The action potential in *Chara*: Ca\(^{2+}\) release from internal stores visualized by Mn\(^{2+}\)-induced quenching of furadextran

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Summary

The action potential (AP) in *Chara* is associated with a transient elevation in the concentration of cytoplasmic-free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{\text{cyt}}\)]. The quenching properties of the fluorescent Ca\(^{2+}\) indicator dye furadextran, in combination with Mn\(^{2+}\), was used to investigate whether this [Ca\(^{2+}\)\(_{\text{cyt}}\)] transient is due to Ca\(^{2+}\) release from internal stores or to Ca\(^{2+}\) influx across the plasma membrane. Adding Mn\(^{2+}\) to the external medium or pre-injection of Mn\(^{2+}\) into the vacuole caused no perceivable quenching of the fura fluorescence, during an AP. This makes it unlikely that Ca\(^{2+}\) influx across the plasma membrane or the tonoplast contributes significantly to the [Ca\(^{2+}\)\(_{\text{cyt}}\)] transient in an excited cell. When cells were pre-incubated in external solutions containing Mn\(^{2+}\) from 25 to 30 mM APs evoked a transient quenching of fura fluorescence in Mn\(^{2+}\)-free solutions. Under these conditions, the quenching must be attributed to an AP-associated release of Mn\(^{2+}\) from internal stores. Based on the finding that exposing cells to millimolar concentrations of Mn\(^{2+}\) caused a progressive quenching of the fura fluorescence in non-excited cells, it can be assumed that some Mn\(^{2+}\) enters the cells during pre-incubation and is loaded into internal stores. During excitation, this stored Mn\(^{2+}\) is released together with Ca\(^{2+}\).

Introduction

The plasma membrane of plant cells is excitable, in a similar manner to that of nerve- or muscle-cells in animals.

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Finally, evidence in support of a Ca$^{2+}$ influx through channels in the plasma membrane comes from electrophysiological measurements. The amplitude of the AP was correlated with the external Ca$^{2+}$ concentration and this was interpreted as evidence for Ca$^{2+}$ influx during the downstroke of the AP (Findlay, 1962; Hope, 1961a,b; Staves and Wayne, 1993). Furthermore, current measurements under voltage clamp control suggested that one component of the complex excitation current is sensitive to the external Ca$^{2+}$ concentration (Beilby and Coster, 1979; Lunevsky et al., 1983; Shiina and Tazawa, 1987). This rendered the current a probable candidate for the Ca$^{2+}$ inward current anticipated during excitation. The latter interpretation, however, is challenged by two arguments. Firstly, the reversal voltage of the Ca$^{2+}$-sensitive excitation current was well negative of the Ca$^{2+}$ equilibrium voltage, which casts doubt on the idea that the current carries Ca$^{2+}$ (Beilby, 1984; Thiel et al., 1997). Secondly, analysis of the putative Ca$^{2+}$ current with respect to the voltage and time dependency of activation revealed that this current activates with a delay (Beilby and Coster, 1979). Thus, even if the channel was carrying Ca$^{2+}$ influx it would be unlikely that it is responsible for the initial sharp rise in [Ca$^{2+}$]$_{cyt}$ (Beilby, 1984).

Other experimental data, albeit more indirect, favour the view for release of Ca$^{2+}$ from internal stores in the course of excitation. Elevation of inositol-trisphosphate (InsP$_3$) in the cytoplasm triggered AP in Chara and Nitella (Thiel et al., 1990). This indicates, by analogy to the situation in animal cells (Berridge and Irvine, 1989), that internal Ca$^{2+}$ stores are present in the algae and that release of Ca$^{2+}$ from these stores is sufficient to trigger excitation.

Another line of evidence for internal release is related to (Ca$^{2+}$)$_{cyt}$ measurements in tonoplast-free Chara cells in which a biphasic Ca$^{2+}$ transient during the AP could be resolved (Kikuyama and Tazawa, 1983). The suggestion that the initial transient reflects release of Ca$^{2+}$ from depletatable internal stores was based on the finding that the initial transient was abolished after multiple AP, even when sufficient Ca$^{2+}$ was present in the external medium (Kikuyama and Tazawa, 1983).

