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Electrical patterns of tobacco cells in media containing indole-3-acetic acid or 2,4-dichlorophenoxyacetic acid

Their relation to organogenesis and herbicide action

A. Goldsworthy and M.G. Mina

Biology Department, Imperial College, London SW7 2BB, UK

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Abstract. A simple, inexpensive, and stable drive-unit for a vibrating probe is described. It was used to measure transcellular electrical currents and their stability in cells from suspension cultures of *Nicotiana tabacum* L. var. *virginica*. The cells were highly variable in size, morphology and current-pattern. The magnitude and pattern of the currents depended on the age of the culture, the morphology of the cells and the auxin in the culture medium. Currents in small cell clusters were weakest during the lag-phase of growth and strongest when the cultures were actively growing. The shape of the cells was related to the electrical pattern surrounding them, electrically polar cells tending to be elongated. The proportion of polar cells depended on the auxin composition of the culture medium. About 75% of the cells from suspensions grown in the presence of indole-3-acetic acid (IAA) were electrically polar. These cells normally divided at right angles to their electrical axes to form filaments. Only around 20% of the cells grown in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) were electrically polar, the remainder had randomly oriented currents and divided in random directions to form irregular clusters rather than filaments. The electrical patterns of cells in 2,4-D were much less stable than those of cells in IAA. When currents were measured repeatedly at fixed locations on cells, those in 2,4-D were about twice as likely to disappear, arise de novo, or change direction as those in IAA. When cells were transferred from 2,4-D to IAA media, the percentage of polar cells increased from 25 to 40 within 1 d, but when they were transferred from IAA to 2,4-D, this percentage decreased from 48 to 26. It is suggested that one of the reasons that 2,4-D suppresses organogenesis in tobacco cultures (and possibly why it also functions as a herbicide) is that it reduces the stability of transcellular currents and disrupts the electrical patterns of cells so that they become less capable of organized polar growth.

Key words: Auxin (cell polarity) – Cell culture – Electric currents (measurement) – *Nicotiana* (cell polarity) – Polarity (cell) – Vibrating probe

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; IAA = indole-3-acetic acid

Introduction

Electric currents, with densities typically of the order of a fraction of a microamp per square centimetre, flow through the cells of both animals and plants (see Jaffe and Nuccitelli 1977; Jaffe 1981). They are believed to be responsible for the establishment of cell polarity (Chen and Jaffe 1978) and are associated with the growth and differentiation of polar structures (Robinson and Jaffe 1975; Robinson and Cone 1980; Brawley et al. 1984).

Rathore and Goldsworthy (1985) reported a severalfold stimulation of shoot formation in tobacco callus after passing weak electric currents through the cultures. This appeared to be associated with an increase in the polar transport of auxin (Goldsworthy and Rathore 1985). They suggested that these effects might be a consequence of the induction of a common polarity in the callus cells which made the formation of organized structures more likely. If this is so, an organised electrical polarity of its component cells may be a prerequisite for organogenesis in tissue cultures, even without the application of an external current.

The present investigation set out to study this possibility by comparing the *natural* currents flowing through tobacco cells suspended either in a medium containing IAA, which favours organogenesis, or one containing 2,4D, which favours disorganized growth.

Materials and methods

Tissue culture. Cell suspensions were derived from callus from seedlings of *Nicotiana tabacum* L. var. *virginica* (seeds from Thompson

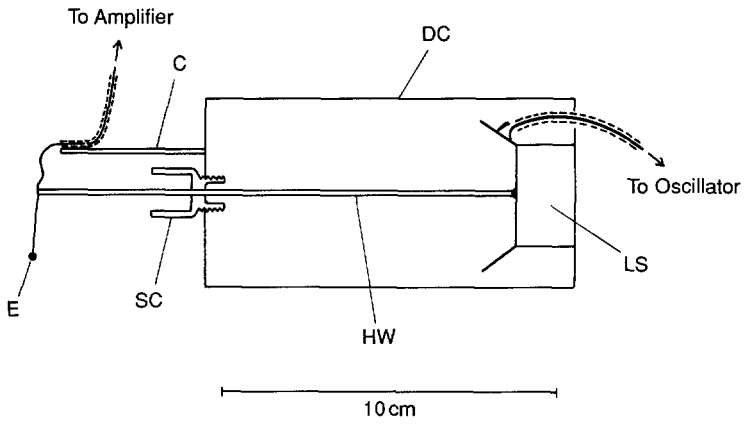

Fig. 1. Vertical section of the probe-vibrating unit. *C*, cable support; *DC*, die-cast box; *HW*, hardwood rod; *LS*, loudspeaker coil and magnet assembly; *SC*, serum cap; *E*, vibrating electrode (electrode not shown to scale)

