FEBS Letters 405 (1997) 390–393 FEBS 18376

Repetitive Ca^{2+} spikes in a unicellular green alga

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Received 2 January 1997; revised version received 12 February 1997

Abstract Cytosolic Ca^{2+} activity ($[Ca^{2+}]_{cy}$) and membrane potential were measured simultaneously in the unicellular green alga *Eremosphaera viridis*. Steady state $[Ca^{2+}]_{cy}$ was about 160 nM. A 'light-off' stimulus induced a transient elevation of $[Ca^{2+}]_{cy}$ ($[Ca^{2+}]_{cy}$ spike) in parallel with a transient hyperpolarization of the plasma membrane. Caffeine and Sr^{2+}, known to release Ca^{2+} from intracellular stores in animal cells, induced repetitive $[Ca^{2+}]_{cy}$ spikes in *Eremosphaera* which were always accompanied by parallel repetitive transient hyperpolarizations. These transient hyperpolarizations could be used as an indicator for $[Ca^{2+}]_{cy}$ spikes. Repetitive $[Ca^{2+}]_{cy}$ spikes in *Eremosphaera* were similar to repetitive $[Ca^{2+}]_{cy}$ spikes in excitable animal cells. The mechanisms underlying these $[Ca^{2+}]_{cy}$ oscillations seem to be comparable in animal and plant cells.

Key words: *Eremosphaera viridis*; Signal transduction; Calcium oscillation; Caffeine; Green alga

1. Introduction

Transient elevations of $[Ca^{2+}]_{cy}$ play a central role in intracellular signal transduction in plant and animal cells [1,2]. Repetitive transient elevations of $[Ca^{2+}]_{cy}$, so-called $[Ca^{2+}]_{cy}$ oscillations, are well established in animal cells [3–5]. In plant cells, repetitive increases of $[Ca^{2+}]_{cy}$, e.g. as induced by phytohormones [6,7], were strongly damped and ceased after a few repetitions [6,7]. Only recently stable $[Ca^{2+}]_{cy}$ oscillations were reported for plant cells [8,9]. The shape and frequency of transient repetitive $[Ca^{2+}]_{cy}$ elevations vary considerably [4]. A regular spiking pattern for $[Ca^{2+}]_{cy}$ elevations was observed in excitable animal cells and recently in a highly specialized plant cell [10]. The physiological significance of Ca^{2+} oscillations is a matter of debate [3–5], but the potential to encode and transduce information in the pattern of an oscillation is obvious.

Very little is known about the occurrence and mechanisms of repetitive transient elevations of $[Ca^{2+}]_{cy}$ in plant cells. The unicellular green alga *Eremosphaera viridis* responds to a 'light-off' stimulus by a transient hyperpolarization of the plasma membrane [11]. The 'light-off' signal is received inside the chloroplast [11]. The hyperpolarization is caused by the transient opening of K^+ channels. It is not known how this signal is transduced to the K^+ channels of the plasma membrane. Different chemical effectors like Ba^{2+}, caffeine, and $InsP_3$, known to release Ca^{2+} from intracellular stores in animal cells [5,12], induce single or repetitive transient hyperpolarizations in *Eremosphaera* [13–15]. This indicates that transient elevations of $[Ca^{2+}]_{cy}$ may be the origin of the transient hyperpolarizations. Here the occurrence of repetitive $[Ca^{2+}]_{cy}$ spikes in an algal cell is shown.

