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Abscisic acid induces a cytosolic calcium decrease in barley aleurone protoplasts

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Cytosolic calcium concentrations (Ca_i) of barley aleurone protoplasts after stimulation with the plant hormone abscisic acid (ABA) were measured by using the calcium-sensitive fluorescent dye Indo-1. The measured basal Ca_i is about 200 nM. Stimulation with ABA induces a strong dose-dependent decrease in Ca_i to a minimal value of about 50 nM. This decrease occurs within 5 s. The Ca^{2+} antagonists La^{3+} and Cd^{2+} inhibit the ABA-induced Ca_i decrease in a dose-dependent manner, while the Ca^{2+} channel blockers verapamil and nifedipine give no inhibition. The induction of Ca_i decrease by ABA is consistent with activation of the plasma membrane Ca^{2+}-ATPase by ABA. The possible role of this ABA-induced Ca_i decrease in ABA signal transduction and in counteracting the effects of gibberellic acid are discussed.

Abscisic acid; Calcium; Ca^{2+}-ATPase; Gibberellic acid; Barley aleurone; Dormancy

1. INTRODUCTION

The plant hormone abscisic acid (ABA) plays an important role in seed development and seed dormancy [1–3]. It has been shown that removing of ABA induces precocious germination in cultured immature wheat embryos [4]. Furthermore, seeds which have been mutated in either sensitivity for ABA or synthesis of ABA show lack of dormancy [5–7]. ABA inhibits the synthesis of specific enzymes necessary for the initiation of germination [8–10], and is able to induce expression of different genes [11–13]. In order to get a deeper insight into the role of ABA in barley seed dormancy it is essential to understand the signal transduction pathway of ABA. However, at present little is known about ABA signal transduction pathways and the relation to dormancy.

ABA counteracts a variety of gibberellic acid (GA)-mediated responses in barley aleurone layers [14,15]. Conversely, the inhibition of germination by ABA can be at least partially reversed by GA in different species [16].

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; ABA, abscisic acid; Des, diethylstilbestrol; GA, gibberellic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Ca_i, cytoplasmic free Ca^{2+} concentration; Tris, tris(hydroxymethyl)aminomethane; Pipes, 1,4-piperazinediethanesulfonic acid; RAB, responsive to abscisic acid

It is known that Ca^{2+} is an important secondary messenger in both plant and animal hormone signal transduction [17]. Both increases and decreases of Ca_i, which play a role in different signal transduction systems (e.g. in regulation of stomatal closure and metabolism), have been demonstrated in plant cells [18,19]. In wheat aleurone tissue, ABA-induced polypeptide synthesis can also be influenced by Ca_i [20]. In barley seed germination, Ca^{2+} is required both for α-amylase activity and stability [21]. α-Amylases are essential hydrolases in seed germination. Therefore, our first aim in the study of ABA signal transduction is to investigate a possible role for Ca_i changes. In this study, we report that ABA induces a rapid decrease in Ca_i which is consistent with activation of plasma membrane Ca^{2+}-ATPases.

2. MATERIALS AND METHODS

2.1. *Materials*

Both Indo-1 and Indo-1/AM were from Boehringer Mannheim (Germany). Des, Mes, verapamil, L-α-phosphatidic acid and ABA were obtained from Sigma Chemical Co. (St. Louis, USA), and cellulase Onozuka R-10 from Yakult Honsha (Japan). Gamborg B5 was from Flow Laboratories (Irvine, Scotland). PVP K25 was from Fluka Chemie (Switzerland). Nifedipine was from Bayer (Germany). The ABA analogues 1'-deoxy-ABA and α-ionyledene acetic acid were kindly provided by Dr R. Hogan (University College of Wales, Dept of Biol. Sci., Aberystwyth, UK). All other chemicals were from Merck (Darmstadt, Germany).

