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The role of the plasma-membrane Ca^{2+}-ATPase in Ca^{2+} homeostasis in *Sinapis alba* root hairs

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Abstract. The regulation of cytosolic Ca^{2+} has been investigated in growing root-hair cells of *Sinapis alba* L. with special emphasis on the role of the plasma-membrane Ca^{2+}-ATPase. For this purpose, erythrosin B was used to inhibit the Ca^{2+}-ATPase, and the Ca^{2+} ionophore A_{23187} was applied to manipulate cytosolic free $[Ca^{2+}]$ which was then measured with Ca^{2+}-selective microelectrodes. (i) At 0.01 μM, A_{23187} had no effect on the membrane potential but enhanced the Ca^{2+} permeability of the plasma membrane. Higher concentrations of this ionophore strongly depolarized the cells, also in the presence of cyanide. (ii) Unexpectedly, A_{23187} first caused a decrease in cytosolic Ca^{2+} by 0.2 to 0.3 pCa units and a cytosolic acidification by about 0.5 pH units. (iii) The depletion of cytosolic free Ca^{2+} spontaneously reversed and became an increase, a process which strongly depended on the external Ca^{2+} concentration. (iv) Upon removal of A_{23187}, the cytosolic free $[Ca^{2+}]$ returned to its steady-state level, a process which was inhibited by erythrosin B. We suggest that the first reaction to the intruding Ca^{2+} is an activation of Ca^{2+} transporters (e.g. ATPases at the endoplasmic reticulum and the plasma membrane) which rapidly remove Ca^{2+} from the cytosol. The two observations that after the addition of A_{23187}, (i) Ca^{2+} gradients as steep as −600 mV could be maintained and (ii) the cytosolic pH rapidly and immediately decreased without recovery indicate that the Ca^{2+}-exporting plasma-membrane ATPase is physiologically connected to the electrochemical pH gradient, and probably works as an nH^+/Ca^{2+}-ATPase. Based on the finding that the Ca^{2+}-ATPase inhibitor erythrosin B had no effect on cytosolic Ca^{2+}, but caused a strong Ca^{2+} increase after the addion of A_{23187}, we conclude that these cells, at least in the short term, have enough metabolic energy to balance the loss in transport activity caused by inhibition of the primary Ca^{2+}-pump. We further conclude that this ATPase is a major Ca^{2+} regulator in stress situations where the cytosolic Ca^{2+} has been shifted from its steady-state level, as may be the case during processes of signal transduction.

Key words: ATPase (calcium, plasma membrane) – Calcium regulation – Cytosol (calcium, pH) – Ionophore (A_{23187}) – Plasma membrane – *Sinapis* (calcium homeostasis)

Introduction

From the literature on animal cells, cytosolic free Ca^{2+} is known to be a link in numerous intracellular processes and responses, and it is generally regarded a second messenger of signal-transduction pathways (Campbell 1983; Carafoli 1987). In recent years, evidence has accumulated that this ion may play a similar role in plants (Gilroy and Trewavas 1990), although their sessile lifestyle may require different strategies to cope with the various external stimuli. In order to learn more about the role of cytosolic Ca^{2+} in plant signal transduction (Hepler and Wayne 1985; Bush et al. 1989), metabolism (Kauss 1987) or gene expression (Thompson and White 1991), the regulatory components leading to Ca^{2+} homeostasis have to be investigated. In particular, cytosolic Ca^{2+} has to be measured under conditions where regulation is required. This can be accomplished through experimentally manipulating the resting Ca^{2+} level, as well as forcing the cells to react to different stimuli. In doing this, Ca^{2+} ionophores (Pressman 1976; Campbell 1983) and Ca^{2+}-ATPase inhibitors (e.g. erythrosin B; Rasi-Caldogno et al. 1989a, b; Evans et al. 1991) can be indispensible tools.

In an attempt to investigate cellular Ca^{2+} homeostasis in plant cells, we tested these agents and focussed our

Abbreviations and symbols: EB = erythrosin B; E_m = membrane potential; pCa = negative logarithm of the Ca^{2+} concentration

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interest on the reaction of the plasma-membrane Ca^{2+}-ATPase to perturbances of cytosolic Ca^{2+}. For this purpose the tip-growing root-hair cells of *Sinapis alba* proved a favourable quasi-unicellular test system.

Material and methods

General conditions. Seedlings of *Sinapis alba* L. (Fa. Hesemann, Giessen, FRG) were grown for 2–3 d on filter paper which was soaked with tap water. The excised roots were mounted in a Plexiglas chamber which was constantly perfused with the basic test medium. This comprised 0.1 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$ and a mixture of 5 mM of 2-(N-morpholino)ethanesulfonic acid/Tris, adjusted to pH 6.1. After 2–4 h the roots were equilibrated and had grown new root hairs. Only these were used for the experiments. All deviations from these conditions are given in the figure legends.

