Functional Characterization and Determination of the Physiological Role of a Calcium-Dependent Potassium Channel from Cyanobacteria1[C][W]

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Despite the important achievement of the high-resolution structures of several prokaryotic channels, current understanding of their physiological roles in bacteria themselves is still far from complete. We have identified a putative two transmembrane domain-containing channel, SynCaK, in the genome of the freshwater cyanobacterium *Synechocystis* sp. PCC 6803, a model photosynthetic organism. SynCaK displays significant sequence homology to MthK, a calcium-dependent potassium channel isolated from *Methanobacterium thermoautotrophicum*. Expression of SynCaK in fusion with enhanced GFP in mammalian Chinese hamster ovary cells’ plasma membrane gave rise to a calcium-activated, potassium-selective activity in patch clamp experiments. In cyanobacteria, Western blotting of isolated membrane fractions located SynCaK mainly to the plasma membrane. To understand its physiological function, a SynCaK-deficient mutant of *Synechocystis* sp. PCC 6803, ΔSynCaK, has been obtained. Although the potassium content in the mutant organisms was comparable to that observed in the wild type, ΔSynCaK was characterized by a depolarized resting membrane potential, as determined by a potential-sensitive fluorescent probe. Growth of the mutant under various conditions revealed that lack of SynCaK does not impair growth under osmotic or salt stress and that SynCaK is not involved in the regulation of photosynthesis. Instead, its lack conferred an increased resistance to the heavy metal zinc, an environmental pollutant. A similar result was obtained using barium, a general potassium channel inhibitor that also causes depolarization. Our findings thus indicate that SynCaK is a functional channel and identify the physiological consequences of its deletion in cyanobacteria.

Detailed structural and mechanistic data now exist for many prokaryotic channels, but their physiological roles remain largely unclear (Martinac et al., 2008). This is especially true for potassium channels. K+ is the most abundant cation in organisms, and in general, it plays a crucial role in the survival and development of cells by regulating enzyme activity and tuning electrochemical membrane potential. Potassium channels in prokaryotes have been hypothesized to contribute to the setting of membrane potential rather than to high-affinity potassium uptake normally achieved thanks to specific ATP-dependent potassium transporters (Kuo et al., 2005). K+ channel genes are found in almost every prokaryotic genome that has been sequenced, but in most of the few studies where their deletion was obtained, no specific phenotype has been observed, suggesting either functional redundancy or that these channels are only required in case of rather specific environmental stresses. Gain-of-function potassium channel (KCh) mutants of *Escherichia coli* failed to grow in millimolar-added K+ but not Na+ (Kuo et al., 2003), and external H+ suppressed the gain-of-function phenotype, supporting the hypothesis that KCh might function to regulate membrane potential. However, a clear-cut role of prokaryotic potassium channels by genetic deletion was demonstrated only in a few cases.

The model organism *Synechocystis* sp. PCC 6803 harbors an intracellular membrane system, the thylakoids, where both photosynthesis and respiration take place. In this work, we have identified in *Synechocystis* sp. PCC 6803 a so-far uncharacterized putative potassium channel, NP_440478, encoded by the open reading frame (ORF) sl00993, with sequence homology to MthK, a Ca2+-activated K+ channel of the archaeon *Methanobacterium thermoautotrophicum*. The structure of MthK in an open conformation has been determined (Jiang et al., 2002). The MthK subunit has two transmembrane segments and one pore region, followed by an extension of approximately 200 amino acid residues, which contains a region called the regulator of conductance of K+ (RCK) domain. RCK of MthK binds divalent cations, such as Ca2+ or Cd2+ (Jiang et al., 2001, 2002). The physiological meaning of the activation of MthK with millimolar Ca2+ (Jiang et al.,

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2002) is, however, unclear, because Ca$^{2+}$ as a second messenger operates at micromolar concentrations in eukaryotes, and the possible signaling roles of Ca$^{2+}$ in prokaryotes are still unclear. Another RCK-containing 160-picoSiemens (pS) K$^+$ channel from the archaeon *Thermoplasma volcanium*, TvoK, was also found to be activated by millimolar Ca$^{2+}$ (Parfenova et al., 2007). Here, we report evidence that, similar to MthK, SynCaK can also be activated by calcium. Furthermore, we show localization of the protein in cyanobacteria and describe a phenotype associated with the lack of the channel in SynCaK-less mutant *Synechocystis* sp. PCC 6803 cells.

