarity to its eukaryotic counterpart, the endo(sarco)plasmic reticulum (SERCA) Ca^{2+} transporters, is the P-type Ca^{2+}-ATPase from *B. subtilis* (Raeymaekers *et al*., 2002). Nine out of 10 critical amino acid residues in SERCA transmembrane domain involved in calcium binding sites were conserved in the *B. subtilis* protein. More recently, a mechanosensitive Ca^{2+} ion channel was reported from the cyanobacterium *Synechocystis* sp. PCC6803, which is capable of releasing Ca^{2+} in response to depolarization of the plasma membrane. It was hypothesized that the Ca^{2+} channel is involved in Ca^{2+} homeostasis under temperature-stress conditions (Nazarenco *et al*., 2003).

In eukaryotic organisms, calcium is known to be involved in cell cycle events such as initiation of DNA synthesis, mitosis and cell division (Whitaker and Larman, 2001). Calcium fluxes activate a series of events leading to phosphorylation reactions, activation of regulatory proteins and cell cycle regulation (Clapham, 1995). In *E. coli*, significant Ca^{2+} mobilization occurs during the cell cycle. Using electron probe analysis, Chang *et al*. (1986) showed that in non-dividing cells the

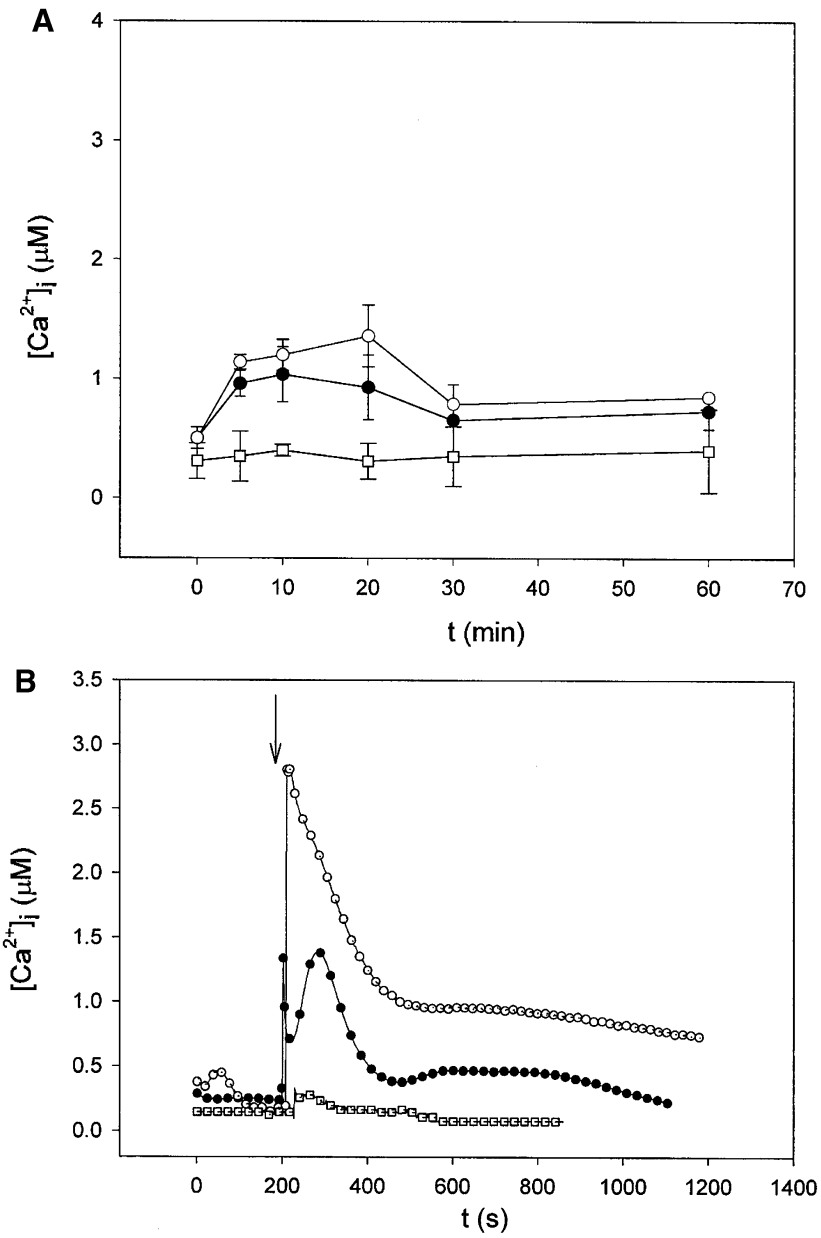

Fig. 2. Changes in $[Ca^{2+}]_i$ in response to cold shock in *Anabaena* strain PCC7120.
A. Continued cold-shock treatment in a water bath at 0°C of coelenterazine-treated cells incubated with increasing external Ca^{2+} concentrations (○, 5 mM Ca^{2+} ●, 0.25 mM Ca^{2+}). Cuvettes were removed from the water bath at specific times to monitor luminescence. 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 was recorded. 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. This material is copyrighted by the American Society of Plant Biologists and is reprinted from Torrecilla *et al.* (2000) with permission.