2. Materials and methods

The coccal green alga *Eremosphaera viridis* de Bary (algal culture collection Göttingen LB 228-1, Germany) was cultured and prepared for measurements according to Köhler et al. [11]. Measurements were performed in artificial pond water (APW, 0.1 mM KNO_3, $MgCl_2$, $CaCl_2$, 2.0 mM MES adjusted to pH 5.6 with NaOH). For measurements at a low external concentration of divalent cations, the medium contained 0.1 mM KNO_3, 0.1 mM EGTA, 2.0 mM MES/NaOH pH 5.6. At pH 5.6 the buffering capacity of EGTA for divalent cations is reduced and the free Ca^{2+} concentration as measured with Ca^{2+} selective microelectrodes was in the range of 300 nM. Sr^{2+}, Gd^{3+}, and La^{3+} were added as chloride salts. Caffeine was directly added to APW. The fluorescent Ca^{2+} sensitive dye fura-2 dextran (M_r = 10,000; Molecular Probes, Leiden, Netherlands) was injected mechanically into the cytosol of the alga [16,17]. Afterwards a microelectrode containing 3 M KCl was impaled to record the membrane potential. Parallel to the membrane potential the Ca^{2+}-dependent fura-2 dextran fluorescence $[Ca^{2+}]_{cy}$ was measured by fluorescence ratio imaging using a CCD camera [16,17]. For in vitro calibrations standard calibration solutions (C3722, Molecular Probes) containing 1.0 mM Mg^{2+} were used [16,17].

3. Results

$[Ca^{2+}]_{cy}$ and the membrane potential of *Eremosphaera viridis* were measured simultaneously in the same algal cell. At steady state the free running membrane potential (E) was

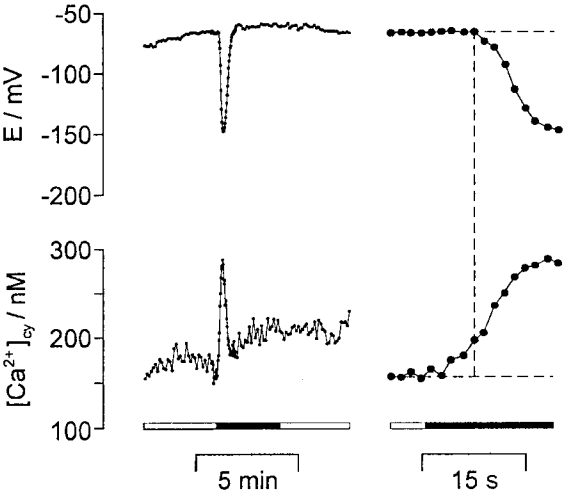

Fig. 1. A transient change of $[Ca^{2+}]_{cy}$ (bottom) and the membrane potential (E, top) after a 'light-off' stimulus (black bar at the bottom). Left, normal time scale; right, the same signals immediately after light off on a 20-fold extended time scale (the dashed lines indicate the baselines and the last sampling interval before hyperpolarization, respectively). Sampling interval was 1.5 s.

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PII S0014-5793(97)00231-7

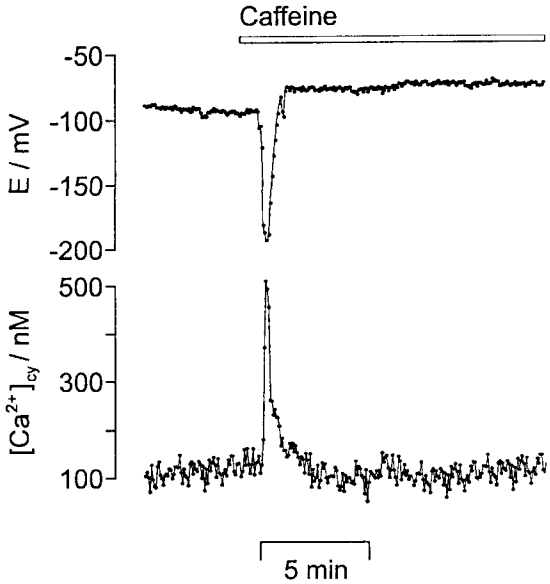

Fig. 2. A transient change of $[Ca^{2+}]_{cy}$ and the membrane potential (E) induced by addition of 20 mM caffeine to the external medium (bar on top gives perfusion protocol). Sampling interval was 3 s.

-85 ± 11 mV and $[Ca^{2+}]_{cy}$ was 163 ± 42 nM ($n = 50$). A 'light-off' stimulus induced a transient hyperpolarization of the plasma membrane in 75% of the measurements. It had a duration of 53.1 ± 10.2 s. As shown in Fig. 1 this hyperpolarization was accompanied by a transient elevation of $[Ca^{2+}]_{cy}$. The $[Ca^{2+}]_{cy}$ increase started a few seconds after 'light-off'. Extending the time scale (Fig. 1, right) demonstrates that the rise in $[Ca^{2+}]_{cy}$ precedes the hyperpolarization. Besides this $[Ca^{2+}]_{cy}$ spike, we did not observe any statistically significant light-dependent changes in $[Ca^{2+}]_{cy}$.

