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ATP-dependent acidification and tonoplast hyperpolarization in isolated vacuoles from green suspension cells of *Chenopodium rubrum* L.

(patch-clamp technique/membrane ATPase/current–voltage analysis/ion channel)

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ABSTRACT **The tonoplast of isolated vacuoles from photoautotrophic suspension cells of *Chenopodium rubrum* L. was studied by means of the patch-clamp technique. In a symmetrical K^+ concentration of 46 mM, similar to *in vivo* conditions, the tonoplast displayed a membrane potential near zero and a linear current–voltage relationship with a mean slope of 1.0 S/m². ATP at 2 mM hyperpolarized the tonoplast (vacuole positive) by 15–20 mV and, in a parallel experiment, acidified the vacuole (outside pH 7.0) to pH 5.0, as monitored by accumulation of acridine orange. Analysis of the voltage-clamp current indicates a 2-fold, ATP-dependent increase of the membrane capacitance, from 4 to 8 mF/m², and an ATP-independent, unidentified ion channel having a mean opening time of about 5 msec and a conductivity of 0.5–1.0 pS.**

Considerable attention currently is devoted to the energetics of substrate transport across the tonoplast, the membrane separating cytoplasm and vacuole in plant cells (1). A membrane ATPase is thought to pump protons into the vacuole and thus to energize the tonoplast by a pH gradient and possibly by a membrane potential. By applying the patch-clamp technique to isolated vacuoles, we have studied the electrical properties of the tonoplast and found that the latter features an ATP-dependent electrogenic pump that creates a small membrane potential but a substantial pH gradient, revealed by parallel experiments with the pH probe acridine orange.

MATERIALS AND METHODS

Photoautotrophic and phytohormone-independent suspension cells derived from hypocotyl cells of *Chenopodium rubrum* L. (2) were cultured as described (3). For protoplast isolation, cells from the exponential growth phase were used (for the growth pattern of the culture, see ref. 4). Cells from 6-day-old cultures were harvested from the suspension by filtration through a 15-μm-pore nylon net (Thoma, Mössingen, F.R.G.). Fifteen grams of cells (fresh weight) was suspended in 50 ml of medium I [20 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)/KOH, pH 5.3/0.3 M mannitol/2 mM $CaCl_2$/10 mM KCl/1 mM DL-dithiothreitol/5 mM $MgCl_2$/0.5% bovine serum albumin]. Fifty milliliters of medium I containing 2.5 g of cellulase TC from *Trichoderma reesi* (Serva, Heidelberg) and 2.5 g of pectinase 5S from *Aspergillus niger* (Serva) was added to the cell suspension and incubated on a gyratory shaker (120 rpm); after 90 min, there was no more Calcofluor white-staining (5) detectable at the cell surface (Calcofluor white ST solution was a gift from U. Seitz, Tübingen, F.R.G.). Protoplasts were harvested by centrifugation (100 × *g*, 15 min) and washed twice with medium II (20 mM Mes/KOH, pH 6/0.3 M mannitol/25 mM KCl/1 mM DL-dithiothreitol/0.5 mM $MgCl_2$/0.1% bovine serum albumin); yield of protoplasts was 81%. For further purification, the protoplasts in medium II were loaded on a step gradient of 10, 7.5, 5, and 2.5% (wt/wt) Ficoll 400 in medium II and centrifuged (100 × *g*, 15 min). Spherical protoplasts (60% of the crude protoplasts) banding at the 7.5/5% (wt/wt) Ficoll 400 interface were used for the isolation of vacuoles.

Vacuole preparation (a modification of the method given in ref. 6) was performed as follows. Purified protoplasts [5 ml of medium II/22% (wt/wt) Ficoll 400 containing 3 × 10^6 protoplasts per ml] were layered under a step gradient containing 2.5 ml of 17% Ficoll 400 in medium III (20 mM Mes/KOH, pH 6/0.3 M mannitol/25 mM KCl/1 mM Na_2EDTA/1 mM DL-dithiothreitol/0.5 mM $MgCl_2$), 9.5 ml of DEAE-dextran medium [20 mM Mes/KOH, pH 6/0.1 M mannitol/15% Ficoll 400/0.8% (wt/vol) DEAE-dextran 500,000/25 mM KCl/1 mM Na_2EDTA/1 mM DL-dithiothreitol/0.5 mM $MgCl_2$], 7.5 ml of dextran sulfate medium [20 mM Hepes/KOH, pH 7/0.3 M mannitol/5% Ficoll 400/0.4% (wt/vol) dextran sulfate 5000/1 mM Na_2EDTA/1 mM DL-dithiothreitol/0.5 mM $MgCl_2$], 6.5 ml of 2.5% (wt/wt) Ficoll 400 in medium IV (50 mM Hepes/KOH, pH 7/0.3 M mannitol/25 mM KCl/1 mM DL-dithiothreitol/0.5 mM $MgCl_2$), and 4 ml of medium IV. Centrifugation (22,500 × *g*, 60 min) was carried out in a Sorvall AH627 swinging-bucket rotor. Four milliliters of vacuole suspension was harvested from the top of the gradient.

