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Signal Transduction in *Sinapis alba* Root Hairs: Auxins as External Messengers

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Summary

In developing root hairs of *Sinapis alba* the effects of externally applied indole-3-acetic acid (IAA) and other auxins have been investigated with respect to membrane potential, membrane conductance, cytosolic Ca^{2+} and pH. Following a delay of roughly 30 s, 10^{-12} to 10^{-10} M IAA slowly hyperpolarize, 10^{-7} M IAA rapidly depolarize the root hairs, while 10^{-9} M has hardly an effect. We show that these voltage responses are not the result of a change in membrane conductance or permeability, but are presumably caused by a change in H^+ ATPase activity. The other tested auxins and analogues yielded comparable effects, but with much lower effectivity (IAA > 1-NAA > 2,4-D \geq 2-NAA > 2,3-D). Cytosolic Ca^{2+} and pH were decreased during depolarization by 0.2 and 0.4 units, respectively. No such changes were observed during hyperpolarization or about 1 h after the first encounter of the root hairs with IAA.

We propose that IAA is a natural external signal for roots while competing with neighboring organisms for nutrients and salts, and suggest a signal chain with the plasma membrane H^+ ATPase as a target protein. The delay in response to IAA, the time dependency, and the extremely low effective IAA concentrations point to the existence of a IAA receptor. Since the IAA-induced shifts in cytosolic pH and Ca^{2+} occur simultaneously with the depolarization, the question whether these ions are cellular messengers and part of an IAA-triggered signal chain is critically discussed.

Key words: Auxin, cytosolic Ca^{2+} , cytosolic pH, membrane potential, root hairs, signal chain, *Sinapis alba*.

Abbreviations: IAA = indole-3-acetic acid; 1-NAA = 1-naphtylacetic acid; 2-NAA = 2-naphtylacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; 2,3-D = 2,3-dichlorophenoxyacetic acid; Em = membrane potential.

Introduction

Synthesis of the plant growth regulator auxin, indole-3-acetic acid (IAA), occurs mainly in the apex of shoots and in young leaves, from where it is transported into the roots. (Torrey, 1976; Goldsmith, 1977). During acropetal transport inside the roots, fractions of the IAA are either inactivated or exported into the soil (Phillips, 1964; Torrey, 1976; Goldsmith, 1977). Inversely, it has been shown that roots may take up exogenous IAA from their immediate environment (Mascarenhas and Canary, 1985). The comparison of roots which

have been cultivated under sterile and nonsterile conditions, resp., has revealed that a fair amount of the IAA extracted from roots must have been synthesized by microorganisms (Pegg, 1985). Such organisms, some of which live in close encounter with plant roots, synthesize not only auxin but evidently also other plant growth substances (Wang et al., 1982; Pegg, 1985). In this so-called rhizosphere plants and microorganisms compete for nutrients and salts (Dowling and Broughton, 1986), and apparently influence each other through other compounds such as IAA. Thus, it may not be surprising that in roots of mycorrhizae symbionts much lar-

ger concentrations of IAA are found than in non-mycorrhizal roots (Mascarenhas and Canary, 1985), and many plants infected by pathogenic bacteria or fungi show typical hyperauxin effects (Pegg, 1985). For instance, rhizobitoxine producing strains of *Bradyrhizobium japonicum* excrete more than $20 \mu\text{M}$ of IAA into their culture medium which then inhibits growth of alfalfa roots even after 100-fold dilution, whereas the culture medium of strains which do not release IAA have no effect on the growth of these roots (Minamisawa and Fukai, 1991). In other phytopathogenic bacteria, like *Pseudomonas syringae* *pv.* *savastanoi* and *Agrobacterium tumefaciens* a set of IAA biosynthetic genes have been found (Morris, 1986).

Not all regions of the roots are equally sensitive to microorganisms. Most of the infections occur between the elongation zone and the emerging root hairs, whereas mature root hairs appear not very susceptible to infection (Vance, 1983). For instance, the infection of roots by nodule bacteria occurs through the just developed root hairs, and auxins apparently play a role in the resulting curling of these root hairs (Mascarenhas and Canary, 1985).

