

SHORT COMMUNICATION

A steep Ca^{2+} -dependence of a K^+ channel in a unicellular green alga

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Abstract

An increase of cytosolic Ca^{2+} in the unicellular green alga *Eremosphaera viridis* activates Ca^{2+} -dependent K^+ channels causing a hyperpolarization of the plasma membrane. Data from parallel calcium, and potential measurements were combined with *I/V* relationships. This yielded a steep Ca^{2+} -dependence of K^+ channels with a co-operativity of 4 and an affinity of 300 nM.

Key words: *Eremosphaera viridis*, plasma membrane, Ca^{2+} -dependent K^+ channel, co-operative binding.

Introduction

Among the second messengers proposed to be involved in the regulation of K^+ channels, cytosolic Ca^{2+} is most frequently mentioned. In animal cells, a large group of Ca^{2+} -activated K^+ channels has been characterized in detail (Latorre *et al.*, 1989). In plant cells little is known about Ca^{2+} -dependent K^+ channels. The inward rectifying K^+ channel of the guard cell plasma membrane is inhibited by increasing cytosolic free Ca^{2+} in the physiological range (Schroeder and Hagiwara, 1989; Blatt *et al.*, 1990; Fairley-Grenot and Assmann, 1992; Lemtiri-Chlieh and MacRobbie, 1994; Kelly *et al.*, 1995). An ion channel resembling large conductance Ca^{2+} -activated K^+ channels in animal cells was characterized in the tonoplast membrane surrounding protoplasmic droplets from *Chara* (Laver and Walker, 1991). Some plant outward rectifying K^+ channels seem to be activated by increasing cytosolic Ca^{2+} as well (Stoeckel and Takeda, 1989; Ketchum and Poole, 1991). Quantitative studies about the effect of

physiological cytosolic Ca^{2+} concentrations on plant plasma membrane K^+ channels have not yet been reported.

In the unicellular green alga *Eremosphaera viridis* different stimuli like darkening, caffeine or Sr^{2+} , or micro-injection of InsP_3 cause characteristic single or repetitive transient hyperpolarizations of the plasma membrane (Schönknecht *et al.*, 1998). Over a wide range of external K^+ concentrations (0.1 mM to 100 mM) the amplitude of the hyperpolarization follows the Nernst potential for K^+ (Köhler *et al.*, 1985; Bauer *et al.*, 1998) indicating that the opening of plasma membrane K^+ channels gives rise to the hyperpolarization. Each artificial increase of the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cy}}$) by Ca^{2+} ionophores (Thaler *et al.*, 1989) or by direct micro-injection of Ca^{2+} (Förster, 1990) induces a transient hyperpolarization. Parallel measurements of $[\text{Ca}^{2+}]_{\text{cy}}$ and free running membrane potential in *Eremosphaera* have shown that transient hyperpolarizations of the plasma membrane after darkening or after addition of caffeine or Sr^{2+} are always accompanied by parallel $[\text{Ca}^{2+}]_{\text{cy}}$ spikes. Recently it was demonstrated that the $[\text{Ca}^{2+}]_{\text{cy}}$ spikes cause transient hyperpolarizations and not the other way round (Bauer *et al.*, 1998). Obviously, transient hyperpolarizations in *Eremosphaera* are due to the Ca^{2+} -dependent opening of K^+ channels (Schönknecht *et al.*, 1998). Here, the relation between the cytosolic free Ca^{2+} concentration and the membrane conductance in *Eremosphaera* was investigated in a quantitative manner. Based on parallel measurements of $[\text{Ca}^{2+}]_{\text{cy}}$ and membrane potential, in combination with data from *I/V* relationships, a quantitative analysis of the Ca^{2+} -

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Abbreviation: $[\text{Ca}^{2+}]_{\text{cy}}$, free cytosolic Ca^{2+} concentration.

dependent opening of the plasma membrane K^+ channel in *Eremosphaera* is presented.

Materials and methods

Plant material and solutions

The coccal green alga *Eremosphaera viridis* de Bary (algal culture collection, Göttingen LB 228-1, Germany) was cultivated as described (Köhler *et al.*, 1983). The external medium contained 0.1 mM KNO_3 , 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM MES adjusted to pH 5.6 by NaOH. Stable oscillations of $[Ca^{2+}]_{cy}$ and membrane potential were induced by the addition of 20 mM caffeine (divalent cations substituted by 0.1 mM EGTA) (Bauer *et al.*, 1997) or by 0.1 mM $SrCl_2$ (Bauer *et al.*, 1998). The measuring chamber was continuously perfused with medium at a flow rate adjusted to exchange the chamber volume within 1 min. Algal cells, which had diameters of at least 150 μm , were fixed by a suction pipette and impaled with the help of mechanical micromanipulators.

