Evidence that fusicoccin and indole-3-acetic acid induce cytosolic acidification of *Zea mays* cells

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Microelectrodes were used to measure simultaneously the effects of fusicoccin on cytosolic pH and membrane potential in a maize root cell. The cytosolic pH began to fall within seconds of adding fusicoccin, whereas the membrane hyperpolarization commenced after a lag of 2 min. The pH microelectrode could not be used with coleoptile cells for technical reasons. However, the dual-wavelength absorbance technique showed that fusicoccin also induced a rapid cytosolic acidification in coleoptile cells. Indole-3-acetic acid lowered the cytosolic pH of these cells. The effect was less pronounced than with fusicoccin and began after 5 min well before membrane hyperpolarization (10 min) and extracellular acidification (30 min) were detectable. Procaine, which penetrates the plasma membrane and gains protons in the cytosol, was shown to depolarize root cells and inhibit indole-3-acetic acid-induced growth of coleoptiles. 1-Naphthylacetate, which acidifies the cytosol, hyperpolarized root cells and stimulated coleoptile growth. The results support the concept that fusicoccin and auxins induce elongation growth by lowering the cytosolic pH.

Auxin *Coleoptile* *Cytosolic pH* *Fusicoccin* *Growth* *Membrane potential* Zea mays

1. INTRODUCTION

The stimulation by auxins of the outwardly-directed electrogenic proton pump present in the plasma membrane of seedling stems and coleoptiles is believed to be related to the stimulation of elongation growth [1]. Since acid solutions also induce growth, the wall acidification following proton pump stimulation by auxins is thought to explain their effect on growth [2–7]. However, there are inconsistencies in the wall-acidification theory of elongation growth [8–15]. Moreover, acids penetrate the plasma membrane and acidify the cytosol [16,17]. The degree of growth stimulation by acids is positively correlated with the extent of their cytoplasmic acidification and stimulation of the proton pump [16].

Auxins may stimulate the proton pump by increasing the substrate levels, i.e. by acidifying the cytosol [13,16,18]. Here we describe experiments directly and indirectly examining the relationship between cytosolic acidification, proton pump stimulation and elongation growth.

2. MATERIALS AND METHODS

Zea mays (Orla 264) coleoptiles and roots were obtained as described [14,16]. The methods used for growth measurements, measurement of membrane potential, preparation of pH-sensitive electrodes and the measurement of cytosolic pH changes, using the dual-wavelength absorbance technique [19] or pH-sensitive microelectrodes, have been detailed in [14,16,20].

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3. RESULTS

3.1. *Effects of fusicoccin on the cytosolic pH of maize root cells*

pH-sensitive microelectrodes could be used to measure simultaneously changes in membrane potential and cytosolic pH in a single maize root cell. Fusicoccin, which leads to acidification of the medium when added to roots and induces elongation growth in many plant tissues [21,22], hyperpolarized the root cells (fig.1). However, the hyperpolarization commenced after a lag of 2 min (fig.2). On the other hand, cytosolic pH began to fall within 15 s of adding fusicoccin. A decrease from pH 7.1 to 7.0 was induced by 8 μg/ml fusicoccin and was completed within 4 min. The membrane potential changed from −130 to −155 mV and was completed in 6 min. Hence, the stimulation of the proton pump brought about by fusicoccin treatment clearly results from cytosolic acidification.

Technical problems prevented us from making similar measurements on IAA-treated coleoptiles.