A similar model involving Ca$^{2+}$ stores that were refilled by Ca$^{2+}$ from the external medium was obtained from patch clamp experiments on the plasma membrane of Chara. In this case it was found that the Cl$^{-}$ channels, which form the elementary basis of the excitation Cl$^{-}$ current, activated without a temporal correlation to the putative plasma membrane Ca$^{2+}$ influx (Thiel et al., 1993). One plausible explanation for these data is that the activation of the Cl$^{-}$ channels was due to a release of Ca$^{2+}$ from internal stores (Thiel et al., 1993, 1997).

In this study, we used fluorescence-ratio imaging to record Ca$^{2+}$ transients during excitation and attempted to resolve the origin of Ca$^{2+}$. An important feature of the Ca$^{2+}$-indicator fura-2 is that its fluorescence is quenched by Mn$^{2+}$ ions under all excitation wavelengths (Gilroy and Jones, 1992; McAinsh et al., 1995; Malhó et al., 1995; Thomas and Dellavalle, 1991; Zottini and Zanoni, 1993). Mn$^{2+}$ is similar to Ca$^{2+}$ and, hence, able to penetrate Ca$^{2+}$ channels and other pores (i.e. K$^{+}$ channels) that are permeable to Ca$^{2+}$ ions (Fasolato et al., 1993; Piñeros and Tester, 1995; Striggow and Ehrlich, 1996). In the case that the external solution contains Mn$^{2+}$, it is possible to discriminate between Ca$^{2+}$ influx and Ca$^{2+}$ mobilization from internal stores. Therefore the Ca$^{2+}$-independent fluorescence of the dye excited by light near the isosbestic point (i.e. F$_{360}$ with $\lambda_{ex} = 360$nm), was measured in addition to the Ca$^{2+}$ dependent wavelengths for ratio measurements of [Ca$^{2+}$]$_{cyt}$ (i.e. F$_{340}$ and F$_{380}$) and inspected for Mn$^{2+}$ evoked quenching (Merrit et al., 1989).

Results

Measurement of action potentials in Chara cells with Mn$^{2+}$ ions in the bathing medium

Figure 1 illustrates the simultaneous recording of the membrane voltage and [Ca$^{2+}$]$_{cyt}$ in a fura-dextran loaded internodal cell of Chara during repetitive excitation in the absence and in the presence of 50 $\mu$M Mn$^{2+}$. As a general observation it appeared that addition of submillimolar Mn$^{2+}$ to the bath solution did not abolish excitability over the period of recording. But the concomitant [Ca$^{2+}$]$_{cyt}$ transients gradually decayed in amplitude with time of exposure to Mn$^{2+}$ (Figures 1; 2a,d; 3c,d), while the magnitude of the AP increased (Figures 1; 2a,b; 3a,b).

Figure 2 shows a close up of the fluorescence signals...
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Figure 2. Examples of APs from Figure 1, without Mn$^{2+}$ in the external bathing medium (a, c, e) and with 50 µM Mn$^{2+}$ in the external bathing medium (b, d, f); membrane potential (a, b); cytoplasmic calcium concentration (c, d); fluorescence signals (e, f); open circles = F$_{340}$; closed circles = F$_{360}$; line without symbols = F$_{360}$.

Figure 2(e) shows that the fluorescence measured at 360 nm near the isosbestic point (F$_{360}$) underwent, at the resting voltage, random variations that were not reflected in [Ca$^{2+}$]$_{cyt}$ (see also Figure 4b; 7c). The amplitudes of variations in F$_{360}$ decreased as the cytoplasmic streaming ceased due to AP (data not shown), and could therefore be assigned to fluctuations in dye concentration generated by moving particles in the cytoplasm (Plieth and Hansen, 1996).

