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Ion Transport in Isolated Protoplasts from Tobacco Suspension Cells

III. MEMBRANE POTENTIAL[1]

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ABSTRACT

The membrane electrical potential difference was measured in cultured cells and isolated protoplasts of tobacco (*Nicotiana glutinosa* L.) by inserting a microelectrode into cells held fast by a suction micropipette. The potential difference (± standard deviation) for unplasmolyzed tobacco cells was −52 ± 12 millivolts, for cells in 0.3 molar mannitol, −50 ± 11 millivolts; and for cells plasmolyzed in 0.7 molar mannitol, −49 ± 12 millivolts all inside negative. The potential difference for isolated protoplasts in 0.7 molar mannitol was −49 ± 16 millivolts, inside negative. In both cultured cells and protoplasts, the addition of 0.1 millimolar KCN caused a depolarization of the membrane potential. It was concluded that plasmolysis and enzymic release of the protoplast had no significant effect on the membrane potential of cultured tobacco cells.

Protoplasts isolated from the cells of higher plants provide a novel system for studying the mechanism of ion transport. The removal of the cell wall eliminates the confounding effects of ion exchange properties of the cell wall on ion adsorption and allows direct access to the plasmalemma. In addition the methods used in studying ion transport become simplified because the protoplasts can be treated as single cells instead of as complex tissues. An important assumption that has some support (13, 14, 17), is that enzymic removal of the cell wall does not significantly alter the transport properties of the protoplast.

The membrane potential provides a sensitive index of cell condition with respect to ion transport (6). An immediate problem that occurs in using microelectrodes to measure the membrane potential in isolated plant protoplasts is that a method must be provided to hold the protoplast steady so that impalement can be achieved. Recently, two methods of cell immobilization have been utilized: one by Racusen *et al.* (15) involves the impaling of protoplasts embedded in an agar block; and the other, originally proposed by Barber (3) for cells of *Chlorella pyrenoidosa* and then modified for use with the larger plant protoplasts of *Acer pseudoplatanus* L. by Rona *et al.* (16), involves the use of a suction micropipette for holding the isolated protoplast steady. Both Racusen *et al.* (15) and Rona *et al.* (16) have reported that isolated plant protoplasts have a positive membrane potential with respect to the outside. In addition, Heller *et al.* (7) showed that liquid suspension cells of *A. pseudoplatanus* L. had a membrane potential of −22 to −40 mv (inside negative); however, when protoplasts were isolated, a membrane potential of +10.4 mv (inside positive) was realized. These observations suggest that protoplast isolation imparts change on the ion transport properties of plant cells and question the validity of transport studies performed with isolated plant protoplasts.

More recently, Rubinstein (17) suggested that because of the fragility and lack of turgor of isolated protoplasts, it may be more reliable to use lipophilic cations with the Nernst equation rather than microelectrodes to estimate the membrane potential. He used the equilibrium distribution of triphenylmethylphosphonium to calculate a Nerst potential of −62 mv, inside negative, for isolated mesophyll protoplasts from oats.

In the research reported here we found microelectrodes to be both useful and reliable for measuring the membrane potential of isolated tobacco protoplasts or plasmolyzed tobacco cells. The negative membrane potentials found in protoplasts were not significantly different from the membrane potentials found in the liquid suspension cells from which they were isolated.

MATERIALS AND METHODS

Source of Protoplasts and Isolation Procedure. Liquid suspension cell cultures were initiated from friable callus of *Nicotiana glutinosa* L. The cell cultures were maintained at 27 C with 16 h daily illumination on a gyratory shaker (New Brunswick Scientific, N. J.) operating at 150 rpm. The cultures were harvested after 4 to 5 days in passage which corresponds to the beginning of the log phase of growth (18).

