J. Membrane Biol. 107, 229–235 (1989)

The Journal of
Membrane Biology

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Quantitative Analysis of Outward Rectifying K^+ Channel Currents in Guard Cell Protoplasts from *Vicia faba*

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Summary. A quantitative analysis of the time and voltage dependence of outward-rectifying K^+ currents ($I_{K^+,out}$) in guard cells from *Vicia faba* is described using the whole-cell patch-clamp technique. After step depolarizations from −75 mV to potentials positive to −40 mV, time-dependent outward currents were produced, which have recently been identified as K^+ channel currents. This K^+ current was characterized according to its time dependence and its steady-state activation. $I_{K^+,out}$ could be described in terms of a Hodgkin-Huxley type conductance. Activation of the current in time was sigmoid and was well fitted by raising the activation variable to the second power. Deactivating tail currents were single exponentials, which suggests that only one conductance underlies this slow outward K^+ current. Rates of channel closing were strongly dependent on the membrane potential, while rates of channel opening showed only limited voltage dependence leading to a highly asymmetric voltage dependence for channel closing and opening. The presented analysis provides a quantitative basis for the understanding of $I_{K^+,out}$ channel gating and $I_{K^+,out}$ channel functions in plant cells.

Key Words stomata · ion channel · rectifier · Hodgkin-Huxley · action potential · repolarization · patch clamp

Introduction

Voltage-gated outward-rectifying K^+-selective channels ($I_{K^+,out}$) have recently been found in patch-clamp studies of various higher plant cells (Schroeder, Hedrich & Fernandez, 1984; Iijima & Hagiwara, 1987; Schauf & Wilson, 1987; Schroeder, Raschke & Neher, 1987; Bush et al., 1988; Moran et al., 1988; Hedrich & Schroeder, 1989). Detailed investigations of $I_{K^+,out}$ channels in guard cells and pulvini have shown that ion transport properties of these channels correlate closely with physiological K^+ release during closing of stomata in leaves (Schroeder et al., 1987; Schroeder, 1988) and leaf movements (Moran et al., 1988).

Properties of the K^+ conductance in the algal cell *Nitella* such as the cation selectivity sequence (Sokolik & Yurin, 1986) are similar to the permeability sequence of $I_{K^+,out}$ channels described in guard cells ($P_{K^+} > P_{Rb^+} > P_{Na^+} > P_{Li^+} \gg P_{Cs^+}$) (Schroeder, 1988). The efflux of K^+ ions in algal cells occurring during action potentials (Gaffey & Mullins, 1958; Mummert & Gradmann, 1976) has been attributed to a K^+ conductance (for reviews *see* Hope & Walker, 1975; Tazawa, Shimmen & Mimura, 1987). This action-potential-associated algal K^+ conductance has been ascribed to outward-rectifying K^+ channel currents in the plasma membrane (e.g., Findlay & Coleman, 1983; Bertl & Gradmann, 1987; for review *see* Tazawa et al., 1987).

Action potentials have important physiological functions both in algal cells (for reviews *see* Hope & Walker, 1975; Tazawa et al., 1987) and in higher plant cells (for reviews *see* Sibaoka, 1966; Simons, 1981). The initial depolarization of algae action potentials is Ca^{2+} and Cl^- dependent (Findlay, 1961; Mullins, 1962; Beilby, 1982; Williamson & Ashley, 1982; for review *see* Tazawa et al., 1987). The Cl^--dependent depolarization has been investigated using a Hodgkin-Huxley approach (Beilby, 1982). It has been implied that an outward-rectifying K^+ conductance controls the repolarization of action potentials (Gaffey & Mullins, 1958). A quantitative description of the time dependence of the outward-rectifying K^+ conductance has, however, been hampered by complications inherent to voltage-clamp recordings with conventional microelectrodes in plant cells (*see* Findlay & Hope, 1976). Nevertheless, in some cases, voltage-clamp recording in algae have been feasible. In the spherical algal cell *Hydrodictyon africanum*, voltage-clamp recordings of the time- and voltage-dependent activa-

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tion of the outward-rectifying K^+ conductance were obtained (Findlay & Coleman, 1983). These K^+ currents correspond closely in their time and voltage dependence to $I_{K^+,out}$ channel currents characterized in guard cells, reflecting sigmoidal activation and exponential-like deactivation on a slow time scale (*see* Fig. 1) (Schroeder et al., 1987). The whole-cell patch-clamp technique (Marty & Neher, 1983) applied to single, isolated guard cell protoplasts circumvents problems inherent to microelectrode recordings and allows well-defined voltage-clamp recordings of $I_{K^+,out}$. Using this technique, a quantitative analysis of the time and voltage dependence of $I_{K^+,out}$ currents is conducted in this report, which may serve as a basis for studies of $I_{K^+,out}$ channel functions.

