A Patch Clamp Study of Tonoplast Electrical Properties in Vacuoles Isolated from *Chenopodium rubrum* Suspension Cells

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The patch clamp technique has been applied to isolated vacuoles from green suspension cells of *Chenopodium rubrum* to record electrical parameters of the tonoplast. In a symmetrical K^+ solution of 46 mM, the membrane displays a near zero voltage, whereas 2 mM ATP will hyperpolarize it to 15 or 20 mV (vacuole positive). The conductance amounts to about one $S \cdot m^{-2}$. Fluctuations of the clamp current are explained by an unknown channel species having opening times of 5–10 ms. Together with previous work on a tonoplast vesicle preparation and unpublished data on vacuoles from our laboratory, the present results indicate an electrogenic membrane ATPase pumping protons from the cytoplasm to the vacuole.

Introduction

Presently transport processes across the tonoplast membrane are intensively studied, particularly whether energy is expended by a membrane ATPase pumping protons from the cytoplasm into the vacuole. Since direct evidence for an electrogenic proton pump, as available through standard electrophysiology with impaled microelectrodes for the plasmalemma [1], is hardly feasible, we have applied the "whole cell"-recording mode of the patch clamp technique [2] to isolated vacuoles from green suspension cells of *Chenopodium rubrum*. We present data on membrane potential and conductance of the tonoplast, including the activity of an ATPase.

Abbreviations: BSA, bovine serum albumin; C_m, membrane capacity [F]; DEAE-, diethylaminoethyl-; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether) N,N,N′,N′,diaminetetraacetic acid; FCCP, *p*-trifluoromethoxycarbonyl cyanid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; I, current [A]; MES, 2-(N-morpholino)ethanesulfonic acid; R_{ser}, serial resistance [Ω]; R_M, membrane resistance [Ω]; Tris, tris(hydroxymethyl)aminoethane; *U*, electrical potential [V].

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Materials and Methods

Cell culture

Photoautotrophic and phytohormone independent suspension cells derived from hypocotyl cells of *Chenopodium rubrum* L. [3] are cultured as described in [4].

Isolation of protoplasts

For protoplast isolation cells from the exponential growth phase were used (for the growth pattern of the culture see [5]); cells from 6 days old cultures were harvested from the suspension by filtering through a 15 μm nylon net (Thoma GmbH, Mössingen, FRG); 15 g cells (fresh weight) were suspended in 50 ml medium I (20 mM MES/KOH, pH 5.3, 0.3 M mannitol, 2 mM $CaCl_2$, 10 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 0.5% BSA); 50 ml medium I with 2.5 g cellulase TC from *Trichoderma reesi* (Serva) and 2.5 g pectinase 5S from *Aspergillus niger* (Serva) were added to the cell suspension and incubated on a gyratory shaker (120 rpm); after 90 min, there was no more Calcofluor white-staining [6] detectable at the cell surface (Calcofluor white ST-solution was a gift from Dr. U. Seitz, Tübingen). Protoplasts were harvested by centrifugation with $100 \times g$, 15 min, and washed twice with medium II (20 mM MES/KOH, pH 6, 0.3 M mannitol, 25 mM KCl, 1 mM DTT, 0.5 mM $MgCl_2$, 0.1% BSA); yield of protoplasts was 81%.

For further purification, the protoplasts in medium II were loaded on a step gradient with 10, 7.5, 5, 2.5% (w/w) Ficoll in medium II and centrifugated for 15 min with $100 \times g$. Spherical protoplasts (60% of the crude protoplasts) banding at the 7.5 to 5% (w/w) Ficoll interface were used for the isolation of vacuoles.

