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REVIEWS

Ca^{2+} Signaling in Prokaryotes

I. V. Shemarova[1] **and V. P. Nesterov**

Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia

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Abstract—The role of Ca^{2+} ions in the regulation of motility, cell cycle, and division of prokaryotes is discussed, as well as their involvement in the pathogenesis of some infectious diseases. The structural and functional organization of the prokaryotic Ca^{2+} signaling system and the mechanisms of Ca^{2+} membrane transport and homeostasis are described. Special attention is paid to the role of Ca^{2+} cation channels, Ca^{2+} transporters, and Ca^{2+}-binding proteins in the regulation of the intercellular Ca^{2+} concentration.

Keywords: prokaryotes, bacteria, Ca^{2+} ions, Ca^{2+}-binding proteins, cation channels, Ca^{2+}/H^+ exchange

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In both higher and lower eukaryotes, Ca^{2+} ions are common intracellular signaling messengers. Along with cyclic nucleotides, diacylglycerol, nitric oxide, signaling oligopeptides, and some other active molecules and ions, Ca^{2+} cations are involved in the regulation of numerous processes in eukaryotic cells, such as cell cycle, nuclear division, nerve impulse conduction, muscle contraction, and others [1–3].

In prokaryotes, the role of Ca^{2+} in intracellular signaling is considerably less studied. Only a few works have considered Ca^{2+} ions as potential regulators of such cellular processes as ion transport, spore germination, heterocyst differentiation, gene expression, cell motility, adaptation to low or high temperatures, virulence, etc. [5–10]. For a long time, investigation of Ca^{2+} signaling in microbial cells was hindered by methodological problems in determining intracellular calcium concentrations ($[Ca^{2+}]_i$), calcium contamination, reagent toxicity, and difficulties in handling individual cells; these questions were discussed in [11–13]. However, the unique optical methods and other approaches to investigation of individual bacterial cells developed in the 1990s, which made it possible to register the changes in Ca^{2+} levels in microbial cytosol in response to various external stimuli [14–16], boosted the studies of the regulatory functions of Ca^{2+} in prokaryotic signaling.

In the present review, we discuss the historical development of ideas concerning Ca^{2+} signaling in prokaryotic cells and the current concepts of the related research in evolutionary biology.

FUNCTIONS OF Ca^{2+} IN PROKARYTIC CELLS

The first studies concerning the role of Ca^{2+} as a secondary messenger in prokaryotic organisms date back to the 1970s, when Ordal found that decreased Ca^{2+} intake by bacterial cells affected the chemotactic behavior in *Bacillus subtilis* [17]. Further research confirmed his observations in other microorganisms. A study on *Escherichia coli* showed that a decrease in $[Ca^{2+}]_i$ could alter the swimming mode in flagellate bacteria. Specifically, the characteristic slow and directed swimming changed to chaotic disordered motion [18]. Measurements of $[Ca^{2+}]_i$ levels in *E. coli* performed using the Fura-2 fluorescent Ca^{2+} indicator revealed that bacterial repellents caused a temporary increase in $[Ca^{2+}]_i$ (to over 100 nM) and inhibited chemotaxis, whereas attractants, in contrast, temporarily decreased $[Ca^{2+}]_i$ levels and stimulated chemotaxis [18].

Experiments on blocking the voltage-gated Ca^{2+} channels (Cav) of different types (L, P/Q, and R) showed that inhibitors added to the incubation medium impaired the chemotactic behavior of bacteria. In prokaryotes, it was expected that the stimuli inducing chemotactic reactions may increase the intake of extracellular Ca^{2+} through the ion channels opened in response to membrane depolarization, as it occurs in protists and even in multicellular eukaryotes. However, there was no direct correlation between the extracellular Ca^{2+} concentration, membrane depolarization, and changes in swimming behavior of bacteria [18]. It is possible that the chemotactic behavior in bacteria is regulated by means of alternative Ca^{2+}-dependent mechanisms involving histidine kinases [19].

Apart from chemotaxis, Ca^{2+} ions affect a number of other processes in prokaryotic cells: cell growth,

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[1] Corresponding author; e-mail: shem@iephb.ru

division and differentiation, or spore formation and viability, as well as in formation of fruiting bodies in myxobacteria and heterocysts in cyanobacteria [8, 15, 20–22].