Materials and Methods

Guard Cell Isolation and Whole-Cell Recordings

The method used for guard cell protoplast isolation was the same as described elsewhere (Schroeder, 1988). This method for enzymatic purification of guard cell protoplasts from *Vicia faba* ("Grünkeimige Hangdown") produced high yields in seals between patch pipettes and the plasma membrane and allowed recordings of stable $I_{K^+,out}$ currents. The ability of protoplasts to swell during light exposure was tested as an indication of their physiological integrity (*see* Raschke et al., 1988). The whole-cell recording technique was applied as described in detail in previous reports (Schroeder et al., 1987; Schroeder, 1988). In the present study, all whole-cells were selected for effective access resistances of ≤10 MΩ. Experiments were performed at temperatures of 22 ± 1°C.

Solutions

The composition of the bathing medium was (in mM): 10 K-glutamate, 2 $MgCl_2$, 1 $CaCl_2$, 1 KOH, 10 MES (2-(N-morphalino) ethanesulfonic acid), pH 5.5. The composition of the pipette solution, which equilibrates with the cytoplasm was (in mM): 100 K-glutamate, 2 $MgCl_2$, 1 EGTA, 5 KOH, 10 HEPES, 2 MgATP, pH 7.2. Solutions were adjusted to final osmolalities of 480 mmol kg^{-1} (bath solution) and to 530 mmol kg^{-1} (internal solution) by addition of D-mannitol and verification with a vapor pressure osmometer (Wescor 5100C).

Data Recording and Analysis

Data were low-pass filtered at 200 or 400 Hz with eight-pole bessel characteristics and were subsequently sampled at five times the filter cutoff frequency and stored on a PDP 11/73 computer (INDEC, Sunnyvale, CA) operating on line. Programs were developed for on-line reading and off-line analysis of data sequences. For a quantitative description of activation and deactivation time courses of K^+ currents, arbitrary functions were fitted to the data by the nonlinear Marquardt algorithm of iterative least squares (Marquardt, 1963). As fit routines were designed to fit 60 data points, running averages of the data were made prior to fitting. All data values and error bars reflect the mean ± SD.

Results

We have shown previously that guard cell protoplasts respond to depolarizing and hyperpolarizing voltage-clamp pulses by developing outward- and inward-rectifying potassium currents through potassium-selective channels (Schroeder et al., 1984, 1987). The steady-state ion-transporting properties of both of these K^+ currents have been described in detail in order to assess their importance for K^+ transport during guard cell movements (Schroeder, 1988). The object of the experiments reported here was to investigate in detail the kinetics of the slowly activating outward K^+ currents ($I_{K^+,out}$).

Sigmoid Activation of $I_{K^+,out}$

In Fig. 1*A*, whole-cell K^+ currents were recorded in response to voltage pulses of 1-sec duration. The sigmoidal rise of $I_{K^+,out}$ at potentials (V_{pulse}) more positive than −40 mV and the exponential decay of whole-cell currents at the end of the pulse (V_{tail} = −115 mV) were clearly resolved. It was generally observed that the outward-rectifying K^+ currents reached steady-state levels within 2 sec of depolarization. Furthermore, it has been demonstrated previously that $I_{K^+,out}$ showed no significant signs of inactivation, even during depolarizations longer than 10 min (Schroeder, 1988).