Protoplasts were isolated by the method of Uchimiya and Murashige (18), as previously described (13). The isolation medium consisted of 1% (w/v) cellulysin and 0.2% (w/v) macerase in 0.7 M mannitol. The cell-enzyme mixture was incubated at 27 C for 4 h with gentle shaking. The protoplasts were filtered through four layers of cheesecloth, a 50-μm pore size nylon cloth, and then pelleted and washed by repeated centrifugation. The final protoplast pellet was suspended in 0.7 M mannitol with 1 mM KCl (pH 5.8) as a 1 to 27 dilution. When membrane potential measurements were performed in agar, the protoplasts were plated in 0.7% agar in 0.7 M mannitol with 1 mM KCl.

Membrane Potential Measurements. A schematic diagram of the apparatus used to measure the membrane potential in isolated plant protoplasts and free suspension cells is presented in Figure 1. The isolated plant protoplast (or suspension cell) was held by means of a suction micropipette controlled by a micromanipulator (Narishige, Japan). Suction was provided by a small syringe. The measurement of the membrane electrical potential difference utilized standard techniques (8) with a model M4A electrometer (W-P Instruments, Hamden, Conn.) and D10 single beam oscilloscope (Tektronix, Beaverton, Ore.). All measurements were carried out at room temperature (about 22 C).

Both microelectrode and suction micropipette were drawn with a vertical microelectrode puller (Research Instruments Ltd., U.

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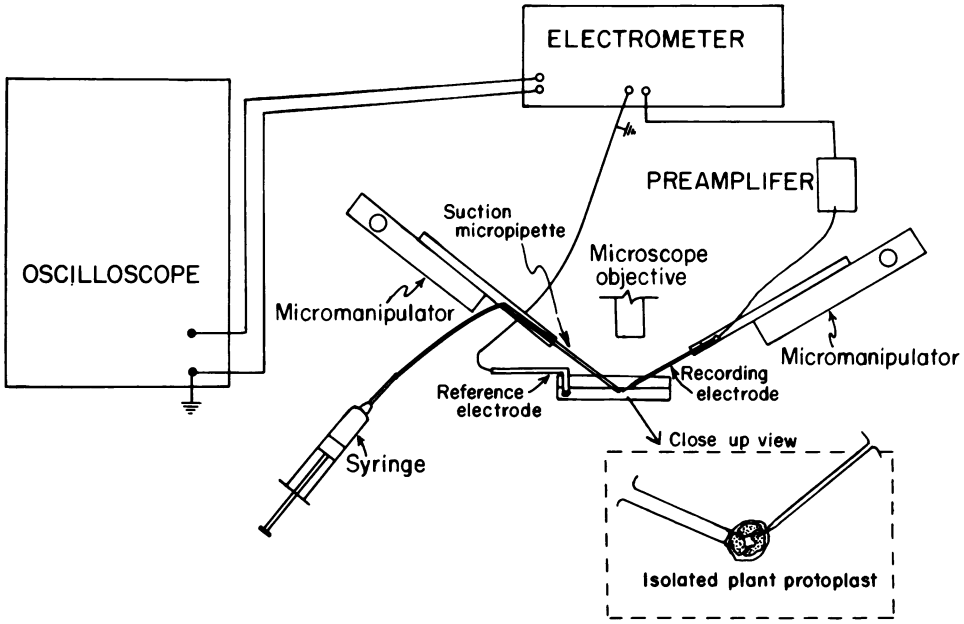

FIG. 1. Schematic diagram of the apparatus used for the measurement of membrane potentials, in solution, of liquid suspension cells and protoplasts. Inset shows relationship between the suction micropipette, recording electrode, and cell during electrical potential measurement.

K.). The tip diameter of the recording electrode was estimated by comparing the electrode resistance to values for electrodes where the tip diameter was measured with the scanning electron microscope. Unless otherwise stated the recording electrodes had a tip diameter of less than 0.5 μm. Electrical contact was achieved with Ag-AgCl wires in 3 M KCl in the recording electrode and in 3 M KCl in 2% agar in the reference electrode.