2.2. *Isolation of protoplasts from barley aleurone*

Barley (Himalaya) aleurone protoplasts were prepared essentially as described by Jacobsen et al. [21], except for incubations of aleurone layers with enzymes which were carried out at 25°C in the

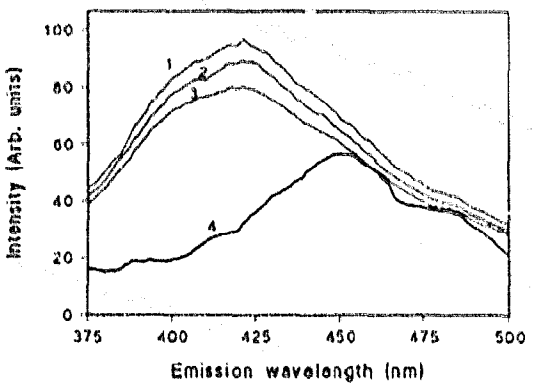

Fig. 1. Emission spectra (excitation wavelength 355 nm) of protoplasts loaded with Indo-1/AM in loading buffer. Loading conditions are described in section 2. Spectrum 1 is that obtained at the end of the loading and washing, and shows a typical Indo-1 curve, indicating that the fluorescent dye Indo-1/AM has been converted into Indo-1. Spectra 2 and 3 have been obtained after stimulation with 200 and 400 μM ABA, respectively. Spectrum 4 shows the emission spectrum (excitation of 355 nm) of Indo-1/AM.

dark for 16 h. The obtained protoplasts were sieved on a 100 μm sieve and were washed three times with a washing buffer (0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 0.5 mM K_2HPO_4, 10 mM Pipes-HCl (pH 6.8)) before use [22].

2.3. *Loading of fluorescent dye Indo-1*

The buffer used to load the fluorescent dye into the protoplasts was the same as the washing buffer [22]. 10 μM Indo-1/AM (dissolved in DMSO, maximal DMSO in sample was 1%) was added to the protoplast suspension (10^6 protoplasts/ml). In control experiments, protoplasts were treated with 1% DMSO. The suspension was incubated in the dark at 30°C for 4 h, then washed three times with washing buffer. Indo-1 fluorescence intensity at 405 nm was measured using a Perkin-Elmer Luminescence Spectrometer LS 50 at an excitation wavelength of 335 nm. The data were analyzed with the use of Perkin-Elmer Fluorescence Data Manager software. During measurements the protoplast suspension (6×10^5 protoplasts/ml) in washing buffer was continuously stirred.

Experiments were performed at room temperature (about 22°C). All given values are means ± SD, with n = number of independent experiments.

3. RESULTS

3.1. *Measurement of intracellular free calcium*

In order to know whether the fluorescent dye (Indo-1/AM) was loaded into the protoplasts and whether it was converted to the Ca^{2+}-sensitive form Indo-1, the fluorescence emission spectra of Indo-1/AM-loaded protoplasts were measured (Fig. 1). The fluorescence emission spectrum of loaded protoplasts was different from the Indo-1/AM spectrum and was identical to the emission spectrum of Indo-1 (Fig. 1; cf. [22]). To further prove that the fluorescent dye was loaded into the protoplasts instead of non-specific binding on the protoplast membranes, the loaded protoplasts were disrupted by sonication and thereafter centrifuged at 10000 × g, 4°C for 10 min. The spectra of the supernatant were identical to the spectrum of Indo-1. The pellets, resuspended in loading buffer with the same volume as the supernatant, showed very low fluorescence intensity and a different spectrum than that of Indo-1 (data not shown).

The cytoplasmic free Ca^{2+} concentration can be calculated from the Indo-1 fluorescence of a sample when the maximal and minimal fluorescence of the sample are known according to the following equation [23]:

$$[Ca^{2+}]_i = K_d \times \frac{(F - F_{min})}{(F_{max} - F)} \quad (1)$$

in which K_d is the dissociation constant for Indo-1 and Ca^{2+} (22°C, 250 nM), F is the measured 405 nm fluorescence intensity at 335 nm excitation, F_{max} is the fluorescence intensity of the dye present in the sample at saturating Ca^{2+} concentration and F_{min} is the fluorescence intensity of the dye present in the sample at zero Ca^{2+} concentration [24]. In order to establish the F_{max} and F_{min} of the samples, protoplasts were disrupted by sonication (Sonics and Moterials, Danburg, CT, USA) at the end of each experiment. After disruption of protoplasts, in extracellular measuring buffer containing 1 mM Ca^{2+}, the fluorescence F_{max} was obtained, which represents the fluorescence intensity of the dye at saturating Ca^{2+} concentration. Subsequently the Ca^{2+} in the medium was chelated with excess EGTA (after addition of a high concentration of Tris-HCl buffer, pH 7.5, to ensure maximal Ca^{2+} binding by EGTA) to obtain F_{min}, the fluorescence of the dye at zero Ca^{2+}. Addition of Mn^{2+} did not further decrease the fluorescence intensity (see below).