**RESULTS**

**Predicted Structural Features of the SynCaK Channel**

A search in the nonredundant protein database at the National Center for Biotechnology Information using the W-BLAST algorithm and the amino acid sequence T-X-G-(Y-F)-G-(D-E) as query revealed a protein in *Synechocystis* sp. PCC 6803 classified as a putative potassium channel (NP_440478). Until now, there is no experimental evidence about the function of this protein, but bioinformatic analysis underlines a sequence homology with MthK (Jiang et al., 2002; Fig. 1). In silico analysis of the primary sequence of NP_440478, denominated SynCaK, indicates that the protein contains two membrane-spanning segments, a recognizable K$^+$ channel selectivity filter signature sequence with only conservative substitutions, and a RCK, similar to MthK (Fig. 1). The RCK region contains two conserved domains, K$^+$ transport systems NAD-binding domain (TrkA-N) and TrkA-C, which occur in many potassium channels and transporters. TrkA-N contains an alternating $\beta$ab$\beta$bab Rossmann-fold motif, which may bind to NAD or NADH and thereby may mediate conformational switches (Roosild et al., 2002). Because TrkA-N and TrkA-C are also present in the MthK channel, a similarity in domain organization between MthK and SynCaK is probable. Structural studies of MthK revealed the presence of an octameric gating ring, composed of eight intracellular ligand-binding RCK domains. Binding of Ca$^{2+}$ to RCK has been shown to regulate the gating ring conformation that in turn leads to the opening and closing of the channel (Jiang et al., 2002).

**Expression and Functional Analysis of SynCaK in Chinese Hamster Ovary Cells**

To prove that SynCaK forms a calcium-sensitive potassium channel as expected, we used heterologous expression in mammalian cells, followed by electrophysiological analysis. Such an approach has been successfully applied by various groups for the study of prokaryotic and even viral channels. To verify the plasma membrane (PM) localization of the channel protein expressed in Chinese hamster ovary (CHO) cells, a prerequisite for the analysis of protein function by patch clamp, SynCaK was expressed in fusion with enhanced GFP (EGFP), a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. Targeting of the channel to the PM was tested by examining colocalization of EGFP with a specific PM fluorescent dye, FM 4-64. Supplemental Figure S1A shows fluorescence microscopy analysis of transfected CHO cells indicating that at least a part of the fusion protein reaches the PM. In the inside-out excised patch configuration, SynCaK-EGFP-transfected cells displayed an ion channel activity, but only in the presence of Ca$^{2+}$ in the bath in the millimolar range. The top section of Figure 2A shows representative consecutive current traces recorded with the voltage ramp indicated after addition of 2 mM calcium to the cytoplasmic face, i.e. to the bath ($n = 15$). Cells transfected with the control vector encoding only for EGFP did not display any current at different potentials under the same ionic conditions ($n = 50$; Fig. 2A, bottom). These experiments were conducted in potassium gluconate, allowing us to state that the slightly rectifying channel we observe is permeable to potassium, given that gluconate does not permeate across chloride channels in CHO cells. Channel conductance was 45 ± 7 pS at positive voltages, while at negative voltages, we observed 29 ± 9 pS. To further prove the selectivity of SynCaK, we performed experiments under asymmetric ionic conditions with sodium gluconate in the pipette and potassium gluconate in the bath, always in the presence of 2 mM calcium ($n = 7$). The observed reversal potential ($–77 ± 12$ mV) is close to the predicted reversal potential for a perfectly selective potassium channel (value of –87 mV, as calculated from Nernst equation). Figure 2B shows a representative current trace under these conditions and shows channel activity on an extended time scale as well. To further prove that the observed channel activity was due to SynCaK, patch clamp experiments were performed also with cells expressing the mutant SynCaK68A-EGFP fusion protein; the introduced single point mutation did not alter PM targeting (Supplemental Fig. S1B) but changed a very conserved amino acid of the pore region, which, in general, is essential for potassium conduction (K$^+$ channels with GAGD sequence are known to be expressed but are unable to conduct a current [Heginbotham et al., 1994]). The mutant protein did not give rise to channel activity, even at 10 mM calcium ($n = 5$). Given the presence of the RCK domain in SynCaK, its sequence similarity to the calcium-activated MthK, and the observation that the channel was not active in the absence of calcium, we finally tested channel activity at 10 mM calcium (Fig. 2C). SynCaK activity was increased when cytoplasmic calcium concentration was increased to 10 mM. In summary, these data indicate that SynCaK is able to function as a calcium-activated potassium channel, and in accordance with reports on MthK, a high
calcium concentration in the millimolar range is required for full activation (Jiang et al., 2002).