During the downstroke of the AP the F$_{360}$ signal generally increased transiently (Figure 2e,f). The reason for this unexpected transient rise in F$_{360}$ during excitation is not known. One possible explanation for this phenomenon could be that the massive loss of KCl during the AP (Kikuyama, 1986, 1987; Kikuyama et al., 1984) produces a short-lasting water loss and consequently a rise in cytoplasmic dye concentration. Another explanation could be that the cytoplasmic environment causes changes in the optical properties of the dye that lead to a shift of the isosbestic point to longer excitation wavelengths in vivo compared with data obtained in vitro (Bancel et al. 1992; Blatter and Wier, 1990; Haugland, 1992). Under these conditions the F$_{360}$ measurements retained a slight amount of Ca$^{2+}$ sensitivity.

The right panel of Figure 2 shows, for comparison, the fluorescence signals obtained during the AP with Mn$^{2+}$ in the bath. Under these conditions the AP evoked similar changes in the three fluorescence signals as those obtained in the absence of Mn$^{2+}$. Compared to the control, only the magnitudes of changes in [Ca$^{2+}$]$_{cyt}$ were reduced by the presence of Mn$^{2+}$ in the bath (Figure 2c,d). Most important, the F$_{360}$ signal indicated no obvious quenching of the fura fluorescence during the AP in the Mn$^{2+}$ containing bath medium (Figure 2e,f).

To uncover potentially small Mn$^{2+}$-related differences of the fluorescence signal, the F$_{360}$ responses during AP with and without Mn$^{2+}$ from Figure 1 were normalized to the time of excitation and averaged (Figure 3). In essence, the mean value of F$_{360}$ remained, for both treatments, with the exception of the initial transient fluorescence increase, constant during AP. The control data (mean F$_{360}$ in the absence of Mn$^{2+}$) were further subtracted from the mean F$_{360}$ signal recorded in the presence of Mn$^{2+}$. This operation should abolish the contribution of Mn$^{2+}$-independent background fluctuations in fluorescence intensity and leave

only Mn$^{2+}$-related effects on F$_{360}$. Figure 3(g) shows the respective F$_{360}$ difference curve, which revealed no perceivable Mn$^{2+}$-related quenching during the AP.

Neither increasing the concentration of Mn$^{2+}$ up to 150 µM (N$_{Exp}$ = 6) nor using Ni$^{2+}$ as another potential quenching ion (Haugland, 1992) in the outer medium (N$_{Exp}$ = 4) produced any significant quenching of F$_{360}$ during an AP (data not shown).

The lack of quenching of F$_{360}$ during the AP in Mn$^{2+}$-containing solution prompts two alternative explanations. (i) The transient rise in [Ca$^{2+}$]$_{cyt}$ may be due to Ca$^{2+}$ release from internal stores without contribution of significant influx across the plasma membrane. (ii) The quenching ion Mn$^{2+}$ may, unlike the situation in other organisms, not permeate Ca$^{2+}$ transporting channels in the plasma membrane of Chara (Fasolato et al., 1993; Píñeros and Tester, 1995; Striggow and Ehrlich, 1996).

To test the latter hypothesis, namely that Mn$^{2+}$ is unable to cross the plasma membrane, fura-dextran loaded Chara cells were exposed to high concentrations of Mn$^{2+}$. Figure 4 shows the response of the electrical and fluorescence parameters to an exposure of 30 mM Mn$^{2+}$. Immediately after adding Mn$^{2+}$ to the perfusion stream, $V_M$ depolarized. The [Ca$^{2+}$]$_{cyt}$ concentration remained unaffected by this treatment. A scrutiny of F$_{360}$ revealed that high concentrations of Mn$^{2+}$ caused a slow progressive quenching of the fluorescence, indicating that Mn$^{2+}$ could enter the cell via plasma membrane-resident transporters. In comparable experiments a mean quenching of 41 ± 6% (mean ± SD; N$_{Exp}$ = 10) of fura fluorescence occurred with 25-30 mM Mn$^{2+}$ in the bath.