Measurement of cytosolic Ca^{2+} activity and membrane potential

The fluorescent Ca^{2+} -sensitive dye fura-2-dextran ($M_r = 10\,000$; Molecular Probes, Netherlands) was injected mechanically into the cytosol of the alga by a laboratory-made injection syringe as recently described (Plieth and Hansen, 1996). After microinjection the glass capillary was removed and a microelectrode filled with 1 M KCl was impaled to register the membrane potential in parallel to the Ca^{2+} -dependent fura-2-dextran fluorescence. The computer-aided membrane potential measurement was performed discontinuously synchronized to the parallel ratiometric $[Ca^{2+}]_{cy}$ measurement. A detailed description of the fluorescence ratio measurement of $[Ca^{2+}]_{cy}$ including ratio imaging and *in vitro* calibration is given in Plieth and Hansen (1996).

Two electrode voltage-clamp measurements

Two electrode voltage-clamp measurements were essentially performed as described in Sauer *et al.* (1993). Amplifier (Axoclamp-2B with headstages HS-2A \times 1L and HS-2A \times 10MG, Axon Instruments, USA) and data-acquisition were controlled by a PC equipped with pClamp 6.0 (Axon Instruments, USA). From a holding potential of about -200 mV voltages were clamped in alternating steps of 30 ms duration with 30 ms steps in between, while the voltage was clamped to the holding potential. Routinely, seven cycles of alternating, increasing voltage steps were applied, to more positive voltages in $+12$ mV steps and to more negative voltages in -27 mV steps. Within the 30 ms voltage-clamps the current did not significantly change, and measured currents were plotted as function of measured clamp potentials. The free running membrane potential was not affected by the voltage-clamp protocol. Up to four I/V curves were measured during a single transient hyperpolarization without altering its kinetics or amplitude. All points of a single I/V curve were measured in less than 1 s. The maximum change of the free running membrane potential in 1 s is <5 mV. The I/V curves are therefore 'snapshots' of the momentary conductivities responsible for the actual free running membrane potential (Sauer *et al.*, 1993).

Data analysis and mathematical modelling

Results are given as mean \pm standard error (SE). Non-linear regression analysis was performed with Grafit (Erithacus

Software, London, UK) by a least square fit routine based on the Marquardt algorithm.

Results

During parallel measurements of the free running membrane potential and $[Ca^{2+}]_{cy}$ in *Eremosphaera* it has been observed that transient hyperpolarizations are always caused by $[Ca^{2+}]_{cy}$ spikes (Bauer *et al.*, 1997, 1998). For a quantitative study of this relationship, data from stable oscillations of $[Ca^{2+}]_{cy}$ and membrane potential induced by 20 mM caffeine were used to plot $[Ca^{2+}]_{cy}$ against membrane potential (Fig. 1). From Fig. 1 it is obvious that already small increases in $[Ca^{2+}]_{cy}$ result in a considerable hyperpolarization of the plasma membrane. At $[Ca^{2+}]_{cy} > 500$ nM the membrane potential is approaching -187 mV, and a further increase in $[Ca^{2+}]_{cy}$ does no longer result in a further hyperpolarization. At a cytosolic K^+ activity in *Eremosphaera* of 130 mM (Bethmann *et al.*, 1995) and an external K^+ concentration of 0.1 mM the maximum hyperpolarization corresponds to the Nernst