**Localization of the SynCaK Channel in Synechocystis sp. PCC 6803 Membranes**

To determine the subcellular localization of SynCaK, a specific antibody was raised against 15 amino acids of the protein. First, we tested for the specificity of the antibody by expressing SynCaK in E. coli. Supplemental Figure S2 shows that the antibody recognized a 43-kD protein only in transfected E. coli where expression was induced by isopropylthio-

β-galactoside, proving that the antibody recognizes the recombinant protein. In whole cyanobacteria cell extracts, the antibody recognized three bands, including a band with the predicted Mr of the monomer, i.e. 40.5 kD. Subfractionation of cyanobacteria was performed as previously described (Zanetti et al., 2010) and revealed the presence of an approximately 41-kD protein in the PM (Fig. 3A). We could also observe a fainter 41-kD band in the thylakoid fraction (TH), whose intensity was consistent with residual contamination during purification of the fractions. The PM marker ABC transporter nitrate binding protein (NrtA) was found also in the isolated thylakoid, indicating contamination of thylakoid by PM. Vice versa, CP43, a protein of PSII, which is present only in the TH membrane (Zanetti et al., 2010), was not revealed in our experiment in the PM fraction (Fig. 3B). Thus, the presence of SynCaK in the PM cannot be due to contamination by thylakoids, while the observed band in thylakoids is most likely due to contamination by PM. A protein with approximately 45-kD weight was also strongly recognized in the soluble fraction, indicating unspecific cross reaction of our antibody. For this reason, our attempts to localize the protein also by electron microscopy did not give reliable results (data not shown).

**SynCaK-Less Mutant Is Characterized by Altered Membrane Potential and Resistance to Zinc**

*Synechocystis* sp. PCC 6803 is capable of integrating exogenous DNA into its genome (present in a dozen of copies) by homologous recombination, thus allowing targeted gene replacement. Almost the entire ORF sil0993, encoding the SynCaK potassium channel, was
substituted by a kanamycin resistance cassette (KanR; Fig. 4A). Knockout mutants were recovered after 10 rounds of subcloning, and complete segregation was verified by PCR on purified genomic DNA. Sites of insertion of the KanR and correctness of flanking regions (ORFs slr0994 and slr1022) were verified by sequencing the products of amplification. Figure 4B shows that, according to PCR analyses, no wild-type DNA molecules were retained in the final clones. In accordance, the absence of the 41-kD band in the mutant confirmed the lack of SynCaK protein expression (Fig. 4C). Therefore, the produced mutant was suitable for further studies to evaluate the physiological role played by SynCaK in this photosynthetic organism.

In most microorganisms, high-affinity K⁺ uptake occurs through ATP-dependent transport systems, and in Synechocystis sp. PCC 6803, the potassium-translocating subunit of the potassium-uptake system KtrAB (KtrB) transporter was shown to prevalently mediate K⁺ uptake (Matsuda and Uozumi, 2006). Recently, the inward rectifier potassium channel (KirBac6.1) has also been proposed to contribute to low-affinity K⁺ uptake in Synechocystis sp. PCC 6803 (Paynter et al., 2010). In the case of SynCaK, no significant difference could be observed in the potassium content between the wild-type and ΔSynCaK organism, as determined by atomic absorption spectroscopy, indicating that the channel does not normally contribute to K⁺ uptake (Fig. 5A). Next, we checked whether growth deficiency could be observed in the mutant with respect to the wild type under various conditions typically used to characterize cyanobacterial transporter mutants, including increasing light intensity (Fig. 5B), salt stress at 100 mM KCl, osmotic stress with 0.5 M sorbitol, lack of glucose (Glc), alkaline external pH (pH 10), and 10 mM calcium in the growth medium (Fig. 5C). None of these conditions evidenced any significant difference in growth, indicating that this channel is not involved in protection against osmotic and alkaline stress and is not indispensable for phototrophic growth. Involvement of the channel in the regulation of photosynthesis was further excluded by analysis of chlorophyll fluorescence using pulse-amplitude modulation. No differences in maximum photochemical efficiency of PSII in the dark-adapted state (Fv/Fm) were revealed between

Figure 2. SynCaK functions as a potassium channel when expressed in CHO cells. A, Representative current traces recorded under symmetrical ionic conditions (150 mM potassium gluconate on both sides plus 2 mM CaCl₂ in the bath) in the inside-out excised patch configuration. The voltage ramp protocol shown (top) was used to elicit channel activity. Two representative, consecutive current traces are shown. The bottom section shows activity recorded at -170 mV on an extended time scale. Under these conditions, reversal potential is at 0 mV. B, Representative current trace recorded under asymmetrical ionic conditions (150 mM potassium gluconate solution with 2 mM CaCl₂ in the bath and 150 mM sodium gluconate solution in the pipette; top). Current recording on extended time scale from the indicated part of the top section (middle). Representative trace recorded under the same ionic condition from CHO cells transfected with mutant SynCaK (bottom). No activity can be recorded; the observed current is due to leak. C, Current trace recorded under the same ionic conditions except that bath, i.e. the intracellular side, contained 10 mM CaCl₂ (top). Traces in bottom section show activity on extended time scale from the indicated region and at +100 mV. [See online article for color version of this figure.]
wild-type and mutant organisms, indicating that photosynthetic efficiency was not dependent on the presence of the channel (data not shown).