It was observed that, in the absence of Ca^{2+}, *E. coli* cells stopped dividing, became spherical in form, unviable, and eventually underwent lysis and died [7]. At the same time, an increase in extracellular Ca^{2+} concentration from 0.3 to 3.0 mM resulted in doubling the growth rate of soil bacterium *Rhizobium melitoti* [23]. It was supposed that Ca^{2+} affected the growth of bacteria by regulating their cell cycle, primarily by affecting phosphorylation of the proteins involved in initiation of chromosome replication [24]. Apparently, one of such proteins mediating the effects of calcium on the prokaryotic cell cycle is an autophosphorylating protein DnaK, which is homologous to the eukaryotic heat shock protein p70 and contains a fragment showing structural similarity (60% homology) to the canonical CaM binding site [25]. In *E. coli*, the DnaK effector is required for the activation of another protein, DnaA, which plays a key role in initiating chromosome replication [26].

There is evidence suggesting that Ca^{2+} ions are involved in pathogenesis of certain infectious diseases. For instance, in some *Yersinia pestis* strains, increased extracellular Ca^{2+} concentrations trigger expression of the genes responsible for their virulence [27]. *Streptococcus mutans* cells are involved in the pathogenesis of caries via Ca^{2+}-activated aggregation and adhesion [28]. Pathogenic strains of *Bacillus anthracis* produce a Ca^{2+}/CaM-dependent adenylate cyclase toxin, which acts by binding cytokine receptors and inhibiting T-cell chemotaxis, thus suppressing the immune response [29]. In *Pseudomonas aeruginosa*, increased $[Ca^{2+}]_i$ levels induce biofilm formation and production of virulence factors [16].

MOLECULAR BASIS OF Ca^{2+}-DEPENDENT REGULATION OF CELL FUNCTIONS IN BACTERIA

Similar to eukaryotic cells, Ca^{2+} signaling in bacteria is based on local changes in the concentrations of free calcium ions ($[Ca^{2+}]_i$) in the cytoplasm. It was found that $[Ca^{2+}]_i$ levels in viable bacteria are sustained low, and may be approximately 1000 times lower than in the environment [22]. A number of studies have focused on the problems of Ca^{2+} transport and the mechanisms of Ca^{2+} homeostasis in prokaryotes [4, 5, 30–35].

In most detail, Ca^{2+} homeostasis was studied in *E. coli*. Analysis of the *E. coli* transcriptome identified 110 genes affecting cytosol concentrations of Ca^{2+}. Among them, 41 transcripts acted to increase $[Ca^{2+}]_i$, and 69 transcripts downregulated $[Ca^{2+}]_i$ [20]. Experiments using Fura-2 and aequorin showed that *E. coli* cells maintained constant $[Ca^{2+}]_i$ levels of approximately 90 nM [12, 15]. The low Ca^{2+} concentration in the cytosol of bacterial cells is maintained due to three factors: selective permeability of the cell membrane (which is finely tuned to control the Ca^{2+} intake), the high buffer capacity of the cytosol, and the efficient system of Ca^{2+} export.

Calcium intake by bacterial cells. While the molecular mechanisms of Ca^{2+} intake by eukaryotic cells have been characterized fairly well [1, 2, 36–38], their prokaryotic counterparts are currently insufficiently understood. In bacterial cells, two types of voltage-gated Ca^{2+} channels have been discovered: low-selectivity Ca^{2+} protein channels (Cav) [39, 40] and combined Ca^{2+} channels composed of poly-β-hydroxybutyrate/calcium polyphosphate membrane complexes [41]. Based on their electrochemical characteristics, both types of channels—similar in function, although different in origin—were classified as VOCCs Ca^{2+} channels. It had been previously assumed that Cav channels were present only in eukaryotic cells.

A comparison of primary structures of the Cav channels of in pro- and eukaryotic cells showed that, while the main subunits of eukaryotic Ca^{2+} channels were composed of four repeat domains of 300 to 400 amino acid residues, in prokaryotes the protein subunit forming the channel pore consisted of only one domain comprising six transmembrane α-helical segments S1–S6 (Fig. 1) [40, 42]. In bacteria, Cav channels show the features of L-type channels [39]. It was found that, similarly to classical L-type voltage-gated channels (Cav1.m) [43], bacterial Cav channels (e.g., in *B. subtilis*) could be activated by membrane depolarization and in response to BAY K8644, a 1,4-dihydropyridine derivative, a known Cav1.m agonist, and blocked by Cav1.m antagonists: phenylalkylamines and 1,4-dihydropyridine, as well as by La^{3+} ions [39]. However, the genes encoding the protein subunits of this channel in *B. subtilis* have not been identified so far. In *Bacillus halodurans*, the gene encoding the protein forming the NaChBac ion channel was cloned. The molecular structure of this channel was similar to that of L-type Ca^{2+} channels from vertebrate skeletal muscle cells, but differed from them in its higher selectivity towards Na^+ and lower selectivity towards Ca^{2+} [40, 42]. Two of the further eleven bacterial NaChBac-like proteins, Na(v)PZ from *Paracoccus zeaxanthinifaciens* and Na(V)SP from *Silicibacter pomeroyi*, also exhibited the structural and functional properties of mixed-type voltage-gated channels [44]. Investigation of NaChBac-like channels showed that their selectivity towards Na^+ or Ca^{2+} depended on only three amino acid residues located at the pore entrance; substitution of these residues changed the channel specificity from sodium to calcium [45]. Moreover, in some extremophilic microorganisms, even a single amino acid substitution in a key