Since it can be assumed that in natural environment IAA plays a signalling role in the interaction between plants and microorganisms, between neighboring plants, or even amongst roots of their own, we were interested in the sensitivity of growing root hairs to exogenous IAA. It has been shown that electrophysiological techniques are optimally suited to quickly characterize basic reactions of plant cells or tissues to auxins. This is because in the systems tested so far, auxins are not only transported electrophoretically across the plasma membrane, but also affect the activity of the electrogenic plasma membrane H^+ ATPase (Hager et al., 1971; Cleland, 1973; Brummel and Hall, 1987).

Material and Methods

General conditions

Seedlings of *Sinapis alba* were grown for 2–3 d in Petri dishes on filter paper which was soaked with tap water. The roots were mounted in a Plexiglass chamber which was constantly perfused with the test medium. This comprised 0.1 mM KCl, 0.1 mM NaCl,

0.1 mM CaCl_2 and a mixture of 5 mM of Mes/Tris adjusted to the respective pH. Unless otherwise stated external pH was 6.0. After transferring the roots into the test medium, within 3–4 h new root hairs had emerged. Only these were used for the experiments.

System-kinetics

In order to find out, whether the cells respond to the tested agents immediately upon encounter or with a delay, the KCl-test has been applied. This is based on the fact that the so-called diffusion potential of many plant cells is dominated by K^+ . In the moment external K^+ is altered and comes in contact with the membrane, the diffusion potential changes without measurable delay. The time between the addition of KCl and the reaction of the cells is determined and compared with the response times to other agents.

Electrophysiology and ion-selective microelectrodes

The electrical setup for the impalement of the root hairs and membrane potential measurements has been described repeatedly (Felle, 1982, 1987). The test chamber is open on both sides and allows a simultaneous horizontal approach of 2 separate electrodes.

The fabrication of the pH- and the Ca^{2+} -selective microelectrodes has been described in detail recently (Felle and Bertl, 1986; Felle, 1988, 1990). These electrodes were connected to high-impedance amplifiers (FD 223 or Duo 773; WP-Instruments, New Haven CT. USA), and were equilibrated for approx. 1 h before calibration.

Membrane conductance and current-voltage measurements

The membrane conductance was measured by inserting two separate electrodes into the tip of the same root hair, one being the current injecting electrode, the other the voltage electrode. Rectangular pulses were fed into the cells using a constant current amplifier (S-7000 WP-Instruments). The deviation from the resting potential due to the current flowing to ground across the membrane was monitored. No cable analysis (Cole, 1968; Felle, 1978) of these longitudinal cells has been carried out in order to determine the inner resistance; therefore, the current-voltage curves shown are from individual cells (input values), and as such may differ from cell to cell according to the actual cell surface.

Chemicals

The auxins and analogues have been purchased from Fluka. The final concentrations were dilutions from aqueous stock solutions (1 mM).

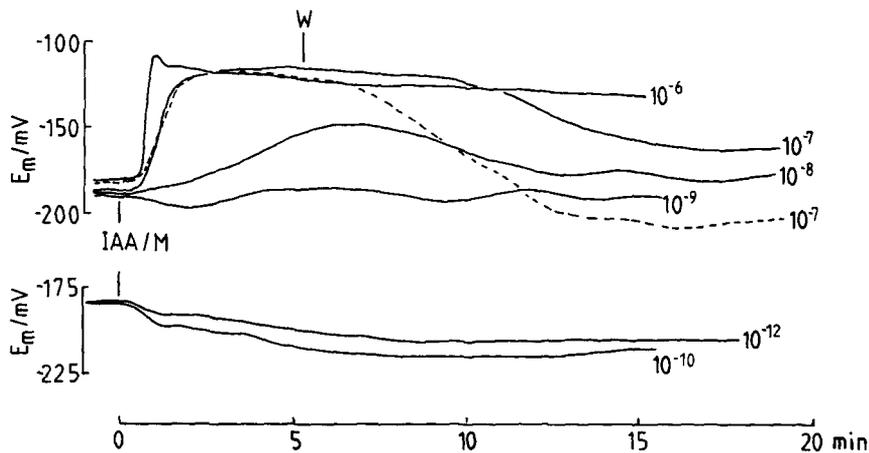


Fig. 1: Membrane potential (E_m), measured on 3 to 4 h grown *Sinapis alba* root hairs before and after externally added IAA at the indicated concentrations. W indicates the removal of the IAA (dashed curve). Given traces are representative of 4–6 equivalent measurements (10^{-12} to 10^{-8} M IAA) and 23 measurements for 10^{-7} M IAA.