Proton pumping and sodium motive force are crucial in determining the resting membrane potential in bacteria, but K+ gradient across the cell membrane is also expected to contribute to membrane potential. In cyanobacteria, both potassium efflux and influx could be detected (Reed et al., 1981). Given the very small capacitance of the cytoplasmic membrane of bacterial cells, only a very small number of ions flowing through an ion channel would be sufficient to cause a substantial change in the resting membrane potential (Martinac et al., 2008). To test the hypothesis that the lack of SynCaK might alter the membrane potential, we used the ratiometric fluorescent cyanine dye 5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazolocarbocyanine iodide (JC-1), efficiently exploited to measure very negative membrane potentials in mitochondria and bacteria (Novo et al., 1999). JC-1 is a cationic dye that exhibits potential-dependent accumulation in bacteria, indicated by a fluorescence emission shift from green (approximately 525 nm) under blue light excitation to red (approximately 590 nm) under green light excitation. Consequently, membrane depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. Our measurements, shown in Figure 6, suggest that the ratio between green versus red forms of the dye is significantly different between wild-type and mutant cells, indicating a depolarized membrane potential in the mutants (Fig. 6, A and B). The ratio of green to red fluorescence is dependent only on the wild-type and mutant organisms, indicating that photosynthetic efficiency was not dependent on the presence of the channel (data not shown).

Figure 3. Localization of SynCaK in cyanobacteria. A PM, soluble (S), thylakoid membrane (TH), and outer membrane (OM) fractions were isolated from Synechocystis sp. PCC 6803 (2 μg of proteins per lane). The channel protein was detected using α-SynCaK antibody at the expected molecular weight of 41 kD. B, The purity of PM and TH fractions were checked by using antibodies against the PM marker NrtA and the thylakoid marker CP43 (2 μg of proteins per lane).

Figure 4. Construction of a SynCaK-deficient Synechocystis sp. PCC 6803 strain. A, Schematic diagram of construction of the mutant organism. ΔSynCaK was obtained by inserting a KanR into the Synechocystis sp. PCC 6803 genome. Forward and reverse primers, introducing mutagenic sites, were used to generate a PCR product containing the SynCaK gene in the central position and two flanking regions (see “Materials and Methods” for details). B, PCR analyses indicated lack of wild-type gene and correct insertion of the KanR gene in the mutant organism. Agarose gel electrophoresis of analytical PCR amplifications performed on genomic DNAs from kanamycin-resistant control and ΔSynCaK strains: molecular mass marker (1-kb ladder, Promega; 1); wild-type DNA amplified with VC9 and CV10 primers (2); ΔSynCaK DNA amplified with VC9 primers (3); wild-type DNA amplified with VC9 and DISP2 primers (4); ΔSynCaK DNA with VC9 and DISP2 primers (5); wild-type DNA with DISP3 and CV10 primers (6); ΔSynCaK DNA with DISP3 and CV10 primers (7); wild-type DNA amplified with DISP2 and DISP3 primers (8); and ΔSynCaK DNA with DISP2 and DISP3 primers (9). C, Western blotting of protein extracts (optical density at 730 nm = 0.3) using the SynCaK antibody, showing no detection of SynCaK channel (41 kD, arrow) in the mutant strain. Unspecific recognition of two other bands indicates equal loading. WT, Wild type.
membrane potential and not on other factors, such as size, shape, and density, that may influence single-component fluorescence signals (see JC-1 data sheet of Invitrogen). The use of fluorescence JC-1 ratio detection therefore allowed us to make comparative measurements of membrane potential. To further study the involvement of bacterial potassium channels in setting the membrane potential, we used a general potassium channel blocker, barium. Addition of barium to E. coli expressing SynCaK reduced overexpression-

Figure 5. Wild-type and mutant organisms do not differ in potassium content, and their growth is comparable at various light intensities and under osmotic and salt stress. A, The potassium concentration was determined by atomic absorption spectroscopy. The amount of K⁺ was normalized to the chlorophyll concentration of the respective cultures (P > 0.05). B, Wild-type and SynCaK-less cells were grown on solid BG11 medium supplemented with 5 mM Glc (except when indicated without Glc [No Glc]) at the indicated light intensities. Photos were taken at day 4. The experiment was repeated two times giving similar results. C, Cells were grown as in B under the indicated conditions at 20 μE m⁻² s⁻¹ light intensity. WT, Wild type. [See online article for color version of this figure.]