As described above, the calibration was carried out by disrupting the protoplasts at the end of the experiment. However, after the sonication of the protoplasts, the fluorescence intensity was somewhat lower than the fluorescence intensity at the beginning of the experi-

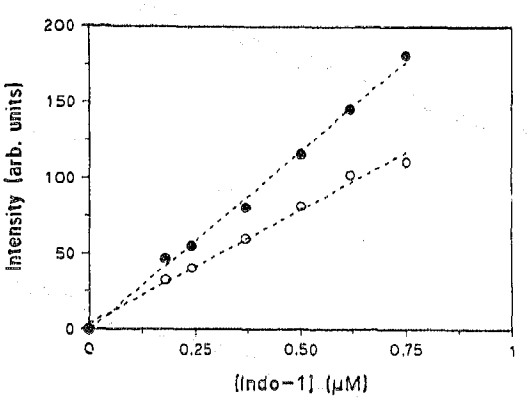

Fig. 2. Fluorescence intensity of different Indo-1 concentrations in a suspension of protoplasts (6×10^5 cells/ml) in loading buffer before (●) and after (○) sonicating the cells. Fluorescence intensity was measured at 405 nm at an excitation wavelength of 355 nm. Dashed lines show linear regression with correlation coefficients >0.995.

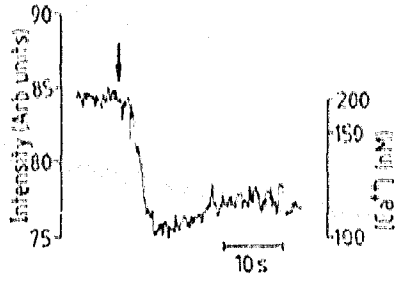

Fig. 3. Response of Ca_i of barley aleurone protoplasts upon stimulation with 200 μM ABA (the moment of stimulation indicated by arrow). Response of one experiment is shown.

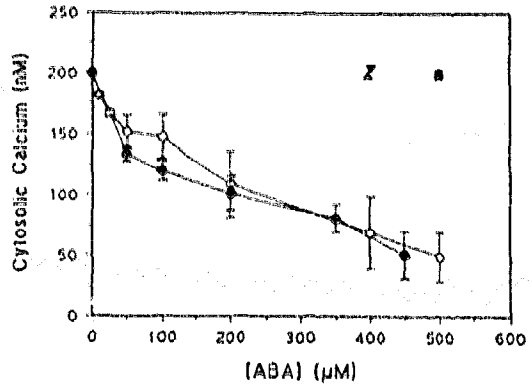

Fig. 4. Mean Ca_i decrease after stimulation of protoplasts as a function of the ABA concentration. Results of 4 independent experiments are shown. The open circles represent the experiments performed with protoplasts in normal loading buffer. The closed circles represent the experiments performed with protoplasts in a buffer without added Ca^{2+} but with 10 mM EGTA. (■) represents Ca_i after stimulation with ABA carrier (NaOH) (equal to the amount used for 500 μM ABA); (▲ and ▼) represent Ca_i after stimulation with 400 μM L-α-phosphatidic acid and 400 μM 2,3-dihydroflavanone, respectively. Bars indicate ± SD.

ment. Apparently some of the fluorescence intensity was quenched due to the presence of disrupted protoplasts (this may also explain the absence of a further decrease in fluorescence intensity after addition of Mn^{2+} in establishing F_{min}). Therefore a series of control experiments were carried out to determine the real F_{max} value. Fig. 2 shows the correlation between the amount of Indo-1 in the presence of 6×10^5 protoplasts/ml and the fluorescence intensity before and after sonication. This shows that disrupted protoplasts indeed cause a decrease in Ca^{2+}-saturated Indo-1 fluorescence. Hence, F_{max} values had to be corrected for the fluorescence quenching due to protoplast disruption.

