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Communication

Electrical Noise Measurements on Red Beet Vacuoles[1]

ANOTHER WAY TO DETECT THE ATPase ACTIVITY

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ABSTRACT

The vacuolar potential (V_{vac}) and its fluctuations were recorded in red beet vacuoles (*Beta vulgaris* L.). Measurements with vacuoles in their suspension medium gave $V_{vac} = 10 \pm 2$ millivolts (referred to the external medium) when 3 molar KCl microelectrodes were used. Buffering the microelectrode filling solution at pH 7.7 reversed the sign of the potential: $V_{vac} = -7 \pm 2$ millivolts. The magnitude of the potential fluctuations was lowered by dilution (5–1000 times) with the suspension medium containing components released by the cells during the mechanical preparation. Fluctuations were decreased by 50 millimolar KNO_3 while they were enhanced by 5 millimolar ATP-Mg. No noticeable change in membrane resistance was detected. The presence of an ATPase bound to the tonoplast may explain the recorded noise spectra. These spectra imply a close connection between the rate of ATPase functioning and the magnitude of ionic fluxes across the tonoplast. It is suggested that noise analysis could be used to detect ATPase (or related enzyme) activity in vacuoles. Possible use of H^+ diffusion through a buffered microelectrode, to modify intravacuolar pH, is also suggested.

The existence of a vacuolar ATPase has now been proved in several cases (6, 7, 9, 15) and especially in the red beet vacuole (1, 9). ATPase activity has been measured in isolated vacuoles or in tonoplast vesicles. The rate of hydrolysis by the ATPase was shown to be dependent on the transport of ionic species through the tonoplast. Gramicidin, by increasing the tonoplast permeability to cations, increased the rate of ATPase hydrolysis in vacuoles (4). If enzymic activity produces variations in ionic fluxes through a membrane, it should be possible, at least in principle, to detect these variations by electrical measurements, using noise analysis or the patch-clamp technique (12, 16). Applications of the latter to vacuoles are still rare, because of experimental difficulties (10, 13). It is easier to record the intravacuolar potential and its fluctuations using a microelectrode. However, the interpretation of the data remains difficult as it will depend on the overall properties of the vacuole (tonoplast and noncontrolled internal medium). It is not easy to analyze potential variations in response to changes in the external medium because changes in V_{vac}[2] may indicate either a charge redistribution of ions reaching a new equilibrium, or a real variation of ionic fluxes. In the red beet vacuoles (*Beta vulgaris* L.) which we are using in our experiments, V_{vac} seems to be especially difficult to characterize (5). We expected that fluctuations of the potential would be a more sensitive indicator of changes in ionic fluxes; in a biological membrane important fluctuations are generated by the 'chopping action' of channels on ionic fluxes and the magnitude of these fluctuations is controlled by ionic gradients through this membrane (16). Although the recorded noise spectra of the red beet vacuole remain complex, this report indicates that (a) reproducible spectra may be obtained and (b) significant modifications in these spectra allow us to detect the effects of metabolic effectors on the ATPase.

MATERIALS AND METHODS

Red beet storage roots were purchased commercially. They were stored for up to 3 months at 5°C. Vacuoles were prepared by mechanical slicing of the beet roots according to the procedure of Leigh and Branton (8) in a slightly modified medium (1 M Sorbitol, 50 mM Tris [pH 7.7], 5 mM EDTA). Various media were used in the experiments. A given volume of the preparation medium, including a few selected vacuoles, was diluted in one of these media. Medium A is 1 M Sorbitol, 20 mM KCl, 50 mM Tris (pH 7.7); medium B: 1 M Sorbitol, 5 mM KCl, 50 mM KNO_3, 50 mM Tris (pH 7.7); medium C: 1 M Sorbitol, 50 mM KCl, 5 mM ATP Mg, 50 mM Tris (pH 7.7). We used micromanipulators (2) to transfer a given sample of vacuoles with their preparation medium, in controlled media A, B, or C. In this way the preparation medium was diluted 5 or 1000 times, depending on the experiment. In a first set of experiments, V_{vac} (referred to the external medium) was recorded with 3 M KCl microelectrodes. In other experiments we used microelectrodes filled with 0.6 M KCl, 0.6 mM Sorbitol, 50 mM Tris (pH 7.7). Details concerning the measurement of $S_I(f)$ have already been given elsewhere (2). Two sets of experiments were used to compare $S_I(f)$: (a) in a first set of experiments, the magnitude of the spontaneous fluctuations of V_{vac} was recorded in the frequency range 0.1 to 3000 Hz; (b) in a second set of experiments, a small current (square wave) was injected through the microelectrode and Fourier analysis of the response to this signal allowed computation of the modulus of the small signal impedance of the membrane-electrode system. These experiments give $S_I(f)$ in three bands of frequencies (0.1–10 Hz; 1–100 Hz; 10–1000 Hz). In each band 17 samples, each containing 1024 values in the time domain, are used for computation. The vacuolar resistance was estimated by the difference in the low frequency value of the impedance recorded inside the vacuole and its value outside. A settling time of up to 20 min was often necessary to reach steady state values for the potential.