A typical loading of Chara with fura results in a cytoplasmic dye concentration of about 5 µM (Plieth and Hansen, 1996). Considering that 40% of the fluorescence intensity (i.e. 2 µM fura) is quenched during Mn$^{2+}$ incubation, we can, based on the high affinity of Mn$^{2+}$ to fura (K$_{d}$ (fura-Mn$^{2+}$) ≈ 5 nM) estimate that the cytoplasmic concentration rose by ca. 2 µM.

In control experiments cells were also exposed to comparable concentrations of Ni(NO$_3$)$_2$, an operation that similarly produced membrane depolarization but no quenching of F$_{360}$. Hence, the loss of F$_{360}$ fluorescence upon exposure to Mn$^{2+}$ appeared specific for Mn$^{2+}$ influx and was not due to osmotic effects or due to the concomitant membrane depolarization.

After pretreatment in Mn$^{2+}$, AP cause fluorescence quenching

In the presence of millimolar Mn$^{2+}$ concentrations, the cells were not excitable. However, cells gradually regained excitability after washing the heavy metal out of the bath solution (Figure 5). AP elicited after pre-treatment with Mn$^{2+}$ were somewhat reduced in amplitude and duration. The recovery of excitability was also associated with progressive reappearance of [Ca$^{2+}$]$_{cyt}$ transients (Figure 5b).

The most important observation with respect to the fluorescence signals in cells pretreated with 25 mM Mn$^{2+}$ is detailed in Figure 6. Under control conditions, F$_{360}$ remained largely constant during the AP (Figure 6e). After pretreatment with high Mn$^{2+}$, the F$_{360}$ signal decreased in all cases during the AP, concomitantly with the rise in [Ca$^{2+}$]$_{cyt}$ (Figure 6f). The amplitude of quenching varied from 8% to 12% between different AP, with no apparent decay during continuous excitation. Figure 6(g) shows the mean quenching of F$_{360}$ during AP in Mn$^{2+}$-pretreated cells (Figure 6f) after subtraction of the background variations obtained before Mn$^{2+}$ exposure (Figure 6e).

Similar quenching of the F$_{360}$ signal in parallel with the elevation of [Ca$^{2+}$]$_{cyt}$ was obtained after Mn$^{2+}$ pretreatment.
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Figure 6. Average of five time series during action potentials. Membrane potential (a, b); \[\text{Ca}^{2+}\] (c, d); F360 fluorescence quenching (e, f); before (a, c, e) and after (b, d, f) incubation in 25 mM Mn2\(^{1+}\); the difference curve (g) was calculated by subtracting the curve in (e) from that in (f) (compare Figure 3). An example for the fit of the biphasic fluorescence response (h), the fitted curve (= line) was fitted to the scaled data (= circles) by a sum of two exponentials (equation 1). The time constants here are \(\tau_1 = 27.5\) sec and \(\tau_2 = 266\) sec; ca. 30 sec after the AP the scatter increases due to the recovery of cytoplasmic streaming.

Measurement of action potentials in Chara cells with Mn2\(^{2+}\)-loaded vacuoles

In plant cells the vacuole is believed to be a Ca\(^{2+}\) store. It contains several mM of Ca\(^{2+}\) (Okihara and Kiyosawa, 1988), some of which might be released upon stimulation. We therefore tested whether the vacuole was the store releasing Ca\(^{2+}\) during the AP. In order to investigate potential vacuolar Ca\(^{2+}\) efflux during excitation, the vacuoles of Chara cells were loaded with 50 \(\mu\)M, \([\text{Mn}^{2+}]_v\), 100 \(\mu\)M (for details of vacuolar loading see the Experimental procedures). The AP recorded under these conditions were associated with the typical rise in cytoplasmic \([\text{Ca}^{2+}]_{\text{cyt}}\). Inspection of the F360 signal showed no evidence for AP-associated quenching (Figure 7). The same absence of quenching was found in all experiments conducted (\(N_{\text{Cells}} = 3, n_{\text{APs}} = 16\)). This makes Ca\(^{2+}\) release from the vacuole during excitation unlikely.