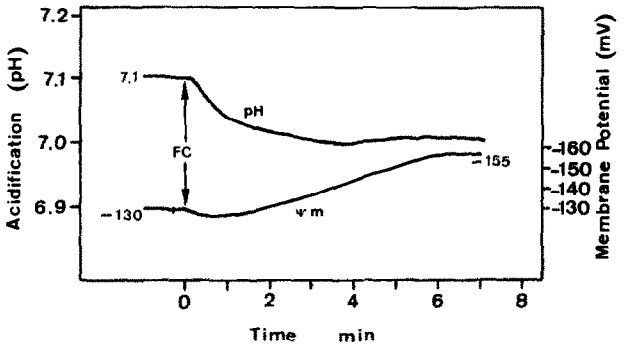

Fig.1. Effects of fusicoccin on the cytosolic pH of maize root hair cells. Roots were incubated in 2 mM Tris-Mes, 0.1 mM $CaCl_2$, pH 6.5. FC, 8 μg/ml fusicoccin. Membrane potential was recorded simultaneously with a second electrode within the same cell. The variability of the absolute values between experiments was: pH_{cyt} = 7.1 ± 0.1 (n = 15); ΔpH (FC) = 0.1 ± 0.01 (n = 4); ψ_m = −130 ± 3 mV (n = 9); $\Delta\psi_m$ = −25 ± 6 mV (n = 5).

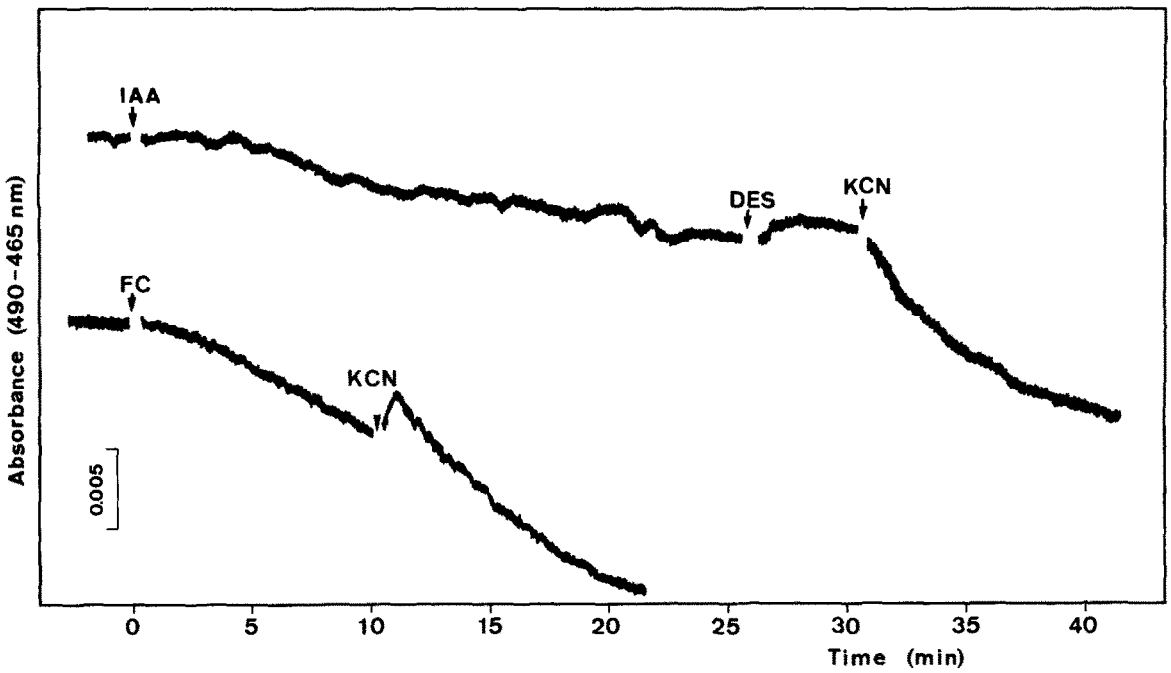

Fig.2. Effects of IAA and fusicoccin on cytosolic pH. Nine maize coleoptile segments were loaded with FA_2, washed and changes in absorbance measured using the dual-wavelength technique. Decreases in absorbance are indicative of acidification. Segments were incubated in 50 mM Mes-NaOH, 0.1 mM $CaCl_2$, pH 6.2. Substances added at arrows were: 20 μM IAA, 50 μM diethylstilboestrol (DES), 1 mM KCN, 5 μg/ml fusicoccin (FC).

