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Intracellular microelectrode membrane potential measurements in tobacco cell-suspension protoplasts and barley aleurone protoplasts: interpretation and artifacts

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Abstract

Intracellular microelectrode measurements in plant cell protoplasts have been widely used to study hormone signal transduction processes. However, the interpretation and reliability of such measurements are largely dependent on a detailed evaluation of the measurement conditions, as investigated in the present paper. Upon microelectrode penetration of tobacco cell suspension protoplasts and of barley aleurone protoplasts a fast negative going impalement-induced potential transient of less than a few ms duration could be observed. After reaching a steady-state potential at the ms time scale the measured potential hyperpolarized again and, in most cases, subsequently depolarized to a new steady-state value. Analysis of the electrical equivalent circuit of the measurement configuration showed that the occurrence of the impalement-induced potential transient indicates that these measurements suffer from a microelectrode-induced shunt resistance which loads the measurement. In addition, it is shown that the peak-value of the potential transient is the most reliable indicator of the true membrane potential and of true membrane potential changes of the protoplast, since this value is rather membrane resistance independent. For correct interpretation of steady-state measurements of membrane potential and stimulus-induced membrane potential changes data on membrane and shunt resistance are essential. As an example of the measurement of membrane potential changes the effects of 1-NAA on measured potential values in tobacco protoplasts and the effect of extracellular pH changes on barley aleurone protoplasts are analyzed with regard to the above described conclusions.

Key words: Auxin; Membrane potential; Microelectrode; (*N. tabacum*); (*H. vulgare*)

1. Introduction

The plasma membrane potential and plasma membrane potential changes may play an important regulatory role in different cellular processes and in the functioning of plant cells. In addition, the membrane potential can be seen as an indicator for ion fluxes across the plasma membrane, and can be used to assay the sensitivity of cells for compounds affecting the electrophysiological properties of the membrane, such as plant hormones (e.g., Refs. [1–4]).

In general the membrane potential of plant cells in a tissue can be relatively easily measured with intra-

Abbreviations: C_e, microelectrode capacitance (after capacitance compensation); C_m, membrane capacitance; E_d, diffusion potential across the ion unselective microelectrode-induced shunt; E_m, true membrane potential across undisturbed plasma membrane; E_{max}, most negative potential value reached after E_p; E_p, most negative value of the microelectrode-induced potential transient; E_s, initial steady-state potential value reached just after E_p; E_{ss}, final steady-state potential value reached during the measurement; R_e, microelectrode resistance; R_i, input resistance of impaled protoplast; R_m, membrane resistance; R_s, microelectrode-induced shunt resistance; V_e, potential value measured by microelectrode; V_m, shunted membrane potential.

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cellular microelectrodes. However, since many experimental approaches require the use of a protoplast system, e.g., when antibodies against plasma membrane proteins are used, it has been shown useful to perform membrane potential measurements in plant cell protoplasts [1,2,5–7]. Since membrane potential values of around −50 mV for tobacco suspension cells and protoplasts have been reported [4,8], the relatively small potential values (in the order of 0 to −10 mV), reported more recently for tobacco protoplasts with the use of intracellular microelectrodes (e.g., Refs. [1,2,5, 7]), may cause some scepticism about the reliability of this method in protoplasts. Therefore, a careful interpretation of results obtained with intracellular microelectrodes in protoplasts and a detailed analysis of such measurements is essential to draw valid conclusions. In contrast to intracellular microelectrode measurements in whole plant cells, these measurements are technically difficult in protoplasts. Especially the introduction of a (in time variable) shunt resistance upon microelectrode penetration complicates interpretation and analysis of this type of measurements.

The patch-clamp technique in the whole-cell configuration provides an alternative method to measure the membrane potential without interference of a shunt resistance. However, several disadvantages in the use of this technique for routine membrane potential measurements exist (see, e.g., Refs. [9–11]). For example, only just after obtaining the whole-cell configuration by breaking the membrane under the patch-electrode, the membrane potential of the undisturbed protoplast can be measured. Perfusion of the cytoplasm with the patch-pipette content and diffusion of cytoplasmic compounds out of the protoplast into the pipette will influence cellular properties and likely affect signal transduction cascades. So, in fact only a single time point measurement of the membrane potential of the undisturbed cell can be made with the patch-clamp technique, unless the 'slow whole-cell' configuration can be used (e.g., Ref. [12]). Other alternative methods for membrane potential measurements in protoplasts include, e.g., the use of potential sensitive fluorescent indicators and measurements of the distribution of lipophilic cations. These methods, however, have many disadvantages as well (e.g., calibration problems, compartmentalization of the indicators etc.) and only are indirect indicators of the membrane potential.