and Morgan, Ipswich, UK). The callus was subcultured every four weeks in Gamborg's B5 medium containing 2% sucrose and 1% agar at pH 5.5 (Gamborg et al. 1968). The medium also contained 1 $mg \cdot l^{-1}$ 2,4-D and 0.1 $mg \cdot l^{-1}$ kinetin (N^6-furfurylaminopurine) to give rapid growth but suppress differentiation. These, as well as other hormones and vitamins, were purchased from Sigma Chemical company, St. Louis, Mo., USA. Other chemicals were purchased from BDH Chemicals, Poole, Dorset UK. Stock suspension cultures were prepared by cutting inocula weighing about 1 g from actively growing regions of the parent callus and placing them in 100 ml of sterilised liquid B5 medium with the same hormone composition in 250-ml conical flasks. The flasks were incubated on an orbital shaker at 120 rpm in a temperature-controlled room at 24° C with a 16-h daily period of diffused illumination (7.2 $W \cdot m^{-2}$) from warm-white fluorescent lamps. The suspensions were subcultured by transferring 10 ml of the parent culture into 100 ml of similar medium at three-week intervals. Prior to making electrical measurements, cells were subcultured into B5 media, containing 0.1 $mg \cdot l^{-1}$ kinetin and either 1 $mg \cdot l^{-1}$ 2,4-D (favouring disorganized growth) or 0.03 $mg \cdot l^{-1}$ IAA (favouring shoot-formation).

Current measurements. Transcellular currents were made with a modified version of the vibrating probe, originally described by Jaffe and Nuccitelli (1974). The probes were made from parylene-insulated tungsten electrodes (0.5 mm diameter, 75 mm long; Clark Electromedical Instruments, Pangbourne, Berks., UK). They were cut to a length of about 2.5 cm and attached to a gold-plated connector using silver-loaded epoxy adhesive (both from RS Components, Corby, Northants, UK). The electrode tip was plated electrolytically with gold and platinum in succession, up to a maximum diameter of 25–30 μm. Tip-capacitance was monitored using the protocol of Scheffey (1986). Probes with values less than 50 nF were either re-plated or discarded.

Because many of the currents measured in cultured cells were very small and many of the measurements had to be made repeatedly over long periods, a good signal-to-noise ratio and freedom from drift was important. The piezoelectric bender used in preliminary experiments to vibrate the probe proved a source of problems in these respects. A considerable improvement was found when this was replaced by a loudspeaker. Loudspeakers have already been used successfully as vibrating units, even for two-dimensional probes (see Freeman et al. 1986) and have several advantages over piezoelectric benders, which have unwanted resonances (Freeman et al. 1986) and in our hands were extremely sensitive to even minor changes in temperature so as to affect the probe output. Problems can also arise because prezoelectric benders normally have a high impedance and require relatively high voltages to drive them. This leads to comparatively strong electric fields near the probe at the frequency of vibration. These can be picked up by the signal circuitry, are not rejected by the lock-in amplifier, and appear as spurious readings at the output. On the other hand, a loudspeaker is a low-impedance device, operates at lower voltages, generates smaller electrostatic fields and can be mounted at a greater distance from the probe to further minimize the effects of any stray fields.