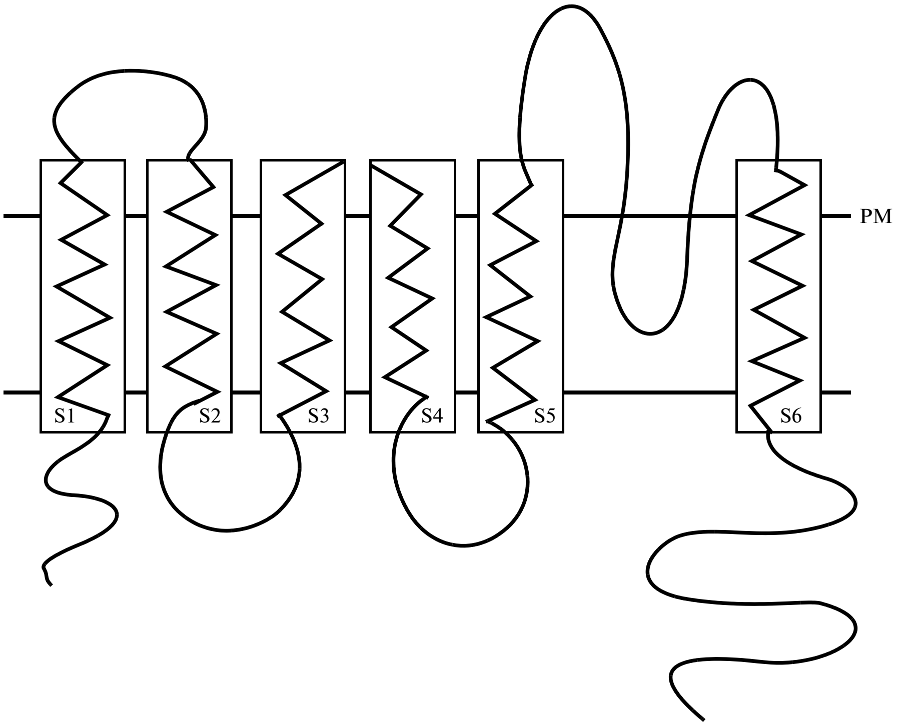

Fig. 1. The hypothetic primary structure of the voltage-gated Na_vPZ channel for Na^+ and Ca^{2+} ions from *Paracoccus zeaxanthinifaciens.* Blocks show teh S1–S6 transmembrane domains [44].

position of the selective pore filter could generate a Ca^{2+}-specific channel [46]. Voltage-gated Ca^{2+} ion channels were also found in *E. coli*; however, their pharmacological characteristics indicated that they were closer to vertebrate T-type channels [47]. Characteristically, *E. coli* channels of this type are significantly more sensitive to ω-conotoxin than to verapamil and gallopamil, which block the L-type Ca_v channels.

The non-protein bacterial calcium channels have been studied in more detail [41, 48]. These channels were detected in *E. coli* cells using artificial membrane constructs. The poly-β-hydroxybutyrate/calcium polyphosphate membrane complexes extracted from bacterial membranes was incorporated into an artificial bilayer. In this construct, the reconstructed Ca^{2+} channels (PHB/polyP channels) exhibited the properties of classical VOCCs channels. They were activated by a membrane potential (60 mV), had a considerable conductivity (9 pS), and were highly selective towards Ca^{2+}, Sr^{2+}, and Ba^{2+}, but not towards Mg^{2+}. Their selectivity towards monovalent ions was affected considerably by local pH values [48]. The relationship between the blocking of Cav channels and La^{3+} concentrations in bacteria was similar to the dependence observed for classical eukaryotic VOCCs channels [41].