3.2. *Effects of abscisic acid on cytosolic calcium level*

Resting Ca_i of barley aleurone protoplasts was calculated from the Ca^{2+}-dependent fluorescence intensity of Indo-1-loaded protoplasts after determination of F_{max} and F_{min}. Resting Ca_i was 200 ± 50 nM (n = 16). Upon addition of the lipophilic acid ABA Ca_i immediately decreased. This decrease reached its minimal level in about 5 s, and then increased slightly. After about 10 s, Ca_i reached a new steady-state level (Fig. 3). Protoplasts incubated with the solvent of ABA gave no Ca_i decrease (Fig. 4). In order to investigate the specificity of the Ca_i decrease for ABA the effects of the ABA analogues 1'-deoxy ABA and α-ionyledene acetic acid on Ca_i were studied. Table II shows that both analogues could only induce a very small Ca_i decrease (as compared to ABA). Furthermore, both the lipophilic acids L-α-phosphatidic acid and 2,3-dihydroflavanone had no effect on Ca_i (Fig. 4). The Ca_i decrease was dependent on the ABA concentration, as shown in Fig. 4. Half-maximal decrease in Ca_i was found at about 125 μM ABA. 10–50 μM ABA was able to induce a significant decrease of Ca_i. The induction of Ca_i decrease was independent of the extracellular Ca^{2+} concentration, since protoplasts in loading buffer without Ca^{2+} containing 10 mM EGTA did not show a significantly different response to ABA (Fig. 4). This result suggests that plasma membrane Ca^{2+} channels do not play a significant role in the ABA-induced Ca_i decrease.

Fig. 1 shows the changes of the emission spectrum of the Indo-1-loaded protoplasts after addition of ABA. These changes are consistent with the Indo-1 spectra at different Ca^{2+} concentrations in the range 50–200 nM (cf. [22]).

Since the affinity of Indo-1 to Ca^{2+} is pH-dependent [25], it is essential to know whether the decrease in fluorescence intensity of Indo-1 in protoplasts by ABA is due to an ABA-induced change of the intracellular pH. Therefore, fluorescence intensity of Indo-1 was measured at different pH values (excitation 355 nm, emission 405 nm). Fig. 5 shows that the fluorescence of 0.25 μM Indo-1 in loading buffer at different pH values is strongly affected by pH between pH 4 and pH 6. However, there was almost no difference in fluorescence intensity between pH 6 and 8 (Fig. 5). The

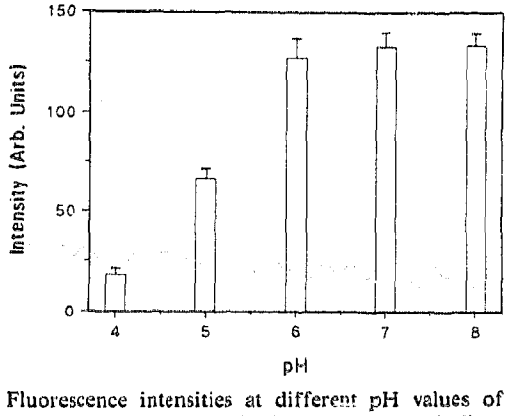

Fig. 5. Fluorescence intensities at different pH values of Indo-1 (0.25 μM). Emission 405 nm, excitation 355 nm. Bars indicate ± SD.