In view of the above mentioned complications of different methods to measure membrane potentials in plant cell protoplasts and the fact that a number of 'key' studies in plant hormone signal transduction research are based on intracellular microelectrode measurements a detailed analysis of such measurements is necessary.

In this paper we present data obtained from intracellular microelectrode measurements on tobacco cell suspension protoplasts and barley aleurone protoplasts. These data are analyzed and interpreted with regard to the electrical behaviour upon microelectrode impalement as predicted from electrical equivalent circuit simulations of the measurement configuration. The possible pitfalls, essential checks and precautions for performing microelectrode measurements in protoplasts and reliable interpretation of such measurements are discussed.

2. Materials and methods

2.1. Protoplast isolation

Tobacco protoplasts. Tobacco suspension cells (*Nicotiana tabacum* L. cv Bright Yellow) were grown in Linsmaier and Skoog medium [13] as described before [14]. Protoplasts were prepared from the cells by incubation for 4 h in a solution containing 0.4 M mannitol, cellulase and pectolyase as elsewhere described [15]. Protoplasts were collected after three times washing with enzyme free solutions by centrifugation (3 min, $100 \times g$).

Barley aleurone protoplasts. Barley (*Hordeum vulgare* L. cv. Himalaya, harvest 1985; Department of Agronomy, Washington State University, Pullman, WA) aleurone protoplasts were prepared as described by Jacobsen and Chandler [16], except for the imbibition of the half grains which was carried out in H_2O and incubations with enzymes which were carried out at 25°C in the dark for 16 h. Protoplasts were sieved (100 μm sieve) and washed with B-ECS (see solutions).

2.2. Solutions

Tobacco protoplasts were incubated in a standard extracellular solution (T-ECS) consisting of 10 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KOH, and 10 mM MES (pH 5.5). The osmolarity of T-ECS was adjusted with mannitol to about 570 mosM.

The standard extracellular solution (B-ECS) for barley aleurone protoplasts consisted of 0.5 mM K_2HPO_4, 10 mM KCl, 1 mM $MgCl_2$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 10 mM PIPES-HCl (pH 6.8) and was adjusted to about 830 mosM with mannitol.

2.3. Intracellular microelectrode measurements

Fine tipped, wide taper, 3 M KCl filled intracellular microelectrodes with a resistance between 30 and 70 MΩ were used for membrane potential measurements essentially as described before [17]. A microelectrode amplifier with capacitance compensation (WPI Series 700 Micro Probe Model 750, WP Instruments, New Haven, CT) was used. Microelectrode capacitance was

compensated to obtain rise times (= time to reach 66% of the potential response upon a current pulse) faster than 0.05 ms. All potential values were measured with respect to the microelectrode tip potential. To ensure rapid (4 μm/0.1 ms) and reproducible impalements of protoplasts with minimal lateral vibration, a piezo-stepper device (Piezo-stepper P-2000, Physik Instrumente (PI) Gmbh Co., Waldbronn-Karlsruhe, Germany) was used. This device proved to give minimal variation in the impalement-induced shunt resistance. For microelectrode measurements protoplasts were kept in a glass bottom Teflon culture dish [18] with 2 ml extracellular solution and observed with 40 × or 100 × objective magnification.

2.4. Statistics

Data are presented as means ± S.E., with n the number of measurements. Differences between values were tested with Student's t-test.

3. Results and discussion

3.1. Response upon microelectrode impalement

Impalement of free protoplasts with a microelectrode is not easy, but in clean preparations, protoplasts tend to adhere to a carefully cleaned glass surface which makes microelectrode penetration possible with the methodology described (see Materials and methods). Upon penetration of both tobacco protoplasts and barley aleurone protoplasts a potential transient could be observed within the first milliseconds upon impalement (Figs. 1A,B). The occurrence of such an impalement-induced transient in animal cells has first been described by Lassen et al. [19] and was thereafter experimentally and theoretically analyzed in more detail in human monocytes and *Dictyostelium discoideum* amoeba (see, e.g., Refs. [17,20]). The impalement with a microelectrode can be simulated with the use of an electrical equivalent circuit of the measurement configuration, by instantaneous introduction of R_s (the microelectrode-induced shunt resistance) and R_e (the