Results

Basic phenomena: IAA affects the membrane potential of *Sinapis* root hairs in different ways

Sinapis root hairs which were grown for about 3 h in the test solution at pH 6 typically have a membrane potential of -175 to -185 mV. These cells are extremely sensitive to externally applied IAA: as shown in Fig. 1, they react to already 10^{-12} M IAA with a clear-cut hyperpolarization of 10 to 15 mV; a maximal hyperpolarization of 30 ± 5 mV ($n = 6$) is observed at 10^{-10} M IAA, whereas in the presence of 10^{-9} M IAA the membrane potential just oscillates around its resting value. After a lag-phase of roughly 30 s, 10^{-7} M IAA transiently depolarizes the cells to the so-called diffusion potential. Whereas the spontaneous repolarization is slow, upon removal of the IAA the membrane potential recovers more rapidly and may exceed the original resting potential by about 25 to 30 mV. A second or third addition of 10^{-7} M IAA then results in much smaller responses, which after about 1 h following the first encounter, almost completely vanish (see below, Fig. 5). But even without repetitive addition of IAA the response is time-dependent and is optimal within the second and fourth hour after the root hairs started to emerge. Fully grown root hairs then only weakly respond to IAA (not shown).

Other auxins

From *Zea mays* coleoptiles we know that the physiological response to auxins, viz. the stimulation of the H^+ ATPase (Felle et al., 1991) is rather unspecific, i.e. all tested auxins and their structural analogues had a more or less pronounced effect. Since the response of *Sinapis* root hairs to IAA evidently differs from that in *Zea*, it was necessary to test other auxins also. We found that *Sinapis* root hairs react to other auxins, but much weaker than to IAA (Fig. 2). Without

wanting to describe this figure in detail, it seems that 2,3-D is the least effective of all, since even at 10^{-6} M hardly a reaction was to be observed. The selectivity sequence from Figs. 1

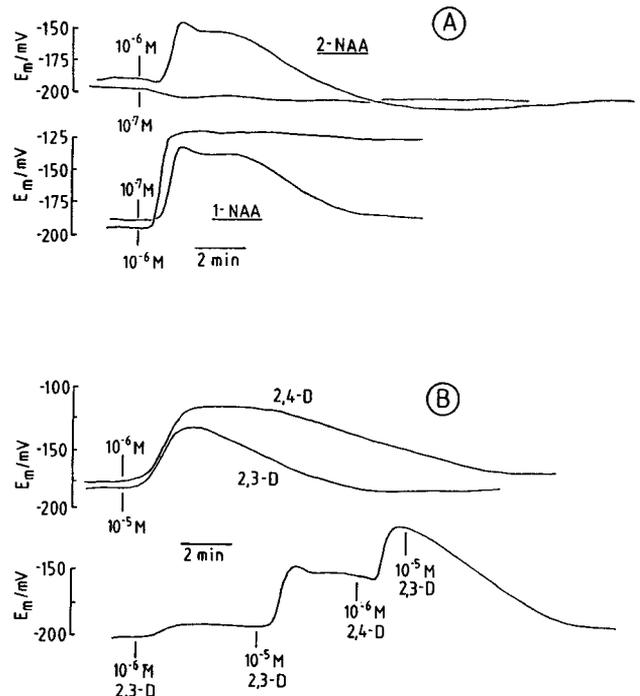


Fig. 2: Effect of different synthetic auxins on the membrane potential (E_m) of *Sinapis alba* root hairs. (A) Comparison of the response to the naphtylacetic acids 1-NAA and 2-NAA; (B) response to the dichlorophenoxy acids 2,3-D and 2,4-D. In the lower curve the different auxins were exchanged without returning to the standard solution first. Traces are representative examples of 5–6 measurements each.

