Plant Physiol. (1978) 62, 927-929

Use of Lipophilic Cations to Measure the Membrane Potential of Oat Leaf Protoplasts[1]

Received for publication January 23, 1978 and in revised form July 26, 1978

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ABSTRACT

Uptake of the lipophilic cation triphenylmethylphosphonium into mesophyll protoplasts of oat (*Avena sativa* L. cv. "Garry") approaches equilibrium at 3 to 4 hours. The resulting external and internal concentrations are then used with the Nernst equation to obtain a membrane potential of −62 millivolts, inside negative. Potentials calculated in this manner are depolarized by adding 2 mM sodium azide and 50 μM carbonyl cyanide *m*-chlorophenylhydrazone as well as by increasing the external proton and potassium concentrations. The depolarizations are qualitatively similar to those seen when oat mesoyphll cells are measured *in situ* with microelectrodes. It is concluded that due to the lack of turgor and fragility of protoplasts, estimations of their membrane potential may be made more reliably, under some conditions, with lipophilic cations than with microelectrodes.

It is becoming increasingly evident that protoplasts are suitable material for studies of physiological properties of membranes. One reason is that free space determinations are usually unnecessary for these easily manipulated cells; thus, more quantitative estimates can be made for membrane-regulated events such as proton, mineral ion, and solute fluxes as well as for investigations into hormone transport and mode of action.

An important parameter for these studies is the membrane potential which is both a result of, and tends to regulate, transport of charged and uncharged species (6). Membrane potentials of +10 to +15 mv, inside positive, have been obtained for *Acer pseudoplatanus* protoplasts (5) and for protoplasts of six other species (14). These values were obtained by impaling cells with microelectrodes, and are strikingly different from membrane potentials of cells in intact tissues which are negative inside.

Even though the results for seven species of protoplasts were consistent (5, 14), the possibility exists that these cells cannot withstand microelectrode penetration as effectively as fully turgid cells *in situ*. Furthermore, fluxes of Rb^+ and amino acids, which depend in part on a membrane potential which is inside negative (2, 8), showed similar characteristics in both intact tissues and protoplasts (ref. 13; and Rubinstein and Tattar, in preparation). I decided, therefore, to estimate protoplast membrane potential without using microelectrodes.

The procedure selected was described first for organelles by Grinius *et al.* (3) and has been used subsequently for bacteria (4, 15), *Thermoplasma* (9), and *Chlorella* (11) among other cell types. It involves the passive uptake of a lipophilic cation whose concentration ratio across the cell membrane at equilibrium is converted to membrane potential by the Nernst equation[2]:

$$E_m = 59 \log \frac{C_o}{C_i}$$

activity coefficients are omitted and a temperature of 25 C is assumed. It will be shown that the radioactive lipophilic cation triphenylmethylphosphonium ($TPMP^+$) is accumulated in oat leaf protoplasts against a concentration gradient and the membrane potential is estimated to be in the order of −62 mv, inside negative.

MATERIALS AND METHODS

Protoplasts were isolated and purified by a modification of the procedures of Kanai and Edwards (10). Leaves of 7-day-old light-grown oats (*Avena sativa* L. cv. "Garry") with the lower epidermis removed were plasmolyzed for 15 min on a solution containing 0.6 M sorbitol, 29 mM sucrose, 20 mM MES-KOH (pH 5.5) and 5 mM $MgCl_2$. Cellulysin (Calbiochem) and DTT were then added to a final concentration of 0.5% (w/v) and 2 mM, respectively, for 2.5 hr at 33 C. The released protoplasts were filtered through 70-μm nylon mesh and centrifuged at 400*g* for 2.5 min. The pellet was first resuspended in 0.6 M sorbitol, 29 mM sucrose, 50 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$; then 1.2 ml was added to 10.8 ml of a mixture of PEG 6000 (Baker), Dextran T_{20} (United States Biochemicals), Na-phosphate buffer (pH 7.5), and sorbitol [final concentrations: 5.5% (w/w), 2.5 mM, 400 mM, 0.6 M, respectively], and centrifuged at 300*g* for 5 min. The purified protoplasts banding at the interface were removed and washed twice by centrifuging at 400*g* for 60 sec in the buffer to be used for the uptake experiments. The cells were then stored overnight in the refrigerator. Cell counts were made with a hemocytometer.