3.2. *Effects of fusicoccin and indole-3-acetic acid on the cytosolic pH of maize coleoptile cells*

Since we are not yet able to use the pH microelectrode on coleoptile cells, the dual-wavelength absorbance technique (using fluorescein) was employed [14,19]. As with root cells, fusicoccin caused a decrease in cytosolic pH that commenced almost immediately (fig.2). As shown in other systems [23], KCN led to a rapid cytosolic acidification. A precise estimation of the pH decrease was not possible since determination of cytosolic pH values by the digitonin-induced null-absorbance change [19] was not feasible due to the large central vacuole.

When IAA was added to coleoptiles the cytosolic pH began to drop after approx. 5 min (fig.2). The addition of diethylstilboestrol, which inhibits the proton pump [24], stopped the decrease. IAA treatment consistently resulted in a decrease, even when lower concentrations (down to 1 μM) were used. Hence, cytosolic acidification resulting simply from IAA uptake is unlikely. Furthermore, we have not been able to detect changes in the cytosolic pH of root cells with the pH microelectrode when similar concentrations (20 μM) of weak acids were added.

Membrane potential measurements on the coleoptiles showed that, following IAA treatment, hyperpolarization commenced after a lag of 10 min (not shown), in agreement with the work of others [25–27]. Wall acidification was first detectable after 30–60 min (not shown).

3.3. *Effects of procaine and 1-naphthylacetate on membrane potential and growth*

If a decrease in cytosolic pH is important for elongation growth, substances which interfere with its regulation will be expected to influence growth.