The Ca^{2+} ionophore A_{23187} was obtained from Calbiochem (Frankfurt, FRG) and erythrosin B (EB) from Sigma (Deisenhofen, FRG).

Electrophysiology and ion-selective microelectrodes. The electrical setup for the impalement of root hairs and for measurements of membrane potential (E_m) has been described previously (Felle 1982, 1987). The test chamber is open on both sides and allows the simultaneous horizontal approach of two separate electrodes. The fabrication and intracellular use of the pH- and Ca^{2+}-sensitive microelectrodes has been described in detail recently (Felle and Bertl 1986; Felle 1989). These electrodes were connected to high-impedance amplifiers (FD 223 or Duo 773; WP-Instruments, Sarasota, Fla., USA).

Presentation of the data and statistics. Since the ion-selective microelectrodes primarily record the voltage sum of the free-ion concentration plus E_m, the signal of the voltage electrode was simultaneously subtracted (differential amplifier) from the signal of the ion-selective electrode to obtain the net free-ion concentration (for a detailed description, see Felle and Bertl 1986). For the sake of clarity in presentation, only the traces of the free-ion concentrations are shown or, wherever interesting to the argument, the net trace and the E_m are given in the figures. The number of experiments is stated in the figure legends. Typical chemical time resolutions ($T_{1/2}$) of the ion-selective electrodes were 2–5 s for the pH-electrode and 5–8 s for the Ca^{2+}-electrode.

Since cytosolic changes of pH and Ca^{2+} were recorded, the sensitivities of these electrodes were cross-checked. All calibrations were carried out in solutions containing 100 mM K^+ which closely represents the cytosolic $[K^+]$ of the root-hair cells. The Ca^{2+}-electrodes gave a reading of 1–3 mV in response to pH changes from 6 to 8; the pH-electrode did not react at all to changes in pCa from 5 to 7.

Results

Effect of the Ca^{2+} ionophore A_{23187} on the plasma-membrane potential of Sinapis alba root hairs. Figure 1A shows the effect of different A_{23187} concentrations on the E_m of freshly grown *S. alba* root hairs. In the presence of 0.1 mM external Ca^{2+}, 0.01 µM A_{23187} had hardly any effect on E_m, but it markedly stepped-up the sensitivity of the plasma membrane to Ca^{2+}. This is shown in Fig. 18 where, after the addition of 0.01 µM A_{23187}, the cells reacted to an increase in external Ca^{2+} from 0.1 to 10 mM by a two to threefold enhanced depolarization, in spite of the proton-pump activity which tends to repolarize the cells. In order to show the impact of this ionophore on passive membrane parameters only, the proton pump was deactivated by cyanide (Fig. 1C). It is known that cyanide depresses the E_m to the so-called diffusion potential which basically depends on the ion gradients present on each side of the plasma membrane

