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A patch clamp study on the electro-permeabilization of higher plant cells: Supra-physiological voltages induce a high-conductance, K^+ selective state of the plasma membrane

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ABSTRACT

Permeabilization of biological membranes by pulsed electric fields ("electroporation") is frequently used as a tool in biotechnology. However, the electrical properties of cellular membranes at supra-physiological voltages are still a topic of intensive research efforts. Here, the patch clamp technique in the whole cell and the outside out configuration was employed to monitor current–voltage relations of protoplasts derived from the tobacco culture cell line "Bright yellow-2". Cells were exposed to a sequence of voltage pulses including supra-physiological voltages. A transition from a low-conductance (~0.1 nS/pF) to a high-conductance state (~5 nS/pF) was observed when the membrane was either hyperpolarized or depolarized beyond threshold values of around −250 to −300 mV and +200 to +250 mV, respectively. Current–voltage curves obtained with ramp protocols revealed that the electro-permeabilized membrane was 5–10 times more permeable to K^+ than to gluconate. The K^+ channel blocker tetraethylammonium (25 mM) did not affect currents elicited by 10 ms-pulses, suggesting that the electro-permeabilization was not caused by a non-physiological activation of K^+ channels. Supra-physiological voltage pulses even reduced "regular" K^+ channel activity, probably due to an increase of cytosolic Ca^{2+} that is known to inhibit outward-rectifying K^+ channels in Bright yellow-2 cells. Our data are consistent with a reversible formation of aqueous membrane pores at supra-physiological voltages.

1. Introduction

The (transient) permeabilization of the cellular membrane by exposure of cells to a pulsed electric field of high intensity, usually known as "electroporation," is frequently used in biotechnological applications, for instance, in genetic engineering as a tool for introducing DNA into target cells [1]. Electroporation was also successfully employed to optimize the release of cellular ingredients from plant tissues, e.g., sugars from sugarbeet [2], or to decontaminate waste water from bacteria [3,4]. Despite numerous experimental and theoretical studies, many aspects of "electroporation" are still poorly understood, including the physical nature and the molecular properties of the pores generated by membrane charging [1,5–8].

Usually, electroporation is studied by exposing a cell suspension to a homogeneous electric field generated by two external electrodes. Under these conditions, however, membrane charging is highly inhomogeneous. At the poles of a spherical cell, maximum deflections of the native membrane potential occur that are opposite in sign, depending on the orientation towards the cathode and the anode, respectively [9]. By contrast, the membrane potential remains unaffected at the cell equator. To study the electrical properties of cellular membranes at supra-physiological voltages, it would be most adequate to record trans-membrane currents for *homogeneous* charging of the membrane. This can be achieved conveniently by applying the patch clamp technique in the whole cell configuration [10]. However, surprisingly few attempts have been made so far to make use of the patch clamp technique in research on electroporation. O'Neil and Tung [11] used voltage ramps in the cell-attached configuration of the patch clamp technique to determine threshold potentials for membrane breakdown on cardiac tissue. Threshold potentials were highly variable, ranging from 0.6 to 1.1 V. Interpretation of the data was hampered by highly non-stationary conditions during voltage ramp application; moreover, the status of the cellular membrane in series with the patch, and the status of the seal (attachment of the membrane surface to the glass), were not well defined. Tovar and Tung [12,13] repeated these patch clamp experiments

Abbreviations: BSA, bovine serum albumin; BTP, Bis–Tris propane; BY-2, Bright yellow-2; EGTA, ethylene glycol tetraacetic acid; $E_{rev.}$, reversal potential; NTORK, *Nicotiana tabacum* outward rectifying K^+ channel; SD, standard deviation; SE, standard error; TEA, tetraethylammonium

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using rectangular pulses of 5 or 10 ms duration, but the same critical points with respect to the status of the seal and the cellular membrane apply to those studies. More recently, Pakhomov et al. [14,15] used the patch clamp technique to monitor effects of pulsed electric fields on membrane conductance, but these referred to membrane properties *after* pulse application.

So far, electroporation has been investigated with a medical background and a focus on mammalian cells, and much less work has been dedicated to plant cells, even though practical application of the technique also includes plant cells and tissues. In a series of early studies, Coster et al. [16,17] investigated the effect of supra-physiological voltages on Characeen cells with voltage and/or current clamp technique using intracellular electrodes. They observed a strong increase in membrane conductance to chloride (see also reference 18) when the membrane was hyperpolarized beyond a threshold potential (−300 to −400 mV, depending on the temperature). This phenomenon is known as the "punch-through" effect. Later, Coster and Zimmermann [19,20] found a strong reversible increase in membrane conductance in the giant marine alga *Valonia utricularis* when the membrane voltage exceeded a (temperature-dependent) "breakdown potential" of about +800 mV. So far, only few attempts have been made to study the effect of supra-physiological voltages on protoplasts derived from higher plant cells with the patch clamp technique. Meissner [21], using isolated plant vacuoles in the "vacuole attached" (analogous to the "cell attached") configuration, observed electroporation of the patch enclosed by the pipette tip when the membrane was charged beyond about −300 mV and +250 mV. At these voltages, the rest of the membrane remained intact; further charging led to a complete electroporation of the vacuole.

In this study, we made use of the patch clamp technique to analyze the response of the plasma membrane to homogeneous charging, including supra-physiological voltages. We used protoplasts that were prepared from the tobacco cell line "Bright yellow-2" (BY-2) by enzymatic digestion of the cell wall [9]. We chose this cell line because it is a widely used model system in plant cell biology [22]. Patch clamp experiments were performed in the whole cell and the outside out configurations to avoid limitations associated with working on cell attached patches (see above). Current–voltage curves of the plasma membrane were obtained from 10 ms-rectangular pulses in order to identify the threshold potential for membrane permeabilization. Moreover, the ion selectivity of the electro-permeabilized membrane was studied using fast voltage ramps.