The sigmoid activation and exponential deactivation of transmembrane K^+ currents was first described by Hodgkin and Huxley (1952) in the squid giant axon by a scheme of the form:

$$I_{K^+} = n^p \, I_{K^+,max} \quad (1)$$

where n is the activation variable and $I_{K^+,max}$ is the completely activated current. The value of the exponent p can be interpreted as the number of independent membrane-bound gating particles, which control the opening of $I_{K^+,out}$ channels (Hodgkin & Huxley, 1952). The sigmoidal rise of $I_{K^+,out}$ in Fig. 1*A* indicates that $p > 1$. To determine the value of p, currents elicited by steps from the holding potential (V_H = −75 mV) to V_{pulse} = −20 to +81 mV were fitted by the equation:

$$I_{K^+,out} = I_L + I_{K^+_\infty}[1 - \exp(-t/\tau_n)]^p \quad (2)$$

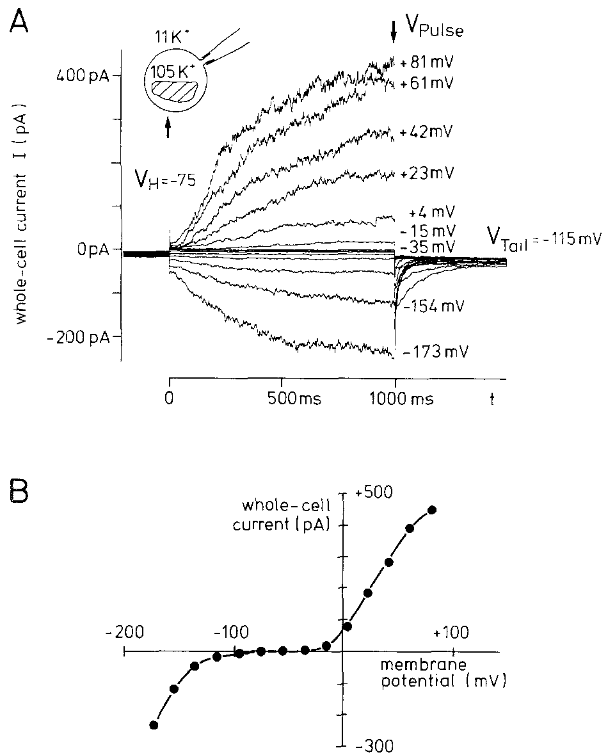

Fig. 1. Recordings of outward ($I_{K^+,out}$) and inward K^+ currents across the plasma membrane of a single guard cell protoplast with 105 mM K^+ (100 mM K-glutamate + 5 mM KOH) in the cytoplasm and 11 mM K^+ (10 mM K-glutamate + 1 mM KOH) in the bath (*see* insert top left. (*A*) The superposition of 14 sequential recordings of K^+ currents in response to individual voltage pulses. Each voltage pulse started at the upward pointing arrow and terminated at the downward pointing arrow. The applied voltages (V_{pulse}) are shown to the right of elicited currents. Upward deflecting traces have been previously shown to correspond to K^+ efflux and downward deflecting traces correspond to K^+ influx through K^+ channels (Schroeder et al., 1987). The group of more rapidly deactivating tail currents at the end of the pulse, $V_{tail} = -115$ mV, corresponds to deactivation of $I_{K^+,out}$. Between each pulse the membrane was held at −75 mV (V_H) for 12 sec. *B* shows the plot of K^+ current measurements at the end of the pulse in *A* as a function of the pulse potential

where I_L is a leakage component contributed by the whole-cell membrane resistance ($R_m \approx 10$ GΩ), $I_{K^+_\infty}$ is the steady-state current after activation and τ_n represents the activation time constant. The time course of activation of $I_{K^+,out}$ was fitted by Eq. (2), with $I_{K^+_\infty}$, γ_n and p as free running parameters. The data were best described by Eq. (2) when the value of the parameter p was approximately 2 (2.05 ± 0.4, $n = 20$). Therefore, in Eq. (2), the parameter p was fixed to $p = 2$. Figure 2 demonstrates that the activation of $I_{K^+,out}$ is well described by Eq. (2) for $p = 2$. The activation time constants τ_n plotted as a function of the membrane potential (Fig. 3) reflect that τ_n is only slightly dependent on the membrane

