Discontinuous single electrode voltage-clamp measurements: assessment of clamp accuracy in *Vicia faba* guard cells

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Abstract

The use of a discontinuous single electrode voltage-clamp (dSEVC) offers an attractive alternative to the patch-clamp technique, since whole-cell measurements can be performed with a single sharp electrode. Comparison of current–voltage relations, however, revealed a weaker voltage dependence of channels measured with the dSEVC compared to patch clamp. The accuracy of the dSEVC was tested on *Vicia faba* guard cells impaled with double-barrelled electrodes. The actual clamp potential was measured independently of the dSEVC, at the second barrel. The weaker voltage dependence of ion channels appeared to be due to an overestimation of the clamp potential by the dSEVC. The deviation between the intended and actual clamp potential showed a linear relationship with the injected current; on average a 126 mV deviation was found for a clamp current of 1 nA. The deviation was probably caused by a slow settling capacity at the electrode, not compensated by the dSEVC amplifier. It is concluded that the dSEVC method in its current state is only suited for the study of small ion conductances in plant cells.

Key words: Discontinuous single electrode voltage clamp, plant cell, guard cell.

Introduction

The discontinuous single electrode voltage-clamp (dSEVC) technique allows the membrane potential of a cell to be clamped with a sharp single-barrelled electrode. In electrophysiological studies on animal cells, this method is typically used to study cell types that would have been difficult to reach with patch-clamp electrodes (Richter et al., 1996). The method is of a more general use for plant cells, since the cell wall obstructs a direct application of patch-clamp electrodes. The patch-clamp technique can only be used after enzymatic digestion of the cell wall (Elzenga et al., 1991; Hedrich, 1995) or its removal with a laser (Taylor and Brownlee, 1992; Henriksen et al., 1996). The use of the dSEVC method is an attractive alternative to the whole-cell configuration of the patch-clamp technique, since whole-cell voltage-clamp measurements can be conducted on cells with an intact cell wall.

Voltage-clamp measurements with sharp glass microelectrodes are ideally carried out with two electrodes that separate the current injection from the potential measurement (Eisenberg and Engel, 1970; Finkel and Gage, 1985). The dSEVC separates the current injection from potential measurement in time, by rapid switching between a current injection mode and potential measuring mode (Brenneke and Lindemann, 1974; Finkel and Redman, 1985; Halliwell et al., 1987). The method takes advantage of a slower time constant of the plasma membrane (\(r_m = R_m \times C_m\)) compared with the electrode. Provided the switching frequency between the current injection- and voltage measuring-mode is high enough, the plasma membrane can be clamped to a steady membrane potential.

The method was first developed for large cells with a low conductance, since these cells have a high capacity and discharge slowly (Brenneke and Lindemann, 1974; Wilson and Goldner, 1975). Physical analysis showed that the method is best used on large cells with a small conductance (Sala and Sala, 1994) and requires...
microelectrodes with fast settling times (Finkel and Redman, 1985). Recent developments, however, have provided amplifiers that run at high switching frequencies (up to 60 kHz) and enable voltage clamp of much smaller cells (Juusola, 1994).

The single-electrode current clamp technique was first applied on plant cells by Anderson et al., who used the method to study the membrane conductance of root cortical cells of *Pisum sativum* (Anderson et al., 1974). The method was criticized by Etherton et al. who compared the performance of a single-electrode current clamp with that of a conventional two-electrode current clamp in a single cell (Etherton et al., 1977). The assumption that the time constant of the electrode was small compared to that of the membrane was taken into question. Furthermore, the authors recognized that the capacitance of a microelectrode can never be fully compensated by a microelectrode amplifier. A later report (Schefczik et al., 1983) used the same approach, but a modified analysis procedure was used. The latter authors concluded that the method can be used accurately, provided pulse protocols are used that are adapted to the electrode properties.