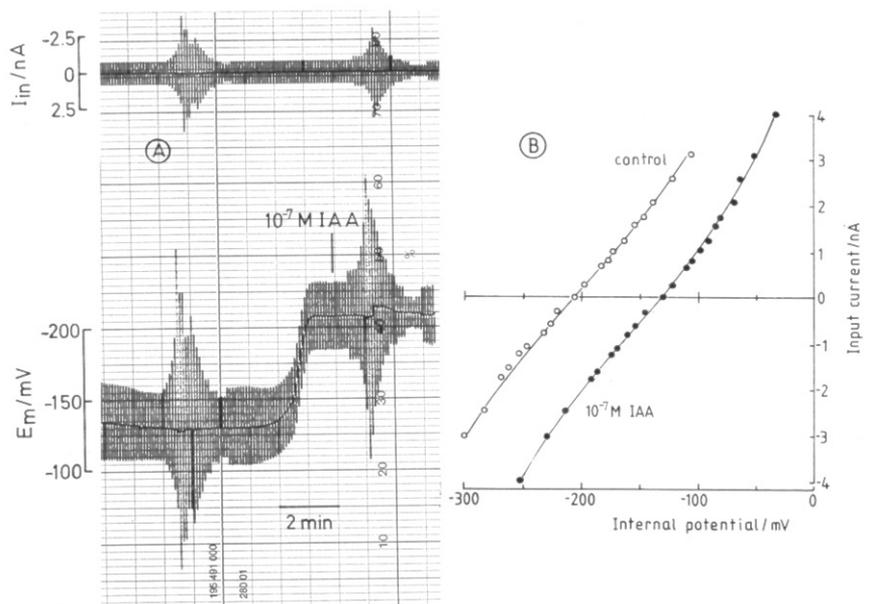


Fig. 3: Effect of 10^{-7} M IAA on the membrane conductance of *Sinapis alba* root hairs. (A) The solid line denotes the membrane potential (E_m), the deflections from the membrane potential are the responses to the defined current (I_{in}), injected through a second microelectrode (same cell). Larger response means smaller conductance. (B) Current-voltage curves obtained from the measurement of A. The two curves were taken before (control) and in the presence of 10^{-7} M IAA in the moment of maximal depolarization; slope of the curves denotes the conductance (see Material and Methods). Example of 7 equivalent current injection measurements on different cells.

and 2 is: 2,3-D < 2-NAA \leq 2,4-D < 1-NAA < IAA. So, it appears that in contrast to *Zea mays* coleoptiles, where both 2,4-D and 1-NAA appear as physiologically strong auxins, to root hairs of *Sinapis* IAA is by far the most effective.

The membrane conductance

The observed changes in membrane potential may indicate an activation of a specific signal chain (delay), but could just as well arise from unspecific effects of the auxins on the membrane. In the latter case, the hyperpolarization should be accompanied by a decrease in conductance, whereas an increase in conductance should be the cause of the observed depolarization. This has been tested: (i) No significant change in membrane conductance was found before, during or after the addition of 10^{-12} to 10^{-10} M IAA (not shown). (ii) The membrane conductance decreased in the presence of 10^{-7} M IAA by 10 to 15% (Fig. 3 A). Since these changes could theoretically be a voltage-dependent phenomenon, current-voltage (IV-)curves have been taken before (control) and in the presence of 10^{-7} M IAA (Fig. 3 B). A comparison of both curves yields no indication of an unspecific IAA effect on the plasma membrane, i.e. no significant change in slope (= conductance) occurs in any part of the curves which would warrant the interpretation that the depolarization was a trivial membrane effect. In fact, quite the opposite is true: the IAA-induced slight decrease membrane conductance would rather favour a hyperpolarization.

Cytosolic Ca^{2+} and pH

Since there is a delay of about 30 seconds between the moment of IAA presence and the onset of depolarization, it appeared obvious to assume the activation of a specific signal chain which could involve cytosolic Ca^{2+} , as well as pH. Whereas we were unable to detect a clear effect on either ion activity during hyperpolarization (low external IAA concentration), during depolarization (higher IAA) we measured clear changes. Adding IAA for the first time to the root hairs we observed an increase in cytosolic pH by 0.4 to 0.5 units (Fig. 4) which spontaneously recovered by about 0.2 units. When IAA is removed from the medium, the cytosolic pH slowly and completely recovered. The cyanide experiment serves to determine the so-called diffusion potential and it proves the validity of the pH measurement: the cytosolic acidification in the presence of cyanide follows exactly the pattern known from earlier measurements on *Sinapis* root hairs (Felle, 1987).