The yield of vacuoles was 7.5%, based on the number of protoplasts layered under the gradient. The viability of each preparation was checked by monitoring ATP-dependent H^+-translocation into the vacuoles. For this purpose, 0.5 ml of vacuole suspension was diluted 1:2 with medium IV; after addition of 10–20 μl of 3 mM acridine orange, the absorption at 490 nm was monitored before and after addition of 10 μl of 100 mM ATP (disodium salt; Merck, Darmstadt, F.R.G.) in 100 mM $MgCl_2$/7 mM Hepes, pH 6.5, in a photometer (Gilford Response; Corning Medical, Giessen, F.R.G.).

The patch-clamp technique was employed in the "whole-cell-attached" mode as described (7, 8). During the measurement, the vacuoles were bathed in 500 μl of medium IV with 0.1 mM NaCl added. The electrodes were filled with 30 μM EGTA in medium IV. Under these conditions, the electrodes had a resistance of 10–20 MΩ. ATP (Tris salt; Sigma, Munich) dissolved in equimolar $MgCl_2$ at pH 6 was added as a 50-fold concentrated stock solution.

All experiments shown were conducted with the voltage clamped to the given values and the current registered on a 4-track tape recorder. For current vs. potential (I–V) curves, only the stationary current more than 10 sec after the last change in the clamp voltage was considered. For analysis of

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the current fluctuations, the recorded data were low-pass filtered at 400 Hz, digitized by a Nicolett 1170 digital oscilloscope, and evaluated with a Hewlett-Packard 9830A calculator.

RESULTS AND DISCUSSION

Vacuoles isolated from photoautotrophic suspension cells of *C. rubrum* show a strong ATP-generated H^+ translocation across the tonoplast (Fig. 1). Within 20 min the vacuolar pH has fallen two pH units to pH 5.0. This pH has been calculated from the final absorbance loss (ΔA_{490}) of 0.51 units, using a recently developed quantitative formalism, which takes into account intra- and extravacuolar volumes, as well as the pH- and concentration-dependent equilibria between the different acridine orange species (3). Five different samples, with vacuolar volumes between 0.09 and 0.35 μl and ΔA_{490} between 0.11 and 0.51 units, gave an ATP-generated mean vacuolar pH of 5.0 ± 0.1.

Fig. 2 shows that the patch-clamp technique (7) is well applicable to an isolated vacuole of *C. rubrum*: a "gigaseal" of >50 GΩ is formed after the vacuole has been sealed to the pipette (*Inset*, trace a). The establishment of the open connection between the interior of the patch pipette and the vacuole ["whole-cell-attached" recording mode (7)] is indicated by appearance of the current spikes in trace b. These spikes are due to current flow to and from the membrane capacitance. We have routinely analyzed the current signals of each vacuole, using the usual analysis (7); the given vacuole (25 μm diameter) displayed a membrane capacitance of 7 mF/m^2. The current–voltage curve of Fig. 2 is linear and passes almost through the origin (zero membrane potential). Since the tonoplast faces media with identical K^+ concentrations (46 mM; i.e., the *in vivo* concentration), the linear current–voltage curve passing near the origin is consistent with K^+ diffusion governing the electrical properties of the isolated, nonenergized vacuole. Fig. 2 yields a slope conductance of 1.5 nS, representing a specific conductance of 0.8 S/m^2 for this vacuole of 25 μm diameter. This is a typical value; the observed range of 0.8 to 1.2 S/m^2 resembles the range reported for the plasmalemma of plant cells (9), including *C. rubrum* (10).