More recently, the dSEVC has been applied to electrically isolated plant cells. The method enabled the analysis of ionic currents of (i) laticifer protoplasts of *Hevea brasiliensis*, a natural rubber-producing plant (Bouteau et al., 1996), (ii) root hair cells of *Medicago sativa* (Kurkdjian et al., 2000) and (iii) guard cells of several species (Forestier et al., 1998). The plasma membrane conductance of the latter cell type has been studied in detail with the patch-clamp technique (Dietrich et al., 1998) and a voltage-clamp technique that uses double-barrelled electrodes (Blatt, 1992). The data obtained with these two techniques, however, differ from those measured with the dSEVC, with respect to the activation times and voltage dependence of inward- and outward K⁺-channels. Therefore, an experimental approach was undertaken to measure the accuracy of the dSEVC in guard cells of *Vicia faba*. The difference in voltage dependence of ion-channels measured with single- or double-barrelled electrodes was quantified.

**Materials and methods**

**Tissue preparation**

3–6-week-old *Vicia faba* L. cv. Grünkernige Hangdown plants, were used, which were grown in the greenhouse. The abaxial epidermis was peeled from the leaves and attached to a microscope slide using Medical Adhesive (VM 355, Ulrich AG, St Gallen, Switzerland). The microscope slide was mounted in an experimental chamber filled with bath solution, 50 mM KCl and 1 mM Ca(OH)₂ buffered to pH 6.0 with Mes. Guard cells were impaled on an upright microscope (Axioskop 2FS, Carl Zeiss, Göttingen, Germany), at an angle of 30°. The guard cell’s long axis had a length of 38 μm (SD = 2 μm), while their diameter was 13 μm (SD = 1 μm). Assuming a geometry of a twisted cylinder with hemispherical caps on its ends the surface area was 2070 μm² and the plasma membrane capacitance was ~21 pF.

**Electrodes and electrical system**

All electrodes were pulled from borosilicate glass capillaries with a wall thickness of 0.21 mm (GC100F-10, Clarc Electromedical Instruments, Pangbourne Reading, UK) and filled with 300 mM KCl. Single-barrelled electrodes were pulled on a horizontal laser puller (P2000, Sutter Instrument Co., Novato, CA, USA). Double-barrelled electrodes made from two capillaries that were aligned, heated and twisted 360° on a horizontal puller (PD-5, Narashige, Tokyo, Japan), the electrode-tip was pulled in a two-step procedure. The tip resistance of the electrodes was measured before impedance and ranged from 40 to 120 MΩ, the capacitance of the electrodes was 16 pF (SD = 2, n = 4).

Single-barrelled electrodes were connected to a dSEVC amplifier (SEC 05 L/H, NPI Electronic, Tamm, Germany). In case double-barrelled electrodes were used, one barrel was connected to the dSEVC amplifier, while the other was connected to a regular microelectrode amplifier (VF-102, Bio-Logic, Claix, France). Voltage-clamp protocols were fed into the dSEVC amplifier using Pulse software (Heka, Lambrecht, Germany) and an ITC-16 interface (Instrutech, Corp., Elmont, NY, USA). The data were filtered with 8-pole Bessel filters present at the dSEVC amplifier or externally (type 902, Frequency Devices, Haverhill, MA, USA). The low pass filters were run at 0.3 or 1.3 kHz, while data were sampled at 1 or 10 kHz. The dSEVC amplifier was run at a switching frequency of 20 kHz, while current injection and voltage measuring time intervals were equally long (duty cycle = 0.5). The capacity compensation of the dSEVC amplifier was adjusted just before impalement, with the following procedure. The amplifier was set to its switching current-clamp mode. Current pulses of 1 nA were fed through the amplifier, the capacity compensation was adjusted until deflections in the voltage signal had disappeared. At this point, the current applied during the current-injection time-interval has no effect on the potential recorded in the voltage-measuring time-interval. The capacity compensation of the regular microelectrode amplifier was set to a sub-critical level.

**Numerical analysis**

The voltage dependence of the inward rectifying K⁺-channel was determined by fitting a Boltzmann equation to the conductance–voltage relationship. The conductance was calculated as $G_m = \frac{I_m}{(V_m - E_{rev})}$, where the reversal potential $E_{rev}$ was assumed to be at −30 mV. The following equation was used:

$$G_m = G_{max}(1 + e^{F(V_{1/2} - V_m)/RT})$$

where $G_{max}$ is the maximum conductance, $z_g$ the effective gating charge and $V_{1/2}$ the half maximum activation potential. In an ensemble fit of several conductance–voltage plots, $G_{max}$ and $V_{1/2}$ varied for each individual cell, while a single value for $z_g$ was obtained.