Caffeine applied externally in the millimolar range is known to release Ca^{2+} from internal Ca^{2+} stores in animal cells, and $[Ca^{2+}]_{cy}$ oscillations induced by caffeine were repeatedly reported [18,19]. Fig. 2 shows the effect of 20 mM caffeine on $[Ca^{2+}]_{cy}$ and on membrane potential in *Eremosphaera*. Caffeine induced a $[Ca^{2+}]_{cy}$ spike and a simultaneous transient hyperpolarization in five out of six experiments. The transient changes of $[Ca^{2+}]_{cy}$ and membrane potential had a duration of 67.4 ± 12.6 s. Steady state $[Ca^{2+}]_{cy}$ was not changed by the perfusion of caffeine. The probability of inducing a hyperpolarization by caffeine was concentration dependent. It decreased from 81% at 20 mM ($n = 16$) to 53% at 10 mM ($n = 17$) and to 33% at 1 mM ($n = 18$). When divalent cations were omitted from the external medium (0.1 mM EGTA, no divalent cations added) the addition of 20 mM caffeine induced fast repetitive $[Ca^{2+}]_{cy}$ spikes and parallel repetitive transient hyperpolarizations (Fig. 3) in 50% of the measurements ($n = 20$). In 20% of the measurements, only one or two $[Ca^{2+}]_{cy}$ spikes accompanied by transient hyperpolarizations were observed. The caffeine-induced $[Ca^{2+}]_{cy}$ spikes and hyperpolarizations had a frequency of 1.0 ± 0.4 per minute and a duration of up to 60 min. The repetitive $[Ca^{2+}]_{cy}$ spikes resulted in a significant increase of the $[Ca^{2+}]_{cy}$ baseline (249 ± 43 nM; $n = 10$) compared to 160 nM under control conditions. The addition of 100 μM $GdCl_3$, a well-known Ca^{2+} channel blocker, reversibly inhibited the $[Ca^{2+}]_{cy}$ spikes and the transient hyperpolarizations (Fig. 4). Beside 100 μM $GdCl_3$, the addition of 100 μM $LaCl_3$ ($n = 6$) or 10 μM verapamil ($n = 7$) also reversibly blocked caffeine-induced oscillations (not shown).

Beside caffeine, Sr^{2+} is an established effector for $[Ca^{2+}]_{cy}$ oscillations in animal cells [20]. In *Eremosphaera* the addition of 1.0 mM Sr^{2+} to the external medium induced repetitive $[Ca^{2+}]_{cy}$ spikes and parallel repetitive transient hyperpolarizations of the membrane potential (Fig. 5) in nearly all experiments (95%, $n = 27$). Neither the probability of inducing oscillations by Sr^{2+} nor their duration or frequency were influenced by the external concentration of Ca^{2+} (or Mg^{2+}) in the range between 1 mM EGTA (no Ca^{2+} or Mg^{2+} added) and 1 mM Ca^{2+} or 10 mM Mg^{2+}. The Sr^{2+}-induced $[Ca^{2+}]_{cy}$ spikes and hyperpolarizations had a frequency of 0.5 ± 0.2 per minute and a very long lifetime. Durations of more than 2 h were frequently observed. The baseline of the repetitive $[Ca^{2+}]_{cy}$ spikes (168 ± 43 nM) was identical with the steady state $[Ca^{2+}]_{cy}$ under control conditions (160 nM). Compared to caffeine, the repetitive transient changes induced by Sr^{2+} displayed an about twofold lower frequency. The duration of a single $[Ca^{2+}]_{cy}$ spike during Sr^{2+}-induced repetitive changes was comparable (35 ± 8 s) to caffeine-induced repetitive changes (30 ± 2 s). The higher frequencies were mainly caused by shorter intervals between the single caffeine-induced $[Ca^{2+}]_{cy}$ spikes compared to Sr^{2+}-induced spikes.