Uptake was measured by adding an aliquot of protoplasts (1–5 × 10^5) which had been at room temperature for 1 hr to a buffered solution (usually at pH 7.0) of sugars and ions [5 mM each of Tris, HEPES, and MES, 0.6 sorbitol, 29 mM sucrose, 1 mM glucose, 1 mM KH_2PO_4, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$] containing ^3H-triphenylmethylphosphonium bromide (310 mCi/mmol; final concentration, 12.6 μM; supplied by New England Nuclear); the volume of incubation medium was usually 250 μl. After the desired incubation period at 25 C under fluorescent light of 200 ft-c, a 200-μl aliquot was removed and suspended in a calibrated tube containing 10 ml of cold incubation buffer. For the experiments in Table I, the tubes were poured onto 0.45-μm filters, the filters were then dried and placed into scintillation vials to which 4 ml of Scintisol (Isolab) had been added. For all other experiments, the tubes were centrifuged at 45*g* for 6 to 8 min, the supernatant aspirated to 0.2 ml of the pellet, and, after adding 10 ml more cold incubation buffer, it was centrifuged again. A final aspiration to 0.2 ml was performed, 0.2 ml of buffer added, the tube vortexed, and 0.3 ml was placed in scintillation vials. Counts were converted to dpm with a quench curve for the isotope and the radioactivity associated with cells at zero time (about 3–5% of total uptake by 3 hr) was subtracted from each time point. Internal concentrations were calculated on the basis of a 40-μm diameter for the average protoplast.

[1] Supported by National Science Foundation Grant PCM 76-00439.

[2] Abbreviations: E_m: membrane potential; C_o: External concentration; C_i: internal concentration; J_i: influx; J_o: efflux; $TPMP^+$: triphenylmethylphosphonium; CCP: carbonyl cyanide, *m*-chlorophenylhydrazone.

Membrane potentials of leaf cells *in situ* were determined by inserting a 3 M KCl-filled microelectrode (tip diameter less than 1 μm; resistance less that 25 megohm) into a peeled leaf segment. The signal was amplified and recorded as previously described (16). During these measurements, tissues were bathed in a flowing solution of 1 mM HEPES-KOH buffer (pH 6.5) containing 1 mM $CaCl_2$.

RESULTS

Two typical time courses for $TPMP^+$ uptake are shown in Figure 1. The isotope approached equilibrium somewhat beyond 3 hr, but the curves were usually not flat even after 6 to 8 hr. Experiments were terminated at 3 to 4 hr, however, since protoplast numbers began to decrease sharply at that time. If the C_i and C_o for $TPMP^+$ are determined at 210 min for 11 different experiments, the average membrane potential using the Nernst equation is −62 ± 9 mv, inside negative. This value is thus an underestimate since uptake may still not be at equilibrium and the cells may become depolarized somewhat during the uptake period.

To see if the $TPMP^+$ was free within the cells or was tightly bound to or dissolved in the cell components, protoplasts were osmotically broken and filtered before or after the uptake period (Table I). All of the radioisotope within the protoplast is probably not tightly bound since counts associated with cells ruptured at the start of the uptake period were not significantly different from counts associated with cells ruptured 210 min later.

External concentrations of H^+ and K^+ were altered to see if changes in E_m which are known to result (6) could be detected by measuring concentrations of $TPMP^+$ close to equilibrium. A pH

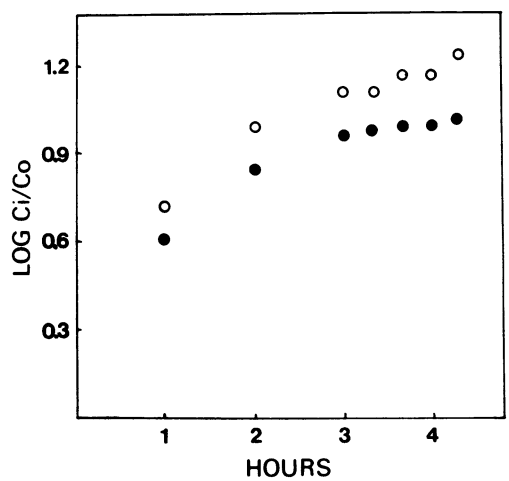

FIG. 1. Time course of [^{3}H]$TPMP^+$ uptake into oat leaf protoplasts. Each point represents the average of two replications. Data shown are for experiments performed on 2 days with different preparations of protoplasts. (○): 1.6 × 10^5/ml; (●): 2.1 × 10^5/ml.

Table I. Association of ^{3}H-$TPMP^+$ with the non-filterable portion of oat leaf protoplasts

At the end of the uptake period, an aliquot of protoplasts to be burst (200 μl, 5.1 x 10^5/ml) was transferred to 10 ml of distilled water; a similar aliquot of cells to remain intact was transferred to 10 ml of isoosmolar buffer. The supernatant was removed by filtration and the filters were placed in scintillation fluid for counting.