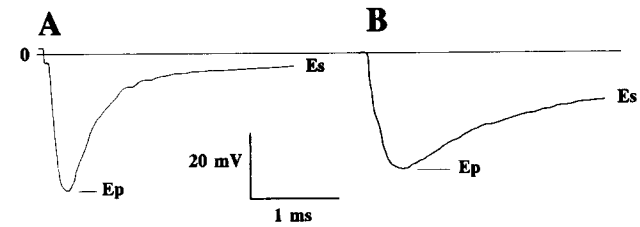

Fig. 1. Microelectrode impalement-induced potential transients observed upon penetration of a tobacco protoplast (A) and a barley aleurone protoplast (B). Upon touching the protoplasts with the microelectrode a small positive prepotential was seen in many cases. The time-course of depolarization after reaching the peak-value, E_p, is mainly determined by the time constant of the penetrated protoplasts membrane. E_s indicates the steady-state level reached at a millisecond timescale.

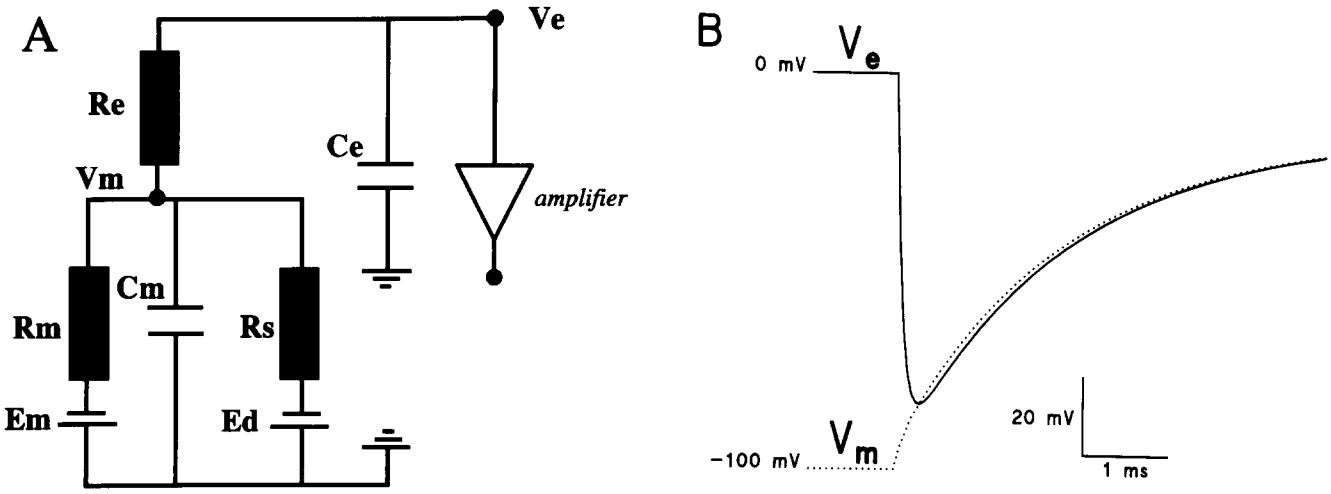

Fig. 2. (A) Electrical equivalent circuit of the intracellular microelectrode measurement configuration. The membrane resistance R_m and membrane capacitance C_m determine the electrical membrane properties, and describe together with the membrane potential E_m the electrical equivalent circuit of the undisturbed protoplast. Upon microelectrode penetration a shunt resistance R_s in parallel with R_m and C_m is introduced. E_d represents the diffusion potential across the ion unselective shunt. R_e and C_e represent the microelectrode resistance and capacitance (after capacitance compensation), respectively. V_m is the shunted membrane potential and V_e is the potential that is actually measured. (B) Simulation of the potential response of both V_e and V_m upon penetration of the protoplast membrane with the electrical equivalent circuit shown in (A). Upon introducing the microelectrode, and the microelectrode-induced shunt resistance in the circuit, V_m decays to a new, depolarized, steady-state level (described by Eq. (1)), while V_e follows a transient potential change. The used circuit parameters in the shown simulation are in the same order of magnitude as the estimated or measured parameter values in real measurements: $C_m = 20$ pF, $C_e = 1$ pF, $R_m = 1$ GΩ, $R_s = 100$ MΩ, $R_e = 80$ MΩ, $E_m = -100$ mV, $E_d = -5$ mV.

resistance of the microelectrode) in the electrical equivalent circuit of the protoplast, resulting in the circuit in Fig. 2A. In first instance we have to look at the steady-state behaviour of this circuit. In this circuit the measured potential, V_e, in the steady-state condition is given by the following equation:

$$V_e = V_m = \frac{E_m \cdot R_s + E_d \cdot R_m}{R_m + R_s} \quad (1)$$

in which E_m is the true membrane potential, E_d is the diffusion potential across the microelectrode-induced shunt, V_m is the shunted membrane potential, R_m is the membrane resistance and R_s is the microelectrode-induced shunt resistance. From Eq. (1) it can be seen that in the steady-state condition V_e depends on the ratio between R_m and R_s, and only will be close to the value of the real membrane potential, E_m, if $R_s \gg R_m$.