The arrangement developed for the present investigation (Fig. 1) was made by cutting the outer part of the cone and its supporting framework from a 3.5-inch 35-Ω shielded-magnet loudspeaker (from RS Components). The magnet and voice-coil assembly were glued with epoxy resin at one end inside an electrically earthed, oblong, aluminium die-cast box which was itself screwed to a micromanipulator. A hardwood rod, about 2 mm thick and 150 mm long, was glued to the centre of the voice-coil and passed through the thin septum of a rubber serum cap fitted into a hole drilled at the other end of the box. The probe was mounted at right angles to the rod in a connector fixed with epoxy resin at its distal end. (Hardwood was selected because it is stiff, has no unwanted resonances, and does not become electrostatically charged. A kebab stick is suitable.) The 35-Ω loudspeaker was connected directly by a screened cable to a probe-vibrator power supply (Model N-801; Vibrating Probe Company, Davis, Cal., USA). Speakers of other impedances or other oscillator units may need a matching transformer or an additional stage of amplification.

The reference electrode for the probe was made from about 3 cm of platinum wire submerged in the medium. The signal from the vibrating electrode was processed by a lock-in amplifier (Model 5101; EG & G Princeton Applied Research, Princeton, N.J., USA), mostly with a 10-s time-constant, and displayed on a chart recorder. The probe was calibrated in the experimental medium by vibrating it in a standard current from a glass capillary ("Kwik-fill" standard-range single capillary with inner filament from Clark Electromedical Instruments) pulled to a tip diameter of 1–5 μm and filled with 3 M KCl. A typical trace showing the stability and signal-to-noise ratio is shown in Fig. 2.

Electrical measurements of cell cultures were made in a 4-cm-diameter glass Petri dish on the stage of an inverted microscope (Olympus, Tokyo, Japan). Cell material was selected from the suspensions using a sterile wide-bore pipette and transferred to the Petri dish which contained B5 culture medium in which the inorganic salts were reduced by a factor of ten to increase its resistivity. The medium also contained 0.1 $mg \cdot l^{-1}$ kinetin and either 1 $mg \cdot l^{-1}$ 2,4-D, or 0.03 $mg \cdot l^{-1}$ IAA, or was auxin-free as appropriate. Small clusters of about four to eight cells were held by suction at the fine-tapered end of a capillary in a micromanipulator. The temperature was maintained at 24° C. Currents were measured with the probe placed 15–20 μm from the cell surfaces (there was no measurable difference in current density within this range). The probe was also routinely vibrated near the tip of the capillary with no cells in position to show that there was no barrier artifact or current caused by the capillary itself. Drawings were made of each cell cluster showing the location of the points of measurement. Initially, these were evenly spaced around each cell, but extra readings were frequently intercalated in regions of special interest.

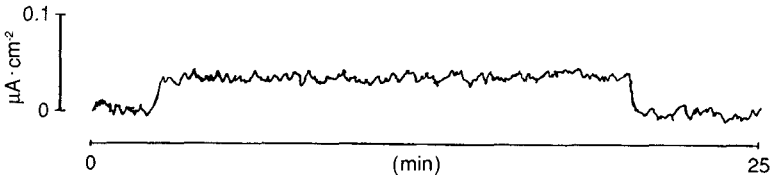

Fig. 2. A typical chart recording of current emerging from a single point on a tobacco cell, measured over about 17 min with a 10-s time-constant. The current was stable over this period, as was the base line, which is shown at each end of the trace and was obtained when the probe was moved away from the cell

In some experiments, repeated measurements were made at the same locations over a period of about 24 h, the calibration being checked before and after each group of measurements. Precautions were also taken to minimize contamination by working in a small draught-free room and enclosing the whole probe assembly in a solid-walled "Faraday cage" fitted with a blind which covered the front when access was not required. Under these conditions, there was no obvious contamination of the medium during the period of the experiment. There was also no measurable change in the resistivity of the experimental medium as a result of evaporation.

Table 1. Electrical polarity of tobacco cells measured during days 3–7 following subculture into media containing 0.03 $mg \cdot l^{-1}$ IAA or 1 $mg \cdot l^{-1}$ 2,4-D. Polar cells are defined as cells with their inward and outward currents grouped at opposite ends. Polar filaments are defined as those in which all the cells were polar and shared the same polarity

Medium	Cells examined	Polar cells		Filaments examined	Polar filaments	
		No.	%		No.	%
IAA	72	53	73	16	9	56
2,4-D	40	9	22	6	1	17

Results and discussion

Tobacco suspension cultures are highly heterogeneous. They consist of individual cells and groups of cells of various shapes and sizes, usually in the form of filaments or irregularly-shaped cell clusters. Growth and division occurs mainly in the groups and this investigation concentrated on the electrical patterns surrounding groups consisting of between three and fifteen cells.