concentration of calcium ions was 25 times higher in the membrane than in the cytosol. But during division, the intracellular calcium concentration increased significantly (approximately fourfold). Eukaryotic-like protein kinases and cytoskeletal elements like FtsZ and MreB/Mbl are known to be present in bacteria suggesting a possible involvement of calcium in the prokaryotic cell cycle. FtsZ is a key protein in the bacterial cell division. Its GTPase activity is closely related to the cytoskeletal protein tubulin (Addinall and Holland, 2002). *In vitro* studies showed that Ca^{2+} stimulate FtsZ polymerization *in vitro* (Yu and Margolin, 1997). However, currently the role of calcium in the cell cycle and Z ring formation remains unclear (Addinall and Holland, 2002).

Conclusions

From the literature presented here, it is evident that prokaryotic cells like eukaryotes are equipped with all the elements to maintain Ca^{2+} homeostasis. Unfortunately, over the years calcium in bacteria has not been studied systematically and many important investigations have not been followed up. This could possibly result from the difficulties encountered in Ca^{2+} research such as the lack of reliable methods to measure calcium in bacteria, toxicity of reagents, difficulties in loading Ca^{2+} indicator dyes into bacterial cells and other. To prove that a Ca^{2+} transient is an essential intermediate in a stimulus–response phenomena requires the demonstration not only that a stim-

ulus evokes a change in $[Ca^{2+}]_i$ but additional criteria: (i) artificial induction of changes in $[Ca^{2+}]_i$ should induce a physiological response, (ii) blocking the Ca^{2+} transient should block the downstream response and (iii) cells should have mechanisms to sense $[Ca^{2+}]_i$ changes. Even though limited work has been done in investigating the role of calcium in bacteria, there is evidence that some of these requirements appear to be fulfilled in prokaryotic cells. However, there is not clear or enough evidence that all three criteria are already fulfilled in bacterial cells. Much more work has to be done in prokaryotes to demonstrate that Ca^{2+} is a modulator of a specific function or the generator of a response. For example, does Ca^{2+} directly affect the affinity of a protein for its target or substrate? or do Ca^{2+}-levels affect gene/protein expression? Indeed, EGTA treatment of either *E. coli* or *B. subtilis* induces marked synthesis of several proteins (Laoudj *et al*., 1994; Herbaud *et al*., 1998). The issue of whether Ca^{2+} is a 'chemical switch' or whether the divalent cation encodes specificity has been controversial. Whereas in mammalian cells it is well documented that Ca^{2+} signalling patterns encode specific cellular responses, in plant cells Ca^{2+} specificity has been more difficult to demonstrate (Scrase-Field and Knight, 2003). It would be interesting to see what function Ca^{2+} plays in bacteria.

A role of ion condensation in signal transduction cannot be discounted because various stimuli alter the charge parameter to cause either condensation or decondensation depending on the magnitude and nature of the perturbation. This process of condensation/decondensation is in a feedback relationship with phosphorylation/dephosphorylation which may lead to signal transduction (Ripoll *et al*., 2004).

Recently, Michiels *et al*. (2002) have identified many candidate CaBPs in the database of bacterial genomes including EF-hand proteins. Could such proteins function as sensors or storage proteins? or is it possible that some of these proteins may be associated with Ca^{2+} channels/Ca^{2+} pumps regulating Ca^{2+} fluxes?

It is clear that evidence in support of the role of Ca^{2+} as a regulator in prokaryotes is accumulating. However, the extent and importance remains unclear. A full assessment of the processes involving calcium awaits further analysis. Only a detailed, systematic investigation will reveal the molecular basis of the role of calcium in bacteria.

Acknowledgements

The support of The Ford Foundation and The National Academies is gratefully acknowledged. I thank I.B. Holland for advice and encouragement and R.H. Krestinger and F. Fernadez-Pinas for reading the manuscript. I am also grateful to V. Norris for introducing me to the theory of ion condensation.

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