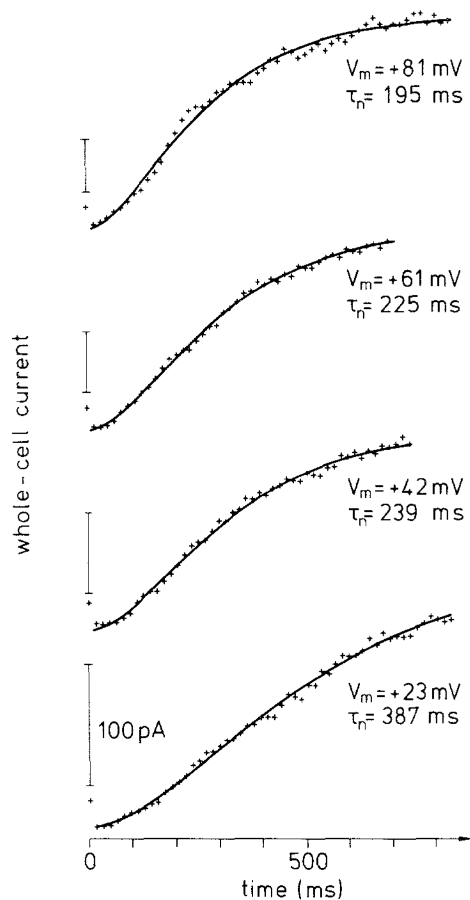

Fig. 2. Nonlinear least squares fit of the Hodgkin-Huxley Eq. (2) for $p = 2$ to $I_{K^+,out}$ currents. Membrane potentials during the pulse (V_m) and activation time constants (τ_n) are indicated to the right of each trace. Crosses represent the data points, which were obtained by computing the running average in the vicinity of each cross and continuous lines show the fitted curves

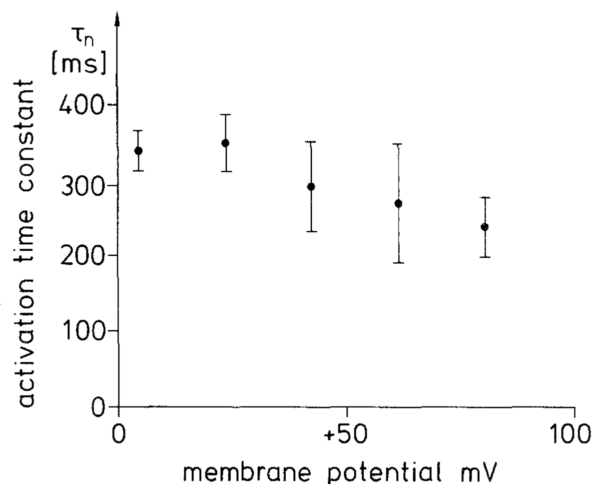

Fig. 3. Voltage dependence of activation time constants deduced by fitting of Eq. (2) for $p = 2$ as shown in Fig. 2 ($n = 5$ cells, error bars ± SD)