Experiments in which cells were removed from cultures of varying ages after subculture and examined with the vibrating probe indicate a relationship between the strength of the currents and the speed of growth. The cultures were initiated from three-week-old parent suspensions which were in their stationary phase of growth. Measurements were made of the mean current density at about 100 random locations on samples of about 15 cells withdrawn at different times.

Inward and outward currents were normally found to be approximately in balance for each cell cluster and the mean for the whole sample was calculated from all of the measurements but ignoring the sign. Inevitably, there was some variability attributable to the heterogeneous nature of the population, but the results fell into a clear pattern. For the first 1–2 d after subculture (corresponding approximately to the lag phase) the currents were very weak with mean values ranging from the immeasurably low to around 0.05 $\mu A \cdot cm^{-2}$. They then rose appreciably as the cultures entered their phase of rapid growth and showed visible signs of cell division. During this period (2–9 d after subculture) 85% of the cells had measurable currents, with mean values in the region of 0.05 to 0.15 $\mu A \cdot cm^{-2}$ in both IAA and 2,4-D media. Cells were selected during this period for measurements of current pattern and polarity.

Although the magnitudes of the currents were broadly similar in the two auxins, the patterns of current-flow were quite different. In particular, there was a marked difference in the degree of electrical polarity between the cells grown in IAA and those grown in 2,4-D. Polarity was determined by making 6–10 measurements around each cell (depending on its size). A cell was designated as polar if all the measured currents were inwardly directed at one extremity and outwardly directed at the other. It must be emphasised that the present technique gives only a two-dimensional picture of what is really a three-dimensional electrical pattern around each cell. However, it is still a useful basis for comparing the relative degree of polarity of cells measured under otherwise identical conditions.

Table 1 shows the results of a series of experiments in which cells were transferred from a medium containing either IAA or 2,4-D to an auxin-free medium and the electrical pattern measured during the next 2–3 h. Of the cells from the IAA medium which had measurable currents, 73% had them entering at one end and leaving at the other, but only 22% of the cells from the 2,4-D medium showed this sort of polarity. The remainder had currents entering and leaving at several random locations. Since the measurements were all made under identical auxin-free conditions, it implies that external auxin is not directly involved in the generation of transcellular currents. However, the nature of the auxin previously supplied seems to influence the location and-or the activity of the ion pumps and channels responsible for cell polarity.

The electrical pattern surrounding the cells appeared to be related to their pattern of growth. When isodiametric cells growing in IAA medium were compared with elongated cells growing in the same culture, the elongated cells showed a much stronger electrical polarity with currents usually entering at one end and leaving at the other. In the isodiametric cells, the currents typically entered and emerged at random locations and had densities only about half those of elongated cells (Fig. 3). This is consistent with the concept that the direction of cell growth is controlled by the direction of the transcellular currents (Lund 1923; Jaffe 1966; Robinson and Jaffe 1975; Jaffe and Nuccitelli 1977).

There is also evidence that the plane of cell division

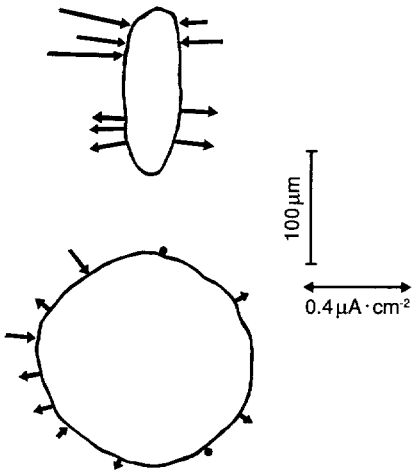

Fig. 3. Typical current patterns from an elongated and an isodiametric cell from an IAA-grown culture. The elongated cell has strong polar currents whereas those of the isodiametric cell are weaker and more randomly oriented. *Arrows* indicate the size and direction of the current. *Dots* on the cell surface mark positions where measurements were made but no current detected

may be controlled by the direction of the transcellular currents in these cells. The predominantly polar cells from the IAA medium usually divided transversely to their electrical axis to form filaments in which most of the cells shared the same polarity. In 56% of the filaments studied, *all* of the constituent cells had the same polarity. This sharing of a common polarity compares with the electrical pattern found by Lund (1947) in the filamentous alga *Pithophora* and indicates that the filamentous condition may be the consequence of a common unidirectional current flowing through all cells, with cell elongation being parallel to the current and division at right angles to it.