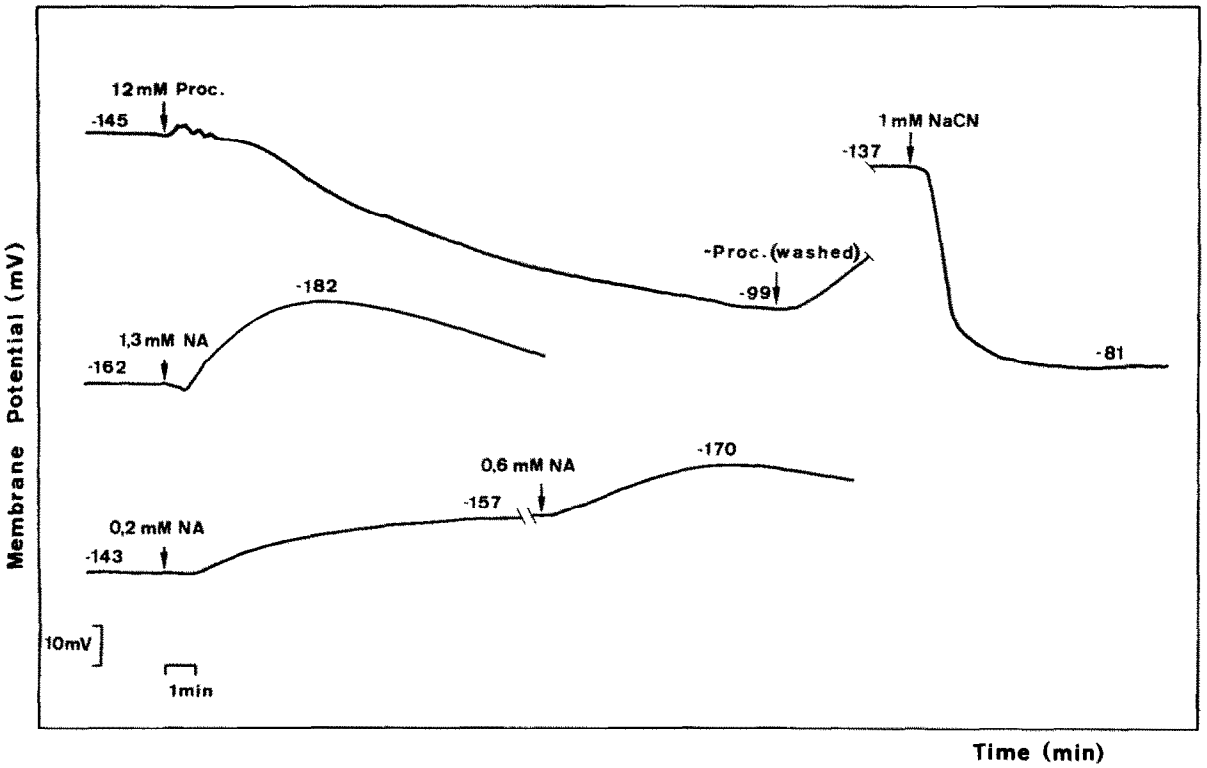

Fig.3. Effects of procaine and 1-naphthylacetate (NA) on the membrane potential of maize root rhizodermis cells. Roots were isolated from 4–5-day-old seedlings and fixed horizontally in a chamber that was continually perfused by buffer. At different times (arrows) substances to be tested were added at the concentrations shown. Buffer for procaine was 5 mM Hepes-NaOH, pH 8.2, 1 mM KCl, 0.1 mM $CaCl_2$. Buffer for NA was 2 mM Tris-HCl, 1 mM KCl, 0.1 mM $CaCl_2$. The numbers indicate the membrane potential measured.

Procaine is a weak base with a pK_a higher than the cytosolic pH and can cross the plasma membrane. A correspondingly high external pH (pH_o) is required to generate sufficient free base externally [23]. Procaine reionizes in the cytosol by gaining protons. Hence, the cytosolic pH increases and less protons are available for the pump.

Fig.3 shows that depolarization occurs when maize roots are treated with procaine. When added with IAA to coleoptiles, 10 mM procaine completely inhibited growth induction (fig.4a). Following addition to coleoptiles elongating in response to IAA, procaine inhibited growth completely after a lag of approx. 1 h.

1-Naphthylacetate (NA) penetrates the plasma membrane and is hydrolyzed by a cytosolic esterase [28]. This leads to acidification of the cytosol. Hence, stimulation of the proton pump occurs which results in membrane hyperpolarization (fig.3). Concentrations of 0.2 mM NA resulted in both hyperpolarization (fig.3) and growth rates similar to 20 μM IAA. At 0.1 mM NA still caused a significant stimulation of growth (fig.4b, see [28]).