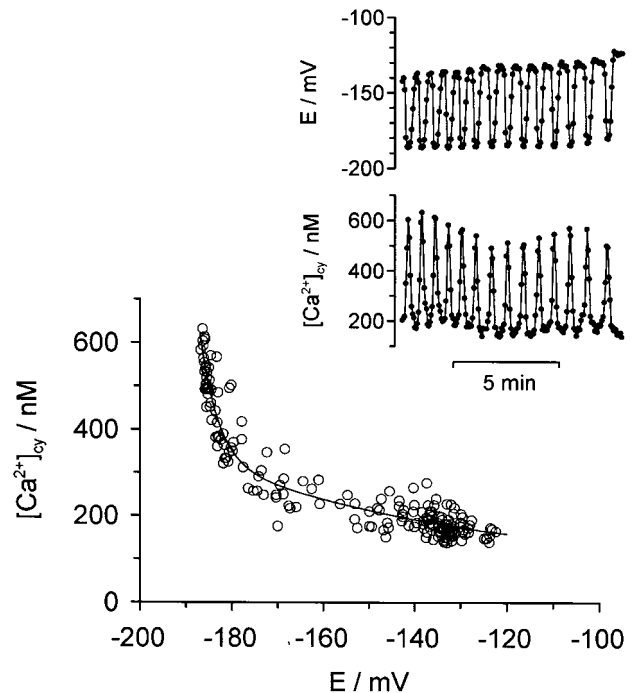


Fig. 1. Relation between cytosolic free calcium concentration ($[Ca^{2+}]_{cy}$, nM) and membrane potential (E , mV). The inset in the upper right corner shows membrane potential (E , mV) and cytosolic Ca^{2+} ($[Ca^{2+}]_{cy}$, nM) measured simultaneously in the same algal cell as a function of time at a sampling interval of 1.5 s. Oscillations were induced by 20 mM caffeine. A plot of cytosolic Ca^{2+} ($[Ca^{2+}]_{cy}$, nM) against membrane potential (E , mV) illustrates a close relation. The solid line represents a two exponential fit of the data points according to $[Ca^{2+}]_{cy} = 258 \text{ nM} \times \exp(-0.016 \text{ mV}^{-1} \times (V + 187 \text{ mV})) + 293 \text{ nM} \times \exp(-0.25 \text{ mV}^{-1} \times (V + 187 \text{ mV})) + 71 \text{ nM}$. The offset was fixed at 187 mV, the other parameters were determined by non-linear regression analysis. For the regression analysis $[Ca^{2+}]_{cy}$ was plotted on the y-axis and E on the x-axis although $[Ca^{2+}]_{cy}$ initiates the membrane potential changes.

potential for K⁺ (−184 mV). Obviously, an increase in [Ca²⁺]_{cy} results in an increase of the plasma membrane K⁺ permeability of *Eremosphaera*.

For a further characterization of the permeability changes of the plasma membrane during hyperpolarization, two electrode voltage-clamp measurements were performed. As already documented in more detail earlier (Sauer *et al.*, 1994), *I/V* curves, measured during a transient hyperpolarization, displayed increasing outward currents at increasing negative free running membrane potentials (Fig. 2a, insert). This outward current is carried by K⁺ and reflects the increase of the conductance of the plasma membrane for K⁺ during hyperpolarization (Sauer *et al.*, 1994; Schönknecht *et al.*, 1998). To estimate the K⁺ conductance, *G*, of the plasma membrane the slope of *I/V* curves was calculated for clamp potentials between −130 and −140 mV. At these clamp potentials currents are carried by K⁺ and the contribution of other conductances which occur at more negative clamp potentials (Sauer *et al.*, 1994), is negligibly small. In Fig. 2a the conductance, *G*, is plotted as a function of the corresponding free running membrane potential, *E*. Since the relation between free running membrane potential and estimated membrane conductance did not differ for different stimuli, data were pooled from 22 cells which were exposed to 20 mM caffeine, 0.1 mM SrCl₂ or darkening. Finally, the data relating membrane potential, *E*, and membrane conductance, *G*, (Fig. 2a) were combined with the data relating membrane potential, *E*, and [Ca²⁺]_{cy} (Fig. 1). For this purpose, data points in Fig. 1 were fitted by two exponentials, and the resulting mathematical equation ([Ca²⁺]_{cy} = *f*(*E*)), was used to recalculate in Fig. 2 the values for membrane potential, *E* (Fig. 2a) into [Ca²⁺]_{cy} values (Fig. 2b). In Fig. 2b the conductance, *G*, of the plasma membrane for K⁺ is shown as a function of [Ca²⁺]_{cy}. It is obvious, that at resting cytosolic Ca²⁺ activities which are about 160 nM in *Eremosphaera* (Bethmann *et al.*, 1995; Bauer *et al.*, 1997), the K⁺ conductance of the plasma membrane is rather low. A slight increase in [Ca²⁺]_{cy} is already sufficient to mediate a considerable K⁺ conductivity, and at [Ca²⁺]_{cy} > 500 nM no further increase in the K⁺ conductivity seems to be possible.