When this is not the case, e.g., in cells with a relatively high R_m or when the membrane does not seal off around the microelectrode (R_s will be small), V_e will only be a fraction of E_m (i.e., V_m decays). When we look at the dynamic behaviour of the circuit upon introducing microelectrode impalement we see that, due to the presence of different capacitors in the circuit, the decay of V_m is not stepwise, but follows a time-dependent decrease. Under favourable conditions (a small microelectrode rise-time among others, see Ince et al. [20]) the introduction of R_s can be observed in the occurrence of a fast peak-shaped potential transient in V_e and a decay of V_m which reflects the discharge of the membrane capacitance, C_m, to the new steady-state potential (as seen in a simulation with circuit parameter values in the same order of magnitude as found in the real measurements, Fig. 2B). The most negative value of the microelectrode-induced potential transient, E_p, is determined by all circuit components [20], while the value of the steady-state potential reached thereafter, E_s, is described by Eq. (1). Most importantly, the value of E_p is very *un*sensitive for variations in R_m, whereas E_s is very sensitive for R_m variations [20]. The occurrence of such a peak-shaped potential transient upon impalement already shows that a shunt-resistance is introduced which loads the measurement. Hence, in measurements where this transient can be observed, the value of E_p is considered to be the best estimate of the membrane potential of the protoplast before impalement. This was, for instance, shown in whole-cell patch-clamp measurements in the current clamp mode in combination with intracellular microelectrode measurements in human monocytes [20] and in measurements in *D. discoideum* cells of different sizes [17].

From the above we conclude that the occurrence of the peak-shaped potential transient in both tobacco and barley aleurone protoplasts indicates that a shunt resistance, which is loading the measurement, is introduced upon the impalement in both types of protoplasts. The mean value of E_p in tobacco protoplasts was about −28 mV, and about −45 mV for barley aleurone protoplasts (Table 1). The mean E_s values were −10 mV and −16 mV for tobacco and barley aleurone protoplasts, respectively (Table 1). The occurrence frequency of a peak-shaped potential transient

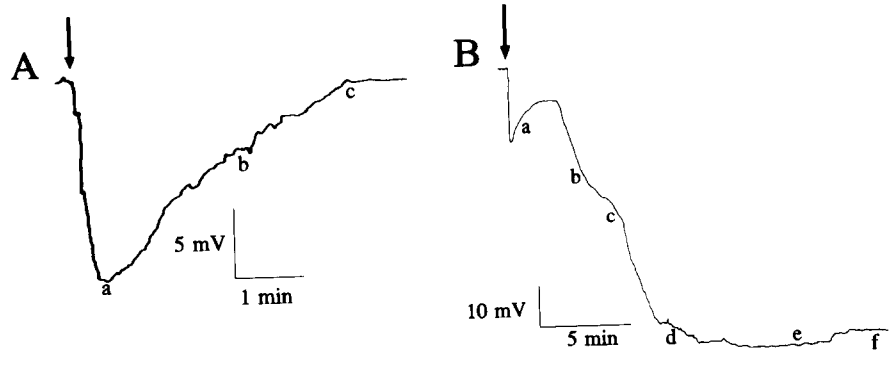

Fig. 3. Slow time-scale recording of membrane potential measurement with intracellular microelectrode. Note the differences in time scale as compared to Figs. 1A,B. (A) Recording from a tobacco protoplast. The arrow indicates the moment of microelectrode impalement. The impalement-induced potential transient is not visible in this figure due to the low cut-off frequency of the chart recorder. Simultaneous recording on an oscilloscope showed that the impalement-induced potential transient had an E_p of −26.7 mV and an E_s of −1.5 mV. After reaching the E_s value the measured potential hyperpolarized again rapidly, as shown in the figure, to a maximal potential value indicated by (a). Thereafter a slower depolarization was recorded to a steady-state level of about −1.5 mV (c). At different time points the resistance of the penetrated protoplast was measured by application of −48 pA current pulses. R_i values at the indicated points were at (a) 57 MΩ, (b) 41 MΩ and (c) 29 MΩ. Microelectrode resistance was 52 MΩ. (B) Recording from a barley aleurone protoplast. As in (A) the impalement-induced potential transient is not visible in this figure. E_p of this protoplast was −62.0 mV, and E_s was −23.1 mV. After reaching E_s the measured potential hyperpolarized to a steady-state level which could be maintained for about 30 min. In this case E_{max} was equal to E_{ss}. The R_i values measured at the different indicated points were at (a) 5 MΩ, (b) 30 MΩ, (c) 53 MΩ, (d) 183 MΩ, (e) 239 MΩ, (f) 208 MΩ. Microelectrode resistance was 40 MΩ.

