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MINIREVIEW

Calcium Signalling in Bacteria

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In eukaryotic cells, variations in the levels of cytosolic free calcium regulate processes as important and disparate as chemotaxis, chromosome segregation, fertilization, ion transport, muscle contraction, passage through cell cycle transition points, proteolysis, secretion, and substrate uptake (7). Cytosolic free calcium concentration is tightly controlled by the action of specific pumps and channels in the plasma membrane and subcellular organelles (8, 83). Response to increased cytosolic free calcium concentration is mediated by either direct binding to calcium-sensitive enzymes, such as protein kinase C (49) and calpain (72), or activation of a protein transducer, such as calmodulin (15).

In prokaryotic cells, an equivalent important role for calcium has been harder to demonstrate but is now becoming evident (53, 59, 69). Research on a variety of bacterial processes has passed from the phase of demonstrating a likely involvement of calcium to clarifying the nature of this involvement. In this minireview, recent evidence on the existence of bacterial components (both proteinaceous and nonproteinaceous) concerned with calcium regulation is evaluated, since investigation of these components is one of the surest routes to confirming the involvement of calcium in a process. These components include voltage-gated calcium channels responsible for influx that can be formed from poly-3-hydroxybutyrate–polyphosphate complexes, primary and secondary transporters responsible for efflux, and calmodulin-like proteins responsible for mediating responses to calcium. Such calcium-dependent regulation may be exerted directly by changes in nucleoid structure or indirectly by phosphorylation or proteolysis of target proteins. Despite the problems sometimes associated with studies of calcium, this ion is increasingly implicated in a number of bacterial functions, including heat shock, pathogenicity, chemotaxis, differentiation, and the cell cycle.

INTRACELLULAR CALCIUM LEVELS

Estimates of the intracellular free calcium concentration of 0.1 and 1 μM in the model organism *Escherichia coli* have been obtained with Fura-2 {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid} (24, 77) and aequorin (85), respectively. Such levels are similar to those in eukaryotic cells and are a 1,000 times less than those typically found outside the cell. Three factors are considered responsible for this low level: the low permeability of the envelope with tightly controlled influx mechanisms, a high buffering capacity, and effective export systems.

CALCIUM INFLUX

In eukaryotic cells, a number of mechanisms for gated entry of calcium have been characterized. Families of calcium channels have been identified, which can be classified broadly by the stimulus for channel opening into voltage-operated, receptor-operated, mechanically operated or tonically active calcium channels (83). In particular, eukaryotic L-type, voltage-operated calcium channels (VOCCs) are activated by membrane depolarization and the 1,4-dihydropyridine agonist BAY K 8644 and inactivated by dihydropyridine and phenylalkylamine antagonists and lanthanum ions. In *Bacillus subtilis*, calcium uptake into right-side-out vesicles mediated by a protein displaying these "L-type" calcium channel properties has been reported (45); however, the cloning of the gene encoding this protein has yet to be described.

A most exciting recent development concerns a nonproteinaceous calcium channel (63). A complex of the lipidic polymer poly-3-hydroxybutyrate and calcium polyphosphate was extracted from the membrane of *E. coli* and incorporated into artificial bilayers where it revealed characteristics of VOCCs including voltage dependence, selectivity for the divalent ions calcium, strontium, and barium over magnesium, and concentration-dependent block by lanthanum. These channels were activated by voltage steps of >60 mV and had a conductance of 9 pS (63). There are considerable differences between the activation and inactivation characteristics of these complexes and eukaryotic VOCCs. For example, these complexes are activated in depolarized membranes and are not inactivated in a voltage-dependent manner. The complexes are present in the cytoplasmic membrane at concentrations that are low during exponential growth, higher during stationary phase, and particularly high during suspension in ice-cold calcium buffers (reference 63 and references therein). This last treatment results in the acquisition of genetic competence (29) which is associated with cytoplasmic alkalinization, stimulation of glycolysis, synthesis of a set of competence factors, DNA transport, and activation of membrane endonuclease. Although transformability is correlated with increased concentration of poly-3-hydroxybutyrate–calcium polyphosphate complexes in the membrane (10), it is unclear whether this is due to their activity as calcium channels (29). In contrast, it is clear that a calcium transporter is involved in calcium-DNA uptake, lysis, and competence in *Streptococcus pneumoniae* following inhibition studies with the amiloride derivative 2′,4′-dimethylbenzamil (81, 82). A very different mechanism can also lead to