For a mathematical description of the data points in Fig. 2b, a straightforward assumption is a direct interaction of Ca²⁺ with a K⁺ channel. From the steep Ca²⁺-dependence it is immediately obvious, that a simple Michaelis-Menten type of binding is not applicable. Yet, a co-operative Ca²⁺ binding may well explain the increase of the plasma membrane K⁺ conductivity. Accordingly, the resulting data points were described by the following equation

$$G = \frac{G_{\text{Max}} [\text{Ca}^{2+}]_{\text{cy}}^n}{K^n + [\text{Ca}^{2+}]_{\text{cy}}^n}, \quad (1)$$

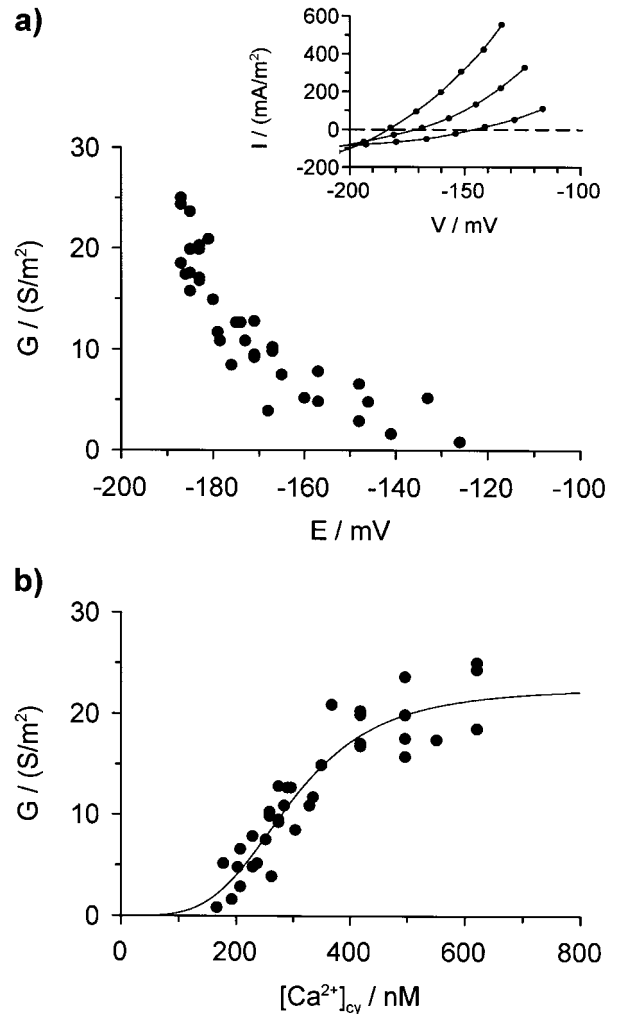


Fig. 2. Relation between membrane conductance *G* (S m⁻²) and (a) membrane potential *E* (mV) and (b) cytosolic free calcium concentration [Ca²⁺]_{cy} (nM), respectively. (a) The insert in the upper right shows current-voltage relationships *I* (mA m⁻²) as a function of *V* (mV) measured at different free running membrane potentials during transient hyperpolarizations. Only clamp potentials positive of −200 mV are shown for clarity. The conductance *G* (S m⁻²) as determined at clamp potentials *V* (mV) between −130 mV and −140 mV (*n* = 38, 22 different cells) was plotted against the free running membrane potential *E* (mV). Of the 38 data points shown, 22 result from caffeine-induced transient hyperpolarizations, 10 from dark-induced and 6 from transient hyperpolarizations induced by 0.1 mM Sr²⁺. With the function [Ca²⁺]_{cy} = *f*(*E*) determined in Fig. 1, x-axis values were recalculated from *E* (mV) in (a) to [Ca²⁺]_{cy} (nM) in (b). (b) The resulting plot displaying the membrane conductance *G* (S m⁻²) as a function of the cytosolic free calcium concentration [Ca²⁺]_{cy} (nM) was fitted by equation 1, resulting in a maximum conductance *G*_{Max} = 23 ± 2 S m⁻², an average Ca²⁺ affinity *K* = 297 ± 18 nM, and a co-operativity *n* = 3.9 ± 0.7.

where *G*_{Max} is the maximum conductance at high [Ca²⁺]_{cy}, *K* is the [Ca²⁺]_{cy} value where *G*_{Max}/2 is reached, indicating the Ca²⁺ affinity, and *n* is the co-operativity (Hill coefficient). A non-linear regression analysis yielded a maximum conductance *G*_{Max} = 23 S m⁻², a Ca²⁺ affinity *K* = 300 nM, and a co-operativity *n* = 3.9 (Fig. 2b).

