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Auxin Effect on the Transmembrane Potential Difference of Wild-Type and Mutant Tobacco Protoplasts Exhibiting a Differential Sensitivity to Auxin[1]

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ABSTRACT

The effects of 1-naphthaleneacetic acid (NAA) and other auxin analogs on the transmembrane potential difference (Em) were compared on tobacco protoplasts isolated from two genotypes differing in their sensitivity to auxins. For both types, NAA modifies Em by inducing at low doses a hyperpolarization, the amplitude of which increased with auxin concentration. Above an optimal concentration this hyperpolarization was reduced and even nullified. However, for the mutant type, this electrical response was shifted toward higher NAA concentrations, as its growth response. In the presence of structural analogs of auxin which have been showed to modify the dose-response curve for growth, the Em was altered: the growth-stimulatory molecule (picloram) initiated hyperpolarization, whereas the growth-inhibitory substance (4-bromophenylacetic acid) caused depolarization. These results provide evidence for a specific action of auxin at the membrane level related to its biological activity.

There is considerable evidence for interactions between auxin and membranes. Ultrastructural alterations were induced by auxin in isolated and *in situ* soybean plasma membranes (22). Modifications of the physical properties of membranes after auxin treatment have been reported. These include an increase in the microviscosity of lipid bilayers (17) or plasma membrane vesicles (13) and changes in electrostatic surface properties of protoplasts (29). Finally, a specific binding of auxin to membranes isolated from maize coleoptiles (9) and tobacco cell suspensions (30) has been described.

Auxin has also been demonstrated to affect functional properties of membranes, *e.g.* the regulation of ionic fluxes. The passive permeability to cations is enhanced on artificial lipid vesicles (15). An H^+-excretion, probably linked to the plasmalemma proton pump is stimulated in several tissues, including pea stem segments or maize coleoptiles (20), oat coleoptile sections (7) or rape leaf protoplasts (28). This effect is usually considered to account for measured changes in the transmembrane potential difference (Em)[2] following auxin treatment of pea internode segments (19), oat coleoptiles (1), or maize coleoptiles (24).

Causal links between these effects of auxin on membrane properties and the biological activity of auxin are difficult to establish. Very recently, binding of auxin at the plasmalemma of epidermal cells has been reported to be related to the elongation and the curling of corn coleoptiles (18). In other studies, the auxin-induced proton extrusion has been correlated to the elongation rate of shoots (12, 14, 25) and the control of root growth (10, 21) on the basis of similarities between the timing of the responses, the dependency upon auxin concentration, and the specificity of the response to auxin analogs. Such results provide evidence for the existence of a relationship between the action of auxin on membranes and the overall activity of this hormone on cell enlargement. Up to now, the involvement of hormone-induced modifications of membranes into the control of cell division and differentiation has not been directly demonstrated.

A novel opportunity to study the basis of the biological activity of auxins at the cellular level was recently offered by the selection of a NAA-tolerant mutant from tobacco mesophyll protoplasts (5, 23). The wild-type (clone D8) and the mutant (clone 36) differ in their sensitivity to auxins as measured by the ability of the cells derived from protoplasts to proliferate in the presence of NAA. In this paper we test the hypothesis that the wild-type and mutant protoplasts differ in their membrane properties. A comparative study of the effect of NAA and other analogs on the transmembrane potential difference of protoplasts isolated from the two genotypes is reported.

MATERIALS AND METHODS

Protoplast Isolation. Mesophyll protoplasts from plants of *Nicotiana tabacum* (cv Xanthi) were prepared as described previously (4). The washed protoplasts were resuspended as a stock suspension in medium To (4) depleted of NAA and adjusted to pH 5.7. The protoplast suspension (1×10^6 protoplasts$\cdot$ml^{-1}) was stored at 4°C prior to the experiment (1 d) and diluted to 1×10^4 protoplasts$\cdot$ml^{-1} just before use.

Measurement of the Transmembrane Potential Difference. Em was measured under the microscope on freshly isolated protoplasts by the microelectrode technique. Protoplasts were immobilized in a microholder and impaled with a glass microelectrode according to the method described by Rona *et al.* (26). Measurements were carried out on aliquots of the stock suspension in To medium minus NAA to obtain a reference value and in To medium with various concentrations of auxin analogs added. Em variations (ΔEm, mV) from the reference value are plotted as a function of the concentration of auxin molecules.