Discussion

A key finding of the present work is that the fura fluorescence near the isosbestic point is not quenched during an AP when Mn\(^{2+}\) is present in the external medium at

Table 1. Time constants (in sec) and amplitude factors (see equation below) of the F360 quenching during AP in Mn\(^{2+}\)-pretreated Chara cells (mean ± SD; \(n_{\text{AP}} = 8; n_{\text{Cells}} = 3\)). The fluorescence signals were normalized to 1 (Figure 6h).

| \(\tau_1\) | 27.5 ± 11.2 |
| \(\tau_2\) | 266 ± 79 |
| \(k_0\) | 1.01 ± 0.04 |
| \(k_1\) | -0.31 ± 0.15 |
| \(k_2\) | 0.66 ± 0.38 |

Kinetic analysis

The AP-associated quenching of F360 was fitted by the sum of two exponentials

\[F = k_0 + k_1 \cdot \exp \left(-\frac{t}{\tau_1}\right) + k_2 \cdot \exp \left(-\frac{t}{\tau_2}\right)\]

The resulting first time constant of the biphasic F360 response provides a measure for the release kinetics of Mn\(^{2+}\) from the internal stores (an example is given in Figure 6f). This operation provides a time constant of \(\tau_1 = 27.5\) sec for the release of Mn\(^{2+}\) into the cytoplasm. The second time constant was found to be \(\tau_2 = 266\) sec (Table 1).

Figure 6(h) shows that the noise in the fluorescence signal increased ca. 30 sec after the AP. This was due to the inhomogeneous dye distribution in the cell and recovery of cytoplasmic streaming, which ceased during excitation (Plieth and Hansen, 1996).
concentrations of up to 150 µM. The conclusion, therefore, is that the bulk of the Ca²⁺ responsible for the transient rise in [Ca²⁺]cyt during the AP must be due to release from internal stores. Ca²⁺ influx across the plasma membrane (PM) does not add significantly to the rise in [Ca²⁺]cyt. The idea of Ca²⁺ release from internal stores during excitation is further supported by the finding that APs were associated with a quenching of the fura fluorescence after prolonged pretreatment of the cells with high Mn²⁺ concentration-containing solution. In this case quenching was detectable even after thorough washing of the heavy metal out of the bath solution. Under these conditions only release of Mn²⁺ from internal stores can possibly provide a source for the fluorescence quenching.

So in summary, the data are consistent with a model that Chara contains cytoplasmic stores that are slowly filled under resting conditions with divalent ions. In the course of an AP, these stores discharge Ca²⁺, which is responsible for the transient rise of [Ca²⁺]cyt during excitation. If the stores contain Mn²⁺, discharge during the AP leads to a quenching of the fluorescence signal.

It is widely believed that the vacuole serves in plant cells as a site of internal Ca²⁺ release via InsP₃-dependent or -independent mechanisms (Allen et al., 1995; Ward et al., 1995). Loading the vacuole in Chara with Mn²⁺ prior to excitation caused no detectable quenching of the fura fluorescence during an AP in the present study (Figure 7). Therefore the vacuole can be excluded as relevant source of Ca²⁺ discharge during excitation, and the Ca²⁺ transporters activated upon excitation do not appear to reside in the tonoplast. Hence, Chara cells must have Ca²⁺ stores other than the vacuole. A possible candidate is the endoplasmic reticulum (ER), which in animal cells contains InsP₃ receptors with Mn²⁺ permeability (Striggow and Ehrlich, 1996).

With these properties, the stores could, as in the present case, be loaded with Ca²⁺ or Mn²⁺ and discharge their content upon stimulation.