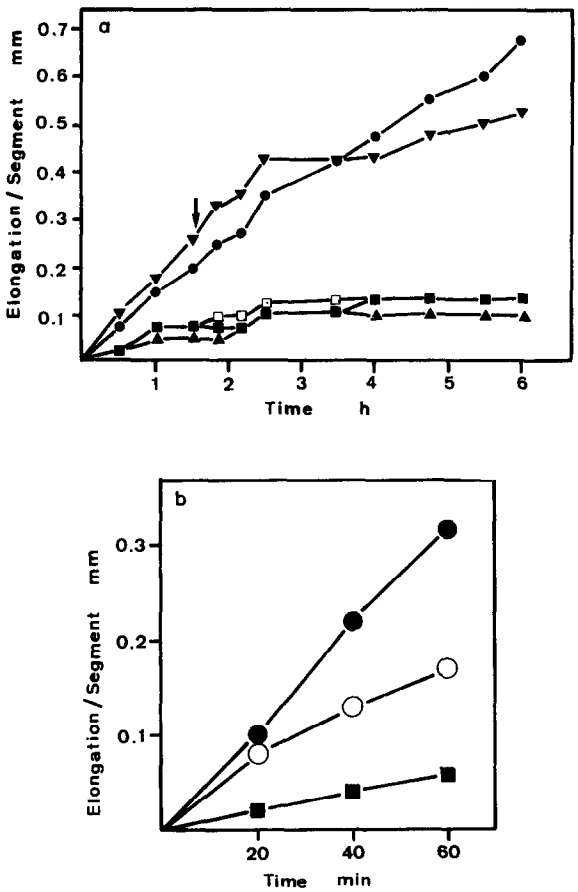

Fig.4. Effects of procaine and NA on maize coleoptile elongation growth. (a) Buffer was 20 mM Hepes-KOH, pH 8.2. Control (■), 10 mM procaine (□), 20 μM IAA (●), 20 μM IAA and 10 mM procaine (▲), 10 mM procaine added after 92 min (arrow) to 20 μM IAA treatment (▼). (b) Buffer was 1 mM Pipes-NaOH, pH 6.7, 2 mM KCl. Control (■), 10 μM IAA (●), 100 μM NA (○).

4. DISCUSSION

The results with the pH-microelectrode clearly showed that the fungal toxin, fusicoccin, caused an immediate decrease in cytosolic pH followed by membrane hyperpolarization in root cells. Hence, cytosolic acidification stimulates the proton pump with subsequent acidification of the cell wall.

Rapid cytosolic acidification by fusicoccin in coleoptile cells could be demonstrated with the dual-wavelength absorbance technique. The measured response was slower than that measured with the pH microelectrode in root cells, probably because the coleoptile cells would not be expected to receive simultaneously the same fusicoccin concentrations.

The results are consistent with the hypothesis that fusicoccin stimulates the proton pump in coleoptile and root cells by lowering the cytosolic pH. The effects are probably related to the stimulation of elongation growth.

The effect of IAA on the cytosolic pH of coleoptile cells could only be measured using the dual-wavelength absorbance technique. Cytosolic acidification was detected, commencing after about 5 min. Membrane hyperpolarization and proton extrusion were first detectable after 10 and 30–60 min, respectively. Unfortunately, the pH changes observed by the dual-wavelength absorbance technique are difficult to calibrate in plant cells [14]. As a result the actual decrease in cytosolic pH is not known. Clearly, the decrease was considerably greater with fusicoccin than with IAA. However, this reflects differences in the extent of proton pump stimulation by the 2

substances. The decrease in wall pH was between 0.4 and 0.6 units with 20 μM IAA, whereas 5 μg/ml fusicoccin led to a decrease of 1.7 pH units (not shown). Whether the decrease in cytosolic pH induced by IAA is sufficient to account for the pump stimulation observed is not known. Experiments with pH microelectrodes (when the technical problems have been solved) should resolve this question.

Manipulating the cytosolic pH of coleoptile cells provided indirect evidence that pH_c is involved in elongation growth. Thus, substances which raised the cytosolic pH (procaine, NH_3, see [29]) inhibited growth stimulation by IAA. Conversely, acidification of the cytosol by NA hyperpolarized the membrane and induced elongation growth.

Fusicoccin and IAA are thought to induce growth in different ways [30], although the existence of 2 completely different growth mechanisms is open to doubt. It is likely that IAA and fusicoccin lower cytosolic pH by different mechanisms. The fusicoccin effect is very rapid and may involve changes in membrane permeability, whereas the slower IAA response may implicate, for example, protein synthesis [30] and changes in cytosolic Ca^{2+} levels [13].

Very recently a paper appeared showing that fusicoccin lowers the cytosolic pH of *Avena* coleoptile cells [17]. The dual-wavelength absorbance method was used and again no pH values could be given. The membrane potential was not measured. Using ^{31}P NMR signals, fusicoccin treatment of maize root tips resulted in some spectra indicating a slight decrease in cytosolic pH [31]. It was suggested that the cytosolic pH may be slightly lower in cells extruding large amounts of protons, but the apparent decrease (about 0.1 pH unit) was close to the limit of resolution of the technique.

Since it is impossible to separate cytosolic acidification from pump stimulation in vivo (inhibiting the pump with specific inhibitors has pleiotropic effects on metabolism) we cannot say whether pump stimulation is vital for growth or simply reflects the cytosolic acidification. Nevertheless, we have proposed that elongation growth depends on changes in ψ_m and transmembrane ion gradients brought about by pump stimulation [13].

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