For each molecule, a complete range of concentrations was

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[2] Abbreviations: Em, potential difference; NAA, 1-naphthaleneacetic acid; picloram, 4-amino-3,5,6-trichloropicolinic acid; 4-BPAA, 4-bromophenylacetic acid.

tested on the same protoplast suspension. Twenty individual Em measurements (about 20 protoplasts) were performed for each concentration.

RESULTS

Effect of NAA on the Transmembrane Em of Wild-Type and Mutant Protoplasts. In the culture medium To without any auxin, the transmembrane Em values were generally positive for both wild-type and mutant protoplasts, mean Em values ranging from −2 to +20 mV according to seasonal fluctuations.

For the wild-type, Em was lower when the medium was supplied with NAA; a maximal effect (ΔEm = −11.5±0.4 mV) was reached with 5 μM NAA (Fig. 1). Increasing NAA concentrations above this level reduced the hyperpolarization until it was decreased by 50% in the presence of 35 μM NAA and nullified for 100 μM. No detailed kinetic study of Em modifications induced by NAA was carried out, but it was observed that Em was modified within less than 2 min after NAA addition and reached a value stable during the time necessary for one series of measurements (about 40 min). Em variations induced by auxin were quite reproducible for experiments performed over a period of 1 year.

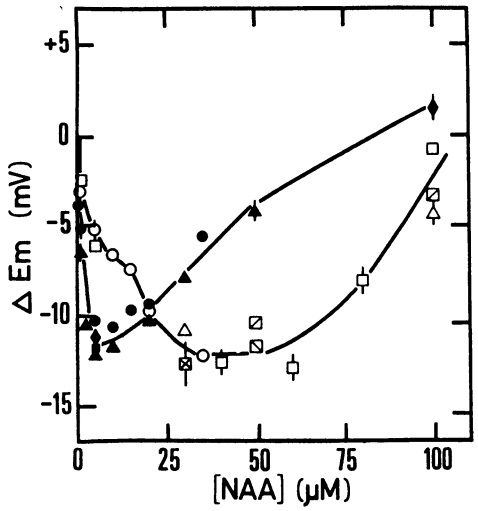

FIG. 1. Effect of NAA on the transmembrane potential difference of wild-type (black symbols) and mutant (open symbols) protoplasts of tobacco mesophyll. The different symbols represent independent experiments performed over 1 year. Em was measured by the microelectrode technique. Measurements were done in a complete culture medium immediately after the addition of various amounts of NAA. Em variations induced by NAA (ΔEm) were calculated from the reference value measured in the absence of auxin. For clarity, standard errors inferior to 0.5 mV were omitted.

The mutant displayed an electrical response of the same intensity (Fig. 1). No difference in the timing, the stability and the reproducibility of the response was noted between wild-type and mutant protoplasts. Both genotypes exhibited in the presence of NAA a non-monotonous response to NAA, a hyperpolarization and a relative depolarization. However, for the mutant, a marked shift in the dose-response curve was observed for the effective concentrations of NAA, as the maximal Em decrease (ΔEm = −12.6±0.6 mV) was obtained at concentrations about 40 μM; at 85 μM NAA the hyperpolarization was reduced by 50% and totally inhibited at 100 μM.

Effect of Structural Analogs of Auxin on the Transmembrane Em. In order to appreciate if the membrane response described for NAA was related to the biological activity of this growth regulator, molecules displaying different abilities to affect the cell growth were tested for their effect on Em. Two molecules were chosen among those already tested for growth in the same concentration range as NAA (6). Picloram exhibits a marked stimulatory activity on cell proliferation at low concentrations but is slightly toxic at higher concentrations. 4-BPAA is toxic and does not promote division.