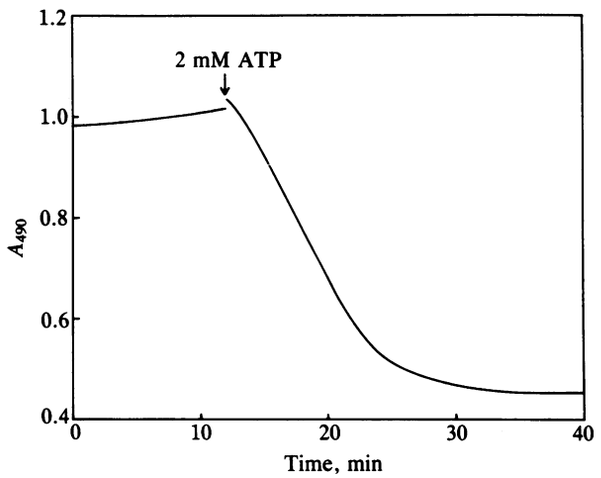

FIG. 1. Kinetics of ΔpH formation across the tonoplast of isolated vacuoles from *C. rubrum* suspension cells upon addition of 2 mM ATP. The absorbance loss at 490 nm indicates the pH-dependent accumulation and polymerization of acridine orange in the vacuole. The 1-ml sample, with a vacuolar volume of 0.35 μl, contained 60 μM acridine orange at pH 7.0. See text for calculation of vacuolar pH.

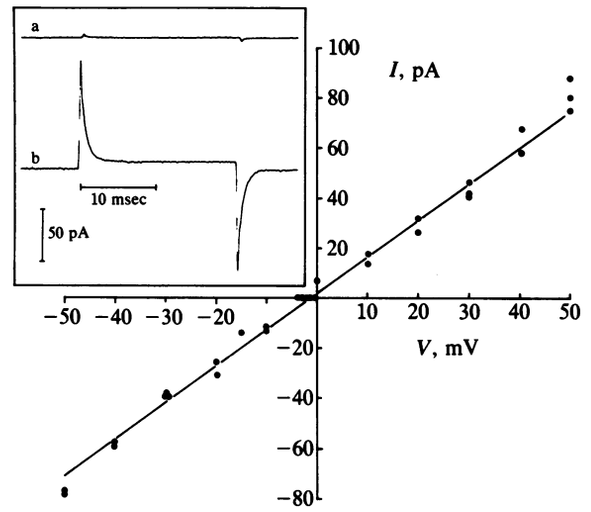

FIG. 2. Current–voltage diagram from a *Chenopodium* vacuole attached to a patch-clamp pipette. The membrane current (*I*) was recorded after the membrane potential (*V*) had been clamped to the abscissa values for at least 10 sec. (*Inset*) Original current traces to 5-mV voltage-clamp pulses. Trace a: electrode attached to the tonoplast (seal resistance > 50 GΩ). Trace b: after whole-cell-attached recording mode was established by removing the membrane patch between the pipette and the vacuolar interior by suction.

Addition of ATP to an isolated *Chenopodium* vacuole monitored by the patch-clamp apparatus hyperpolarizes the tonoplast to a stationary membrane potential of about 15 mV (vacuolar interior positive; Fig. 3). This finding confirms more or less satisfactory voltage data of impaled microelectrodes, obtained from giant algal cells (11) and higher plant cells (12). In general, therefore, the tonoplast seems not to develop a potential as substantial as is known from the plasmalemma.

The findings illustrated in Figs. 1 and 3 strongly suggest the existence of a proton-pumping ATPase in the tonoplast. In both experiments, addition of the uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) dissipated the ATP-generated signal (data not shown). The difference between the curves in Fig. 3 constitutes a constant current of 20 pA generated by ATP. A constant current source is the mode

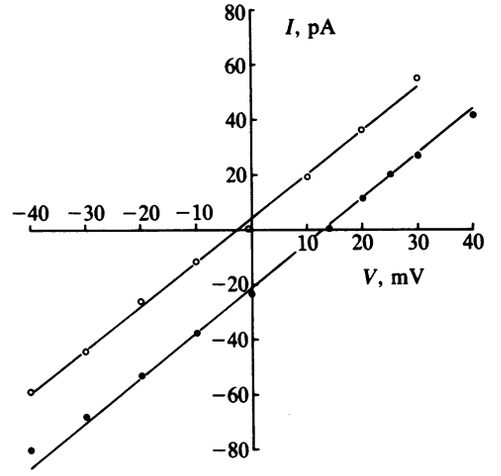

FIG. 3. Current–voltage diagram before (○) and 15 min after addition of 2 mM ATP (●) to the medium bathing the isolated vacuole. Points represent mean values from 47 single recordings (compare with Fig. 2).

of operation suggested for the proton-pumping ATPase in the plasmalemma of some plants analyzed previously (9), as well as for proton pumping in a tonoplast-containing membrane-vesicle preparation from *C. rubrum* (3).