To construct the suction micropipette, the tip of a microelectrode was carefully broken under a high power light microscope. The broken electrode tip was then flared and fire-polished by passing it in and out of a hot electrical element coil.

When membrane potentials were measured in 0.7% agar, protoplasts were plated into a 3.5-cm plastic Petri dish. The membrane potential was recorded by impaling protoplasts embedded in agar directly in the Petri dish.

Application of Metabolic Inhibitor. KCN (1.0 mM) was applied to the medium surrounding isolated protoplasts and suspension cells by allowing a volume of 1.0 mM KCN solution to pass down the outside of the suction micropipette (Fig. 1) so that the final concentration of KCN in the Petri dish was 0.1 mM. With protoplasts, the 1.0 mM KCN solution was in 0.7 M mannitol and with liquid suspension cells in double-distilled H_2O. KCN was applied 4 min after impalement of cells and protoplasts and the value of the membrane potential recorded at 1-min intervals.

Measurement of the Membrane Potential of *Elodea densa*. The membrane potential in leaves of *E. densa* was measured according to the method of Racusen *et al.* (15). Leaves of *E. densa* were maintained in a 1× nutrient solution (10) and impaled in a Lucite chamber under a light microscope. As indicated, the membrane potential in cells of *E. densa* was measured in 0.3 or 0.7 M mannitol in 1 mM KCl.

RESULTS AND DISCUSSION

Membrane Potential in Tobacco Suspension-cultured Cells and Protoplasts in Solution. Impalement of cells and protoplasts in solution was achieved by first moving the microelectrode tip up to a cell held fast by the suction micropipette so that the electrode tip just made contact with the cell. Then the micromanipulator holding the recording electrode was gently tapped. This drove the microelectrode tip cleanly into the cell and established stable electrical contact without cell disruption. This tapping technique and the use of recording electrodes with small (less than 0.5 μm) tip diameter were essential for successful impalement. Cells and protoplasts of *N. glutinosa* L. could not be impaled by slowly driving the microelectrode tip through the cell as described by Rona *et al.* (16) for cells of *A. pseudoplatanus* L. Therefore, the existence of a series of electrical potential plateaus (16) occurring during the gradual impalement of liquid suspension cells and protoplasts could not be confirmed in these experiments.

Table I. *Membrane Potential of Suspension-cultured Cells and Protoplasts of N. glutinosa under Various Experimental Conditions*

All media contained 1 mM KCl (pH 5.8).

Cell Type	Condition	No. of Trials	Electrical Potential	
			$\bar{X}$	SD
			mv	
Intact cells	Unplasmolyzed (no mannitol)[a]	10	−52	12
Intact cells	Unplasmolyzed (0.3 M mannitol)[a]	15	−50	11
Intact cells	Plasmolyzed (0.7 M mannitol)[a]	15	−49	12
Protoplasts	Plasmolyzed (0.7 M mannitol)[a]	12	−49	16
Protoplasts	Plasmolyzed (0.7 M mannitol) and embedded in 0.7% agar	30	+10	3

[a] Cells or protoplasts held in place by the suction micropipette for impalement by the recording electrode.

There was no significant difference between the membrane potential of protoplasts and the cells from which they were isolated when the measurement was performed on cells in solution using a suction micropipette (Table I). When 0.1 mM KCN was supplied to tobacco cells or protoplasts in solution and the membrane potential measured using the suction micropipette technique, a depolarization of the resting potential occurred (Fig. 2). For liquid suspension cells the depolarization represented a potential drop from −41 to −13 mv and for protoplasts, −40 to −5 mv (Fig. 2). These results constitute strong evidence for a metabolically driven component of the measured electrical potential difference (9) in both intact cells and isolated protoplasts of tobacco.