Previous luminescent measurements have explained the rise in [Ca²⁺]cyt during the AP as the result of Ca²⁺ influx across the PM or a combination of internal release and influx (Kikuyama and Tazawa, 1983; Williamson and Ashley, 1982). In contrast to the present study, the latter measurements of [Ca²⁺]cyt in tonoplast-free cells detected not one but two separable Ca²⁺ peaks during excitation (Kikuyama and Tazawa, 1983). The first of the two peaks appeared to reflect Ca²⁺ release from internal stores, like the Ca²⁺ transient in the present study. Similar to the present data, these internal stores seemed to require refilling with Ca²⁺ from the external medium. The second Ca²⁺ transient detected by Kikuyama and Tazawa (1983) was not observed in the present study. Also, luminescent Ca²⁺ measurements in intact Chara cells only observed a single Ca²⁺ transient (Williamson and Ashley, 1982). Therefore, it seems likely that the second long-lasting [Ca²⁺]cyt transient, which is probably due to Ca²⁺ influx across the plasma membrane (Kikuyama and Tazawa, 1983), is related to specific properties of the tonoplast-free cell system.

In most experiments it was noted that after quenching during the AP the F₃₅₀ fluorescence signal slowly reversed back near the resting level, which lead to the biphasic response (see τ₂, Table 1).

It is of course tempting to interpret the second (slow) time constant as an indirect measure for the refilling of the Ca²⁺ stores. However, Mn²⁺ binds fura with a roughly 50 times higher affinity than Ca²⁺ (dissociation constants: K_d(Mn²⁺-fura) \(\approx\) 5nM; K_d(Ca²⁺-fura) \(\approx\) 250nM). Therefore, fura binding to Mn²⁺ must be considered quasi irreversible in the cytoplasmic environment. This renders retrieval of fura-bound Mn²⁺ into the stores unlikely. This is supported by the finding that the fluorescence quenched after the influence of high Mn²⁺ concentrations (Figure 4) never recovered its previous magnitude.

An alternative explanation for the slow time constant could be that electrical stimulation does not cause excitation of the entire membrane and hence increases [Ca²⁺]c only in parts of the cell (Homann and Thiel, 1994). Thus, during recovery of Ca²⁺-sensitive cytoplasmic streaming after an AP, cytoplasm with elevated [Ca²⁺]c, in the window of recording is mixed with the part of cytoplasm that has been left unaffected. Such a dilution of Mn²⁺-quenched with non-quenched fura could explain the recovery of the F₃₅₀ signal. On this background the mean time of streaming recovery, \(\tau_2\) = 266 sec (Table 1) could be seen as the time of streaming recovery.

In the past it has been observed that AP-associated Ca²⁺ transients in Chara cells gradually reappeared after replacing external Mg²⁺ with Ca²⁺ (Williamson and Ashley, 1982). This was interpreted as a role of Ca²⁺ influx in the
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immediate process of excitation. In the context of the present data, it can be argued that sufficient filling of internal stores with Ca\(^{2+}\) from the external medium is necessary for transient elevation of [Ca\(^{2+}\)]\(_{cyt}\). Such a temporal uncoupling of events would explain the observation that regeneration of Ca\(^{2+}\)-transients does not occur immediately after transfer of cells from a Mg\(^{2+}\)- to a Ca\(^{2+}\)-containing medium but only gradually over several tens of minutes (Williamson and Ashby, 1982).

The same arguments hold true for the effect of the putative Ca\(^{2+}\) channel blocker La\(^{3+}\). The unusually long exposure of cells to the blocker required to abolish excitability (Beilby, 1984; Tsutsumi et al., 1986, 1987) and the lack of recovery from La\(^{3+}\) treatment (Beilby, 1984; Kourie, 1994) are better understood in terms of a reduced loading of internal Ca\(^{2+}\) stores than in terms of a direct block of plasma membrane Ca\(^{2+}\) channel activity during the AP. In the same context, the enhanced influx of \(^{45}\)Ca\(^{2+}\) into excited cells (Hayama et al., 1978; Reid and Tester, 1992) could therefore be due to a stimulated tracer uptake between AP into stores rather than influx during the AP.