The membrane capacitance, emerging from the current-pulse analysis mentioned above, also is ATP-dependent. Fig. 4 yields a value of 13.5 pF after addition of ATP, whereas the mean value before addition of ATP and after addition of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, and from other vacuoles, was about 7.5 pF. The ATP-generated capacitance increase could indicate a more compressed hydrophobic membrane core resulting from increased charge separation across the membrane (13), as well as from the pH-dependent protonation of prevalent negative surface charges of the inner membrane face. Alternatively this capacitance increase could be due to generation of mobile charges inside the membrane (14); however, the measured increase correlates with the intravacuolar acidification, whereas the mobile charges reported in ref. 14 decline with more acidic pH.

Finally, we have analyzed the recorded membrane current fluctuations (Fig. 5) with respect to single-channel conductance (15). Whereas a patch-clamp study on a plant membrane, the plasmalemma of *Vicia* guard cells (16), revealed a K^+ channel with a single conductance around 30 pS (at a K^+ concentration of 225 mM), our analysis yielded a value of 0.8 pS per closing probability for an as yet unidentified channel.

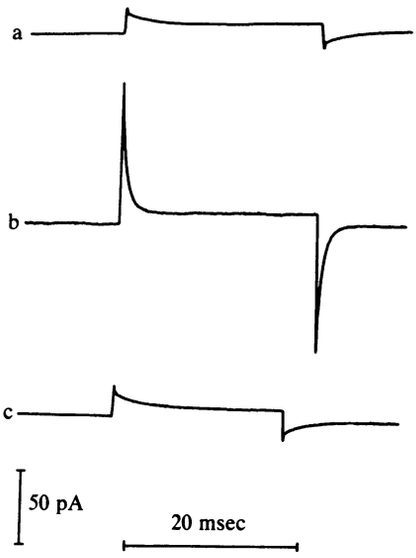

FIG. 4. Current response of the tonoplast of a *Chenopodium* vacuole to a 20-msec voltage-clamp pulse of 5 mV (average from 16 sweeps each). Trace a: zero current (baseline) of the membrane potential was 0 mV, membrane input resistance (R_m) = 440 MΩ, and membrane capacitance (C_m) = 7.7 pF. Trace b: 12 min after addition of 2 mM MgATP (13 min after trace a); zero current at +15 mV, R_m = 610 MΩ, and C_m = 13.5 pF. Trace c: 20 min after addition of 32 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (50 min after trace a); zero current at +3 mV, R_m = 530 MΩ, and C_m = 7.6 pF.

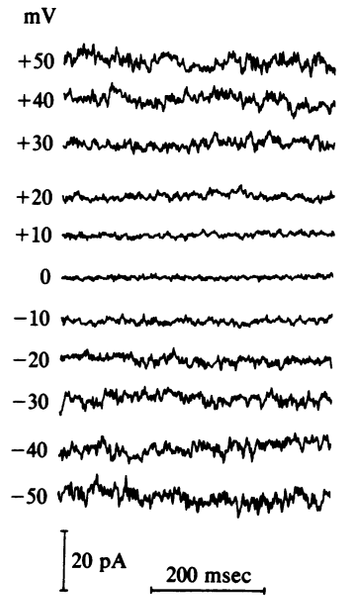

FIG. 5. Clamp current recorded from the tonoplast of a *Chenopodium* vacuole. Numbers at left denote the voltages to which the tonoplast was successively clamped.

On the other hand, the mean channel-opening time, 4–9 msec, from the analysis of the current fluctuations (Fig. 5) agrees with the values of 7–10 msec obtained for the K^+ channel in the plasmalemma of *Vicia* (16). However, since the current fluctuations observed for the tonoplast of *Chenopodium* turned out not to be ATP-dependent, they do not reflect the activity of the proton-pumping ATPase.

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