Discussion

The data presented show a steep Ca^{2+} -dependence of the K^+ conductance of the plasma membrane of *Eremosphaera* (Fig. 2b). Since it is still not possible to perform $[\text{Ca}^{2+}]_{\text{cy}}$, and two electrode voltage-clamp measurements at the same time with the same alga, data were combined from different experiments, as shown in Fig. 1 and Fig. 2. This combination is legitimate, as both the relationship between $[\text{Ca}^{2+}]_{\text{cy}}$ and membrane potential (Fig. 1), and the relation between K^+ conductance and membrane potential (Fig. 2a) were reproducible from cell to cell, i.e. the variation from cell to cell did not exceed the variation between different measurements with the same cell. Even if different stimuli (like darkening, caffeine, Sr^{2+}) are compared the shape and amplitude of $[\text{Ca}^{2+}]_{\text{cy}}$ spikes and transient hyperpolarizations are very uniform (Bauer *et al.*, 1997, 1998).

The parameters resulting from the non-linear regression analysis in Fig. 1 have no further meaning and were solely used to transform the data in Fig. 2 from $G=f(E)$ (Fig. 2a) into $G=f([\text{Ca}^{2+}]_{\text{cy}})$ (Fig. 2b). In contrast to this, the parameters resulting from the non-linear regression analysis in Fig. 2b, allow some insight how $[\text{Ca}^{2+}]_{\text{cy}}$ regulates the plasma membrane K^+ conductance of *Eremosphaera* giving rise to hyperpolarization. The mathematical description of the relation between K^+ conductance, G , and $[\text{Ca}^{2+}]_{\text{cy}}$ (Fig. 2b) was based on the assumption of a co-operative Ca^{2+} binding. The model of a co-operative binding which nicely describes the data in Fig. 2b, is indicative for a direct regulation of a plasma membrane K^+ channel by cytosolic Ca^{2+} , however, further mediators between Ca^{2+} and the K^+ channel can not be ruled out. A maximum K^+ conductance of $G_{\text{Max}} = 23 \text{ S m}^2$ for a spherical alga with a diameter of $150 \mu\text{m}$ corresponds to a conductance of $1.6 \mu\text{S}$ for the whole plasma membrane. This is equivalent to a K^+ channel density of $1 \mu\text{m}^{-2}$ at a single channel conductance of 23 pS . Preliminary single channel recordings with the plasma membrane of *Eremosphaera* (Schönknecht *et al.*, unpublished results) showed the existence of a K^+ channel with a single channel conductance of 18 pS in 100 mM KCl . The resulting affinity for Ca^{2+} of $K=300 \text{ nM}$ indicates that 50% of the maximum K^+ conductance (G_{Max}) is activated at $[\text{Ca}^{2+}]_{\text{cy}}=300 \text{ nM}$. Compared to resting $[\text{Ca}^{2+}]_{\text{cy}}$ of about 160 nM (Bethmann *et al.*, 1995; Bauer *et al.*, 1997) this is just a doubling of $[\text{Ca}^{2+}]_{\text{cy}}$. A co-operativity of $n=3.9$ means that four different Ca^{2+} -binding sites strongly interact with each other, or more Ca^{2+} -binding sites with a smaller interaction exist. Since known plant K^+ channels comparable to animal K^+ channels form tetrameric structures (Daram *et al.*, 1997), it is tempting to speculate that the co-operativity of 3.9 may reflect the interaction of four Ca^{2+} binding sites, one on each of the four K^+ channel subunits.

Up to now, two different types of K^+ channels displaying a steep Ca^{2+} -dependence have been characterized on the molecular level in animal and plant cells, respectively. Small-conductance, Ca^{2+} -activated K^+ channels from rat (rSK2) and human brain (hSK1) expressed in *Xenopus* oocytes display a Ca^{2+} affinity of $K=430 \text{ nM}$ and $K=710 \text{ nM}$ and a co-operativity of $n=4.8$ and $n=3.9$ for rSK2 and hSK1, respectively (Köhler *et al.*, 1996). These channels are voltage-independent, and present an evolutionary distinct branch within the K^+ channel superfamily with six transmembrane domains and one putative pore domain region (Köhler *et al.*, 1996). An outward rectifying K^+ channel from *Arabidopsis* (KCO1) expressed in insect (*Spodoptera frugiperda*) cells also displays a steep Ca^{2+} -dependence and a high Ca^{2+} affinity (Czempinski *et al.*, 1997). Besides its voltage-dependence, KCO1 has a different putative topology with four transmembrane domains and two pore domain regions. The cellular location of KCO1 in *Arabidopsis* is not known (Czempinski *et al.*, 1997). At the moment there is no information about the molecular structure of the Ca^{2+} -dependent K^+ channel in *Eremosphaera*. The quantitative analysis presented here indicates that the Ca^{2+} -activated K^+ channel in the plasma membrane of this unicellular green alga is activated by tetra-co-operative Ca^{2+} binding at physiological cytosolic Ca^{2+} activities.

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