Investigation of the biological functions of Cav channels in *E. coli* showed that their functional characteristics were different at different growth phases of the culture. For instance, in exponentially growing cells, membrane depolarization caused Cav activation at significantly lower external Ca^{2+} concentrations than in the stationary phase cells [41], probably suggesting involvement of different mechanisms in ion-dependent regulation of growth processes in prokaryotes. Bacterial Cav channels also play a certain role in antiviral protection. In their study of *Salmonella typhimurium*, Ter-Nikogozyan et al. [49] showed that bacteriophage infection was accompanied by depolarization of the cell membrane (10–30 mV) and a rapid reversible intake of protons. These processes decreased the cell permeability for bacteriophages and, as a result, the extent of viral infection in bacteria. However, the described processes can take place only in the environments containing Ca^{2+} ions.

Calcium binding by target proteins. An important prerequisite for the signaling function of Ca^{2+} ions is their ability to bind their target proteins sequentially and reversibly. Many of these targets belong to the group of so-called calcium-binding proteins capable of transferring the calcium signal further to the effector protein, which induces an immediate cell response, or to a transcription factor triggering a chain of programmed cell events.

It was long assumed that calcium-binding receptor proteins exist only in the cells of higher eukaryotes, including humans; however, proteins capable of binding Ca^{2+} with high affinity, including calmodulin-like (CaM-like) proteins were later also discovered in

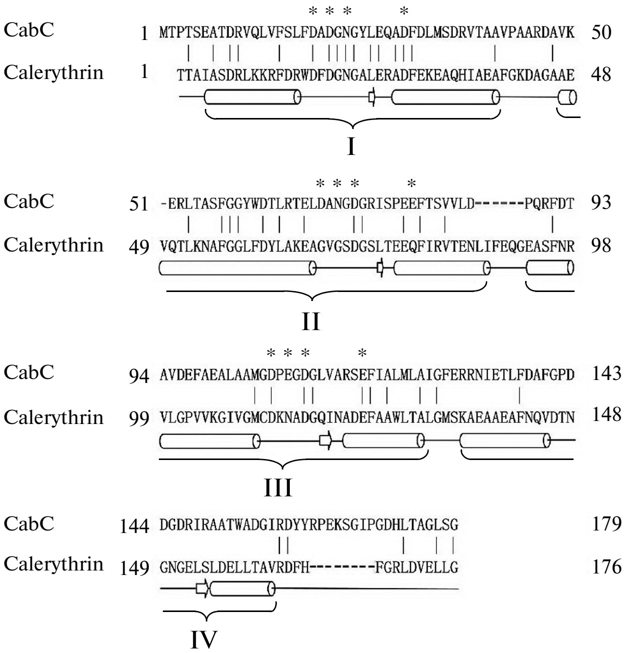

Fig. 2. Amino acid sequences of CaM-like proteins CabC from *Streptomyces coelicolor* and calerythrin from *Saccharopolyspora erythraea*. Secondary structure elements are shown with cylinders (α helices), arrows (β sheets), and lines (loops). Four EF hand subdomains are numbered I to IV; the conserved Ca^{2+}-binding amino acid residues at positions 1, 3, 5, and 12 of EF hand subdomains are marked with asterisks [57].

lower eukaryotes and even in bacteria [50–55]. Moreover, some of these proteins were found to contain two or several structural fragments resembling the EF hand motif [56, 57]. Currently, several bacterial CaM-like proteins have been characterized and the corresponding genes have been cloned [55–60]. A large group of CaM-like proteins was described in *Streptomycetaceae*, although only a few of them contained four EF hand motifs [57]. Their number includes CaM-like proteins of actinomycetes, CaMs from invertebrate muscle cells, calerythrin from *Saccharopolyspora erythraea*, and CabC from *Streptomyces coelicolor* (Fig. 2).

Whereas calcium-saturated calmodulin molecules of vertebrates are dumbbell-shaped, the Ca^{2+}-bound CaM-like proteins of bacteria (e.g., *S. coelicolor*) have a cylindrical shape [55]. The structural differences between eukaryotic CaMs and prokaryotic CaM-like proteins were found to lie in their C-terminal parts. Comparison of the domain structures of calmodulins from different sources showed that the primary sequences of prokaryotic Ca^{2+}-binding domains contained mainly neutral amino acids, such as glycine, alanine, and serine, which contributed insignificantly to Ca^{2+} binding. At the same time, Ca^{2+}-binding sites of bacterial CaM-like proteins contain negatively charged residues, such as glutamic and aspartic acids, which participate in calcium binding by eukaryotic calmodulins; moreover, their sequence and frequency are largely the same as in CaM EF hand domains of higher eukaryotes (Fig. 2). This fact further confirms the importance of these residues for Ca^{2+} binding and shows that Ca^{2+}-binding domains are highly conserved in calmodulins from different types of cells [58].