In essence, the present evidence for Ca\(^{2+}\) release from internal stores during excitation is in line with recent electrophysiological data. In these studies it was found that elevation of the cytoplasmic InsP\(_3\) concentration triggered excitation (Thiel et al., 1990). In combination with the present data it underlines that cytoplasmic Ca\(^{2+}\) stores are present in Chara and that triggered release of Ca\(^{2+}\) is associated with excitation.

Furthermore, in patch clamp investigations it was found that the activation of excitatory Cl\(^-\) channels was not correlated in time with the activity of a putative Ca\(^{2+}\) permeable channel (Thiel et al., 1993, 1997). It was therefore proposed that for activation of Ca\(^{2+}\)-sensitive Cl\(^-\) channels internal stores must be filled with Ca\(^{2+}\) from the external solution. Upon stimulation, the stores discharge their content and transiently activate Cl\(^-\) channels. In this sense both fluorescence optic measurements and the electrophysiological data support the same model for the rise of [Ca\(^{2+}\)]\(_{cyt}\) in membrane excitation in Chara. At this stage we cannot directly interpret the measured [Ca\(^{2+}\)]\(_{cyt}\) transients as those anticipated from electrophysiological measurements. Thus, it is not clear whether the stores discharging Ca\(^{2+}\) during the AP are indeed the same ones that are InsP\(_3\)-sensitive. Furthermore, the anticipated kinetics of [Ca\(^{2+}\)]\(_{cyt}\) transients underlying Cl\(^-\) channel activation is significantly faster than the global Ca\(^{2+}\) changes recorded with Ca\(^{2+}\) dyes (Thiel et al., 1997). However, in analogy to studies in oocytes (Yao et al., 1995), it could be assumed that single short-lived release events initiate global longer lasting Ca\(^{2+}\) waves by increasing the frequency of Ca\(^{2+}\) release from individual release sites. In this sense the measured [Ca\(^{2+}\)]\(_{cyt}\) signal would reflect the statistical superposition of many single events.

The absence of an apparent Ca\(^{2+}\) influx in the present studies raises the question of how an electrical trigger (depolarization) initiates the release of Ca\(^{2+}\) from internal stores. It may be suggested that a tiny Ca\(^{2+}\) influx reaches stores adjacent to the membrane, causes a calcium-induced release (Berridge 1990) and starts a propagating wave of Ca\(^{2+}\) release throughout the cytosol. The detection of this putative influx would be difficult. In the studies here, quenching of the fluorescence near some membrane-adjacent stores would not cause a detectable change of the overall signal. In the case of patch clamp studies, the suggested restriction to parts of the cell (Hommann and Thiel, 1994) may prevent the detection of depolarization-induced Ca\(^{2+}\) influx. However, if the reason for not finding the depolarization-induced Ca\(^{2+}\) influx is its non-existence, it has to be questioned whether the concept of depolarization-induced ion fluxes as known from animal cells (Hille, 1992) can be transferred to excitable plant cells (Beilby and Coster, 1979). An alternative approach is offered by integrins. These membrane-bound proteins are connected to the cytoskeleton and are known to be involved in the transduction of mechanical signals into a biochemical response (Ingber, 1991). Wayne et al., 1992) assume the involvement of integrins in gravisingening in characean cells. Integrins bearing electrical charges could be moved by membrane potential similar to the S4 helices of Shaker-type K\(^+\) channels (Durell and Guy, 1992) and open internal Ca\(^{2+}\) stores in the ER by mechanical signal transduction without involvement of voltage-sensitive ion channels.

Experimental procedures

Plant material

Chara corallina was grown in the laboratory in plastic basins filled with APW (0.1 mM KCl, 1.0 mM NaCl, 0.5 mM CaCl\(_2\)) as described in Plieth and Hansen (1992).

Loading procedures

In order to prevent dye sequestration into the vacuole and other cell compartments, the dextran derivative of fura-2 was used (i.e. fura-dextran; MW = 10 kDa; F-3029; Molecular Probes, Eugene, OR; for emission and excitation spectra see Plieth et al., 1997). A solution of 1 mM fura-dextran was injected into the cytoplasm of the cells by manual micropressure injection or by loading via neighbouring cells, as described in detail by Plieth and Hansen, 1996.