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[2] Abbreviations: V_{vac}, vacuolar potential; $S_I(f)$, fluctuation intensity spectra.

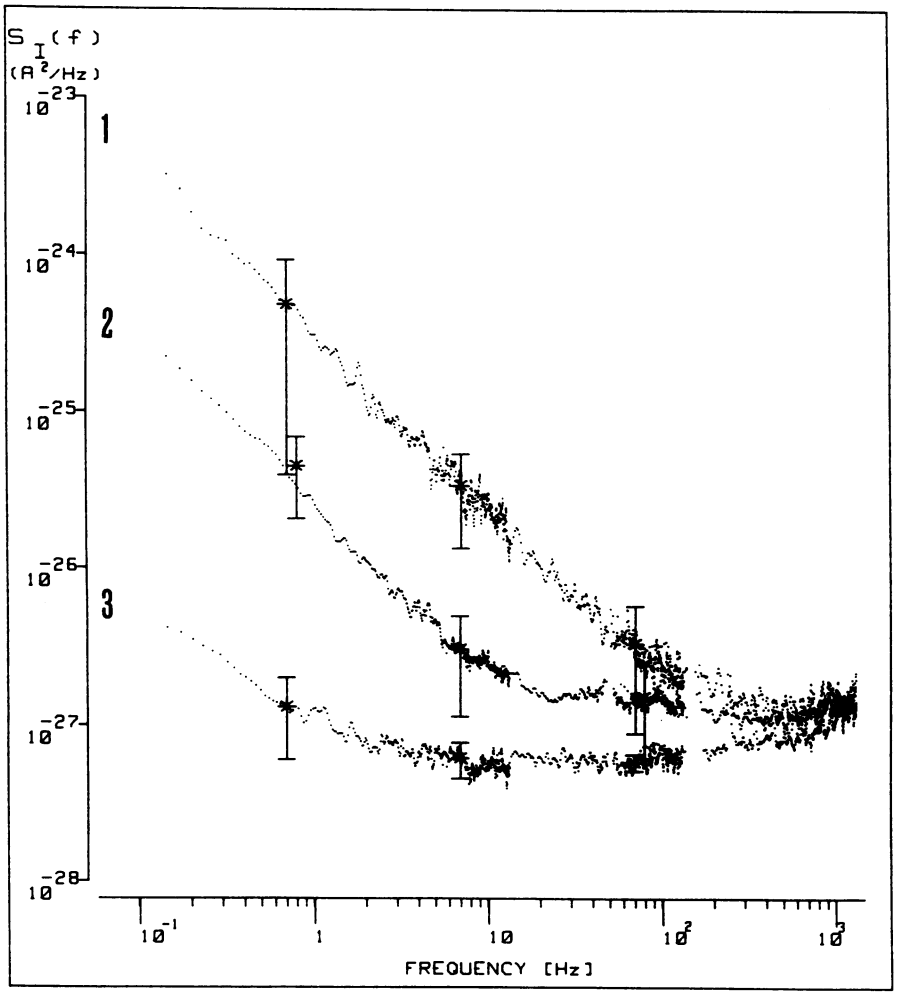

FIG. 1. Fluctuation intensity spectra $S_I(f)$ of the vacuolar current as a function of frequency. Effect of diluting the preparation medium on the spectra. 1, Preparation medium was diluted 5 times in medium A; $V_{vac} = 10 \pm 2$ mV (13) R $= 4.1 \pm 0.3$ KΩ cm² (13). 2, Preparation medium was diluted 1000 times in medium A; $V_{vac} = -7 \pm 2$ mV (5); R $= 4.6 \pm 0.9$ KΩ cm² (5). (Values given as mean ± SD). 3, Background noise of the electrode in the recording media for experiments 1 and 2. Microelectrodes were buffered at pH 7.7 except for the experiments illustrated by 1 (KCl 3 M only). In Figures 1 and 2 bars on the spectra represent the standard deviation at various frequencies (0.7, 7, 70 Hz); V_{vac}, vacuolar potential referred to the external medium; R, specific resistance of the tonoplast. Peaks in Figures 1 and 2 are artefacts due to external 50, 100, and 150 signals.