Time after adding Isotope	Treatment	
	Intact	Burst
Min	dpm·10^{-4}	
0.5	1.14	2.00
210	3.63	2.05

Table II. Effect of pH on $TPMP^+$ uptake into oat leaf protoplasts

After purification, the protoplasts (2 x 10^5/ml) were refrigerated overnight at the pH to be used for the uptake study. Uptake was determined for each pH at 0-time and 210 min by centrifugation as described in Materials and Methods.

pH	C_i/C_o	E_m
		mv
7.3	11.7	-63
6.3	10.0	-59
5.5	7.3	-51

Table III. Effect of KCl concentration on $TPMP^+$ uptake into oat leaf protoplasts

KCl was added with ^{3}H-$TPMP^+$ to the protoplasts (4.5 x 10^5/ml). The radioactivity associated with the cells was determined at 0-time and after 210 min by centrifugation as described in Materials and Methods.

KCl added	C_i/C_o	E_m
mM		mv
1	10.2	-60
10	7.5	-52
30	5.6	-44
100	3.7	-33

Table IV. Effect of respiratory poisons on $TPMP^+$ uptake into oat leaf protoplasts

The inhibitors were added with ^{3}H-$TPMP^+$ to the protoplasts (1.8 x 10^5/ml) at the start of the uptake period. Radioactivity associated with the cells was determined at 0-time and after 210 min by centrifugation as described in Materials and Methods.

Addition	C_i/C_o	E_m
		mv
None	15.3	-70
2mM NaN_3	5.0	-41
50μM CCCP	4.2	-37

study (Table II) shows that the $TPMP^+$-determined E_m is depolarized 12 mv as the pH decreased from 7.3 to 5.5. This depolarization can be compared with depolarizations determined with microelectrodes of 23 mv (from −148 mv to −125 mv) for oat mesophyll cells over the same pH span and 25 mv (from −120 to −95 mv) between pH 6.5 and 4.0 for oat coleoptile cells (2).

The addition of KCl depolarizes the $TPMP^+$-determined E_m from −60 mv at 1 mM KCl to −44 mv at 30 mM KCl in the experiments shown in Table III. These data can be compared with a 58 mv depolarization (from −140 to −82 mv) seen with microelectrode methods when the KCl concentration around oat mesophyll cells is increased from 1 mM to 25 mM and a 60 mv depolarization for oat coleoptiles over the same concentration range (7).

Using microelectrodes, depolarizations of the E_m have been reported after the addition of respiratory poisons (6). In the experiment shown in Table IV, addition of 2 mM NaN_3 or 50 μM CCCP for 210 min decreased the $TPMP^+$-determined E_m 29 mv and 33 mv, respectively. These results can be compared with a 30-mv depolarization (−146 mv to −116 mv) for 1 mM NaN_3 and with a 45-mv depolarization (−148 mv to −103 mv) for 50 μM CCCP measured by impaling mesophyll cells *in situ* with microelectrodes.

DISCUSSION

One criterion for using the Nernst equation with a passively accumulated ion to estimate the E_m is that the internal concentration is determined after equilibrium has been attained. While the time courses (Fig. 1) show that uptake does not always level off

completely by 3 to 4 hr (the time at which the protoplasts begin to deteriorate visually), the potential value being obtained may still be reasonably close to the actual value. This is because E_m is related to the log of the concentration ratio; thus, if the $TPMP^+$ concentration at equilibrium were two times higher than the concentration at 210 min, the E_m would change −18 mv. Even if the $TPMP^+$ concentration within the cell at 210 min were considerably below the equilibrium value, it is obvious that the E_m is negative inside and it is possible that information could still be gathered regarding relative differences between inhibitory and stimulatory treatments.

Another criterion for the use of $TPMP^+$ is that the cation is distributed uniformly within the cell. It appears that the cation is not bound to cellular components (Table I), but a uniform distribution within a multicompartmented structure such as a protoplast is unlikely; it must be assumed, therefore, that the value for internal concentration of $TPMP^+$ represents the average of all compartments and is largely that of the vacuole which occupies over 90% of cell volume. Similar problems regarding the identity of the membrane across which potential measurements are made also plague investigations using microelectrodes.

Compared to the microelectrode-determined E_m of mesophyll cells *in situ* (−140 to −150 mv), protoplasts at −62 mv are depolarized, but they are still electronegative with respect to the external solution. An E_m with the inside negative is consistent with predictions made from uptake studies of Rb^+ (13) and α-aminoisobutyric acid (Rubinstein and Tattar, in preparation), but is opposed to literature values showing the inside of the protoplast to be electropositive compared to the outside (5, 14). This contradiction may be related to the method of measurement, *i.e.* the insertion of a microelectrode. It is possible that protoplast turgor pressure, which is near zero, changes the elasticity of the plasma membrane such that it makes an incomplete seal around the microelectrode; if the microelectrode tip were in the vacuole, the E_m would then be measured across the tonoplast. Positive potentials have been recorded for isolated vacuoles (12).