2. Materials and methods

2.1. Cell cultivation and protoplast preparation

As a model system, protoplasts derived from the tobacco cell line "Bright yellow-2" (BY-2) were used. Cell cultivation and protoplast isolation were basically performed as described previously [9]. Briefly, cells were grown at 25 °C in a culture cabinet on 4.3 g/l Murashige–Skoog medium (basal salt mixture) complemented with 100 mg/l inositol, 30 g/l sucrose, 200 mg/l KH_2PO_4, 1 mg/l thiamin, 0.2 mg/l 2,4-dichlorophenoxyacetic acid, and solidified with 0.6% (wt./vol.) Phytagel™ (Sigma-Aldrich, Taufkirchen, Germany). The pH was adjusted to 5.8. Cells were sub-cultivated every 3 weeks.

Cells were harvested for protoplast preparation at the age of 18–20 days and transferred to an enzyme cocktail consisting of (% in wt./vol.) 1 cellulase, 0.1 pectolyase, 2 BSA, and 1 mM $CaCl_2$. Osmolality was adjusted to 480 mosmol/kg using mannitol, as verified with a vapor–pressure osmometer (VAPRO 5520; Wescor, Logan, UT, USA). After 10 h incubation at 28 °C in the dark, the suspension was diluted with washing solution containing 1 mM $CaCl_2$ and 480 mM mannitol and the suspension was centrifuged at 100*g* for 10 min (Heraeus Primo R centrifuge; Heraeus, Hanau, Germany); the sediment was re-suspended in washing solution and washed in the same way a second time. Subsequently, protoplasts were allowed to equilibrate for at least 3 h in the bath medium used for patch clamp experiments (see below) to which 10 mM glucose and 10 mM sucrose had been added. In some cases, protoplasts were additionally purified using a sucrose gradient as described by Flickinger et al. [9] before they were transferred to the bath medium.

2.2. Patch clamp procedure

For patch clamp experiments, a standard setup was used including an EPC-10 amplifier (HEKA, Lambrecht, Germany) and an inverted microscope (Axiovert 200 M; Zeiss, Oberkochen, Germany). Micropipettes fabricated from borosilicate glass capillaries (Kimble No. 34500 99; Gerresheimer, Rockwood, TN, USA) were pulled with a 2-step-procedure on a Narishige puller (PE21; Narishige, Tokyo, Japan) and filled with a solution containing (concentrations in mM) 120 K-gluconate, 10 EGTA, 2.68 $MgCl_2$, 3.91 $CaCl_2$, 2.13 Mg-ATP, 2 Mes, with free Ca^{2+} and Mg^{2+} concentrations being 0.1 μM and 2 mM, respectively, as calculated with the computer program "Calcium" [23]. Mannitol was added to a final osmolality of 472 mosmol kg^{-1}. The pH was adjusted to 7.2 with Bis–Tris–Propane (BTP). The bath medium was composed as follows (concentrations in mM): 30 K-gluconate, 5 $CaCl_2$, 2 $MgCl_2$, 10 Mes/BTP, pH 5.8. Although the composition of the solutions did not directly mimic physiological conditions, relevance of patch clamp data for the electroporation of intact cells was ensured by adjusting parameters like ion strength, pH, and free intracellular Ca^{2+} concentration adequately. Standard patch clamp procedures in the whole cell and outside out configuration were applied [10]. Data were recorded with a personal computer (Maxdata) using the PatchMaster v2 ×32 software (HEKA). To explore the electrical membrane properties at supra-physiological voltages, rectangular pulses of 10 ms length were administered with negative and positive voltages at an alternating sequence. Starting at the maximum of −1000/+1000 mV, the amplitude was successively decreased in 40 mV-steps. Alternatively, voltage ramp protocols were used, recording the current response of the plasma membrane. Low-pass filtering of the data at a cutoff frequency of 7.2 MHz was performed; the sampling frequency was adjusted to 66 MHz. In addition, K^+ channel activity was screened with 1.5 s-rectangular pulses ranging from −80 to +160 mV (2.7/10 MHz cutoff/sampling frequency). Liquid junction potentials were corrected for using the procedure of Neher [24].

2.3. Processing of patch clamp data

No attempt was made in whole cell experiments to compensate for cellular capacitance or series resistance (R_s) during data acquisition when 10 ms-pulses were applied. Instead of making use of the "R_s compensation" function of the "PatchMaster" software, the effective voltage drop across the cellular membrane ($V_M(t)$) was estimated *ex post* from the change in command voltage (ΔV_{comm}), the amplitude of the capacitive spike induced by the rectangular voltage drop (I_0), and the current amplitude at the end of the pulse ($I(t)$) according to

$$V_M(t) = V_{comm} - (I(t) / I_0) * \Delta V_{comm} \quad (1)$$

I_o was extrapolated by fitting the capacitive current relaxation with an exponential function:

$$I_{cap}(t) = I_0 * \exp(-t / \tau) \quad (2)$$

A voltage step of several hundred millivolts usually induced a sudden drop in membrane resistance (see below). In the majority of the experiments, the membrane resistance (R_M) roughly equalled the access resistance (R_S) under these conditions; as a consequence, R_M and R_S that are connected in series acted as a voltage divider, and only ~50% of the command voltage dropped across the cellular membrane.