RESULTS AND DISCUSSION

Computing $S_I(f)$ usually implies experiments in which vacuoles are impaled by a microelectrode for several hours. Classical (3 M KCl) microelectrodes very often gave us (20 times out of 33 experiments) osmotic-like artefacts, sometimes visible (formation of a clear spot near the tip of the electrode), sometimes almost invisible but producing unexplained 'bumps' in the $S_I(f)$ spectra. However, recording of V_{vac} for a short period of time in medium A and estimation of membrane resistance by injection of a constant current through the electrode gave us values for V_{vac} (Fig. 1) similar to those obtained by previous workers (3). The average specific membrane resistance for a set of vacuoles ranging from 40 to 80 μm in diameter (optical estimation) was R $= 4.1 \pm 0.3$ KΩ cm² (13).

To avoid the above described 'osmotic' problems we used microelectrodes buffered for pH and osmolarity ("Materials and Methods"). Surprisingly enough, V_{vac} turned out to be negative, while the resistance did not change in any significant way (Fig. 1). We are still unable to propose a simple relation between the pH value inside the microelectrode and V_{vac}. However, when the pH buffer inside the electrode was suppressed V_{vac} regained positive values, although the gross ionic concentrations remained unchanged in the bathing medium of the vacuoles (Fig. 1). This change could be due to proton diffusion between the vacuole and the pipette, resulting in a change in the vacuolar pH (the mobility of H^+ ion in solution is about 10 times higher than the mobility of other small ions). More data are needed to verify this.

With nonbuffered pipettes and a five times dilution of the preparation medium in medium A we had to discard 20 out of 33 experiments because the spectra were obviously nonreproducible. Figure 1.1 shows one of the 13 remaining records which satisfied our criteria of stationarity and reproducibility. The other spectra in Figures 1 and 2 represent an average from *n* similar experiments (*n* is given in the Fig. legends). These experiments were performed with buffered microelectrodes. In these conditions, we did not have to reject any of our data and the standard errors on $S_I(f)$ were smaller.

In the absence of a plateau or corner frequency, spectra remain difficult to interpret in a quantitative way (2). If we ignore the well known artefactual peaks at 50, 100, and 150 Hz no characteristic spectrum occurs, even if we subtract in Figure 2 the NO_3-treated $S_I(f)$ from the ATP-treated one. This may indicate that different channels are involved in the conductance mechanism of the tonoplast. However, significant and reproducible modifications of these spectra were induced by parameters of biological interest. We know from the literature (*e.g.* 1, 4) that an ATPase inhibited by NO_3^- and activated by ATP-Mg exists in the tonoplast of the red beet vacuole. Spectra in Figure 2 show the effect of NO_3 and ATP-Mg on the noise spectra. They suggest that there is a strong dependency between the rate at which the ATPase functions and the magnitude of the ionic fluxes (*i.e.* the fluctuations) across the tonoplast. Fluctuations express in another way the fact already cited (4) that an increase in the cation permeability of the tonoplast increases the rate of hydrolysis by the ATPase. The effect of diluting (Fig. 1) the preparation medium was less expected. Since there is no source of energy in medium A we have to assume that energy brought by the vacuole