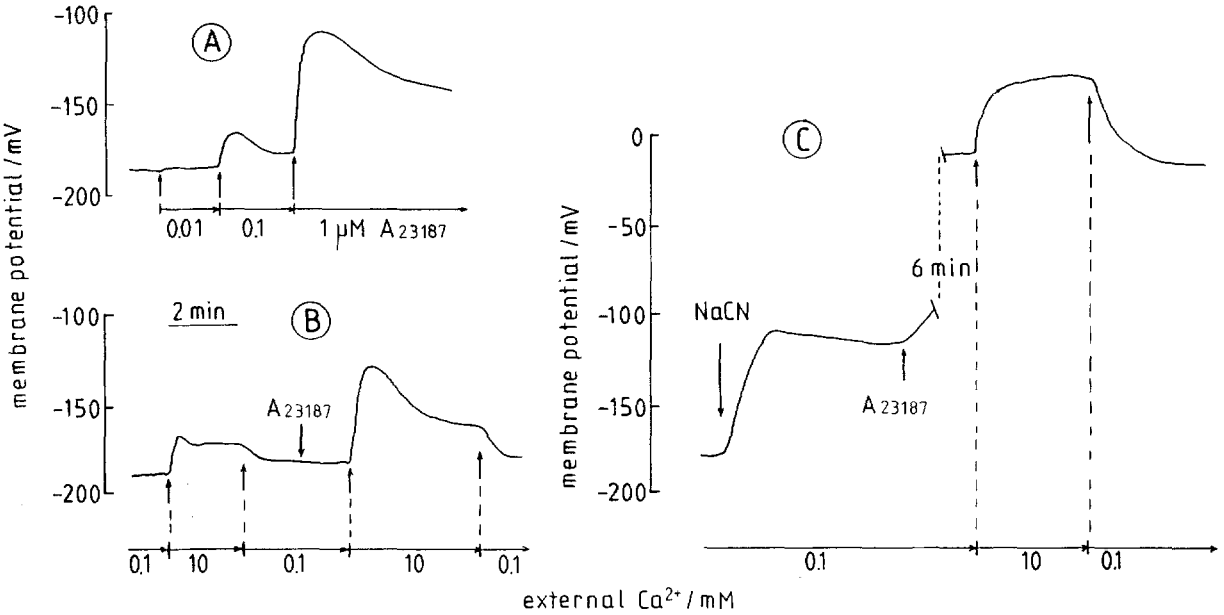

Fig. 1A–C. Effect of the Ca^{2+} ionophore A_{23187} on the E_m of freshly grown root hairs of *Sinapis alba*. **A** Different A_{23187} concentrations, as indicated, were added in the presence of 0.1 mM external Ca^{2+}. **B** Effect of 10 mM Ca^{2+} on the E_m before and after the addition of 0.01 µM A_{23187}. **C** Action of 0.5 µM A_{23187} on the diffusion potential in the presence of 1 mM NaCN and at the indicated external Ca^{2+} concentrations. Representative curves of six to ten equivalent measurements

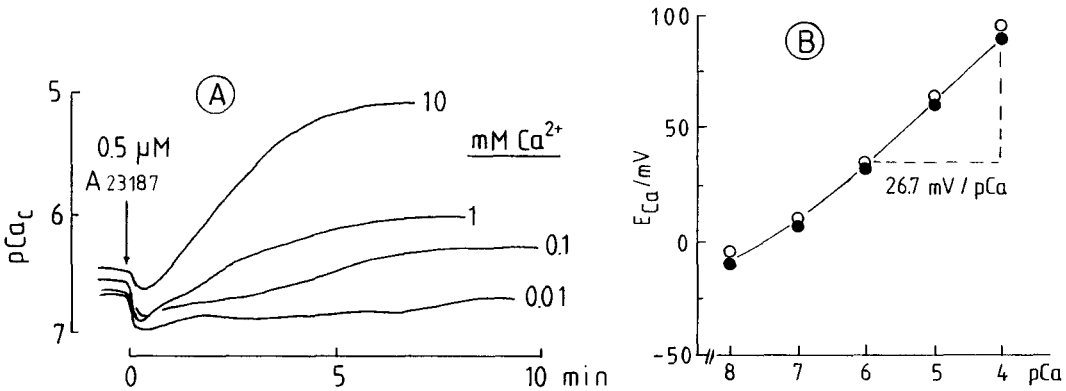

Fig. 2. A Effect of 0.5 µM A_{23187} on cytosolic free Ca^{2+} (pCa_c), measured with a Ca^{2+}-selective microelectrode. The curves are individual reactions of different *Sinapis alba* root hairs to the application of the ionophore in the presence of different external Ca^{2+} concentrations, as indicated. All curves are difference traces obtained by measuring and simultaneously subtracting the voltage trace from the trace of the Ca^{2+}-selective microelectrode (see *Material and methods*). Each curve is representative of three to five experiments, carried out under equivalent conditions. **B** Calibration of the Ca^{2+} selective microelectrode used for the tests given in **A**. E_{Ca} is the net potential difference obtained by subtraction of the voltage electrode signal from the Ca^{2+} electrode signal. Precalibration (●), recalibration (○). The reference buffer filling the Ca^{2+}-electrode had a pCa of 7