**Results**

In a first series of experiments, guard cells were impaled with single-barrelled electrodes. The plasma membrane conductance was tested, by clamping the membrane
potential from a holding potential of −100 mV to more negative and positive potentials (data not shown, but as in Fig. 1A). The currents measured were similar to those of Fig. 1C, at potentials negative of −100 mV the activation of inward rectifying channels was found, while outward rectifying channels activated at more positive potentials. Current–voltage plots of these cells confirmed a smaller voltage dependence of both channels measured with the dSEVC, compared to patch- and double-barrelled voltage-clamp techniques (data not shown). The difference was quantified for the inward rectifying channel by comparison of the gating charge of the channel. Using the dSEVC method a value for \(z_g\) of 0.52 (SE = 0.04, \(n = 9\)) was found, while \(z_g\) was 1.5 in patch clamp (Dietrich et al., 1998) and 1.4 in double-barrelled voltage-clamp (Blatt, 1992). The difference in gating charge may originate from an overestimation of the clamp voltage by the dSEVC amplifier, a possibility tested in a second series of experiments.

Guard cells were impaled with double-barrelled electrodes, one barrel being used to clamp the membrane potential with the dSEVC amplifier. The second barrel was used to measure the membrane potential independently. During voltage clamp, the voltage recorded by the dSEVC did not deviate from the intended clamp potential (\(V_{int}\)) (Fig. 1A). The potential measured at the second barrel (\(V_{meas}\)) was close to \(V_{int}\) at the start of the voltage clamp (Fig. 1B), but began to deviate from \(V_{int}\) with time. Considering \(V_{meas}\) as a representative of the true clamp potential, the cells apparently were not clamped to a steady membrane potential, but to a slowly changing potential instead. The clamp potential reached a stable value only after period of \(\sim 0.5\) s (Fig. 1B).

The change in \(V_{meas}\) strongly correlated with the change in clamp current (Fig. 1C). The relationship between the clamp current and the deviation in voltage was determined for values measured at the end of the 2 s test pulses. A linear relation was found for currents up to 1 nA (Fig. 2A), but in some cells the relationship differed for inward and outward currents. On average, the deviation of \(V_{meas}\) from \(V_{int}\) was 126 mV nA\(^{-1}\) (SD = 98, \(n = 6\) cells).

The steady-state current–voltage relation of the cell depicted in Fig. 1 can be drawn either using \(V_{int}\) or \(V_{meas}\). In Fig. 2B both options are displayed. At potentials with a low conductivity of the plasma membrane, both current–voltage relations overlap. However, the curves deviate at potentials where ion channels activate, as a result of the difference between \(V_{int}\) and \(V_{meas}\). The voltage dependence of inward and outward rectifying channels is higher when currents are plotted against \(V_{meas}\) compared with \(V_{int}\). For the inward rectifier a value for \(z_g\) of 0.72 (SE = 0.05, \(n = 6\)) was found with \(V_{int}\), while \(z_g\) was 1.73 (SE = 0.08, \(n = 6\)) based on \(V_{meas}\). Note that the value of \(z_g\) based on \(V_{meas}\) is closer to the patch-clamp and double-barrelled voltage-clamp values, than that based on \(V_{int}\). This indicates that the dSEVC overestimates the membrane potential, during voltage clamp.

A dSEVC amplifier switches fast between current injection and voltage measurement mode. Before the voltage is sampled, the electrode needs to be discharged. Any charge remaining on the tip of the electrode will be added to the membrane potential and thus result in an overestimation of the clamp potential. An accurate functioning of the system therefore depends on a correct setting of the capacity compensation. For the experiments presented the capacity compensation was set with the electrode close to the cell. However, the resistance of the microelectrode very likely changes during impalement.
and the capacity compensation may need readjustment. The capacity compensation was therefore stepwise increased, while monitoring the deviation between \( V_{\text{int}} \) and \( V_{\text{meas}} \). A linear relationship was found between \( V_{\text{diff}} \) and \( I_{\text{m}} \), however, for some cells a different relationship was found for positive values of \( I_{\text{m}} \) compared to negative values. In the absence of \( C_{e2} \), such voltage jumps did not occur (data not shown). Using this circuit, the voltage at \( V_{\text{m}} \) was clamped with the dSEVC amplifier and measured independently with a second microelectrode amplifier. In the presence of \( C_{e2} \), \( V_{\text{meas}} \) deviated from \( V_{\text{int}} \), while \( V_{\text{meas}} \) and \( V_{\text{int}} \) were identical in the absence of \( C_{e2} \).