By contrast, tobacco cells from the 2,4-D medium had relatively random currents and relatively random planes of division which gave rise to amorphous cell clusters, filament formation being rare. Where filaments were formed in 2,4-D, the vast majority of cells still had random currents. Perhaps in these cases, the filament was only a chance orientation of what could have been a random cell cluster. There was no obvious correlation between the direction of current flow at the time of measurement and the planes of previous cell divisions in 2,4-D. This indicates that the currents may have been unstable in the 2,4-D medium and that their locations had changed between the time of cell division and the time of measurement.

The latter possibility was investigated by comparing the stability of the transcellular currents of cells growing in either the IAA or the 2,4-D medium. This was done by observing how the currents *changed* at fixed locations on individual cells after transfer from one auxin to the other.

Cell cultures were grown in either IAA or 2,4-D medium and measurements made when the cell cultures were still growing rapidly. Samples of a few drops were transferred to 7 ml of the measuring medium in the Petri dish on the stage of the vibrating probe. The measuring medium was the same as the original growing medium (Gamborg et al. 1968) except that its mineral content had been reduced by a factor of ten. Different treatments had an auxin regime either the same or different from the original growth medium.

Table 2 shows the effects on the current densities after such transfers. In most cases, there was a tendency of the current densities to fall somewhat following transfer, but only in those transferred to 2,4-D was the decline statistically significant. The reasons for the fall are likely to be complex and may include the reduced ionic concentration of the measuring medium (needed to increase its resistivity) and the dilution of the cells inducing a lag phase in growth. Whatever the reason for the fall, IAA seems more effective than 2,4-D in minimizing it. This indicates that IAA may be better than 2,4-D in stabilizing pre-existing transcellular currents.

More direct evidence for the increased stability of transcellular currents in IAA was obtained when their variability at individual locations was studied. When transcellular currents were measured repeatedly at fixed

Table 2. Transcellular currents of tobacco cells following transfer between media containing either 1 $mg \cdot l^{-1}$ 2,4-D or 0.03 $mg \cdot l^{-1}$ IAA. Each value is the mean ± SE of the current density (ignoring the sign) in $\mu A \cdot cm^{-2}$ for 300–400 measurements on about 20 cells. The figure in parenthesis is this value expressed as a percentage of the original at 1–2 after transfer. Values marked ** are significantly different from the original at $P < 0.01$

Original auxin	New auxin	Time after transfer (h)			
		1–2	2–4	15–18	20–26
IAA	2,4-D	0.074 ± 0.010 (100)	0.053 ± 0.007 (72)	0.054 ± 0.009 (73)	0.040 ± 0.004** (54)
2,4-D	2,4-D	0.075 ± 0.009 (100)	0.064 ± 0.010 (85)	0.060 ± 0.008 (80)	0.046 ± 0.004** (61)
2,4-D	IAA	0.068 ± 0.007 (100)	0.075 ± 0.007 (110)	0.055 ± 0.005 (80)	0.060 ± 0.008 (88)
IAA	IAA	0.090 ± 0.012 (100)	0.073 ± 0.009 (81)	0.082 ± 0.010 (91)	0.088 ± 0.009 (98)

Table 3. Stability of transcellular currents at specific locations on tobacco cell clusters after transfer between media containing either 1 $mg \cdot l^{-1}$ 2,4-D or 0.03 $mg \cdot l^{-1}$ IAA. Measurements were made at the four times indicated in Table 2. The variance is a measure of the variability with time of the current density at each location. The mean of these gives the average variability for the treatment. Pairs of figures labelled a or b were significantly different at $P<0.05$ and those labelled c at $P<0.02$. Counts were also made of when a new current appeared, when an old current fell below the limit of reliable detection (0.02 $\mu A \cdot cm^{-2}$) or when its direction reversed. The "percent unstable" value was calculated from the sum of these. Regardless of the medium from which they came, or the criterion used to assess stability, currents from cells transferred to 2,4-D medium were about twice as unstable as those transferred to IAA