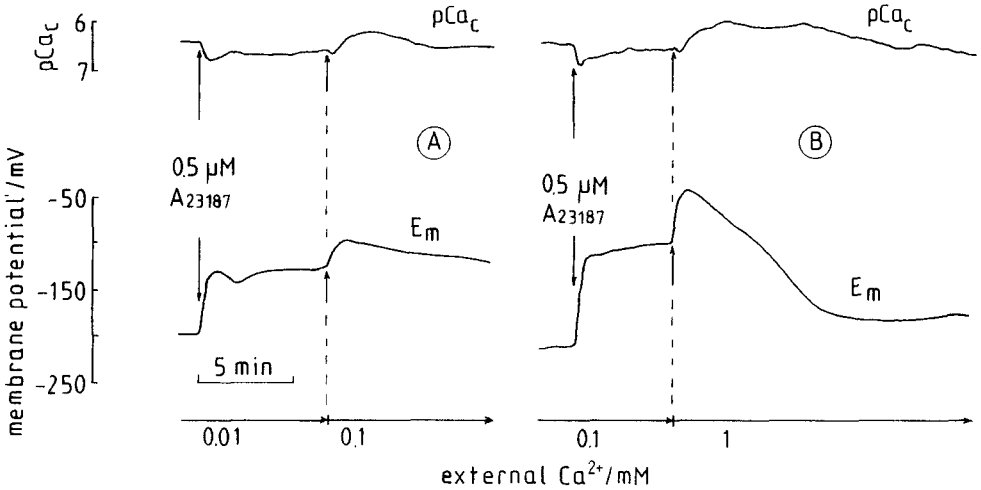

Fig. 3A, B. Cytosolic free Ca^{2+} (pCa_c), measured in the presence of 0.5 µM A_{23187} and different external Ca^{2+} concentrations *(horizontal bars)*. The recordings demonstrate the ability of the *Sinapis alba* root-hair cells to remove from the cytosol Ca^{2+} which had first leaked in through the action of A_{23187}. Simultaneously E_m was measured. Initial external Ca^{2+} concentrations were 0.01 mM (**A**) or 0.1 mM (**B**), added together with the ionophore, followed by 0.1 mM (**A**) or 1 mM Ca^{2+} (**B**). Representative recordings from three (**A**) or four (**B**) equivalent experiments

and the respective ion-permeability ratios (Slayman 1965; Felle 1981). After the cyanide induced depolarization to −115 mV, 0.5 µM A_{23187} further depolarized the cells to about −20 mV. The following increase in external Ca^{2+} from 0.1 to 10 mM drove the E_m to +30 mV which strongly indicates a shift of the permeability ratios of the plasma membrane in favour of Ca^{2+}.

Cytosolic free [Ca^{2+}]. In the tips of *Sinapis* root hairs the cytosolic free [Ca^{2+}] rests between 378 and 831 nM ($n=23$; see also Tretyn et al. 1991b). In the basal parts of these cells towards the cortex we found significantly lower free [Ca^{2+}], viz. 98 to 253 nM ($n=9$; data not shown in figures). Since A_{23187} is known as a Ca^{2+} ionophore (Pressman 1976), its external application would be expected to cause Ca^{2+} intrusion into the cytoplasm, resulting in an increase of cytosolic [Ca^{2+}]. We found this increase but, most surprisingly, it occurred after an initial decrase which strongly depended on external [Ca^{2+}]. As shown in Fig. 2, this Ca^{2+} depletion was only very short termed in the presence of 10 mM Ca^{2+}, but lasted about 4–5 min in the presence of 0.1 mM Ca^{2+}, before an increase of cytosolic Ca^{2+} was observed. With 0.01 mM Ca^{2+} in the external medium no A_{23187}-induced increase in cytosolic free [Ca^{2+}] occurred over the time period of the measurements. In the presence of 10 mM external Ca^{2+} the cytosolic [Ca^{2+}] rose rapidly to about pCa 5.

Figures 3 and 4 give representative examples of the cells regulative power, as tested in the presence of 0.5 µM A_{23187} and at different external Ca^{2+} concentrations. The initial decrease in cytosolic free [Ca^{2+}], measured in