Table I

Effect of Ca^{2+} channel blockers and Des on the ABA-induced Ca_i decrease[a]

Condition	Resting Ca_i (nM)	Ca_i after ABA stimulation[b] (nM)
Control	200 ± 26	109 ± 11
Nifedipine 35 μM	199 ± 16	107 ± 12
Verapamil 150 μM	211 ± 22	113 ± 15
Des 40 μM	203 ± 24	95 ± 10

[a] Results of at least 4 independent experiments for each condition are shown. [b] Stimulation with 200 μM ABA

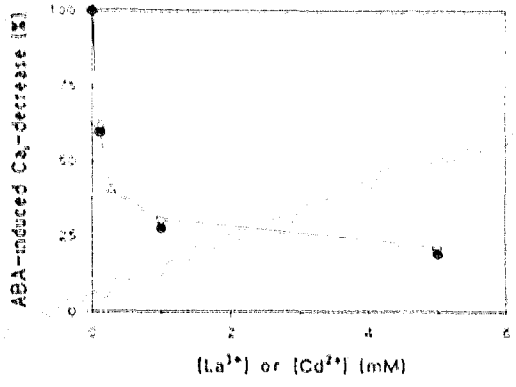

Fig. 6. Effects of La^{3+} (○) and Cd^{2+} (●) on (200 μM) ABA-induced Ca_i decrease. Four individual experiments gave essentially the same results, data of 1 experiment are shown.

cytoplasmic pH (pH_i) in plant protoplasts is about 7.3 [26]. It is unlikely that, under physiological conditions, the pH_i will drop below 6.0. This means that in case ABA induces subtle pH_i changes, these changes cannot cause the observed changes in fluorescenec intensity. Furthermore, we have investigated the effect of the proton pump inhibitor Des on the ABA-induced decrease of fluorescence intensity (Table I). Literature data show that Des (at 100 μM) is able to completely inhibit plasma membrane H^+-ATPase activity [27]. The inhibition of H^+-ATPase activity will lead to a decrease in pH_i. Our data (Table I) demonstrate that Des not only had any effect on resting fluorescence intensity of Indo-1-loaded protoplasts but that it also had no effect on ABA induced decrease in fluorescence intensity. These results provide additional evidence that the decrease of fluorescence intensity induced by ABA was not caused by changes of cytosolic pH. Our data also show that the ABA-induced Ca_i decrease was not affected by H^+-ATPase inhibition. We conclude that the ABA-induced changes in fluorescence of Indo-1 loaded protoplasts indeed represent changes in Ca_i.

We tried to find out some more about the working mechanism by which ABA induces a decrease in Ca_i. Since ABA effects can be water stress-related, an ABA-induced Ca_i decrease may be due to uptake of water. However, protoplast volume measurements by microscopy show that the diameters of protoplasts before (32.0 ± 4.8 μm) and after (32.5 ± 5.2 μm) 100 μM ABA treatment were the same (measurements of 114 protoplasts for each condition). Furthermore, experiments with $[^3H]H_2O$ uptake show that there was no significant change in protoplast volume upon ABA stimulation (100 μM) (in preparation).

Both La^{3+} and Cd^{2+} have been shown to be Ca^{2+}-antagonists [28]. They block both plasma membrane Ca^{2+} channels and Ca^{2+}-ATPases. We investigated the effects of La^{3+} on the ABA-induced cytosolic calcium decrease. Fig. 6 shows that La^{3+} strongly inhibited the ABA-induced decrease in Ca_i. Half-maximal inhibition by La^{3+} of ABA-induced Ca_i decrease was 200 μM, which is in agreement with values obtained in other cell types [28]. In case Cd^{2+} was used, similar effects were observed (Fig. 6).

The extracellular Ca^{2+} had no effect on the ABA-induced Ca_i decrease (Fig. 4) and the Ca^{2+} antagonist La^{3+} and Cd^{2+} strongly inhibited the ABA-induced Ca_i decrease (Fig. 6) but did not influence resting Ca_i (resting Ca_i of La^{3+}-treated protoplasts was 206 ± 26 nM (n = 4) and resting Ca_i of Cd^{2+}-treated protoplasts was 210 ± 30 nM (n = 4), with treatment up to 20 s). Therefore, we concluded, assuming that La^{3+} and Cd^{2+} do not enter the protoplasts, that the ABA-induced Ca_i decrease is due to the activation of membrane Ca^{2+}-ATPase by ABA rather than to activity changes of Ca^{2+} channels (see section 4). To further support this conclusion, the effect of the specific Ca^{2+} channel blockers verapamil and nifedipine (which represent two classes of Ca^{2+} channel blockers) was studied [28,29]. Table I shows that these channel blockers had no effect on both resting Ca_i and the ABA-induced Ca_i decrease.