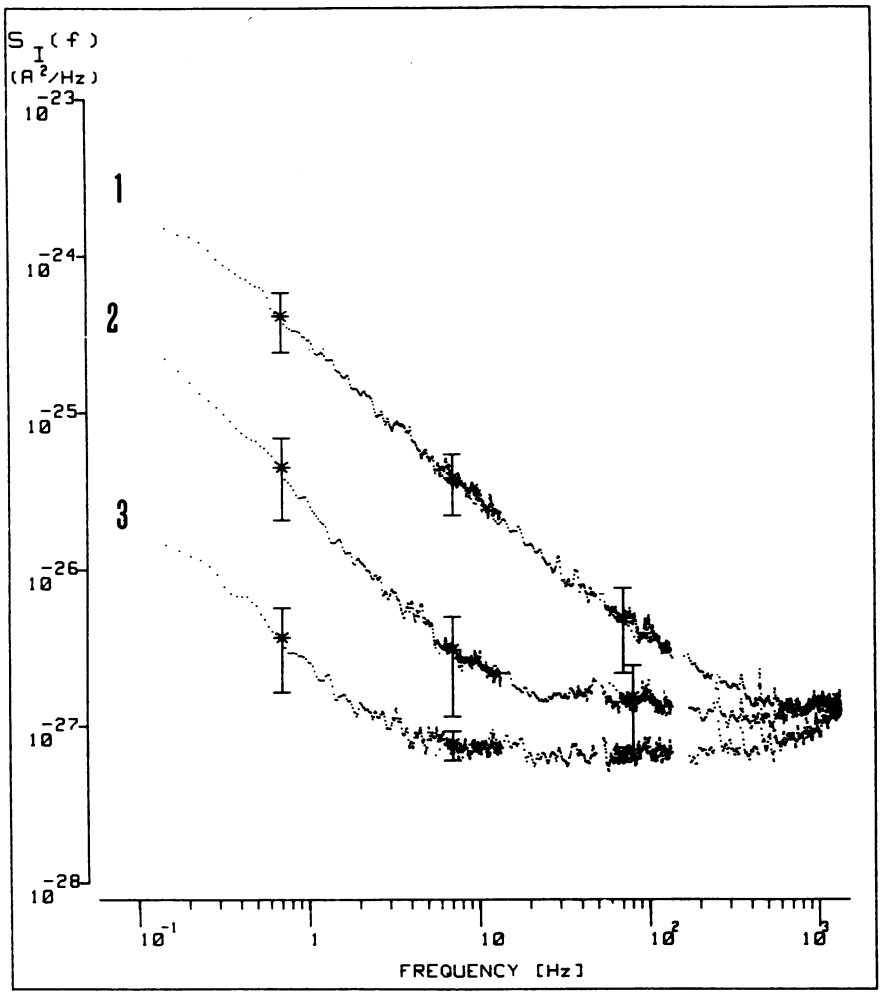

FIG. 2. Fluctuation intensity spectra $S_I(f)$ of the vacuolar current as a function of frequency. 1, In medium C: effect of 5 mM ATP-Mg; V_{vac}, -7 ± 1.8 mV (7); R, 4.5 ± 1 KΩ cm² (7). 2, Reference spectrum (see Fig. 1.2). 3, In medium B: effect of 50 mM KNO_3; V_{vac}, 0 ± 1 mV (3); R, 4 ± 0.7 KΩ cm² (3). (Values given as mean ± SD). For explanation of symbols see Figure 1.

and/or by the preparation medium is still available. Figure 2.3 confirms this interpretation since NO_3^- decreases the fluctuation level in vacuoles that were previously in medium A. ATP is not expected to remain active after 1 h in our media but it has been shown that tonoplast vacuole ATPases can use ADP or PPi as a substrate at a reasonable hydrolysis rate, compared to ATP, namely 30% and 14% (4). In our experiments only vacuoles in medium B (50 mM NO_3^-) give negligible fluctuation spectra and potentials. A null value for V_{vac} in this case can be expected from the equilibrium distribution of ions across the tonoplast. However, it seems difficult to interpret in a noncontroversial way our changes in V_{vac}: (a) there is a pH effect brought by our electrodes which is still difficult to quantify; (b) the recorded values of V_{vac} are low and we were unable to correlate in a precise way variations of V_{vac} with ionic changes in the external medium. In our estimation of R, the resistance of the tonoplast, we assumed that *R* was given by the limiting value of the impedance at low frequencies, assuming a constant RC network for the membrane. This may be an over simplification (11). Our resistance measurements did not show any significant changes in R with the various media used and with recorded V_{vac} varying from +10 mV to −7 mV. This confirms the fact that fluctuation changes must be induced by ionic changes in vacuolar concentrations rather than by permeability changes in the membrane. The results presented here, although mainly qualitative, may be interesting since they provide another way of looking at active transport in intact vacuoles. This approach is experimentally simple enough to allow testing of effectors assumed to activate or inhibit ionic transport through the tonoplast.

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