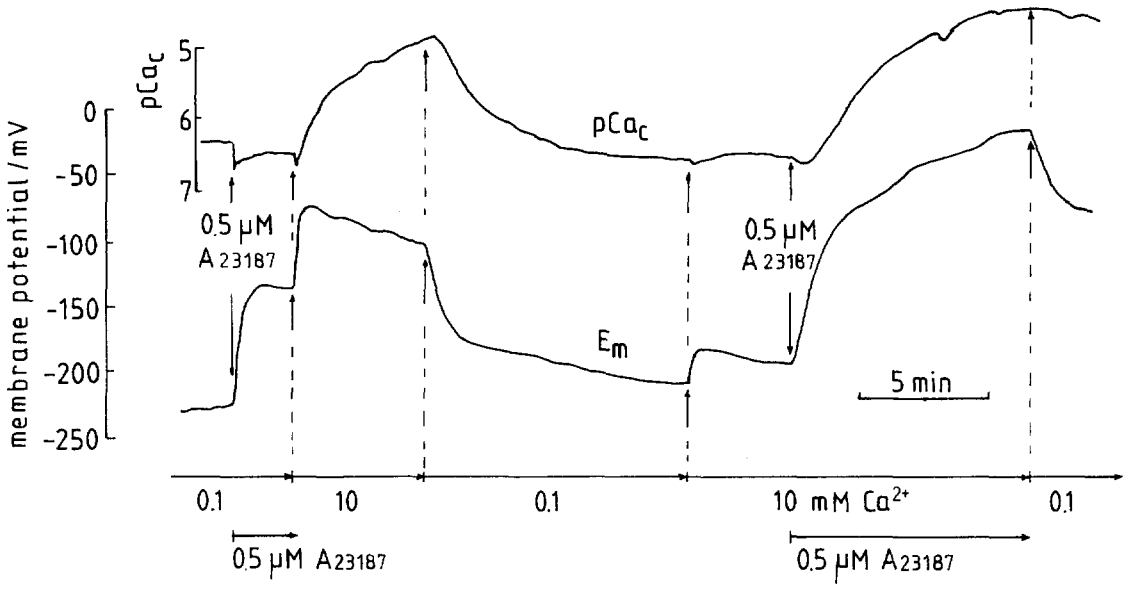

Fig. 4. Changes in cytosolic free $[Ca^{2+}]$ (pCa_c) of *Sinapis alba* root hairs before and after increasing the external Ca^{2+} from 0.1 mM to 10 mM Ca^{2+} in the presence and absence 0.5 µM A_{23187}. After the first addition of 10 mM Ca^{2+}, the increase in cytosolic $[Ca^{2+}]$ was interrupted intentionally to prevent a Ca^{2+}-overload of the cytosol. The intruded Ca^{2+} is exported again after removal of the ionophore and reduction of external Ca^{2+} to 0.1 mM. As a consequence of the following addition of A_{23187} (+10 mM Ca^{2+}), the cytosolic Ca^{2+} again massively increases to concentrations higher than pCa 5, but not into equilibrium with the external Ca^{2+}. Representative for four equivalent experiments

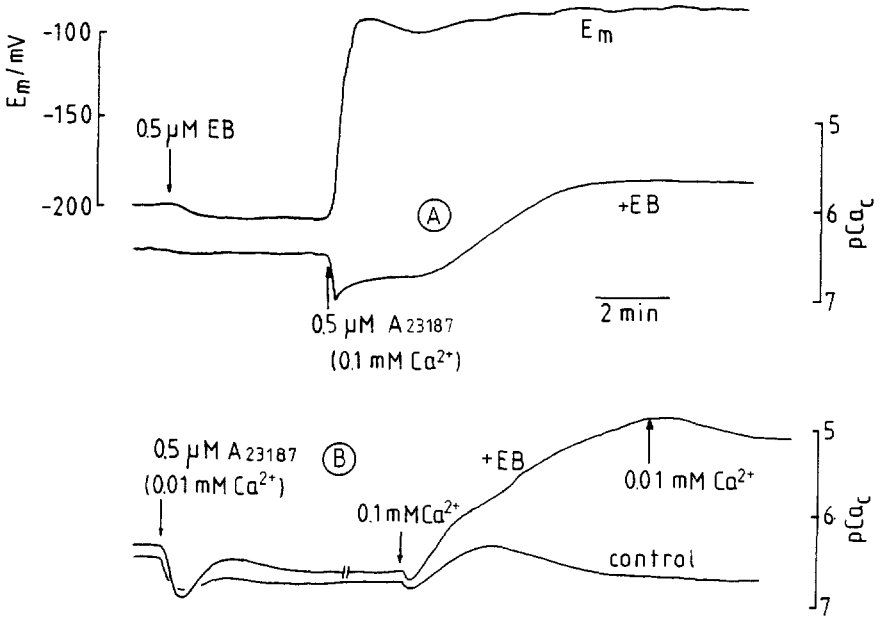

Fig. 5A, B. Inhibition of the plasma-membrane Ca^{2+}-ATPase by 0.5 µM EB. **A** Action of EB on E_m and cytosolic free $[Ca^{2+}]$, before and after the addition of A_{23187}. In **B** 0.5 µM A_{23187} was added in the presence of 0.01 mM external Ca^{2+} and was left working for 8–10 min. After that period the external Ca^{2+} was increased to 0.1 mM and two tests were performed, one with EB (+EB) and one without it (control). Comparison of the two curves demonstrates the ability of the cells to reset cytosolic Ca^{2+} and its loss of this ability in the presence of EB. The curves are each representative of three equivalent experiments

the presence of submillimolar external $[Ca^{2+}]$, was accompanied by a rapid depolarization. Additions of 0.1 and 1 mM external Ca^{2+}, respectively, resulted in an immediate increase in cytosolic Ca^{2+} which slowly (within minutes) recovered. It may be interesting to point out that in Fig. 3B the E_m rapidly repolarized in spite of the increased Ca^{2+} concentration.

In Fig. 4 the limits of the cells ability to restore perturbances in cytosolic free $[Ca^{2+}]$ are demonstrated. An increase in external $[Ca^{2+}]$ from 0.1 to 10 mM (in the presence of A_{23187}) rapidly shifted cytosolic Ca^{2+} to pCa values around 5. From this load the cells only recovered after a reduction in external $[Ca^{2+}]$ and after the removal of the ionophore from the medium.