Both membrane potential and cytosolic pH respond time-dependently to IAA. In Fig. 5 we demonstrate that about 1 h after the first IAA addition the membrane potential only weakly, the cytosolic pH does not respond to IAA any more.

In *Sinapis* root hair tips the cytosolic Ca^{2+} activity with a pCa of 6.2 ± 0.12 ($n = 5$) rests higher than in the other cells tested with Ca^{2+} sensitive microelectrode so far (Felle, 1988, 1989). External addition of 10^{-7} M 1-NAA rapidly increases the pCa from 6.2 to 6.4; as shown for the cytosolic pH, spon-

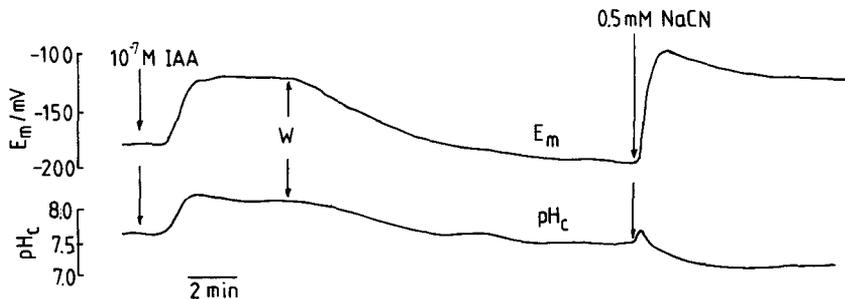


Fig. 4: Cytosolic pH (pH_c) and the simultaneously measured membrane potential (E_m) of *Sinapis alba* root hairs before and after the addition of 10^{-7} M IAA. W = removal of IAA. In order to test the proper function of the pH-sensitive microelectrode, 0.5 mM NaCN has been added to demonstrate the typical cytosolic acidification. Representative example of 4 equivalent measurements each.

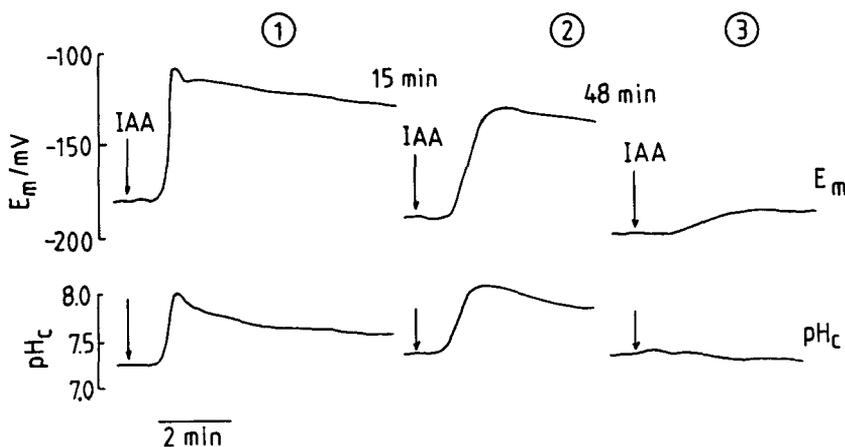


Fig. 5: Time-dependent response of the cytosolic pH (pH_c) and the simultaneously measured membrane potential (E_m) of *Sinapis* root hairs to 10^{-7} M IAA. 1 = first IAA addition, 2 = second IAA addition 15 min after 1, 3 = third IAA addition 48 min after 1. Representative example of 3 equivalent tests each.