It is apparent from Figure 2 that the rates of depolarization with KCN for both cells and protoplasts were slower than the rates reported for other plant cells (1, 2, 11). This was a function of the technique used to apply the inhibitor to the cell and not a characteristic of the depolarization. The inhibitor was applied

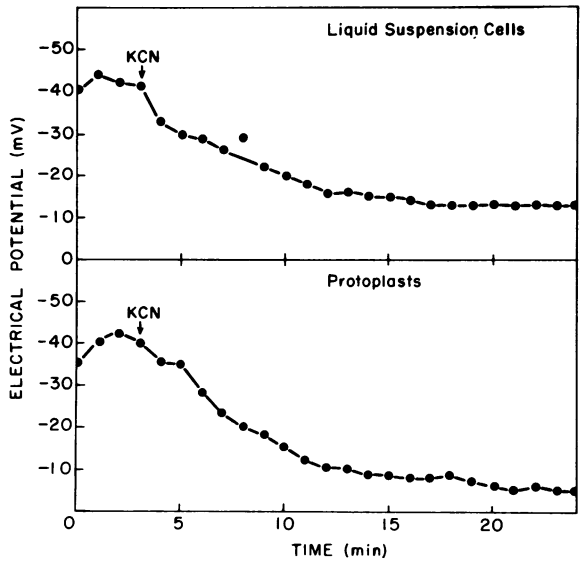

FIG. 2. Time course of KCN-induced depolarization of membrane potential in suspension-cultured cells and isolated protoplasts of tobacco. KCN added to a final concentration of 0.1 mM.

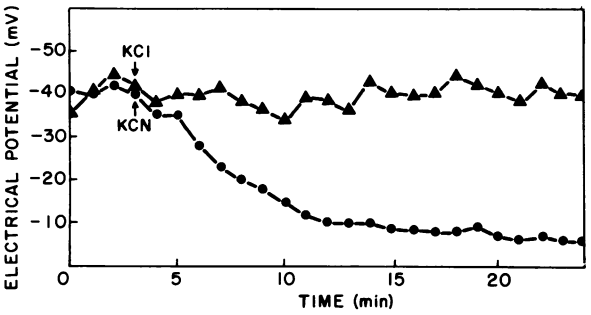

FIG. 3. Time course for effect of 0.1 mM KCl or 0.1 mM KCN on membrane potential of isolated tobacco protoplasts.

slowly down the surface of the suction micropipette to prevent the impaled cell from being washed free from the recording electrode. The diffusion rate of KCN as it entered the solution near the tip of the suction micropipette was rate limiting so that the time course of depolarization was more gradual. The time course of depolarization was still far too rapid to be caused by the running down of a cation diffusion potential resulting from decreased internal ion concentration associated with KCN-induced inhibition of active fluxes (4).

The concentration of K^+ in the external medium can affect the membrane potential in plant cells (5, 10). The addition of KCl did not reproduce the depolarization of the membrane potential for isolated protoplasts observed with KCN (Fig. 3). Figure 3 also shows that the membrane potential measured for isolated protoplast was relatively stable for at least 25 min.

Membrane Potential of Tobacco Suspension-cultured Cells and Protoplasts in Agar. It was very difficult to manipulate the fine-tipped recording electrode in the relatively viscous 0.7% agar gel. Recording electrodes with tip diameters of around 0.5 μm were chosen for the measurements in agar.

Impalement of intact tobacco cells embedded in 0.7% agar was not achieved because the coarser electrodes used in agar tended to push the cell rather than to penetrate the cell wall. The viscosity of the agar was not sufficient to "hold" the cell firmly enough for impalement.

Attempts to impale protoplasts that were embedded in 0.7% agar gave an average electrical potential difference of +10 ± 3 mv (inside positive). This value is in agreement with values found by Racusen *et al.* (15) for protoplasts isolated from various cells and embedded in 0.7% agar.

The positive potential measured for isolated protoplasts in agar is in marked contrast to the negative potential observed for the same cells in solution. The explanation for this difference is not known for sure, but the disparity may be related to the fact that impalement of cells in agar was difficult to achieve. It is quite possible that pushing the recording electrode against the plasma membrane of the relatively flaccid protoplast could give the appearance of impalement without actually penetrating into the interior of the cell. The positive potential recorded may be a function of some surface electrical phenomenon. This explanation may not account for the positive potentials observed by others for isolated plant protoplasts (7, 12, 15, 16).