The effect of erythrosin B. In submicromolar concentrations erythrosin B (EB) is known to specifically inhibit the plasma-membrane Ca^{2+}-ATPase, whereas the H^+-ATPase is inhibited at concentrations about 100 times higher (Rasi-Caldogno et al. 1989a, b). Since the plasma-membrane Ca^{2+}-ATPase is presumed to export Ca^{2+}, an inhibition should have severe effects on cytosolic Ca^{2+}

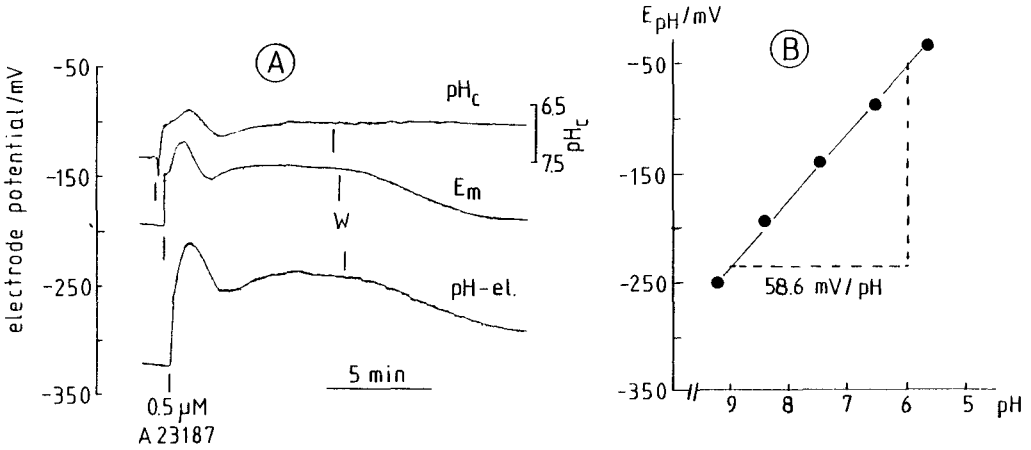

Fig. 6. A Cytosolic pH (pH_c) and E_m, measured before and after the external addition of 0.5 µM A_{23187}. Note that the initial pH change is rapid and does not return to control, although E_m completely recovers after removal of A_{23187} from the medium (*W*). For background information the trace of the pH-electrode (pH-el) also is shown. Representative of five equivalent measurements. **B** Calibration of the pH-sensitive microelectrode used for the tests in **A.** E_{pH} represents the net potential difference obtained by subtraction of the voltage electrode signal from the pH electrode signal. The reference buffer filling of the pH-electode had a pH of 5

homeostasis. As shown in Fig. 5A, 0.5 µM EB slightly hyperpolarized the cells, but it had no appreciable effect on cytosolic free $[Ca^{2+}]$ of the *Sinapis* root hairs. However, as soon as A_{23187} was added (in the presence of EB), the cytosolic free $[Ca^{2+}]$ rose massively after an apparently short period of depletion.

In Fig. 5B it is demonstrated that after the typical A_{23187}-induced decrease in cytosolic $[Ca^{2+}]$, a tenfold increase of external $[Ca^{2+}]$ from 0.01 to 0.1 mM only caused a transient increase in cytosolic free $[Ca^{2+}]$ (control). However, when EB was added together with the Ca^{2+}, there was a massive increase in cytosolic $[Ca^{2+}]$ to about pCa 5 without any sign of spontaneous recovery. Also, a tenfold reduction of external $[Ca^{2+}]$ to the original concentration only partly reduced the cytosolic $[Ca^{2+}]$.

Cytosolic pH. A_{23187} had an immediate effect on cytosolic pH. As shown in Fig. 6, cytosolic pH rapidly decreased from 7.5 to 6.6 following the addition of 0.5 µM A_{23187}. The cytosolic pH remained at 6.8 after a transient increase to about 7.1, although the E_m completely recovered after the withdrawal of the ionophore from the medium.

Discussion

The cytosolic free $[Ca^{2+}]$, recorded from *S. alba* root hairs, is in agreement with the values reported by other authors for plant cells (see Evans et al. 1991). Especially interesting was the finding of an intracellular Ca^{2+} "gradient" with elevated free $[Ca^{2+}]$ in the growing tip (Tretyn et al. 1991b). Such differences in cytosolic free $[Ca^{2+}]$ are not unusual and have been reported for *Lilium* pollen tubes (Jaffe et al. 1975; Reiss and Herth 1978; Nobiling and Reiss 1987), for the *Fucus* rhizoid (Brownlee and Wood 1986), and for *Chara* rhizoids (Hodick et al. 1991). Since it is understood that in tips of longitudinally growing cells an elevated $[Ca^{2+}]$ as well as an intracellular Ca^{2+} gradient may be linked to an increased metabolic activity (Speksnijder et al. 1989), we assumed that for investigations of cytosolic Ca^{2+} regulation these root-hair tips should be well suited.

The meaning of the initial Ca^{2+} depletion. Puzzling, but most interesting from the regulatory point of view, was the observation of the A_{23187}-induced initial depletion of free cytosolic Ca^{2+} which was especially evident in the presence of low external $[Ca^{2+}]$. At first sight one could have assumed this to be an artifact caused by the different time resolutions of the pH- and voltage electrodes, respectively (Felle and Bertl 1986). This is not the case: (i) the changes are Ca^{2+}-dependent (Fig. 2); (ii) the Ca^{2+} depletion is also present in cases where no or very little voltage change occurs; (iii) the Ca^{2+} depletions remain for minutes (Figs. 2–5); if they were just temporal artifacts caused by fast voltage changes across the plasma membrane, the difference traces would return to their starting point after the voltage changes (Felle and Bertl 1986), but they do not. On the other hand, the sharp initial peaks of the Ca^{2+} trace, observed during the first seconds after the addition of the ionophore (Figs. 4, 5), should be treated as such temporal artifacts.