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divalent ion entry whereby uptake of calcium and other divalent ions, such as magnesium, manganese, and cobalt, accompanies uptake of P_i by both the phosphate inorganic transport system, *pit* system of *E. coli*, and by similar systems of *Acinetobacter johnsonii* 210A and probably of other bacteria (84).

EFFLUX

Eukaryotic calcium export systems have been subdivided into two classes: primary exchangers, which are driven by the energy derived from ATP hydrolysis, and secondary exchangers, which use electrochemical gradients of sodium ions or protons. Primary exchange by P-type ATPases is the principal mechanism of export in eukaryotes (8). P-type ATPases form an acyl phosphate with the gamma phosphate of ATP as an intermediate and undergo rapid dephosphorylation under mild alkaline conditions; transport via P-type ATPases is inhibited by orthovanadate and by lanthanum. For over a decade, there has been evidence of primary pumps in bacteria based on ATP-dependent calcium movements across membranes (43). Recently, a calcium pump has been cloned from the cyanobacterium *Synechococcus* sp. strain PCC 7942, sequenced, and characterized as a P-type ATPase (1, 36), while another calcium pump, purified from the soil and water bacterium *Flavobacterium odoratum*, fulfills all the criteria expected of a P-type ATPase and shows a remarkable similarity to the eukaryotic calcium ATPase from sarcoplasmic reticulum (23).

A number of secondary calcium exchangers have been identified in bacteria (43) including the recently discovered ChaA calcium-proton exchanger from *E. coli* (33). ChaA appears to be involved in calcium ion circulation at alkaline pH and possesses a sequence rich in aspartic acid and glutamic acid with an intriguing similarity to sequences found in eukaryotic calcium-binding proteins such as calsequestrin (57). In eukaryotes, sodium/calcium exchange is the preferred strategy, and some bacteria have also adopted this strategy (43). These bacteria include *S. pneumoniae*, in which a sodium/calcium exchanger that is involved in calcium efflux and influx, lysis, and competence for genetic transformation has been identified (82).

REGULATION

In eukaryotes, calmodulin is involved in many processes including the regulation of cell motility and chemotaxis, microtubule disassembly, DNA replication, calcium transport, enzymic activity (stimulating, for example, cyclic nucleotide phosphodiesterase [PDE], adenylyl cyclase, and NAD kinase), and transcription (7, 15). Such regulation is often achieved by interaction of calmodulin with calmodulin-dependent protein kinases. Calmodulin is a heat-stable acidic protein with four characteristic structures (EF hands) that bind calcium. On binding calcium, calmodulin is activated by a large conformational change. When sodium dodecyl sulfate-polyacrylamide gel electrophoresis of calmodulin is performed in the presence of ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), calmodulin migrates with a significantly different mobility (39). These distinctive properties have assisted in the identification of calmodulin-like proteins in a wide range of bacteria including *Myxococcus xanthus* (32), *Saccharopolyspora erythraea* (6), and *B. subtilis* (22). In addition, calmodulin-like proteins have been discovered in several cyanobacteria (for reviews, see references 59 and 69), for example, in *Nostoc* sp. strain PCC 6720 where a protein has been purified on the basis of activation of NAD kinase, antibody cross-reactivity, and gel shift (58). Similarly, in an archaeon, *Halobacterium salinarium*, the first evidence for a calmodulin-like protein has been obtained recently on the basis of pI, calcium binding, and activation of PDE (65). In *E. coli*, calmodulin-like proteins were reported in 1981 on the basis of heat stability and calcium binding (26) as well as calcium-dependent activation and inhibition of PDE (34). More recently, numerous proteins have been observed that are heat stable and acidic, bind calcium, are induced by EGTA, and cross-react with anticalmodulin antibodies, although these characteristics have yet to be associated with a single complete protein sequence (41). A candidate for an EF-hand-containing protein, o396, has been signalled by the genome-sequencing project (5), thus revealing the importance of careful annotation (17). o396 is identical to the integrase of prophage P4, and the open reading frame of o396 is directly downstream of the gene encoding $tRNA_1^{Leu}$. Intriguingly, in view of a possible calcium connection, a search for a calmodulin-like protein in *E. coli*, based on resistance to an antagonist, compound 48/80, yielded two mutants affected in the genes encoding $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$ (9, 13).