4. Discussion

The experiments show the suitability of the unicellular green alga *Eremosphaera* (∅ = 150 μm) for simultaneous measurements of membrane potential and determination of cytosolic Ca^{2+} activity ($[Ca^{2+}]_{cy}$) by means of the fluorescent dye fura-2 dextran. The measured steady state value of $[Ca^{2+}]_{cy}$ of 163 ± 42 nM coincides well with the value of 164 nM obtained with Ca^{2+}-selective microelectrodes in the same species [21]. It is also in the range reported for other plant

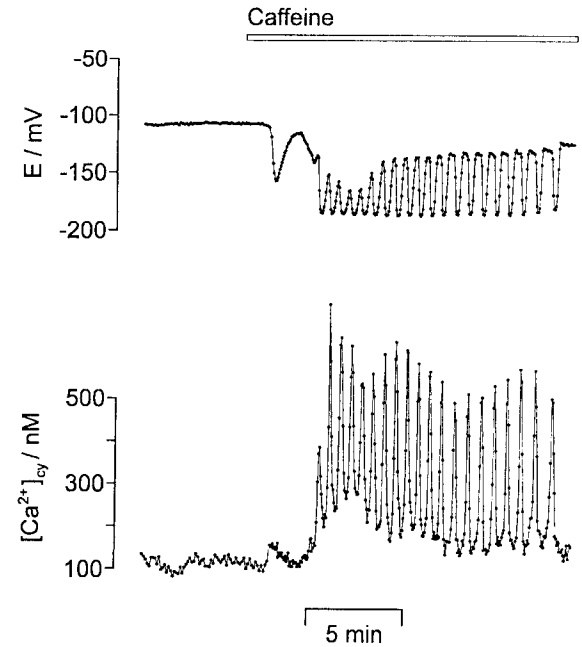

Fig. 3. Repetitive $[Ca^{2+}]_{cy}$ spikes and membrane potential (E) oscillations induced by addition of 20 mM caffeine at a low external concentration of divalent cations (0.1 mM KNO_3, 0.1 mM EGTA, 2.0 mM MES/NaOH pH 5.6) Sampling interval was 1.5 s.

cells [22]. Therefore, the in vitro calibration seems to be reliable under the conditions of these experiments.

The benefit of the greater temporal resolution of fluorescent dyes over Ca^{2+}-selective microelectrodes is demonstrated by the fact that the 'light-off'-induced $[Ca^{2+}]_{cy}$ spike (Fig. 1) was not resolved with electrodes [21] but with the experiments here. The permanent light-dependent changes of $[Ca^{2+}]_{cy}$ reported for *Nitellopsis* [23] were observed neither in *Eremosphaera* (Fig. 1) [21] nor in the liverwort *Conocephalum* [24]. The origin of the light-dependent changes of $[Ca^{2+}]_{cy}$ in plant cells seems to be a release of Ca^{2+} from the chloroplasts. In characean cells, the same time constant was found for light-induced changes in chlorophyll fluorescence and $[Ca^{2+}]_{cy}$-induced changes in plasmalemma resistance [25]. Isolated chloroplasts were shown to release Ca^{2+} upon darkening [26]. Dark-induced Ca^{2+} release from chloroplasts may be involved in the $[Ca^{2+}]_{cy}$ spike observed in *Eremosphaera* upon darkening. Other internal Ca^{2+} stores as the vacuole or the endoplasmic reticulum may contribute as well.