Table 1
Different membrane potential indicators measured with intracellular microelectrodes in tobacco cell suspension protoplasts and barley aleurone protoplasts

	Tobacco protoplast	Barley aleurone protoplast
E_p (mV)	-27.7 ± 2.6 (44)	-45.3 ± 4.7 (31)
E_s (mV)	-10.5 ± 0.8 (69)	-16.1 ± 1.5 (49)
E_{max} (mV)	-14.9 ± 0.9 (69)	-32.6 ± 4.3 (49)
E_{ss} (mV)	-3.8 ± 0.6 (69)	-11.9 ± 2.9 (49)

E_p is the peak value of the impalement-induced potential transient. E_s is 'steady-state' value at the millisecond time scale after the impalement-induced potential transient. E_{max} is the maximal potential value reached during the measurement after the impalement-induced potential transient and E_{ss} is the final steady-state potential value reached during the measurement (stable value for > about 2 min). In a few cases E_{max} and E_{ss} were equal (see Fig. 3B).

upon impalement was somewhat higher than 60% for both type of protoplasts. The other penetrations resulted in an instantaneous measurement of values around E_s. At these penetrations the impalement-induced decay of the potential is too fast to reach a peak-value close to the true resting membrane potential, e.g., due to a too small R_s value (i.e., a too large impalement-induced leak), or penetration was into the vacuole. The more negative E_p and E_s values found in barley aleurone protoplasts as compared to tobacco protoplasts is in agreement with E_m measurements with the patch-clamp technique. Whole-cell patch-clamp measurements showed that the membrane potential of tobacco protoplasts in a similar extracellular solution is about -40 mV [14], and in barley aleurone protoplasts around -60 mV (-58.3 ± 4.9 mV, $n = 8$. Patch-clamp experiments were performed as described before [14]).

3.2. Steady-state behaviour

After reaching E_s, the measured potential, V_e, often hyperpolarized to more negative values in both tobacco and barley aleurone protoplasts. This hyperpolarization was accompanied by an increase in the measured input resistance of the impaled protoplast, R_i, and most likely is due to a sealing of the membrane around the microelectrode, thereby increasing R_s. After this hyperpolarization, V_e depolarized again to a steady-state level a little less negative than the value of E_s (Fig. 3A). In a few cases, mostly in barley aleurone protoplasts, a much more negative potential could be maintained for a longer period (Fig. 3B). The measurement of steady potentials for a longer time could not be improved by using electrodes without Cl^- by filling them with 4 M K-acetate (data not shown). The most negative potential value reached at this slower time scale, E_{max}, and the final steady-state potential, E_{ss}, were measured for both tobacco protoplasts and barley aleurone protoplasts (Table 1). From Table 1 it is clear that the mean value of E_{max} is less negative than the measured E_p in the same cells. The same is true for E_{ss} and E_s.

An increase in R_s may lead to the measurement of a constant potential V_e close to the value of E_m. In this case a hyperpolarizing response is measured which is accompanied by a R_i increase. On the other hand a similar effect on V_e may be obtained by opening of ion channels leading to a decrease of R_m. In this case the hyperpolarization is accompanied by a decrease of R_i (see, e.g., Ref. [21]). Leak of chloride from the microelectrode into the cytoplasm may as well cause changes in E_m leading to changes in V_e, as was shown for non-animal cells [17,22]. The potential increase (hyperpolarization) in both tobacco protoplasts and barley aleurone protoplasts was accompanied by a R_i increase, and the potential decrease (depolarization) by a R_i decrease (Fig. 3).