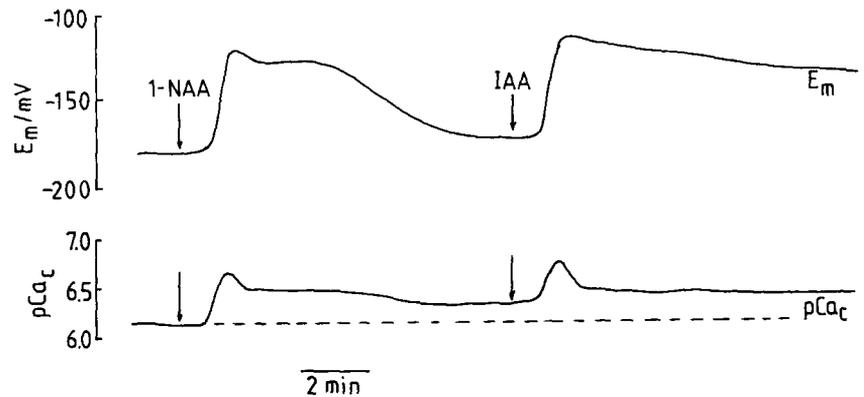


Fig. 6: Cytosolic Ca^{2+} (pCa_c) and the simultaneously measured membrane potential (E_m) of *Sinapis* root hairs to 10^{-7} M 1-NAA, followed by 10^{-7} M IAA. Dashed lines mark the control pCa_c . Part of the conspicuous initial transient on the pCa_c -trace may be caused by the different response times of the fast voltage electrode and the slower Ca^{2+} -electrode during the rapid depolarization. Representative example of 3 equivalent recordings.

tanously this shift only partly recovers, although the membrane potential almost completely returns to the control level without removing the 1-NAA. When 1-NAA is exchanged for IAA, cytosolic Ca^{2+} is further reduced to about 6.5, while the membrane potential again fully depolarizes (Fig. 6).

Discussion

Signal transduction typically requires the sequence of a receptor/transducing system, signal amplification with the involvement of cellular messengers, and a target reaction. Our findings indicate that the IAA response in *Sinapis* root hairs activates such a signal chain: IAA presumably binds to a «receptor», as a result of which the plasma membrane H^+ ATPase changes its activity.

Evidence for an auxin receptor

The main reason why we think of an auxin receptor in these root hairs is the delayed reaction to IAA. Especially the observed rapid depolarization cannot be a simple transport phenomenon. If membrane transport was affected by the IAA directly, an immediate voltage change would be the result, as for instance observed with *Zea mays* coleoptiles, where the so-called influx carrier cotransports IAA^- with nH^+ (Felle et al., 1991; see below). Furthermore, the time-dependent response to IAA and the fast decline in response after the first IAA encounter also strongly support the presence of a receptor.

A third indication for an auxin receptor may be the low effective IAA concentrations. As shown in Fig. 1, a clear hyperpolarization occurs already in the presence of 10^{-12} M IAA, a concentration too low to induce a trivial membrane effect. But even the strong depolarization triggered with 10^{-7} M IAA is quite unusual. There are basically four ways to depolarize a cell: (1) Deactivation of the primary transporter (H^+ ATPase); (2) Shortcircuiting the primary transporter by secondary active transport (cotransport); (3) Alterations of the ion permeabilities (or ratios thereof); (4) Increasing the electrical membrane conductance.

We are able to demonstrate that the conditions 2–4 are not valid here. Although in the presence of cyanide the H^+

ATPase may only partly get deactivated, it is enough to depolarize the plasma membrane to the so-called diffusion potential (Fig. 4). This level is entirely determined by the permeability (ratios) of the ions present on either side of the plasma membrane. IAA depolarizes the membrane potential to this very level which means the permeabilities cannot have changed.

In Fig. 3 we show that the membrane conductance decreases by 10–15% after the application of 10^{-7} M IAA. This means that the depolarization was not caused by effects on passive membrane transport elements, because a decrease in conductance should have had the opposite effect. This leaves the active transporters from which in plants only one is electrogenic, *viz.* the H^+ ATPase. In *Zea mays* coleoptiles (and *Zea* suspension cells) recently we have analyzed a nH^+/IAA^- carrier with an apparent K_m of approx. $5 \cdot 10^{-7}$ M using electrophysiological techniques (Felle et al., 1991). Although the affinity of this carrier to IAA is quite high, it is still about orders of magnitude lower than the IAA concentrations used in this study. In order to short-circuit the H^+ ATPase a rather high carrier-mediated transport of charge across the plasma membrane is needed. In view of the small IAA concentrations this would require an unusually high turnover and carrier density per membrane area. For these reasons we think that the plasma membrane H^+ ATPase is a primary target of the IAA signal. This is interesting because in grass coleoptiles it is also the H^+ ATPase which is affected, although with an apparently different physiology: there the H^+ ATPase is activated by IAA, following a delay of 6–8 min.