One disadvantage for the use of lipophilic cations to measure protoplast E_m is the length of time necessary to reach equilibrium. This has been obviated by Komor and Tanner (11) who plotted a calibration curve of uptake rate *versus* E_m for a range of potentials. Another way to measure E_m at shorter time intervals would be the flow dialysis method described by Ramos *et al.* (15). Finally, estimates of E_m using $TPMP^+$ could be made at shorter times by measuring influx (J_i) and efflux (J_o) from preloaded protoplasts. These values could then be used in the Ussing-Teorell flux-ratio equation which can be written:

$$E_m = 59 \log \frac{C_o \cdot J_o}{C_i \cdot J_i}$$

The chief advantage of measuring protoplast E_m with lipophilic cations rests on the comparative harmlessness of the method. Oat leaf protoplasts are extremely labile (1) and may not be able to survive the trauma of penetration by microelectrodes. Even if the absolute values of E_m cannot be determined accurately because of the length of time for the cation to reach equilibrium, it may still be possible to study the qualitative effects of various treatments on E_m.

Acknowledgments—I am grateful to T. A. Tattar for his aid and to J. A. Lockhart for many fruitful discussions. Expert technical assistance was provided by H. Woodcock.

LITERATURE CITED

1. Altman A, R Kaur-Sawhney, AW Galston 1977 Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. Plant Physiol 30: 570–574
2. Etherton B, B Rubinstein 1978 Evidence for amino acid-H^+ co-transport in oat coleoptiles. Plant Physiol 61: 933–937
3. Grinius LL, AA Jasaitis, YP Kadziauskas, EA Liberman, VP Skulachev, VP Topali, LM Teofina, MA Vladmirova 1970 Conversion of biomembrane-produced energy into electric form. Biochim Biophys Acta 216: 1–12
4. Harold FM, D Papineau 1972 Cation transport and electrogenesis by *Streptococcus faecalis*. I. The membrane potential. J Membr Biol 8: 27–44
5. Heller R, C Grignon, J-P Rona 1974 Importance of the cell wall in the thermodynamic equilibrium of ions in free cells of *Acer pseudoplatanus* L. In U Zimmerman, J Dainty, eds, Membrane Transport in Plants. Springer-Verlag, Berlin, pp 239–243
6. Higinbotham N 1973 Electropotentials of plant cells. Annu Rev Plant Physiol 24: 25–46
7. Higinbotham N, B Etherton, RJ Foster 1964 Effect of external K^+, NH^+, Na^+, Ca^{2+}, Mg^{2+}, and H^+ ions on the cell transmembrane electropotential of *Avena* coleoptile. Plant Physiol 39: 196–203
8. Higinbotham N, B Etherton, RJ Foster 1967 Mineral ion contents and cell transmembrane electropotentials of pea and oat seedling tissue. Plant Physiol 42: 37–46
9. Hsung JC, A Haug 1977 Membrane potential of *Thermoplasma acidophila*. FEBS Lett 73: 47–50
10. Kanai R, GE Edwards 1973 Purification of enzymatically isolated mesophyll protoplasts from C_3, C_4, and Crassulacean acid metabolism plants using an aqueous dextran-polyethylene glycol two-phase system. Plant Physiol 52: 484–490
11. Komor E, W Tanner 1976 The determination of the membrane potential of *Chlorella vulgaris*. Evidence for electrogenic sugar transport. Eur J Biochem 70: 197–204
12. Lin W, GJ Wagner, G Hind 1977 The proton pump and membrane potential of intact vacuoles isolated from *Tulipa* petals. Plant Physiol 59: S-85
13. Mettler IJ, RT Leonard 1977 Ion transport in isolated protoplasts. Plant Physiol 59: S-15
14. Racusen RH, AM Kinnersley, AW Galston 1977 Osmotically induced changes in electrical properties of plant protoplast membranes. Science 198: 405–406
15. Ramos S, S Schuldiner, HR Kaback 1976 The electrochemical gradient of protons and its relationship to active transport in *Escherichia coli* membrane vesicles. Proc Nat Acad Sci USA 73: 1892–1896
16. Rubinstein B, P Mahar, TA Tattar 1977 Effects of osmotic shock on some membrane-regulated events of oat coleoptile cells. Plant Physiol 59: 365–368