Low concentrations of picloram (5 μM) decreased Em of wild-type protoplasts to a value similar to that found in the presence of 5 μM NAA (Fig. 2A). However, increasing the picloram concentration above this level induced no significant reduction of the hyperpolarization up to the highest concentration tested (100 μM). Picloram affected the Em of the mutant protoplasts in a similar manner by inducing only hyperpolarization (Fig. 2B). However, the maximal response was obtained at concentrations of about 50 μM, a value higher than that inducing the same response in wild-type protoplasts. This dose-response effect was thus similar to the hyperpolarizing effect caused by NAA. Treatment with 4-BPAA which does not stimulate growth, induced a response which was different from those exhibited in the presence of NAA or picloram. No hyperpolarizing effect was observed even for low concentrations, for the wild-type (Fig. 2A) and mutant (Fig. 2B) protoplasts. Instead, a clear depolarization response of wild-type protoplasts was obtained in the range 0.25 to 5 μM (Fig. 2A). To further test the depolarizing capacity of the 4-BPAA, protoplasts isolated from the two genotypes were pretreated during 5 min with NAA concentrations inducing the maximum hyperpolarization (*i.e.* 5 μM and 50 μM for wild-type and mutant protoplasts, respectively) before adding various concentrations of 4-BPAA. For both types of protoplasts the NAA effect was reversed by the addition of 4-BPAA (Fig. 3). Again, the mutant protoplasts exhibited a much lower sensitivity to the depolarizing effect of 4-BPAA than the wild-type protoplasts.

DISCUSSION

Effect of Auxin on the Transmembrane Em. We report for the first time an effect of auxin on the transmembrane Em of tobacco protoplasts. The auxin-induced hyperpolarization observed here at low concentrations of NAA is maximal within 2 min and stable for at least 40 min contrary to the time-course of auxin-induced changes of Em in coleoptile segments which is characterized by a hyperpolarization preceded by a slight, transient (10–15 min) and auxin-unspecific depolarization (1, 24). A hyperpolarization has also been reported for parsley cells in a high ionic strength culture medium (2) when treated by low IAA concentrations (<0.1 μM). On tobacco protoplasts, the intensity of the maximal NAA-induced hyperpolarization (about −12 mV for both wild-type and mutant protoplasts) is comparable to that measured on organs in the presence of 10 μM IAA: −17 mV on pea internode segments (19) and about −25 mV on maize coleoptiles (24). Only one recent study (1) reports IAA-induced hyperpolarization (−20 mV) using two concentrations (1 and 100 μM), and it was suggested that the response was concentration independent. In contrast we show here that the NAA-induced Em changes were described by a bell-shaped curve as a function of auxin concentration similar to that described for IAA effects on membrane thickness (22) and calcium binding properties (3) of soybean membranes.

It has been widely suggested that the auxin-induced hyperpolarization involves the stimulation of the proton-pumping ATPase (11, 27), and it would be tempting to speculate that wild-type and mutant protoplasts differ either in the intrinsic properties of this electrogenic system or in its responsivity to auxin. However, modifications of plasmalemma ionic conductances could also account for the Em variations measured.

Such hypotheses involving the plasmalemma in the response of intact cells to auxin should be cautiously extrapolated to tobacco protoplasts which exhibit Em values (mean Em +12.8± 1.5 mV on 16 preparations on the wild-type and mutant geno-

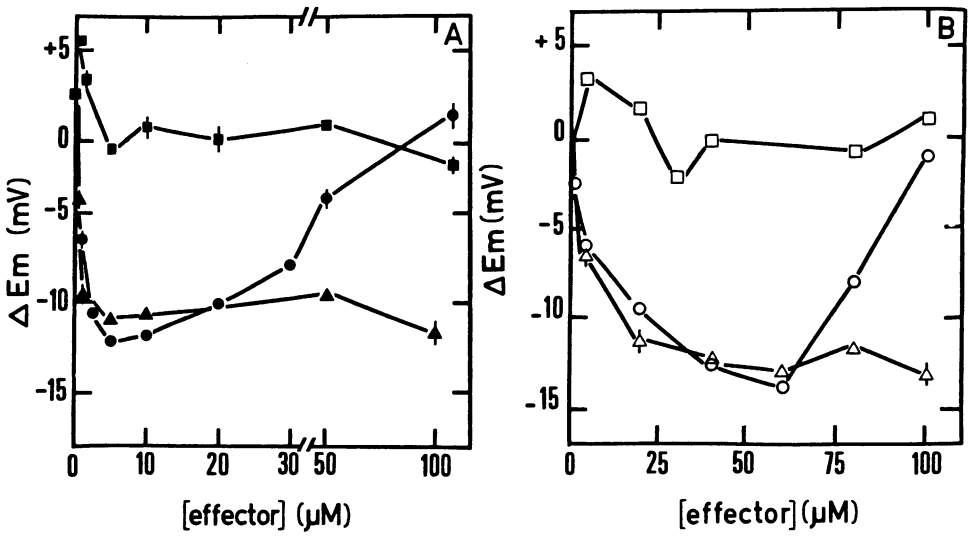

FIG. 2. Effect of NAA (●, ○) and two other auxin structural analogs, picloram (▲, △) and 4-BPAA (■, □) on the transmembrane potential difference of wild-type protoplasts (A) and mutant protoplasts (B). For measurement conditions and standard errors, see Figure 1.