MECHANISMS

There is evidence that calcium plays a role in regulating cellular processes in bacteria through modulation of processes involving nucleoid structure, protein phosphorylation, and alterations in transverse and lateral distributions of membrane lipids (53, 69). Because of its coordination number and variable geometry, calcium has been cited as the ideal bridging ion between DNA and a surrounding acidic protein matrix, and evidence for interaction between calcium and DNA in vivo has been reported (references 53 and 69 and references therein). A direct relationship between nucleoid structure and calcium would be consistent with the conversion of compact nucleoids to axial filaments in *Yersinia pestis* during the low-calcium response where the involvement of calcium-binding proteins has yet to be reported (71). Calcium binds preferentially to G+C-rich regions (2), and a specific interaction between divalent ions, particularly calcium, and GGGCCC-containing DNA increases the coefficient of retardation in gel mobility assays (3). Since this coefficient is the principal measure of DNA curvature in vitro, it is worth considering whether calcium also has a role in determining the expression of certain genes in vivo by binding directly to specific sequences.

Protein phosphorylation is important in the response to changes in osmolarity. EnvZ, the osmotic sensor in the inner membrane of *E. coli* phosphorylates OmpR, a DNA-binding protein that determines the differential transcription of two outer membrane porins, OmpF and OmpC. By using an EnvZ fusion protein, the addition of 60 μM calcium to an in vitro assay stimulated both autophosphorylation of the fusion protein and phosphorylation of OmpR (62). Intriguingly, manganese at similar concentrations had similar effects. Inhibition of these phosphorylation reactions by the calcium chelator EGTA implied an involvement of calcium; however, as discussed below, EGTA is a better chelator of manganese, zinc, and iron than calcium. Thus, reversal of EGTA inhibition of phosphorylation by the addition of calcium does not necessarily imply that the kinase is dependent on calcium. For example, in pursuit of calcium-stimulated kinases in *E. coli* (52), we have observed that EGTA inhibition of protein phosphorylation is reversed more frequently by manganese than by calcium (21), although genuine calcium-stimulated kinase activities do exist (20a).

DnaK, the *E. coli* equivalent of eukaryotic heat shock protein 70, is induced by stress and has an autophosphorylation activity that is stimulated 10-fold by calcium in vitro. In vivo,

phosphorylation of DnaK, albeit on a different residue, increases with temperature and determines binding to denatured substrates (68). The calcium dependence of this phosphorylation is not known, but it may be significant that DnaK has a sequence with 60% identity to the classic 21-residue calmodulin-binding site (70). In L forms of *E. coli*, optimal growth at 32 and 37°C occurs in 0.1 and 1 mM calcium, respectively (reference 60 and references therein). One possible interpretation is that calcium levels serve as part of an intracellular thermometer.