In fact, the initial Ca^{2+} decreases depend on the external $[Ca^{2+}]$ (Fig. 2), i.e. on the amount of Ca^{2+} forming complexes with the ionophore; they could indicate that the intruding Ca^{2+} triggers regulatory forces which initially remove more Ca^{2+} from the cytosol than was originally imported. This is by no means paradoxial, and can be interpreted in different ways. (i) This effect may be caused by the A_{23187} acting as a $2H^+/Ca^{2+}$-antiporter, as proposed by Pressman (1976). Unfortunately, at present we do not have enough information to follow up and discuss this possibility in a satisfactory manner, but it is under investigation in our laboratory. (ii) Calcium ions move rapidly into the cytosol where they are sensed by Ca^{2+} transporters, such as the Ca^{2+}-ATPases at the

ER and the plasma membrane, and are rapidly removed from the cytosol. Apart from the intruding Ca^{2+}, other possible signals to trigger such a reaction could be the rapid depolarization, or the observed fast and strong acidification (Fig. 6).

Recently, a similar effect has been reported for pH regulation: Johannes and Felle (1987) demonstrated that during the initial depolarization of H^+/amino acid (or hexose-) symport in the aquatic liverwort *Riccia fluitans*, cytosolic pH increased just as unexpectedly, although protons as the driving ions obviously must have entered the cells first. Since depolarization and pH increase were found to be related, this effect was interpreted as the result of a voltage-stimulated H^+-ATPase which presumably led to an overcompensated H^+ export. At present we cannot rule out such an effect on the Ca^{2+}-ATPase, but as long as the problem of electrogenicity of this ATPase is not solved (see below), we favour the Ca^{2+}-signal hypothesis. It may not be too speculative to assume that as a consequence of the action of A_{23187}, the $[Ca^{2+}]$ will first rapidly increase in locations close to the plasma membrane. Since the gap between plasma membrane and ER is very small, as shown for instance in etiolated oat coleoptiles (Tretyn et al. 1992), such an increase in $[Ca^{2+}]$ will be sensed by both Ca^{2+}-ATPases. Also, some Ca^{2+}-storage capacity in the mitochondria should be taken into account, since in growing pollen tubes, for example, mitochondria are densely packed in a zone just behind the tip (Steer and Steer 1989). Clearly, both mitochondria and ER as internal compartments have only limited storage capacity: therefore, in stress situations, where Ca^{2+} overfloods parts of the cytoplasm, the plasma-membrane Ca^{2+}-ATPase will take over and export Ca^{2+}, or the surplus will have to be shifted into the vacuole.

The inhibited plasma-membrane Ca^{2+}-ATPase. Ever since ATP-dependent Ca^{2+} transport across the plasma membrane of plant cells was reported by Gross and Marmé (1978), and Dieter and Marmé (1981), the importance of this pump for Ca^{2+} homeostasis has been emphasized (Rasi-Caldogno et al. 1987, 1989a, b; Evans 1988; Dupont et al. 1990), and is demonstrated here through the experiments with EB (Fig. 5). Although EB per se does not affect the resting cytosolic $[Ca^{2+}]$ of *Sinapis* root hairs, its impact on cytosolic Ca^{2+} regulation is massive when it added in situations where Ca^{2+} constantly leaks into the cell, i.e. in the presence of A_{23187}, where the Ca^{2+} ATPase is under stress: then the Ca^{2+} rapidly enters the cytosol down its electrochemical gradient, and within minutes increases the cytosolic level from 0.66 μM to 5.2 μM.

Is the plasma-membrane Ca^{2+}-pump an nH^+/Ca^{2+}-ATPase? In this study we present evidence that A_{23187}, besides its effect on E_m and cytosolic free $[Ca^{2+}]$, also induces an immediate decrease of cytosolic pH which seems to be concomitant with the increased activity of the Ca^{2+}-pump. Miller and Sanders (1987) have pointed out that under conditions where cells build up E_ms of -200 mV or over, a Ca^{2+}-ATPase may not have enough energy from ATP hydrolysis to export Ca^{2+}, i.e. to maintain free Ca^{2+} in the submicromolar range. The inwardly directed Ca^{2+} driving force across the plasma membrane can be formulated as:

$$\Delta\mu Ca^{2+}/F = 2E_m + 59(pCa_o - pCa_c) \quad \text{(Eq.1)}$$

where E_m denotes the membrane potential, $\Delta\mu Ca^{2+}/F$ is the electrochemical Ca^{2+} gradient across the plasma membrane, and pCa_o and pCa_c are the external and cytosolic pCa, respectively. From this equation it is evident that the E_m is by far the largest fraction of the Ca^{2+}-driving force. Inserting a typical E_m of -200 mV, an external pCa of 4, and a cytosolic pCa of 6.5, a Ca^{2+}-driving force of about -550 mV results before the addition of A_{23187}. In Fig. 3B we demonstrate that in the presence of 0.5 μM A_{23187} the cells are well able to regulate cytosolic Ca^{2+} even at 1 mM external Ca^{2+}; moreover, the cells repolarize to -190 mV, i.e. restore and even increase the electrochemical Ca^{2+} gradient to about -590 mV. This provides strong evidence that the Ca^{2+}-regulating system must be connected to some other driving force and, in accordance with Miller and Sanders (1987) and Rasi-Caldogno et al. (1987), we suggest this to be the electrochemical proton gradient, built up and maintained by the electrogenic proton pump at the plasma membrane. This means that for every Ca^{2+} exported one or more protons should enter the cell driven by the same transporter, viz. the Ca^{2+}-ATPase. We give evidence here that this may indeed be the case. As demonstrated in Fig. 6, cytosolic pH decreased immediately after the addition of A_{23187}. Together with the accompanying depletion of cytosolic free Ca^{2+}, this is exactly what must be expected of a stimulated ATPase which exchanges nH^+ for Ca^{2+}. So far, we do not have much information about whether this ATPase is electrogenic or electroneutral, but we found that the addition of EB to the cells caused a hyperpolarization by about 5 mV (Fig. 5A). This is not very impressive, but considering that the activity of the Ca^{2+}-ATPase may be only a small fraction of what the H^+-ATPase exports (Sanders and Slayman 1989), the impact of an electrogenic Ca^{2+}-ATPase on the total E_m can only be of minor importance. Assuming that this Ca^{2+}-ATPase is electrogenic, then the small hyperpolarization could be indicative of more than $2H^+$ being imported for every Ca^{2+} exported, a problem which is under current investigation in our laboratory.

Final remarks

We are well aware of the pitfalls in using substances like EB and ionophores. In particular, the in-vivo test situation requires a careful analysis to separate facts from fancies. Still, we are able to present some new insights into how these compounds may work on a living cell.

(i) We could demonstrate that the inhibition of the Ca^{2+}-ATPase through EB (without A_{23187}) had no impact on the cytosolic $[Ca^{2+}]$. This means that, at least for some time, the cells can keep the cytosolic free $[Ca^{2+}]$ constant without the support of this transporter. On the other hand, cytosolic $[Ca^{2+}]$ rapidly increases when EB

is applied during Ca^{2+} stress. We conclude, therefore, that the main role of the plasma-membrane Ca^{2+}-ATPase is to act in situations where the cytosolic Ca^{2+} gets perturbed, as for instance during cellular signalling (Tretyn et al. 1991b).

(ii) We demonstrate that because of the steep electrochemical Ca^{2+} gradient across the plasma membrane of these cells the hydrolysis of 1 ATP does not provide enough energy to export Ca^{2+} against this gradient or to maintain it. Since we find that the intruded Ca^{2+} is indeed removed from the cytosol, and at the same time cytosolic pH rapidly decreases, we suggest that the plasma-membrane Ca^{2+}-ATPase may be an nH^+/Ca^{2+}-ATPase with n possibly greater than 2, as indicated by the EB-induced hyperpolarization.

(iii) The Ca^{2+} recovery shown in this study appears slow if compared with animal cells where disturbances in cytosolic $[Ca^{2+}]$ are often restored in the subsecond range. Apart from the fact that the presence of the Ca^{2+} ionophore A_{23187} within the plasma membrane may have prevented a faster recovery, plant cells apparently do need longer. In guard cells, Gilroy et al. (1990), McAinsh et al. (1990), and Schroeder and Hagiwara (1990) have shown that the abscisic-acid-induced changes in cytosolic Ca^{2+} recover within minutes, as does the abscisic-acid-induced decrease in cytosolic Ca^{2+} reported in barley aleurone by Wang et al. (1991). This is in agreement with reports on the liverwort *Riccia fluitans*, where cytosolic Ca^{2+} perturbations also recovered within about 1 min (Felle 1991), whereas auxin caused oscillation of Ca^{2+} in *Zea mays* (Felle 1988a, b), and a stable decrease in *Sinapis* root hairs (Tretyn et al. 1991). In our study we found that in spite of the relative slow Ca^{2+} recovery after the A_{23187} treatment, the Ca^{2+}-exporting ATPase reacts quite rapidly, which means that the physiological recovery times are intrinsic properties of the underlying chain of events.

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