Fig. 2. Deviation of the \( V_{\text{meas}} \) from \( V_{\text{int}} \) depends on the clamp current and affects current–voltage relations. (A) The deviation of the measured from the intended clamp potential (\( V_{\text{diff}} = V_{\text{int}} - V_{\text{meas}} \)), plotted against the clamp current (\( I_{\text{m}} \)) for six cells. A linear relationship was found between \( V_{\text{diff}} \) and \( I_{\text{m}} \), however, for some cells a different relationship was found for positive values of \( I_{\text{m}} \) compared to negative values. (B) Steady state current–voltage relations of the same. The steady-state current (measured after 1.9 s) was plotted against the voltage recorded by the dSEVC amplifier (\( V_{\text{int}} \)) and against the voltage recorded independently by the regular microelectrode amplifier (\( V_{\text{meas}} \)). Note, the increased voltage dependence of outward and inward rectifying channels when the current is plotted against \( V_{\text{meas}} \).

Discussion

Based on the present results it was concluded that dSEVC amplifiers in their current state are not suited to study large conductance ion-channels in plant cells. For smaller conductances, however, the method can be used. Under the conditions applied in this report, a plasma membrane conductance of up to 0.8 nS can be measured with an error in the clamp potential of less than 10%.

There are two major problems in studying large conductance K\(^+\)-currents in guard cells with the dSEVC method. First, the voltage measured by the dSEVC amplifier at the end of the 2 s test pulses, deviates from the actual clamp potential. This gives rise to current–voltage plots that display an underestimated voltage dependence of ion-channels. Second, the clamp potential slowly changes in time as voltage-dependent channels activate and the clamp current increases (Fig. 1B, C). The measured activation of voltage-dependent channels is, in fact, a complex mix of changes in membrane conductance and a concurrent change of the clamp potential. Activation and deactivation kinetics obtained with this technique must therefore be interpreted with caution.

The reason that the dSEVC does not function properly at large clamp currents, was most likely due to its inability...
to compensate fully for the electrode capacitance. Fundamental problems of the method described by Etherton et al. are to some extent still relevant (Etherton et al., 1977). The first problem concerns the difference in time constant of the electrode and plasma membrane, which should differ sufficiently to separate settling of the electrode and membrane capacitance in time. These time constants should differ at least two orders of magnitude (according to Etherton et al., 1977). This requirement can be met for guard cells when the membrane potential is clamped to a value at which inward- and outward rectifying channels remain deactivated. At this potential, the resistance of the plasma membrane is high and therefore the membrane will only slowly discharge. Now, in principle, the capacity compensation can be set, based on the voltage response to current pulses.

More problematic is the slow settling capacitance of the electrodes. Electrodes do not behave like simple resistors with a capacitance in parallel, but a change in voltage will cause a redistribution of ions in the electrode, resulting in a slow change of the capacitance (Finkel and Redman, 1985). This feature of microelectrodes has received little attention, but is probably the cause for deviation between the potential measured by the dSEVC amplifier and the actual membrane potential.

The performance of the dSEVC can be improved by the use of microelectrodes with a lower resistance or capacitance. The capacitance may be reduced by coating of the electrode, but for the present experiments the electrode capacitance was already minimized by using thick-walled glass. A lower resistance can be achieved by pulling electrodes with a more blunt tip or by using a higher concentration of electrolyte. Electrodes with a more blunt tip, however, are difficult to impale through the cell wall, while the use of high electrolyte concentrations will load the cells with salt, causing a depolarization of the membrane potential (Blatt, 1987).

Alternatively, dSEVC amplifiers may be further developed. A circuit may be incorporated that corrects for the slow settling capacitance of microelectrodes. Such a circuit will have to be tuned to the electrode properties. The modified dSEVC should be designed in such way that it can correct for the change in electrode resistance, occurring upon impalement.
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References


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