Histone phosphorylation is commonly used to characterize eukaryotic kinases. Phosphorylation of eukaryotic histones by a purified, endogenous kinase from *Legionella micdadei* was stimulated threefold by calcium, suggesting a calcium-dependent kinase activity (66), while other evidence (reference 21 and references therein) for eukaryotic kinase-like enzymes in a variety of bacteria includes calcium-, phosphatidylserine- and tetradecanoyl phorbol acetate-stimulated kinases reminiscent of eukaryotic protein kinase C in *E. coli* and *B. subtilis*, calcium and manganese stimulation and inhibition by antagonists of eukaryotic kinases of phosphorylation in *Streptomyces griseus* (27), and involvement of calmodulin-like proteins in protein phosphorylation in *Clostridium thermohydrosulfuricum* and in the cyanobacterium, *Anabaena* spp. In view of the criticism that few eukaryotic kinase-like enzymes have been pursued as far as DNA sequence, it should be noted that the sequence of at least one of an estimated 25 protein serine/threonine kinases in *M. xanthus* has been determined (47).

Phospholipid metabolism has been associated with calcium in *E. coli* (references 53 and 69 and references therein). This is hardly surprising, given the likely roles for calcium in lipid structures and in transverse and lateral membrane asymmetry (16). The importance of nonbilayer structures in membrane fusion and hence endocytosis, exocytosis, cell division, and the transbilayer movement of lipids and proteins has long been appreciated. Interaction of calcium with phospholipids is implicated in the formation of nonbilayer structures (37) and lipid scrambling (73). In line with structural interpretations of its role, calcium suppresses impaired growth in a *pss* mutant of *E. coli* defective in the synthesis of its principal phospholipid, phosphatidylethanolamine (19). Recent studies of this mutant have revealed that *E. coli* synthesizes its membrane lipids, principally cardiolipin, which binds calcium, so as to maintain a particular proportion of its lipids with a propensity to form type II nonbilayer structures in the presence of divalent ions (37). The formation of lateral domains in membranes occurs in both eukaryotic and prokaryotic cells (44, 80). A role for calcium is suspected since calcium mediates the formation of lateral domains of acidic phospholipids in vitro and possibly in vivo (reference 53 and references therein). One tempting speculation is that a calcium flux reorganizes lateral domains in the cytoplasmic membrane of bacteria at the time of cell division as part of a reset mechanism (see below).

Calcium also appears to have a structural role in stabilizing the lipopolysaccharide monolayer of the outer membrane of gram-negative bacteria and in determining the structure of the S-layer (references 53 and 69 and references therein).

FUNCTIONS

Successful bacterial chemotaxis requires a combination of running and tumbling. In 1977, it was deduced that calcium at $>10^{-7}$ M caused tumbling in *B. subtilis* (61). These studies were based on the calcium specificity of EGTA as a chelator and A23187 as an ionophore (see below). However, a decade later, a variety of calcium channel blockers, which appear to inhibit a VOCC in this organism, were also found to inhibit chemotaxis (references 53 and 69 and references therein). In *E. coli*, the importance of calcium in chemotaxis has been confirmed recently. Inhibition of chemotaxis by prevention of tumbling followed the addition of ω-conotoxins, antagonists of VOCCs, at concentrations as low as 10 nM after permeabilization (78). In electroporated cells, tumbling was similarly prevented by the caged compound, diazo-2 [1,2-bis-(2-amino-5-di-azoacetylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis methyl ester], which on illumination with 340-nm-wavelength light chelates calcium (75). Reciprocally, inhibition of chemotaxis by increasing the frequency of tumbling resulted from the introduction of caged compounds nitr-5 {1-[2-amino-5-(1-hydroxy-1-[2-nitro-4,5-methylene dioxyphenyl]-methyl)-phenoxy-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid} and DM-nitrophen [1-(2-nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-*N*,*N*,*N*′,*N*′-tetraacetic acid], which on illumination release calcium; these calcium-mediated effects required the CheA, CheW, and CheY proteins but not the methyl-accepting chemotaxis proteins (75). A role for calcium signalling in chemotaxis is also consistent with observations of major differences in free calcium between the responses to attractants and repellents of chemotactic and nonchemotactic strains as measured by aequorin (85) and Fura-2 (77) and alterations in chemotaxis in calcium mutants (76).