From Eq. (1) it is clear that the measured steady-state potential is strongly dependent on the ratio of R_s and R_m. In the given measurement configuration (Fig. 2A), it is not possible to distinguish between R_m and

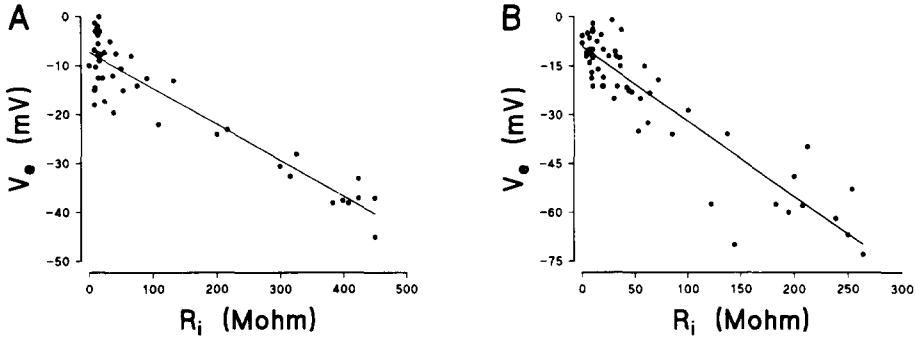

Fig. 4. Measured potential values, V_e, as a function of the measured resistance of the impaled protoplast, R_i. The R_i was measured by application of a small current pulse (-48 pA) and recording of the potential change. R_i values are corrected for electrode resistance. The solid lines are the linear regression fit to the data points. (A) Measurements in tobacco protoplasts. The linear regression line is: $V_e = -0.073R_i - 7.26$ (with V_e in mV, and R_i in MΩ), with a correlation coefficient of -0.92. (B) Measurements in barley aleurone protoplasts. The linear regression is: $V_e = -0.23R_i - 9.01$, with a correlation coefficient of -0.91.

R_s. Both R_m and R_s are recorded as part of the input resistance, R_i, which is the resistance of R_m and R_s in parallel:

$$R_i = \frac{R_m \cdot R_s}{R_m + R_s} \quad (2)$$

Combination of Eqs. (1) and (2) results in:

$$V_e = (E_m - E_d) \cdot \frac{R_i}{R_m} + E_d \quad (3)$$

This predicts a linear relationship between V_e and R_i with a slope of $(E_m - E_d)/R_m$, assuming that variations in R_i are only due to variations in R_s. The V_e value for $R_s = 0$ is equal to E_d (this is for $R_i = 0$), while V_e is equal to E_m when $R_i = R_m$ (this is for $R_s \rightarrow \infty$). When we plot the measured potentials as a function of the measured R_i values for both tobacco protoplasts and barley aleurone protoplasts we, indeed, find a relationship as predicted by Eq. (3) (Figs. 4A,B). From these figures the estimated values of E_d are about −8 mV for both type of protoplasts. From the slopes of these linear relationships we can estimate the minimal value of R_m if we use the E_p values as the best (under)estimation of E_m. This results in minimal R_m values of about 310 MΩ and 160 MΩ for tobacco cell suspension and barley aleurone protoplasts, respectively.

From the above (Eq. (3)) it can be concluded that V_e will only change proportional with E_m changes when R_m and R_s are constant. However, since E_m changes in many cases are caused by or lead to changes in R_m this will not be the case in many experiments. Some examples of these situations are illustrated in Fig. 5, which shows that the measured response at the microelectrode (V_e) on E_m changes is strongly R_m and R_s dependent. We conclude that for a correct estimation of true membrane potential values from steady-state intracellular microelectrode measurements, information about the ratio between R_m and R_s is required. In addition, for correct interpretation of membrane potential changes measured as changes in V_e data on R_m and R_s values are essential.

From the above we conclude that E_s, E_{max} and E_{ss}, in most cases, are not very reliable indicators for E_m changes as they are strongly R_i dependent.

3.3. Measurement of membrane potential changes

For the measurement of stimulus-induced membrane potential changes of plant cell protoplasts with intracellular microelectrodes different strategies can be applied. The membrane potential of a number of protoplasts can be measured before addition of the stimulus. Subsequently the stimulus is added and the membrane potential of a number of stimulated protoplasts