4. DISCUSSION

According to our measurements, resting Ca_i of barley aleurone protoplasts was about 200 nM which is in the range of previously reported Ca_i values for these cells [30]. Instead of loading Indo-1 at acidic pH (extracellular pH 4.5) [30], we loaded protoplasts with Indo-1 at a rather neutral pH (pH 6.8) by using the membrane permeant form Indo-1/AM [22]. Under these conditions, the protoplasts were more viable and the results were more reproducible.

Upon stimulation with the plant hormone ABA, a strong Ca_i decrease in barley aleurone protoplasts was observed (Fig. 2). The experiments with ABA analogues and other lipophilic acids (Table II, Fig. 4) show that this response was ABA-specific. The resting Ca_i was neither affected by La^{3+}, Cd^{2+} nor by the specific Ca^{2+} channel blockers verapamil and

Table II

Effects of ABA analogues on Ca_i

Condition	Ca_i (nM)
Resting	202 ± 6 (n = 6)
200 μM ABA	93 ± 7 (n = 6)
400 μM 1'-deoxy ABA	185 ± 3 (n = 3)
400 μM α-ionyledene acetic acid	170 ± 8 (n = 3)

nifedipine, for up to 20 s of incubation. Hence, it is not likely that an ABA-induced closure of plasma membrane Ca^{2+} channels plays a major role in the ABA-induced Ca_i decrease. This decrease was not affected by extracellular Ca^{2+}, the Ca^{2+} channel blockers nifedipine and verapamil, and Des (Fig. 2 and Table I). However, the ABA-induced Ca_i decrease could be abolished by adding $LaCl_3$ and $CdCl_2$ (Fig. 5). Therefore, our data suggest that the decrease of Ca_i by stimulation with ABA is due to the activation of a Ca^{2+}-ATPase. The absence of any effect of plasma membrane proton pump inhibition on the ABA-induced Ca_i decrease suggests that a possible ABA-induced activation of the plasma membrane H^+-ATPases plays no significant role in the ABA-induced Ca_i decrease. A similar result was found for the light-induced Ca_i depletion in *Nitellopsis* [19]. However, different other ion fluxes (e.g. K^+ and Cl^- fluxes) may play a role in the regulation of this response (e.g. [31]).

The reported amount of ABA used in various systems to induce different responses can vary considerably (1–200 μM) (e.g. [11,13,18,32]). This may be due to a different ABA sensitivity of different cell types [6]. Our data show that in barley aleurone protoplasts 10–50 μM ABA was able to induce a significant decrease of Ca_i. Such concentrations have been reported to be able to induce gene expression and counteract GA-induced responses [11,13,32]. Effects of ABA which have been reported are tissue-dependent [33]. It has been known that ABA can give an opposite Ca_i effect in other tissues, such as leaf guard cells [18]. In leaves and roots, ABA mainly regulates ion concentrations, e.g. osmosis, whereas in seeds and buds ABA regulates gene expression which is related with growth, development and dormancy [3,33]. Therefore, different intracellular responses upon ABA stimulation can be expected to occur in different tissues.

In barley embryos and seedlings, the expression of an α-amylase inhibitor is induced by ABA [34,35], and a group of ABA-responsive proteins and genes have been reported (e.g. RAB-gene) [12,34]. Preliminary results indicate that using the experimental conditions (6×10^5 protoplasts/ml in washing buffer) described in this paper, ABA is able to induce RAB gene expression. Experiments are in progress to investigate the relation between ABA-induced Ca_i changes and RAB-gene expression. The ABA-induced drop of Ca_i in barley aleurone may play a role in the regulation of enzyme activity, as was proposed for the light-induced Ca_i decrease in *Nitellopsis* [19]. The synthesis, secretion, stability and activity of α-amylase are Ca^{2+}-dependent [21,36]. α-Amylase activity is essential in seed germination and is regulated by a balance between GA and ABA [15,36–39]. The ABA-induced Ca_i decrease may be involved in the regulation of α-amylase biosynthesis and activity. Therefore, the regulation of Ca_i by both ABA and GA will be an important line of investigation in our future research.

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