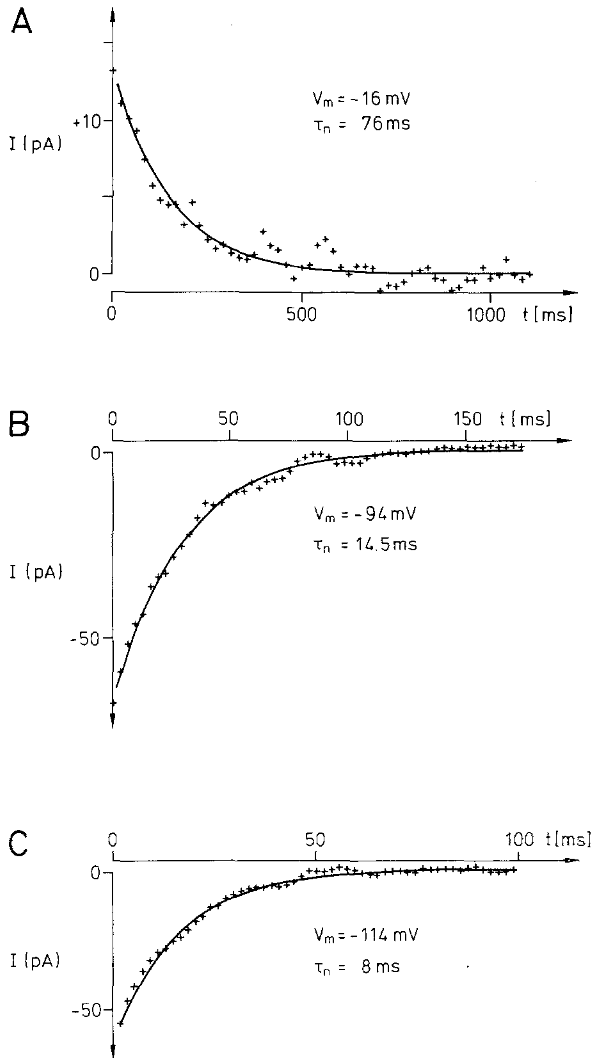

Fig. 4. (*A–C*) Deactivating currents recorded in response to hyperpolarization from a holding potential of V_H = +80 mV. Continuous lines show the fit of single exponentials to the data (crosses). Tail potentials (V_m) and time constants (τ_n for p = 2 in Eq. (3)) are indicated for each trace. (Note that the time scales are different in *A*, *B*, and *C*). Data points for t = 0 were not taken into account when exponentials were fitted to the data

potential. τ_n values showed a variability from cell to cell. In each cell, activation time constants τ_n decreased with growing depolarizations indicating that the voltage dependence (Fig. 3) was significant.

Decay of Currents

Deactivating tail currents were elicited by steps from $V_{pulse} \approx +80$ mV to $V_{tail} = +4$ to -160 mV (*see* Schroeder et al., 1987). These tail currents were fitted to sums of exponentials of the form

$$I_{K^+,tail} = \Sigma I_{K^+,0} \exp(-t/\tau_n)^p \quad (3)$$

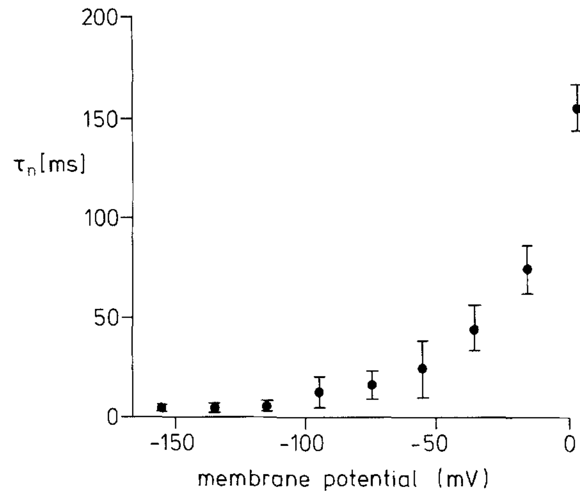

Fig. 5. The deactivation time constants derived from tail current fits are plotted as a function of V_{tail} (n = 5 cells, error bars ± SD)

where $I_{K^+,0}$ were the initial values of exponentials. In 80% of the fits (n = 60), $I_{K^+,tail}$ was described best by a single exponential (Fig. 4). This was also the case for tail potentials more positive than the threshold of activation (−40 mV), although the Hodgkin-Huxley model would predict the sum of two exponentials with time constants τ_n and $\tau_n/2$ (Hodgkin & Huxley, 1952). Possibly two separate time constants could not be distinguished as the predicted two time constants lie close together and as tail currents had a small signal-to-noise ratio in the depolarized range (*see* Fig. 4*A*). The underlying model predicts that τ_n values of activation and deactivation should be equivalent at a given potential. In the range of 0 mV, deactivation and activation time constants converged. The voltage dependence of deactivation time constants is shown in Fig. 5 for p = 2. It is apparent that the deactivation of $I_{K^+,out}$ is strongly dependent on the membrane potential.