In some experiments Mn\(^{2+}\) ions were also loaded into the vacuole by means of micropressure injection. A mixture of 440 mM BCECF–dextran, 20 mM Mn(NO\(_3\))\(_2\) and 100 mM KCl was injected into the vacuole under the control of fluorescence imaging (\(\lambda_{em} = 490\text{nm}, \lambda_{ex} = 530\text{nm}\), using a DMS510 dichroic and BAS20–560 bandpass filter from Nikon, Düsseldorf, Germany) in order to get an estimate of the amount of the [Mn\(^{2+}\)]\(_{vac}\). Loading was stopped when the vacuole showed a BCECF fluorescence corresponding
Preparations of cells and excitation

Individual dye-loaded Chara whorl cells of 5–15 mm length were dissected and transfused to a perfusion chamber (i.e. Petri dish with an outlet as described in Plieth, 1995). The cells were pressed to the bottom with needles sticking in silicon rubber at the bottom of the Petri dish, as previously described (Plieth and Hansen, 1992).

All experiments were carried out in standard medium (SM = 0.1 mM KNO₃, 0.1 mM MgCl₂, 0.1 mM CaCl₂, 2.0 mM Mes adjusted with NaOH to pH = 5.5). This medium was found to be best suited for eliciting AP with large amplitudes. The perfusion flux was 5 ml min⁻¹. Solutions of different ionic composition for eliciting AP with large amplitudes. The perfusion flux was 5 ml min⁻¹. Solutions of different ionic composition (i.e. SM with and without Mn²⁺ ions) could be switched to the perfusion stream by a group of three-way valves (Plieth, 1995).

Two bath electrodes (glass tubes with an inner diameter of 3 mm, filled with 1 M KCl in 3% agar) were positioned near both ends of the cell at a separation of 3 cm. One of them (cathode) was provided with a series resistance of 100 kΩ. The other one (anode) was also used as earth electrode for simultaneous membrane potential measurements. A short DC-pulse (0.5 sec with E = 3 V/cm) was sufficient to elicit an AP. In order to avoid incomplete AP the refractory time (ca. 2 min) had to be waited for before a new stimulus was applied.

Fluorescence ratio imaging

Changes in [Ca²⁺]cyt were measured using a fluorescence ratio imaging system from ImproVision (Coventry, UK), as described by Mühling et al. (1995). Fluorescence values were obtained by averaging the grey levels in a region of interest (i.e. an area of about 100 μm × 200 μm). The ratio of F₃₄₀/F₃₈₀ was used as measure for [Ca²⁺]cyt. Calibration was performed as described below. The images of the sample of the membrane potential were taken every 8 sec. During action potentials the sample rate was increased to 4 ratios per 10 sec. The F₃₈₀ signal was taken because this fluorescence is near the isosbestic point of the dye and is thus nearly insensitive to Ca²⁺ ions. However, it clearly exhibits heavy metal-induced quenching. The optical set-up used here was a DMM04 dichroic mirror and a BAS10 band pass filter (Nikon) for the emitted fluorescent light.

Calibration

In vitro calibration was performed using rectangular glass capillaries (50 μm × 1000 μm; W9005; VitroDynamics, Rockaway, NJ) filled with standard Ca²⁺ solutions (Calibration Kit, C-3722; Molecular Probes; Haugland, 1992) containing 3 μM fura-dextran (Plieth, 1995, Plieth et al., 1997). The saturation of fura-2-dextran above 1 μM needs a comment: fura-dextran has a higher dissociation constant (Kₐ = 350 nM, varying from lot to lot; Haugland, 1992) than the pure pentapotassium salt fura-Kₐ (Kₐ = 140 mM; Lattanzio, 1991). Thus it is not surprising that fura-dextran saturates above 1 μM Ca²⁺ (i.e. pCa < 5).

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