Preparation of vacuoles

Vacuole preparation (a modification of the method given in [7]) was performed as follows: 5 ml purified protoplasts in medium II with 22% (w/w) Ficoll ($3 \cdot 10^6$ protoplasts/ml) were layered under a step gradient containing 2.5 ml 17% (w/w) Ficoll in medium III (20 mM MES/KOH, pH 6, 0.3 M mannitol, 25 mM KCl, 1 mM Na_2EDTA, 1 mM DTT, 0.5 mM $MgCl_2$), 9.5 ml DEAE-Dextran-medium (20 mM MES/KOH, pH 6, 0.1 M mannitol, 15% (w/w) Ficoll, 0.8% (w/v) DEAE-Dextran 500000, 25 mM KCl, 1 mM Na_2EDTA, 1 mM DTT, 0.5 mM $MgCl_2$), 7.5 ml Dextransulfate-medium (20 mM HEPES/KOH, pH 7, 0.3 M mannitol, 5% (w/w) Ficoll, 0.4% (w/v) Dextransulfate 5000, 1 mM Na_2EDTA, 1 mM DTT, 0.5 mM $MgCl_2$), 6.5 ml 2.5% (w/w) Ficoll in medium IV (50 mM HEPES/KOH, pH 7, 0.3 M mannitol, 25 mM KCl, 1 mM DTT, 0.5 mM $MgCl_2$), and 4 ml medium IV; centrifugation ($22500 \times g$, 60 min) was carried out in a Sorvall AH 627-swinging bucket rotor. 4 ml vacuole suspension were harvested from 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 vacuole suspension were diluted 1:2 with medium IV; after addition of 10–20 µl 3 mM acridine orange the absorption at 490 nm was monitored before and after addition of 10 µl 100 mM ATP (disodium salt; Merck, Darmstadt, FRG) in 100 mM $MgCl_2$, 7 mM HEPES (pH 6.5) in a photometer (Gilford Response; Corning, Gießen, FRG). The vacuoles used for further experiments showed an ATP-dependent intravacuolar acidification of more than two pH-units as calculated from the decrease in absorbance at 490 nm with the formalism given in [4] and the pertinent parameters.

Electrophysiological experiments

The patch clamp technique was employed in the "whole cell-attached"-mode as described in [2] and [8]. During the measurement the vacuoles were bathed in 50 µl medium IV with 0.1 mM NaCl added. If not stated otherwise, the electrodes were filled with 30 µM EGTA in medium IV. Under these conditions the employed electrodes had a resistance of 10 to 20 mΩ. ATP (Tris salt; Sigma, München, FRG) solved in equimolar $MgCl_2$, pH 6, and FCCP in ethanol were added as 50-fold concentrated stock solutions.

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

As the current signal ($\Delta I_{(t)}$) to a voltage jump (ΔU) at $t = 0$ for a resistance R_M with parallel capacity C_M and serial resistance R_{Ser} is given by:

$$\Delta I_{(t)} = \frac{\Delta U}{R_M + R_{Ser}} \left(1 + \frac{R_M}{R_{Ser}} \exp(-t/\tau)\right)$$

$$\text{with } \tau = \frac{R_{Ser}\, R_M\, C_M}{R_M + R_{Ser}}$$

and thus $\Delta I_{(t \to 0)} = \Delta U / R_{Ser}$ and $\Delta I_{(t = \infty)} = \Delta U/(R_M + R_{Ser})$; R_{Ser} was calculated from the initial current peak, ($R_M + R_{Ser}$) from the steady state current and C_M from the resistances and τ, neglecting the electrode capacity and the seal resistance; the latter was in the range of 10 to 20 GΩ (as compared to $R_M + R_{Ser}$ in the range of 200 to 1000 MΩ. The thus calculated values differed only slightly from the capacity calculated from the integral of the initial current overshoot and the serial resistance calculated from τ and C_M. For noise analysis the recorded data were low-pass filtered at 400 Hz, digitized by a Nicolett 1170 digital oscilloscope and evaluated with an HP-9830A-calculator.

Results

Immediately after the establishment of the open connection between vacuole and patch electrode interior a "transmembrane potential" of −15 mV was measured. During the first minutes this potential declined to zero corresponding to the exchange of electrode filling and vacuolar sap. This decrease in potential was accompanied by an increase in the serial resistance (Fig. 1). The capacity of the non-energetized vacuole was about 5 $mF \cdot m^{-2}$.

If the patch pipette was filled with 1 mM K^+ instead of 46 mM K^+, the stable transmembrane potential was 15 to 20 mV (inside positive). As Fig. 2 shows, the current-voltage-relationship of the tonoplast was linear in the range from −50 to +50 mV, and indicated a conductance range of 0.88 to 1.18 $S \cdot m^{-2}$ with different vacuoles. Fig. 2 also demonstrates that addition of ATP did not change the slope of the current-voltage-characteristics; however the zero-current potential was shifted from 0 to 17 mV (vacuole positive), indicating a tonoplast hyperpolarization. This hyperpolarization was accompanied by decrease of the serial resistance and an increase of the membrane capacity to 8 $mF \cdot m^{-2}$. After addition of FCCP (32 μM) all of these MgATP-dependent effects were reversed (Figs. 1 and 3).