Effect of Plasmolysis on the Membrane Potential. Plasmolysis is a major perturbation to plant cells during the isolation of protoplasts. Racusen *et al.* (15) reported that plasmolysis caused major changes in the electrical properties of *E. densa* leaf cells resulting in depolarization of the membrane potential from negative to positive values. They found that when the mannitol concentration in the solution bathing the leaf cells was increased from 0.3 M to 0.7 M the membrane potential was depolarized from about −40 to +10 mv. For this reason, the effects of plasmolysis on the membrane potential of cultured tobacco cells and leaf cells of *E. densa* were investigated.

Plasmolysis had no significant effect upon the membrane potential of suspension-cultured tobacco cells (Table I), and had little effect on the membrane potential of *Elodea* leaf cells (Table II). At no time was a positive membrane potential observed in response to plasmolysis.

GENERAL DISCUSSION

The membrane potential for protoplasts isolated from suspension-cultured cells of tobacco was about −50 mv (inside negative) when measured for cells held fast by a suction micropipette in solution. This value did not differ significantly from the membrane potential of the suspension-cultured cells from which the protoplasts were isolated (Table I). The membrane potential of both intact tobacco cells and isolated protoplasts was depolarized to a similar extent by KCN (Figs. 2 and 3). These results support the view (13, 14) that enzymic removal of the cell wall produced no significant alteration in the transport properties of tobacco protoplasts.

The potential difference of about −50 mv observed here for tobacco cells in solution is smaller than the range of potential

Table II. *Effect of Plasmolysis in 0.7 M Mannitol on Membrane Potential in E. densa*

Experiment No.	Condition	No. of Trials	Electrical Potential	
			$\bar{X}$	SD
			mv	
1	Unplasmolyzed[a]	10	−63	16
	Plasmolyzed[b]	10	−62	17
2	Unplasmolyzed	10	−52	13
	Plasmolyzed	7	−52	10
3	Unplasmolyzed	10	−65	18
	Plasmolyzed	10	−58	17
4	Unplasmolyzed	10	−72	23
	Plasmolyzed	10	−50	9
5	Unplasmolyzed	10	−66	16
	Plasmolyzed	10	−53	11
6	Unplasmolyzed	10	−87	41
	Plasmolyzed	10	−49	13

[a] Suspended in 0.3 M mannitol in 1× nutrient solution (1 mM KCl, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$ and 1 mM potassium phosphate, pH 5.6).
[b] Plasmolyzed in 0.7 M mannitol in 1× nutrient solution.

differences commonly observed in various higher plant cells (−80 to −180 mv), but similar to the values reported by others for cultured cells of tobacco (12) and sycamore (7, 16). The relatively small negative potential difference reported here for tobacco cells appears to be characteristic of cultured cells and not a function of the techniques employed for the electrical measurement.

Our results for isolated protoplasts in solution (Table I) confirm the findings of Rubinstein (17) who used the equilibrium distribution of a permeant cation to estimate a Nernst potential of −62 mv for oat leaf protoplasts. However, these results are in marked contrast to those of others who reported positive potentials for protoplasts isolated from various tissues and suspended in solution (7, 16) or in agar (12, 15). We have no explanation for this qualitative difference. The one instance where we measured a positive potential for isolated protoplasts was in agar (Table I), and for reasons discussed earlier, we concluded that the positive potential was an artifact probably associated with a surface electrical phenomenon. We also suspect that the positive potential recorded here for tobacco protoplasts in agar was an artifact because such a change in cell polarity would be expected to alter dramatically the transport properties of the protoplast, but this is not the case (13, 14). The report that plasmolysis induces depolarization of the membrane potential to positive values (15) was not confirmed (Table II).

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