It had been shown earlier that an artificial increase of $[Ca^{2+}]_{cy}$ by Ca^{2+} ionophores or Ca^{2+} injection causes a hyperpolarization in *Eremosphaera* [27,15]. One major issue of our investigations was the demonstration of the close relationship between hyperpolarizations of the plasma membrane and changes in $[Ca^{2+}]_{cy}$. This was found in all experiments, we never observed a transient hyperpolarization in the absence of a $[Ca^{2+}]_{cy}$ spike. Especially during the application of blockers like Gd^{3+} (Fig. 4), the perfect synchronization of the $[Ca^{2+}]_{cy}$ spikes and the repetitive transient hyperpolarizations became obvious. Within our time resolution (1.5 or 3 s per sampling interval) the $[Ca^{2+}]_{cy}$ increase started at the same time as the hyperpolarization or a few sampling intervals in advance (Fig. 1). Obviously, the transient hyperpolarization of the plasma membrane is caused by the $[Ca^{2+}]_{cy}$ spikes,

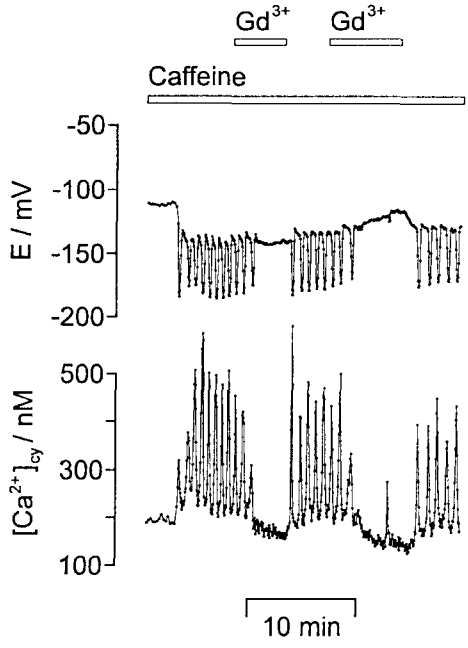

Fig. 4. The repetitive $[Ca^{2+}]_{cy}$ spikes and potential (E) oscillations induced by 20 mM caffeine at a low external concentration of divalent cations were reversibly inhibited by 100 μM $GdCl_3$ applied for 5 min and after wash-out for another 7 min (upper bars give perfusion protocol). Sampling interval was 3 s.

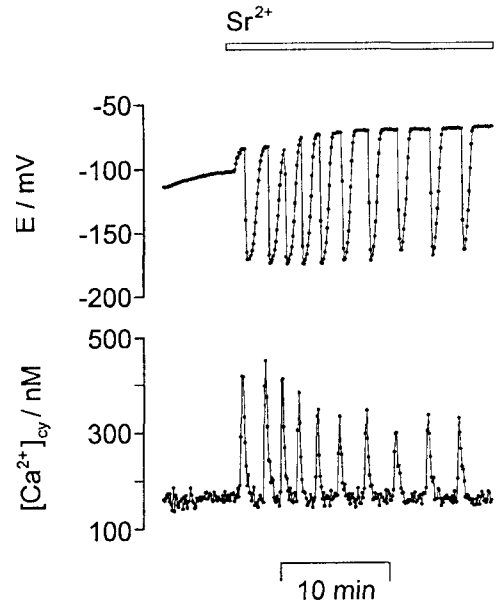

Fig. 5. Repetitive $[Ca^{2+}]_{cy}$ spikes and membrane potential (E) oscillations induced by addition of 1 mM $SrCl_2$. Sampling interval was 3 s.

probably via the opening of Ca^{2+}-activated K^+ channels in the plasma membrane. The hyperpolarization can be used as a qualitative indicator for transient elevations of $[Ca^{2+}]_{cy}$ in *Eremosphaera*.

Little is known about the effect of caffeine or Sr^{2+} in plant cells [14,27]. In animal cells, the involvement of a release of Ca^{2+} from internal stores is known to occur when caffeine and Sr^{2+} enter the cells [3,28,29]. Thus, it is suggested that a similar mechanism applies to the $[Ca^{2+}]_{cy}$ oscillations in *Eremosphaera* which resemble the rhythmic spiking pattern commonly found in excitable animal cells [4]. The rapidly reversible blockage of oscillations by Gd^{3+} indicates that some Ca^{2+} flux across the plasma membrane is necessary for sustained oscillations. If some basic mechanisms of $[Ca^{2+}]_{cy}$ oscillations were developed before plant and animal cells diverged, *Eremosphaera* seems to be an excellent candidate for the study of these common mechanisms.

Acknowledgements: This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 176, TP B11).

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