Naturally, the two reactions, *viz.* hyperpolarization and depolarization cannot have the same cause. Firstly, they are reactions into opposite directions, and secondly, the effective concentration ranges are 4–5 orders of magnitude apart. This means we are observing effects coming either from two «receptors» or at least from two different binding sites (on the same molecule). Binding studies to find this out are in progress in our laboratory.

Are cellular messengers involved?

On first sight «yes». As shown in Figs. 4–6, there are quite substantial changes of both cytosolic Ca^{2+} and pH. In the presence of 10^{-7} M IAA the activity of both ions decreases

by 0.2 and 0.4 units, resp., which could be enough to exert and amplify signals within a cell. Still, these changes may not be the cause of the activity changes of the H^+ ATPase. This is because they occur rather simultaneously to the voltage changes and as such may not be part of that chain transducing the signal to the H^+ ATPase, unless the coupling of these processes is very fast and not resolved. It appears that although the observed changes in H^+ ATPase activity are finally the result of IAA-binding to a still putative receptor, the transduction of the signal to the H^+ ATPase may not involve Ca^{2+} (or pH) directly. The reason for this doubt lies within the observation that depolarization and Ca^{2+} change occur simultaneously (as do the pH changes). Nevertheless, both changes in cytosolic pH and free Ca^{2+} may be important as «secondary» signals to regulate growth, and to change metabolism and transport rates (Johannes and Felle, 1989). The Ca^{2+} was measured in the growing tips and it was found that the activity is higher than in basal part of the same cell (not shown). Such a Ca^{2+} gradient appears to be an important factor for cell development, and has been found in other cells also, e.g. *Fucus* (Brownlee and Wood, 1986) or sea urchin eggs (Jaffe, 1983). So it seems in *Sinapis* root hairs: in the presence of 10^{-7} M IAA this gradient is diminished, the H^+ ATPase is deactivated and growth is stopped. Interestingly, when the IAA is removed again, the activity of the H^+ ATPase not only recovers, but even exceeds its control activity. Since the membrane conductance returns to the control value only, the more negative voltage is a good indication of an increased driving force for membrane transport.

As with Ca^{2+} , we also have a similar problem with the interpretation of the cytosolic pH changes. If these shifts were clearly ahead of the depolarization, it would be easy to argue that the H^+ ATPase was deactivated by increasing pH (= lack of the transport substrate H^+). Unfortunately, this is not so clear, so the possibility remains that the pH changes may be secondary and of metabolic origin. In this context the Ca^{2+} shifts should also be seen. It has been reported recently that cytosolic pH and Ca^{2+} are not independent of each other, i.e. a pH increase may be accompanied by a Ca^{2+} shift into the same direction, simply caused by pH dependent Ca^{2+} binding or buffering (Felle, 1988). Since this seems to be the case here too, we propose that both pH and Ca^{2+} are integral factors of auxin action.

Physiological implications: the IAA is an external signal

Growth is always accompanied with transport of matter across the pertinent plasma membrane. Root hairs are rapidly growing cells and as such translocate cell wall materials especially at the tip. The central and primary transport system is of course the H^+ ATPase which builds up the necessary transport driving force, also known as the electrochemical H^+ gradient. In the presence of 10^{-7} M IAA the growth of root hairs stops (Pilet et al., 1979; Evans et al., 1980; own observations, not shown). Since deactivation of the plasma membrane H^+ ATPase is equivalent with the reduction of transport activity, we believe that this pump must be one of the primary targets of the signal chain under investigation. Based on experiments with brassino-steroids, Romani et al. (1983) came to the same conclusion.

Of course, in natural conditions a sudden increase in external IAA to 10^{-7} M is highly unlikely. In the case that neighboring organisms export IAA as an external signal, then it is a low concentration which reaches the root hairs first. To this the cells react rather «sensibly», namely with an increase in driving force, i.e. a hyperpolarization which helps the root hairs to grow faster. When the root grows closer to the interacting organism, the increasing IAA concentration may indeed reach values which will reduce growth, and as such limit the rhizospheres.

Acknowledgements

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