In *B. subtilis*, it was shown that the rate of spore formation increased significantly in the presence of a CaM-like protein; on the other hand, trifluoroperazine, a CaM inhibitor, decreased this rate considerably [53]. Calcium ions mediated assemblage of the CaM-like protein S on the external surface of the myxospore in *Myxococcus xanthus* and the translocation of the 55 kDa protein to the extracellular matrix in another myxobacterial species, *S. aurantiaca*; thus, they were involved in the formation of the fruiting body [61, 62].

In addition to CaM-like proteins, bacteria were found to contain Ca^{2+}-binding proteins that lack the well-known Ca^{2+}-binding sites with a helix–loop–helix (EF hand) motif. This group of proteins includes, in particular, the CcbP protein from cyanobacteria *Anabaena* sp. A specific feature of this protein is the presence, in addition to two Ca^{2+}-binding sites, of a subdomain that resembles the SH3 (Src homology 3) domain of the signal proteins involved in protein–protein interactions [63]. Another calcium-binding protein detected in *B. subtilis*, YycH, regulates the activity of the YycG histidine kinase and is a part of the signaling pathway responsible for cell viability and growth [64].

Calcium-binding proteins are present in prokaryotic cells throughout their life cycle; they function as Ca^{2+} transporters and play an important role in maintaining Ca^{2+} homeostasis, regulating enzyme activity, and signal transduction to the cell genome [55, 56]. They regulate the rate of cell division by modulating the cell cycle, affect the adaptive behavior, and mediate chemotaxis and differentiation [17, 24, 25, 53, 62, 65].

Export of Ca^{2+} ions. Bacteria possess a wide range of Ca^{2+} transport systems; for instance, three transporters and four membrane-bound proteins participating in calcium transfer were identified in *E. coli* [20]. Eukaryotic systems of Ca^{2+} transmembrane export fall into two groups: primary transporters, which use the energy of ATP hydrolysis, and secondary transporters (ion exchangers), which utilize electrochemical Na^+ or proton gradients.

Primary transporters of the membrane ATPase type have been traditionally considered the main mechanism of calcium ion export [66, 67]. In the late 1980s, it was shown experimentally that bacteria utilized ATP-dependent systems for active Ca^{2+} transfer across the cell membrane against the concentration gradient [68]. A Ca^{2+} pump that resembled eukaryotic P-type ATPases in primary structure and biochemical properties was isolated from cyanobacteria *Synechoccus* sp., and the respective genes were cloned [69, 70]. Another ATPase, which was biochemically more similar to P-type ATPases from the endoplasmic and sarcoplasmic reticulum of eukaryotic cells, was found in the soil bacterium *Flavobacterium odoratum* [71]. Yet another P-type ATPase that pumps cytosolic Ca^{2+} out of the cell was described in *Pseudomonas aeruginosa*; it transfers two calcium ions per each hydrolyzed ATP molecule [16].

Secondary transporters in bacteria are represented by ion exchangers [16]. In *E. coli*, a Ca^{2+}/H^+ exchanger is encoded by *ChaA* [72]. Presumably, this antiporter participates in Ca^{2+} transport at alkaline pH values. In his study of the Na^+/H^+ antiporter in *E. coli*, Dibrov concluded that it could activate the Ca^{2+}/H^+ exchanger, thus stimulating Ca^{2+} export [73]. In eukaryotes, urgent Ca^{2+} export is accomplished using a Na^+/Ca^{2+} antiporter. Antiporters with similar structure, biochemical and functional properties were also found in a number of prokaryotic organisms. It was shown that the Na^+/Ca^{2+} antiporter of *Streptococcus pneumoniae*, in addition to maintaining the intracellular Ca^{2+} homeostasis, performs a number of other important functions, such as controlling the cell wall lysis and acquisition of genetic competence [74].

Thus, the fact that the general organization of intracellular signaling has common traits in pro- and eukaryotes suggests that the Ca^{2+}-dependent signaling mechanisms are evolutionary well-conserved; moreover, it invites the suggestion that Ca^{2+} cations were among the key natural abiotic factors that had a significant impact on the evolution of living organisms.

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