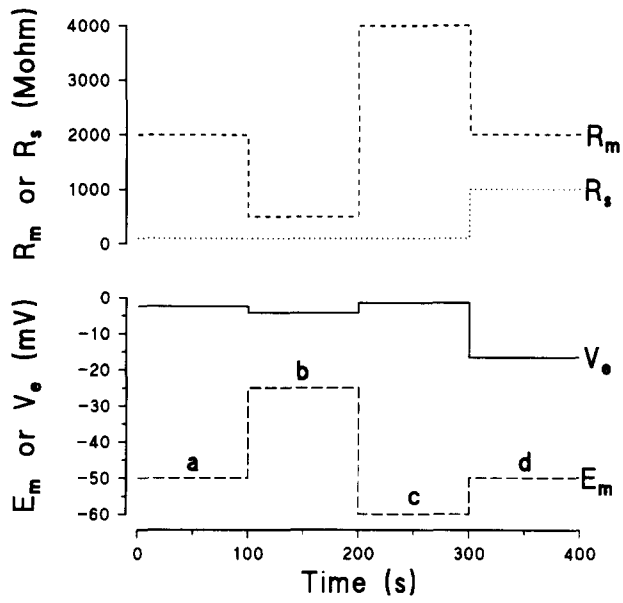

Fig. 5. Simulation of measured potential values, V_e, in the steady-state condition (Eq. (3)) for different given membrane potential E_m, membrane resistance R_m and shunt resistance R_s conditions. In the starting condition (a) R_s and R_m determine the measured potential V_e which is a fraction of the true membrane potential E_m. A membrane depolarization (condition b) accompanied by a membrane resistance decrease (e.g., due to opening of ion channels with a less negative reversal potential), results in the measurement of hyperpolarization of V_e. A membrane hyperpolarization (condition c) accompanied by a membrane resistance increase (e.g., due to closure of ion channels with a less negative reversal potential) leads to the measurement of depolarization of V_e. If in the starting condition (a) the shunt resistance is increasing (condition d), while the other parameters do not change, then a membrane hyperpolarization is measured at V_e.

is measured (e.g., Refs. [1,2]). Although technically most easy, the great disadvantage of this approach is that time-dependent changes are hard to measure, and may interfere with the measurements (i.e., only measurements can be done in steady-state conditions). In an other approach the membrane potential of one protoplast is measured continuously in time and the response upon addition of the stimulus is measured in time. This approach requires stable membrane potential measurements in a single protoplast over a long period, which are technically much more demanding. Both time dependent processes and membrane resistance can be monitored in this type of measurements, which are important for a correct interpretation of the measurements (see analysis above).

As an example of measurements of membrane potential changes we investigated in tobacco protoplasts the membrane potential response upon stimulation with the auxin analogue 1-NAA. The above discussed membrane potential indicators (E_p, E_s, E_{max}, E_{ss}) were measured before and after addition of 1-NAA (Table 2). After addition of 1-NAA at least 15 min was

waited to allow the membrane potential to reach a new steady-state value, to avoid interference with the kinetics of the response [3]. Only the E_p values show a significant ($P < 0.025$) change after 1-NAA stimulation. The E_{max} and E_{ss} values suggest a membrane hyperpolarizing effect of stimulation with 1-NAA, whereas the E_s values suggest a membrane depolarizing action of 1-NAA. In contrast to other reports [2,6] the change in E_{ss} values is not significant. This may be due to different R_s values and the number of measurements. Interpretation of the action of 1-NAA on E_m from E_s, E_{max} and E_{ss} values must be done carefully, since variations in R_i may complicate the measurements as was shown in the analysis described above. With regard to the above, E_p is the most reliable membrane potential indicator, as E_p is almost R_m independent [20] and also shows the largest potential change after 1-NAA stimulation (Table 2). Therefore, it is concluded from these measurements that, at more than 15 min after addition, 1-NAA hyperpolarizes the membrane potential.

Intracellular microelectrode measurements in maize coleoptile cells showed that 1-NAA induces a membrane depolarization which is followed by a membrane hyperpolarization [3]. This initial depolarization could, however, not be measured in maize coleoptile protoplasts as a change in membrane current during whole-cell patch-clamp experiments, whereas the hyperpolarization was seen as in increase in outward directed H^+-current [23]. Maize suspension cells did not show a hyperpolarizing response upon stimulation with 1-NAA as measured with intracellular microelectrodes [3]. These differences found between different cell types may be due to existing differences in response between different cell types, differences between cells and protoplasts, and due to different measuring techniques. We conclude that both intracellular microelectrode measurements and patch-clamp measurements may be necessary to measure the complete electrophysiological response of plant cells and protoplasts to a stimulus.

In addition, the effect of an extracellular pH change on the membrane potential of barley aleurone protoplasts was investigated. The membrane potential indicators E_p, E_s, E_{max} and E_{ss} were measured in the control condition, pH 6.8, and subsequently at a more acid pH of 6.3. Table 3 shows that this decrease in extracellular pH induces a significant decrease of E_p and E_{max}. This change in E_p (about 34 mV/decade) might indicate that E_m is partly determined by the proton diffusion potential (about 59 mV/decade). However, pH dependent proton pump activity changes may lead to similar pH dependent membrane potential changes around pH 6.5 (e.g., Ref. [24]).

The above examples show that, although the E_p value is an underestimation of E_m, it can not only be used as an estimate of the resting membrane potential but also as a reliable indicator of stimulus-induced membrane potential changes.