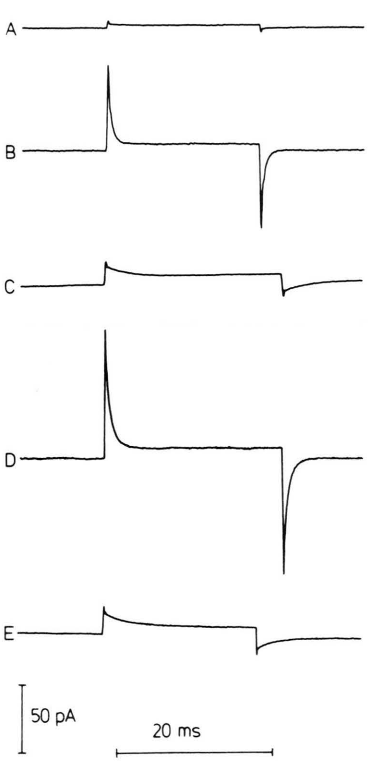

Fig. 1. Current response of a *Chenopodium* vacuole (23 μm diameter) to 5 mV voltage clamp pulses. The displayed current traces are averaged from 16 sweeps each. A. Electrode attached to the tonoplast with a seal resistance of 15.9 GΩ. B. Immediately after connection between electrode- and vacuole-interior has been established by suction; zero current (base line) at −14 mV; R_{Ser} = 71 MΩ, R_M = 884 MΩ, C_M = 7 pF. C. 13 min after B; zero current was at ± 0 mV; R_{Ser} = 278 MΩ, R_M = 438 MΩ, C_M = 7.7 pF. D. 13 min after addition of 2 mM MgATP (25 min after B); zero current at +15 mV; R_{Ser} = 52 MΩ, R_M = 611 MΩ, C_M = 13.5 pF. E. 20 min after addition of 32 μM FCCP (63 min after B); zero current at +3 mV; R_{Ser} = 267 MΩ, R_M = 527 MΩ, C_M = 7.6 pF.

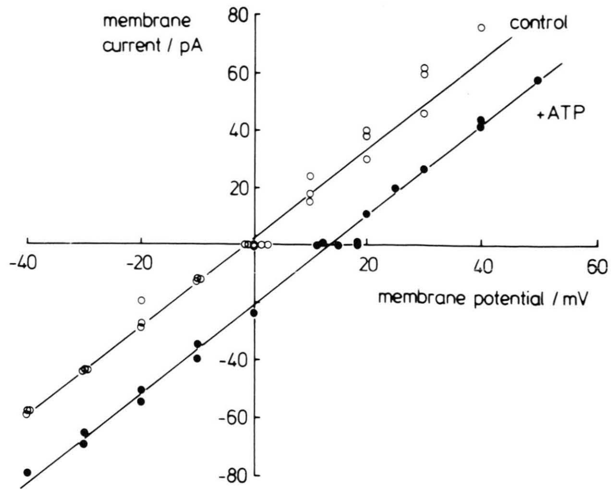

Fig. 2. Membrane current *vs* membrane voltage clamped to the abscissa values before (○) and after (●) addition of 2 mM MgATP to an isolated vacuole of *Chenopodium rubrum*.

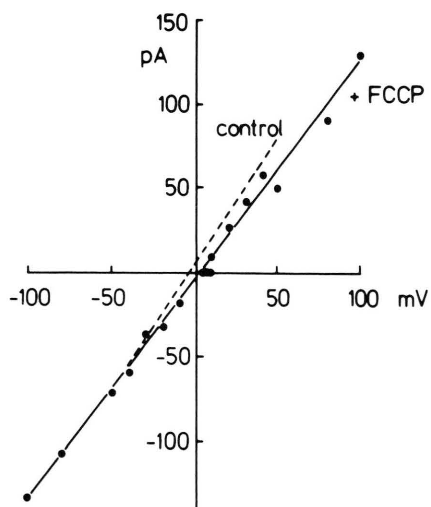

Fig. 3. *U*/*I*-curve after addition of 32 μM FCCP. The dashed line indicates the control curve from Fig. 3. Note the broader voltage range compared to Fig. 2.