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- **A**: Barium addition to E. coli expressing SynCaK reduced overexpression.
related toxicity, suggesting that SynCaK is sensitive to barium (Supplemental Fig. S2; data not shown). One millimolar of barium added to the *Synechocystis* sp. PCC 6803 wild-type culture for 24 h was not toxic for the cells (data not shown) but resulted in a significant depolarization (Fig. 6B).

According to these hypotheses, most microbial channels might be called to function upon peculiar environmental stresses. Zinc is an essential metal but is toxic at high concentrations. Zinc regulation transporter/iron regulated transporter-like proteins (ZIPs) represent a major route for entry of zinc ions into prokaryotic cells, and a recent work demonstrated that bacterial ZIP is a selective electrodiffusional channel (Lin et al., 2010). Thus, ZIP facilitates passive zinc uptake driven by zinc concentration gradients and electrical driving force. Instead, ZitB was proposed to act as an efflux pump, because its overexpression increased zinc tolerance and reduced zinc uptake in *E. coli* (Grass et al., 2001). Zinc transporter B (ZitB) function depends on K\(^+\) and H\(^+\) fluxes (Guffanti et al., 2002), and this molecule was identified as a Zn\(^{2+}/H^+\)
antiporter (Chao and Fu, 2004). In light of these reports, we tested whether the SynCaK strain, having a decreased membrane potential, might become more resistant to zinc. The concentrations tested were in the range of the half maximal effective concentration for the acute toxicity of Zn\(^{2+}\) in the cyanobacteria *Anabena* spp. (Barrán-Berdón et al., 2011). Results of Figure 7A demonstrate that increasing concentration of zinc compromised growth of the wild-type cyanobacteria, in accordance with results of toxicology studies (Zeng and Wang, 2011). SynCaK-less organisms were more resistant with respect to wild-type cells. Resistance to zinc was also observed in cells cultured in liquid medium for two independent SynCaK-less mutant clones, confirming that the phenotype is related to the lack of the channel (Fig. 7B). Because barium caused a significant depolarization of wild-type cells, we tested whether it may mimic the lack of SynCaK and render wild-type cells more resistant to zinc. Wild-type cells cultured in the presence of 1 mM barium and zinc (at the indicated concentration) were significantly more resistant to zinc than those cultured only with zinc (Fig. 7B).

**DISCUSSION AND CONCLUSION**

In this work, we have identified a new putative channel in the genome of *Synechocystis* sp. PCC 6803, demonstrated that it functions as potassium channel, constructed a knockout strain, and identified a growth condition under which the mutant shows a clear phenotype. Our results suggest that SynCaK might be involved in the regulation of membrane potential in cyanobacteria and its lack confers an increased resistance to zinc, an environmental pollutant.

A function related to ion homeostasis has been elucidated only in the case of very few bacterial ion channels. Mechanosensitive channels serve for fast release of osmolytes and ions in bacteria challenged with osmotic stress (Berrier et al., 1992; Martinac et al., 2008), and Large-Conductance Mechanosensitive Channel was proposed to also mediate calcium efflux in cyanobacteria (Nazarenko et al., 2003). In a recent study, a K\(^{+}\)-dependent slight growth defect was observed when the cyanobacterial homolog of KirBac6.1 was deleted from *Synechocystis* sp. PCC 6803, suggesting that KirBac might contribute to low-affinity
uptake of K⁺ (Paynter et al., 2010). Finally, a recent study from our laboratory provided genetic evidence that the voltage-dependent six transmembrane-segment channel SynK, located in thylakoid membranes in *Synechocystis* sp. PCC 6803 (Zanetti et al., 2010), is required for efficient photosynthesis. In particular, the lack of the channel led to an altered partitioning of the proton motive force across thylakoids between the electric and the osmotic component. As a consequence, the SynK-less mutant showed high photosensitivity (Checchetto et al., 2012). In summary, evidence is accumulating on the physiological role of cyanobacterial ion channels.