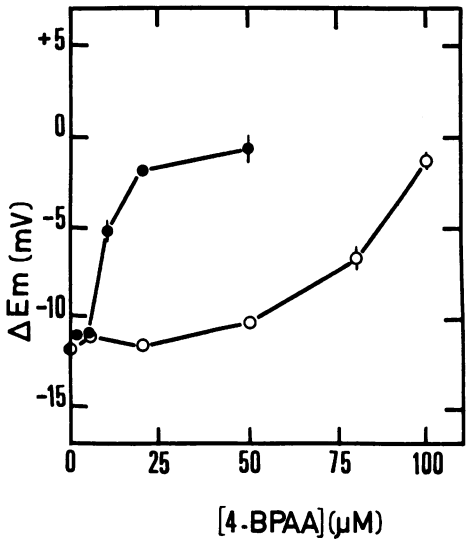

FIG. 3. Effect of 4-BPAA on the transmembrane potential difference of wild-type (●) and mutant (○) protoplasts pretreated during 5 min with 5 μM or 50 μM NAA, respectively (concentrations inducing a maximal hyperpolarization as shown in Fig. 1). Em variations (ΔEm) induced by NAA (in absence of 4-BPAA) or by NAA plus various amounts of 4-BPAA were calculated from the reference value measured in absence of auxin. For standard errors, see Figure 1.

types) very different from those measured in intact tissues (−100 to −130 mV). Positive Em values have already been described on protoplasts isolated from tobacco (16) and sycamore (8). Their significance was discussed by Cornel *et al.* (8) who postulated that the isolated procedure modifies the properties of the plasmalemma leading to a low Em value compared with plasmalemma Em value in cells. As the overall Em value determined on protoplasts is probably the sum of trans-plasmalemma and trans-tonoplast potential differences, their positive polarization would reflect a characteristic of the tonoplast, which is in good agreement with the electropositivity of the vacuoles *in situ* (8). The NAA-induced changes in the overall Em could thus reflect tonoplast as well as plasmalemma Em variations. The effects of NAA on isolated vacuoles of wild-type and mutant genotypes are being investigated.

Despite their peculiar electrical properties, the wild-type and mutant protoplasts exhibited different sensitivities to auxins as revealed by the dose-response curves of Em modifications. Thus, these protoplasts isolated from the two genotypes appear as a good material to study a membrane response to auxins related to their biological activity.

Evidence for a Correlation between the Electrical Response of Protoplasts to Auxins and Growth Response of Protoplast-Derived Cells. The nonmonotonous response described here for Em modifications reminds us of that observed for growth of protoplast-derived cells (5). The comparison of the NAA concentrations effective on these two auxin-induced responses pointed out the existence of a difference, the origin of which has still to be determined. However, the similarity of the shape of the dose-response curves suggests that the hyperpolarization obtained at low doses could be related to the growth stimulation, whereas the relative depolarization observed at high doses could be linked to the supraoptimal toxic effect. In agreement with this hypothesis, hyperpolarization was observed only for growth-stimulatory molecules (NAA at low concentrations and picloram), whereas the growth-inhibitory substances (NAA at high concentrations and 4-BPAA) caused depolarization. Concentrations inducing the maximal hyperpolarization or the complete depolarization were shifted towards higher values for the mutant protoplasts compared to the wild-type ones.

Thus, from the association of a chemical screen (comparison of the activity of various auxin analogs) and a biological screen (comparison of the responses of the two genotypes), this paper provides evidence for a specific action of auxins at the membrane level related to their biological activity at the cellular level. The rapidity of the membrane response (less than 2 min) with regard to the delay of the growth response (3–4 weeks) suggests that the electrical response could be one of the first events involved in the growth response of protoplasts and protoplast-derived cells to auxins.

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