Table 2
Effect of 1-NAA on membrane potential indicators in tobacco protoplasts

	Control	10 μM 1-NAA
E_p (mV)	-24.7 ± 2.2 ($n = 12$)	-32.8 ± 2.6 ($n = 15$)
E_s (mV)	-7.0 ± 1.5 ($n = 14$)	-4.9 ± 1.1 ($n = 16$)
E_{max} (mV)	-12.3 ± 1.0 ($n = 14$)	-14.9 ± 1.4 ($n = 16$)
E_{ss} (mV)	-2.7 ± 0.6 ($n = 14$)	-3.7 ± 0.8 ($n = 16$)

Microelectrode measurements were performed before (control) and more than 15 min after addition of 1-NAA to the bath solution. Only the change in E_p values is significant ($P < 0.025$).

Table 3
Effect of an extracellular pH change on the membrane potential indicators E_p, E_s, E_{max} and E_s as measured in barely aleurone protoplasts

	Control (pH 6.8)	pH 6.3
E_p (mV)	-40.9 ± 6.2 ($n = 20$)	-23.8 ± 4.7 ($n = 15$)
E_s (mV)	-12.5 ± 1.2 ($n = 20$)	-11.5 ± 2.0 ($n = 15$)
E_{max} (mV)	-17.6 ± 1.6 ($n = 28$)	-11.4 ± 1.6 ($n = 20$)
E_{ss} (mV)	-5.6 ± 1.9 ($n = 28$)	-2.8 ± 0.6 ($n = 20$)

The differences between E_p values, and the differences between E_{max} values at the respective pH values are significant ($P < 0.025$).

4. Concluding remarks

In both tobacco suspension protoplasts and barley aleurone protoplasts a peak-shaped potential transient was observed upon microelectrode impalement. The theoretical analysis of the measurement configuration showed that the occurrence of such a transient indicates that a shunt-resistance is introduced which loads the potential measurement. In addition, these peak potential transients showed that the membrane potential of both types of protoplasts is much more negative than the final steady-state potential, E_{ss}, which is measured. The difference in E_p values between tobacco protoplasts and barley aleurone protoplasts is in agreement with a more negative membrane potential of barley aleurone protoplasts than tobacco protoplasts as measured with the patch-clamp technique. The small potential values reported frequently (e.g., Refs. [2,6]) for tobacco mesophyll protoplasts are very likely also due to the introduction of a microelectrode-induced shunt resistance rather than to Cl^- leak from the electrode [6], although a cell type dependent difference cannot be ruled out at the moment. The fact that the true membrane potential of the used protoplasts is much less negative than the usually reported values for intact cells suggests that the procedures (enzyme treatments) to prepare protoplasts affect the electrophysio-

logical properties of the membrane. Hence, conclusions derived from studies on protoplasts are not necessarily valid for intact cells.

In addition, the theoretical analysis of the measurement configuration showed that in membrane potential measurements that suffer from a shunt-resistance the correct interpretation of potential changes is difficult. The steady-state potential is very sensitive for changes in R_m and R_s. Therefore, data on the membrane resistance and the shunt resistance are essential for drawing valid conclusions. A good alternative is the measurement of the value of E_p since E_p is very unsensitive for variations in R_m. Measurements of the effect of 1-NAA on the membrane potential of tobacco protoplasts showed that membrane potential changes can indeed be measured as changes of E_p values. Besides being the most reliable indicator, E_p showed also the largest potential change upon stimulation with 1-NAA as compared to E_s, E_{max}, and E_{ss}.

It turned out to be difficult to perform longer lasting membrane potential measurements at potential values close to the real membrane potential. Specially in tobacco protoplasts stable potential measurements could only occasionally be performed and never longer than for about 10 min. Barley aleurone protoplasts were better suitable for this type of measurements, possibly due to absence of a large vacuole and the presence of a relatively larger cytoplasmic compartment.

Intracellular microelectrode measurements on protoplasts may provide an alternative for patch-clamp measurements when giga-seal formation turns out to be difficult or when large populations of protoplasts have to be examined. However, since membrane potential measurements with intracellular microelectrodes in plant cell protoplasts suffer from an impalement-induced shunt resistance (as can be observed by the occurrence of a peak-shaped potential transient upon impalement) one should use the value of E_p rather than the E_s, E_{max} E_{ss} values as an indicator of true membrane potential changes. For direct measurement of the true absolute membrane potential of the investigated protoplasts the patch-clamp technique has to be used as E_p provides only an underestimation of E_m.

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