Sporulation, fruiting body formation, gliding, and heterocyst formation are all instances of bacterial differentiation that reveal a connection with calcium. In *B. subtilis*, for example, spores are enriched twofold in a heat-stable calmodulin-like activity that stimulates PDE and sporulation is inhibited by the calmodulin inhibitor trifluoperazine at levels at which the endogenous calmodulin-like protein is inhibited (22). Proteins are turned over two or three times during sporulation, and again in *B. subtilis*, lowering calcium levels to 2 μM inhibits sporulation and reduces proteolysis by 60% (56). In line with a calcium requirement for the gliding of both *M. xanthus* and *Stigmatella aurantiaca*, albeit deduced from chelator studies (see below), calcium mediates the assembly of protein S (a calmodulin-like protein) on the surfaces of the myxospores during fruiting body formation in *M. xanthus* (32) and the translocation of a 55-kDa protein to the extracellular matrix in another myxobacterium, *S. aurantiaca* (11). In cyanobacteria, heterocyst cytodifferentiation occurs when nitrates are removed, and in filaments of these organisms, the heterocysts develop every 10 or so cells to protect the labile nitrogen-fixing system from oxygen; a role for calcium is indicated, for example, in *Nostoc* sp. strain 6720, where the frequency of heterocyst formation doubles as external calcium increases 10-fold from 10^{-5} to 10^{-4} M (reference 59 and references therein).

It has been argued recently that the systems underlying the regulation of the prokaryotic and eukaryotic cell cycles are fundamentally the same (51). A role for calcium in the regulation of the eukaryotic cell cycle is widely accepted. However, a role for calcium in the regulation of initiation of chromosome replication in bacteria, first proposed in 1988 (54), remains promising but tenuous, and the involvement of a putative calmodulin-like protein in initiation of DNA replication in *E. coli* is still to be confirmed (35). A role for calcium in cell division might be expected, because the division of one cell into two cells is difficult to envisage without fusion of the inner and outer monolayers of the cytoplasmic membrane. Such fusion should, locally at least, allow calcium to flood down the concentration gradient into the cell and could explain the 20-fold increase in total calcium in the cytoplasm of dividing cells of *E. coli* observed by electron-probe microanalysis (12). Although

an increase in calcium could couple chromosome segregation and cell division (50), the addition of EGTA and EDTA to *E. coli* leads to a premature chromosome segregation and premature cell division (48), rather than the delay predicted by the simple calcium flux models. One protein that might mediate calcium control of the cell cycle is the "cellular thermometer," DnaK, which is autophosphorylated in vitro in response to calcium (see above). DnaK is required for the initiation of both chromosome (67) and plasmid (74, 86) replication in *E. coli* and activates in vitro the key protein in initiation of chromosome replication, DnaA (31). In addition, the *dnaK52* mutant has a defect in segregation and division (46) that is suppressed by overproduction of the key cell division protein, FtsZ (4). Another candidate for this cell cycle control is s396 (see above).

There is some evidence for an involvement of calcium in pathogenesis. In strains of *Y. pestis* harboring the low calcium response plasmid and grown at 37°C, a 20-fold induction of virulence-related genes on this plasmid occurs in the absence of millimolar concentrations of extracellular calcium (71). Under these conditions, major changes in the structure of the nucleoid occur, and the involvement of calcium-binding proteins has yet to be shown as discussed above. *Streptococcus mutans* is involved in dental caries, and recent studies reveal a calcium involvement in plaque formation for this and other streptococcal species where calcium may promote bacterial aggregation and attachment to teeth by interacting with anionic groups of the bacterial matrix and cell wall (64; reference 69 and references therein).