As to the channel activity of SynCaK, it resembles some of the properties of MthK, a calcium-dependent potassium channel. In particular, MthK has been shown to function also at low calcium concentration, but its open probability sharply increased upon addition of calcium up to 25 mM in bilayer experiments to the internal side (Jiang et al., 2002). In accordance, Zadek and Nimigean (2006) observed activation by calcium in the millimolar range. In the case of SynCaK, we also find an increased activity when augmenting the intracellular concentration to 10 mM calcium in inside-out excised patches. The properties of the channel could not be studied in the whole-cell configuration given that cells cannot withstand such a high intracellular calcium concentration. The conductance of the MthK channel was 200 pS in 150 mM KCl at −100 mV when reconstituted into an artificial bilayer (Jiang et al., 2002). In another report, the chord conductance was 240 pS at −200 mV in 200 mM KCl (Zadek and Nimigean, 2006). In both reports, a strong rectification was observed, probably due to a fast block by calcium or to an electrostatic screening effect. However, when expressed in *E. coli* cytoplasmic membrane, MthK displayed an inward rectification and approximately 50 pS conductance in 150 mM KCl (Kuo et al., 2007). SynCaK, when expressed in CHO cells, displays a slight rectification and 45 pS conductance at positive voltages. The reason for the lack of strong rectification is not clear. A more detailed electrophysiological/pharmacological characterization is beyond the scope of this manuscript, which aimed to define the physiological function of such channels. In any case, we show that the overall behavior of SynCaK is compatible with this protein being a calcium-activated potassium-selective channel.

Cyanobacteria are the most abundant photosynthetic organisms on Earth. In general, photosynthetic organisms grow in an environment that is increasingly polluted by heavy metals. In this respect, it is interesting to note that cyanobacteria are also suitable for biosensor and/or bioremediation applications. In the case of a SynCaK-less mutant, our tentative explanation for its increased resistance to zinc derives from the observed depolarized cytoplasmic potential. Assuming an internal potassium concentration of 180 mM based on measurements in cyanobacteria (Reed et al., 1981), under our ionic conditions, the calculated equilibrium potential for potassium is −103 mV. Taking into account the average value of approximately −70 mV for the membrane potential measured in various cyanobacterial strains (Reed et al., 1981; Apte et al., 1987; Ritchie and Larkum, 1998), an efflux of potassium via SynCaK might thus occur. Inhibition of the efflux either by barium or lack of the channel might therefore lead to depolarization. The observed depolarization in turn can cause a decreased electrical gradient for Zn²⁺ uptake and might instead favor Zn²⁺ efflux. However, our results do not exclude other possible explanations. SynCaK is not likely to participate directly in zinc uptake, because the selectivity filter of potassium channels do not allow permeation of zinc ions. Zinc instead has been shown to modulate channel gating and even to block some potassium channels (Mathie et al., 2006). Unfortunately, the exact mechanisms leading to zinc resistance in cyanobacteria are still not completely understood, and both decreased (Zeng and Wang, 2011) or increased zinc accumulation compartmentalization have been proposed to lead to resistance toward this heavy metal. In any case, active regulation of zinc transport rather than genetic adaptation has been proposed to be the most important mechanism for zinc detoxification (Zeng et al., 2009). Earlier studies showed that metal efflux was an important mechanism to regulate the intracellular metal content in bacteria (Nies, 2003; Hassler et al., 2005). Furthermore, the internal metals were found to be partitioned in different subcellular compartments and to be sequestered by metallothioneins (Turner and Robinson, 1995), which may affect the tolerance capability (Wang and Rainbow, 2004). Our attempts to reveal a large difference in zinc content, as determined by atomic absorption spectroscopy, were unsuccessful (data not shown). The situation is further complicated by the fact that our knowledge on the physiological function of potassium channels in bacteria is very limited, so it cannot be excluded that they have an impact, e.g. on signaling pathways, on enzyme activity, on redox state, and on proliferation, similarly to animal cells. Thus, the lack of SynCaK may impact zinc homeostasis by a more indirect way. In conclusion, further advance in the field is necessary to understand the exact mechanism by which SynCaK regulates tolerance to zinc.

In summary, our work demonstrates that SynCaK works as a potassium channel, in accordance with the bioinformatic predictions. Furthermore, we highlight the role of a potassium channel in regulating membrane potential, a hypothesis forwarded long ago concerning the function of prokaryotic potassium channels but, to our knowledge, not proved so far by genetic means. This conclusion is further supported by the depolarizing effect of barium, a general potassium channel blocker. SynCaK, although contributing to the maintenance of membrane potential, does not compromise survival of cyanobacteria and apparently is not involved in protection from various stresses but regulates tolerance to zinc.
MATERIALS AND METHODS

Construction of pSynCaK-EGFP Vectors

The SynCaK gene was amplified by PCR from genomic DNA and cloned into pEGFP-N1 vector (Clontech). Primers were designed to introduce a HindIII site at the initiation codon and a BamHI site abolishing the stop codon so that the gene could be expressed as fusions to the N-terminus of EGFP (SynCaK-EGFP). The HindIII-BamHI restriction fragment of PCR product was introduced into the pEGFP-N1 multiple cloning site to give plasmid pSynCaK-EGFP. The single point mutation F68A was obtained by using the QuickChange II Site Directed Mutagenesis Kit (Stratagene) to give plasmid pF68ASynCaK-EGFP. The recombinant construct was then verified by DNA sequencing. The wild-type and mutant fusion proteins SynCaK-EGFP were expressed in CHO cells and used in electrophysiological analysis, with the mutant form being used as negative control.