Original auxin	New auxin	No of measurements	Mean variance ($\mu A \cdot cm^{-2} \times 10^{-3}$)	No. of currents appearing	No. of currents disappearing	No. of currents reversing	Percent unstable
IAA	2,4-D	340	2.0 a	16	32	24	21.2
2,4-D	2,4-D	408	2.2 bc	21	28	43	22.6
2,4-D	IAA	312	1.4 b	10	9	20	12.5
IAA	IAA	398	1.1 ac	9	11	24	11.1

Table 4. The percentage of polar cells found in three to five separate clusters following transfer between media containing either 1 $mg \cdot l^{-1}$ 2,4-D or 0.03 $mg \cdot l^{-1}$ IAA. Each figure corresponds to measurements made on about 20 cells. Transfer to 2,4-D, almost halved the number of polar cells from the IAA culture, but had little effect on the cells which had already been growing in 2,4-D. Transfer to IAA medium from either IAA or 2,4-D increased the percentage of polar cells

Original auxin	New auxin	Time after transfer (h)			
		1–2	2–4	15–18	20–26
IAA	2,4-D	48	39	26	26
2,4-D	2,4-D	22	27	15	24
2,4-D	IAA	25	45	35	40
IAA	IAA	33	50	78	75

positions, many of them were found to be unstable and varied slowly with time. Some disappeared or even reversed in direction whereas others appeared where none had been found before. To assess current instability, the variance was calculated for the measurements taken at the four different times at each location. The mean of these variance values was then calculated for all locations in each treatment and taken as a measure of current instability. The results are shown in Table 3. Regardless of the culture medium in which the cells were originally grown, the mean variance for the current density of cells measured in 2,4-D was approximately double that for cells measured in IAA, the values being significantly different at the 0.05 level of probability. When cells which were both grown *and* measured in the same auxin were compared, the IAA and 2,4-D treatments were significantly different at the 0.02 level of probability.

Another index of instability was taken to be the percentage of locations which showed either the appearance, disappearance, or reversal of current between measurements. Table 3 also shows these results. Regardless of the culture medium from which they were originally derived, the currents of the cells measured in 2,4-D were about twice as likely to appear, disappear, or change direction as those in IAA.

The changes in current density and direction shown above might be expected to affect the electrical polarities of the cells concerned. Table 4 shows the effect of the different culture media on the percentage of polar cells over the 1-d experimental period. The cells with the highest degree of polarity at the beginning of the experiments were those which had been previously grown in IAA, with between one-third and one-half of them already polar. Only about a quarter of those grown in 2,4-D were polar at this stage. This indicates a greater ability of the IAA medium to support cell polarity.

The *changes* which occurred in the percentage of polar cells in individual clusters after transfer from one auxin regime to the other confirm this conclusion (Table 4). Transfer from IAA to 2,4-D reduced the percentage of polar cells by almost half within 1 d. This implies that 2,4-D can destroy polarity which has already been acquired. This phenomenon may also partially account for the herbicidal action of 2,4-D, since one of the major effects of this substance is to bring about contortions of the plant body and an apparent loss of organized polar growth.

On the other hand, transfer from 2,4-D to IAA increased by about two-thirds the percentage of polar cells. The control, in which cells were transferred from 2,4-D to fresh 2,4-D showed no statistically significant difference. We can therefore conclude that IAA is better than 2,4-D in promoting the electrical polarity of cells. This conclusion is also supported by the increase in the percentage of polar cells after transfer from IAA medium to fresh IAA medium. Because of its relative biodegradability, the IAA in the original culture would have been at least partially depleted. Transfer to fresh medium should therefore increase the availability of IAA to the cells and so increase polarity.

Taken together, our results indicate that the IAA medium supports more stable transcellular currents than the 2,4-D medium, and that the relatively small amount of variability which occurs in IAA still allows this auxin to increase and coordinate cellular polarity. This may explain why IAA is superior to 2,4-D in promoting organogenesis in these cultures and is consistent with the Goldsworthy and Rathore hypothesis that strong and

coordinated cell polarities in tissue cultures are a prerequisite for organogenesis.

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