Although broadly defined as parasitic (42), the relationship between *Rhizobium* spp. and plants is essentially symbiotic. In this relationship, both the bacterium and plant differentiate, the latter to form nodules. Nodulation and host-specific recognition of legumes are encoded by the *nod* genes of *Rhizobium leguminosarum*. One of these genes, *nodO*, encodes an exported protein that binds calcium and that is proposed to have a calcium-dependent interaction with plant root cells (20). The acid tolerance of another soil bacterium, *Rhizobium meliloti*, also appears to be calcium dependent, with growth rates at pH 6 doubling when calcium concentrations were increased 10-fold from 0.3 to 3 mM (79).

CAVEATS

Awareness of the problems associated with studies of calcium (30) is essential for those with an interest in the study of bacterial calcium signalling. These problems include toxicity of chelators, side effects of ionophores, and lack of specificity of hard-to-deliver ion-specific reporter dyes. The ubiquity of calcium contamination in reagents and glassware is likewise a major problem. Several recent articles and reviews (18, 28, 88) provide an entry to these issues.

One of the most important issues relates to measurement of absolute free and total calcium levels, presently carried out in many studies by the use of Fura-2, a ratiometric calcium-sensitive dye. Measuring intracellular free calcium with Fura-2 requires this compound to enter the cell—which may entail harsh treatments with Tris and EDTA, multiple washes, and long incubation periods—and then to remain in the cell; further, effective use of dyes that are delivered in the ester form requires activity of native phosphodiesterases, which may or may not be sufficient to carry out the cleavage. Electroporation may provide superior delivery of the dyes (77). It should also be noted that the dissociation constants of Fura-2 for Zn^{2+} are approximately 1.6 to 2 nM compared with 100 to 300 nM for Ca^{2+} itself; further, zinc shifts the excitation optimal wavelength for Fura as does calcium (25). This could present a problem if calcium levels are measured in the presence of intracellular zinc, particularly if the zinc levels change during the experiment (40).

Measuring intracellular free calcium by expressing a construct encoding the photoprotein aequorin and adding the prosthetic group coelenterazine is a recent technique (38). It offers the enticing prospect of information on calcium levels in growing cells, although users should be aware that aequorin has a low sensitivity in the region of resting cytosolic calcium concentration in eukaryotic cells, is quenched by millimolar concentrations of magnesium, may buffer changes (transients) in calcium levels if overexpressed, and requires the prosthetic group coelenterazine which may perturb cells (14).

Another set of problems concerns the preparation of materials and media. Glassware can provide up to 30 μM calcium, and equipment requires washing with strong acids such as HCl. Reagents should be analyzed for calcium and trace elements. In the preparation of media, it should be realized that amino acid chelate calcium and that autoclaving with phosphate present can result in precipitation of trace elements (87).

ARE THERE MULTIPLE ROLES FOR CALCIUM IN PROKARYOTES?

Like eukaryotic cells, prokaryotic cells maintain a steep calcium gradient across the cell membrane. Like eukaryotic cells, prokaryotic cells appear to have the machinery to exploit the enormous potential for signalling this gradient offers. Therefore, like eukaryotic cells, prokaryotic cells possess a wide variety of systems in which calcium is implicated. Is there a unifying theme(s)? Perhaps. Three classes of actions can be proposed for calcium which may depend on factors such as the amplitude and duration of the calcium transient and the physiological state of the organism. The first is calcium as a primary and indispensable trigger, as in chemotaxis. The second is calcium as a modulator of a specific function. This would be the case if calcium were to advance or retard the timing of a cell cycle event or if calcium were to direct the affinity of a heat shock protein toward a particular set of substrates. The third is calcium as a structural element of, for example, the outer membrane, nucleoid, and spore.

We propose that in all these cases calcium action could also be considered a general reset mechanism that clears the way for new responses. Such a mechanism would be used by cells to allow them to respond more rapidly and effectively to a wide range of new situations. For example, the dismissal of teams of transcription factors and other enzymes, rapid and effective reorganization of chromosomes, membranes, and enzoskeleton (55), and the recruitment of kinases and proteases may all be required for an integrated response. Intracellular calcium has the potential to elicit such a coordinated response.

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