CHO Cell Culture and Transfection

CHO cells were maintained at 37°C and 5% (v/v) CO2 in culture medium (Dulbecco’s Modified Eagle Medium, 10% (v/v) fetal bovine serum, 1% (w/v) penicillin/streptomycin, and 1% (v/v) nonessential amino acids). Cells were treated by standard trypsinization at 70% to 80% confluence. Culture medium was changed every 2 or 3 d to maintain good growth condition. One day prior to transfection, the cells were trypsinized, counted, and confluent layers of cells were grown on coverslips and were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were transfected with 0.5 μg of DNA.

Confocal Microscopy

After transfection (48 h), CHO cells were incubated with FM 4-64 dye (Invitrogen), a dye specific for PM, and analyzed using a Nikon PCM2000 confocal microscope (Bio-Rad). We prepared a working staining solution of 5 μg mL⁻¹ dye in ice-cold Hank’s Balanced Salt Solution (Invitrogen). The coverslip with the cells was washed once in HBSS and immersed in the ice-cold staining solution for 30 s. After removal from the staining solution, cells were then mounted on glass slides and observed with a laser scanning confocal microscope. Fluorescence filters set was as follows: excitation, 485 nm for EGFP and 543 nm for FM 4-64 and emission, 515/530 nm for EGFP and 570-590 nm long pass for FM 4-64. Observations were made with a Plan Apo 63× oil immersion objective with a numerical aperture of 1.4. Image analysis was done with the ImageJ bundle software (http://rsb.info.nih.gov/ij/).

Patch Clamp Analysis

Patch clamp experiments were performed in inside-out patch configuration on control or transfected CHO cells as previously described (Szabò et al., 2000). Bath solution was 150 mM potassium glutamate, 10 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.2, if not otherwise specified. Pipette solution was 150 mM sodium glutamate, 1 mM CaCl₂, and 10 mM HEPES, pH 7.2. Transmembrane voltages were applied, and currents were monitored using an EPC-7 amplifier (HEKA-List). Pulse protocol was applied, and data analysis was performed using the PClamp8 program set (Axon). The pipette resistance was 2 to 5 MΩ. Data were low-pass filtered with an 8-pole Bessel filter with a cutoff frequency of 0.5 kHz and analyzed offline.

Production of Anti-SynCaK Antibody

Anti-SynCaK antibody was produced against the synthetic peptide EQK-VIDERADHYILC, corresponding to a specific sequence of SynCaK, by Agrisera.

Cyanobacterial Strains and Growth Conditions and Isolation of PM, Soluble, and Thylakoid Membrane Fractions from Synechocystis sp. PCC 6803

Strains were cultured in BG-11 medium supplemented with 20 mM TES-KOH (pH 8.2; referred to as BG-11, measuring K⁺ 6 mM). The mutant strain grows in BG-11 containing 50 μg mL⁻¹ kanamycin at 30°C under continuous illumination (20 μmol photons m⁻² s⁻¹) with rotary shaking. Growth on agar plate was obtained under the indicated conditions. Cyanobacteria cells were fractionated as described in Zanetti et al. (2010).

Determination of Chlorophyll and Protein Concentration

Pigments were extracted with 100% (v/v) methanol. The extracts were mixed, and the concentration of chlorophyll a was measured according to the method described in Checchetto et al. (2012). Total protein concentration was determined by the bichromonic acid protein assay.

SDS-PAGE and Western blotting

The electrophoretical separation of proteins in denaturing polyacrylamide gels was carried out as previously described (Bergantino et al., 2003). For immunodetection, proteins were transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with diluted primary antibody (1:2500 for SynCaK and 1:2000 for anti-His tag) in blocking solution for 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit antibody was used as secondary antibody. Proteins were visualized with ECL Western Blot Detection Kit (Pierce).