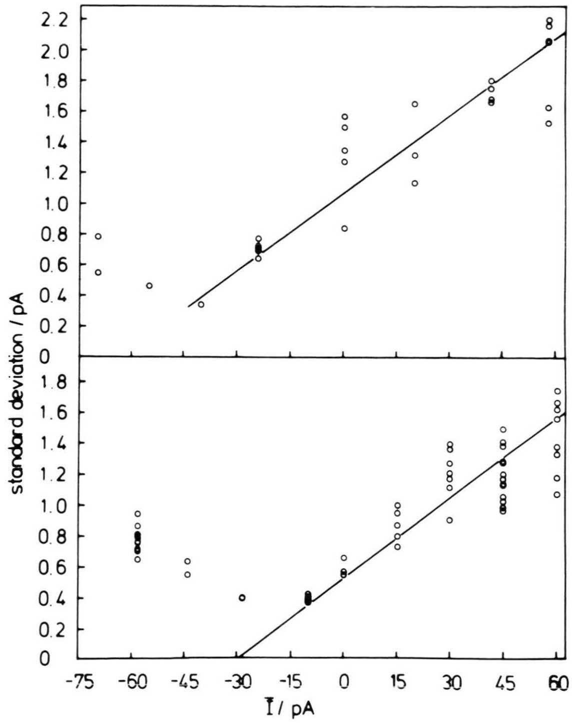

Fig. 4. Standard deviation of the current fluctuations as function of the mean clamp current. Each point resulted from the analysis of one second; sampling rate = 1/ms. The slope of the given lines is the one expected if the individual open channels are ohmic resistors with a conductivity of 0.45 pS/closing probability [9] and the conductivity of the tonoplast as measured (1.5 nS). Upper graph: 2 mM ATP added.

It has not yet been possible to record single channel events from tonoplast patches. However, our whole cell recordings shows very distinct fluctuations of the clamp current necessary to maintain a set transmembrane voltage. The smallest fluctuations appeared not at the zero-current voltage, but at about −10 to −20 mV corresponding to −15 to −30 pA; upon addition of ATP this minimum was shifted to even more negative values (Fig. 4).

Discussion

While the patch clamp technique has been applied already to the plasmalemma of a plant cell [10], this paper communicates the first study on the tonoplast. Isolated vacuoles are obviously well suited for this technique. The membrane potential of the ATP-supplied, *i.e.* normally energized, tonoplast of about 15 to 20 mV (vacuole positive), as indicated by the zero-current voltage of the ATP curve in Fig. 2, is in accord with numerous estimates from microelectrode impalements of whole cells. The tonoplast slope conductance is linear, as expected from a K^+ permeable membrane facing identical K^+ solutions (46 mM); its magnitude of one Sm^{-2} is in the same order as of the intact *Chenopodium* suspension cell [11].

The ATP-generated tonoplast hyperpolarization (Fig. 2) and, from our unpublished data, ATP-generated acidification of isolated vacuoles strongly suggests the existence of a proton pumping membrane ATPase. Its dissipation by the protonophore FCCP (Fig. 3) is consistent with this notion. More interestingly, the difference between the two curves in Fig. 2 constitutes a constant current (about 20 pA), and nicely confirms the conclusion of our previous study on proton pumping in a tonoplast containing vesicle preparation [4]. Previously, this constant current mode has been found with plasmalemma proton pumps [1].

Finally, we analyzed the current fluctuations in terms of single channel conductance [9]. The relationship between the standard deviation of the current fluctuations and the mean clamp current can be explained by a single channel conductance of about 0.5 pS (provided the channel closing probability is assumed to be one). Whereas this value is considerable smaller than 30 pS, reported for the K^+ channel in the plasmalemma of *Vicia* guard cells [10], the mean channel opening times of 5−10 ms emerging from our analysis of the covariance function and 7−10 ms from the K^+ channel in *Vicia* fairly agree. We have not yet identified the channel(s) in *Chenopodium;* however, the ATPase is no likely candidate, as shown by the similarity of the curves in Fig. 4.

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