Expression in Escherichia coli

SynCaK gene was amplified by PCR and cloned into pEGFP-N1 vector (Clontech). This construct was used as template in two separate PCRs with primers C-G_FOR (5′-ATTAATTGGGCGGGATTGGG-3′) and pEGFP-Crick (5′-GACACCGTAACTTGTGG-3′) or pEGFP_Watson (5′-TGTCGTTGGGAGTCTA-3′) and C-G_REV (5′-CCCCCATCGGCCCACCAATT-3′) and high-fidelity polymerase (Finnzyme). A following overlapping PCR was performed using VC7 (5′-CAGGTTCAAGCCCATGGGATTG-3′, inserting a NoI site) and CV8′ (5′-GACCGGTGGCAGGATCTT-3′, inserting a XhoI site) and both previous amplifiers as template. The PCR product and the expression plasmid pET28a(+) were digested with NoI and XhoI and ligated with T4 ligase. The resulting clone pET28a(+)SynCaK was subjected to DNA sequencing and excised to C41(DE3) cells. For protein expression, 1 mL preculture from one freshly transformed colony was grown overnight and used to inoculate 25 mL Luria-Bertani medium containing 50 μg mL⁻¹ kanamycin at 37°C. Culture was grown under continuous shaking to A₅₄₀ of 1, expression was induced by 0.7 mM isopropylthio-β-galactoside, and then culture was divided and further grown for 24 h. Addition of 1 mL barium to the culture medium decreased toxicity. Aliquots of cells equal to A₅₆₀ of 1 were harvested at different times for each culture. Samples were centrifuged at 14,000g for 5 min and recovered pellets were solubilized in Laemmli loading buffer. Yield of expression was evaluated by Western blot using specific antibodies (anti-His tag [Sigma] and anti-SynCaK [Agrisera]).

Determination of Potassium Concentration in Cell Extracts and Culture Medium

K⁺ concentration was determined by atomic absorption spectroscopy with an Analyst 100 spectrophotometer (PerkinElmer Life), using an acetylene/air burner for flame analyses. Cell suspensions of 50 mL were pelleted (5,000 rpm, 10 min). The pellet was resuspended in a final volume of 2 mL with water and then treated with 1 mL of nitric acid overnight at 80°C. After the incubation, the potassium concentration was measured. The amount of K⁺ was normalized to the chlorophyll concentration of the respective cultures.

Production of a SynCaK-Less Synechocystis sp. PCC 6803 Mutant

Forward VC9 (5′-GGCGGCTACTCGGTGCGCTG-3′) and reverse CV10 (5′-ACGCCCCGGCGACACCTT-3′) primers were used to generate a PCR product including the part of sll0994, sll0993, and slr1022 ORFs. The Smal_FOR (5′-TGGCGGTGGCAGAATCCCGGGCTGTTCGAG-3′) and CV8 (5′-GACCGGTGGCAGGATCTT-3′) primers, both intro-
Mut22_REV and Mut33_FOR had been projected with overlapping 5' extension containing a BamHI restriction site. The two fragments were purified from agarose gel and used together as template in amplification with primers Smal_FOR and Smal_REV. The single, 1202-bp-long, obtained fragment was introduced in the pGEM-T Easy vector (Promega). A KanR, derived by BamHI digestion from plasmid pUC4K, was cloned into the unique BamHI site of the latter plasmid to give the final plasmid pSynCaKKO-20. In the new plasmid, the entire insert was completely sequenced to verify that no undesired mutation had been introduced. Lastly, the new plasmid was used to transform wild-type Synechocystis sp. PCC 6803. Transformants were selected by screening for antibiotic resistance on BG-11 plates containing 10 μg mL−1 kanamycin. After repeated subcloning steps, complete segregation of recombination mutants in independent strain was tested by PCR with primers VOS, CV10, DSP2-rev (5'-ATAAAGGCGCCGCGATAATGTCG-3') and DSP3-for (5'-CCGCAAGTGACCGCTAATTGCCTCG-3').

Membrane Potential Analysis in Synechocystis sp. PCC 6803

The dye JC-1 (Molecular Probes) was used to label cyanobacteria in transmembrane potential-dependent manner. A stock solution of JC-1 was prepared at a concentration 1.5 μM in dimethyl sulfoxide (Sigma) and stored at −20°C. Fresh staining solution (7.5 μL) was prepared each time before application by diluting the stock solution in a buffer containing 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES at pH 7.2 (Simeonova et al., 2004) and added to the cells. Imaging and analysis of the mitochondrial transmembrane potential with the JC-1 probe were performed using a Leica LCS-SP5 confocal microscopy.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of SynCaK in CHO cells.

Supplemental Figure S2. Anti-SynCaK antibody specifically recognizes SynCaK protein expressed in CD41 DE3 E.coli.

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LITERATURE CITED


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