The Steady-State Voltage Dependence of $I_{K^+,out}$

The data in Fig. 1*B* indicate the range of membrane potentials at which $I_{K^+,out}$ was activated. The voltage dependence of this gating process must be determined with precision to understand the role of $I_{K^+,out}$ activation. The steady-state activation curve of $I_{K^+,out}$ was obtained by applying longer pulses than those shown in Fig. 1*A* (4-sec or 10-sec duration) to ensure that $I_{K^+,out}$ channel currents had plateaued. The Hodgkin-Huxley (1952) scheme for steady-state activation for p = 2 has the form $n_\infty^2 = G_{K^+}/G_{K^+,max}$ where n_∞ = the steady-state value of the activation variable, G_{K^+} represents the K^+ conductance (chord conductance) of the membrane at a given potential and $G_{K^+,max}$ is the maximum K^+ con-

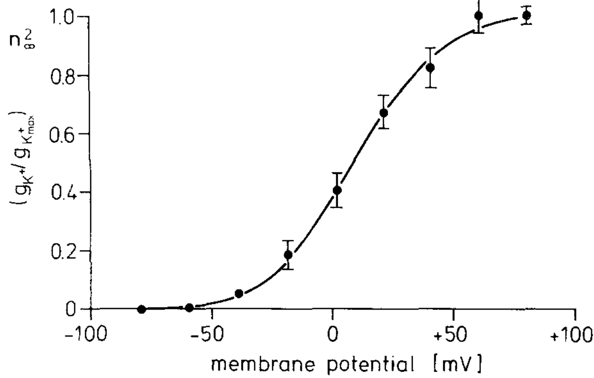

Fig. 6. The steady-state activation curve for $I_{K^+,out}$. The degree of activation n_∞^2 was determined by computing $G_{K^+}/G_{K^+,max}$. The data points were fitted by a Boltzmann distribution (continuous line) (n = 5 cells, error bars ± SD)

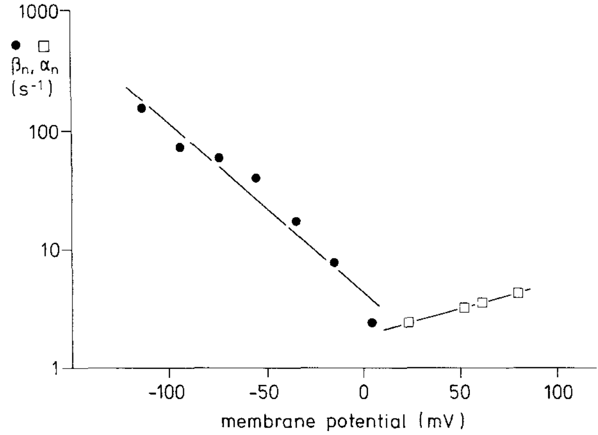

Fig. 7. Semilogarithmic plot of activation rates α_n and deactivation rates β_n as a function of the membrane potential (fitted curves: *see* text)

ductance. Conductance values G_{K^+} and $G_{K^+,max}$ were determined with respect to the K^+ equilibrium potential of −53 mV. The conductance of $I_{K^+,out}$ saturated approximately at potentials more positive than +50 mV. Figure 6 shows n_∞^2 as a function of the membrane potential determined in five cells. A Boltzmann distribution was fitted to the averaged values. The fitted equation was:

$$n_\infty^2 = \frac{1}{[1 + \exp((V_{0.5} - V_m)/S)]^2} \qquad (4)$$

where $V_{0.5}$ represents the potential for $n_\infty = 0.5$ ($n_\infty^2 = 0.25$) and S is the slope factor. Best fits were obtained for $V_{0.5} = -7$ mV and $S = 21$ mV.

Rate Constants for Channel Opening and Closing

The Hodgkin-Huxley model can be used to obtain a kinetic scheme for the transition of single $I_{K^+,out}$ channels from closed states to the open state (Armstrong, 1969). For $p = 2$, a three-state model with two closed states and one open state can be deduced:

$$C_2 \underset{\beta_n}{\overset{2\alpha_n}{\rightleftharpoons}} C_1 \underset{2\beta_n}{\overset{\alpha_n}{\rightleftharpoons}} 0. \qquad (5)$$

The voltage-dependent transition rates for channel opening α_n and for channel closing β_n were calculated from the measured values for n_∞ (Fig. 6) and τ_n (in Figs. 2–5) by the equations (Hodgkin & Huxley, 1952):

$$\alpha_n = n_\infty/\tau_n \qquad (6)$$
$$\beta_n = (1 - n_\infty)/\tau_n. \qquad (7)$$

Figure 7 shows the rate constants α_n and β_n as derived from the steady-state activation curve (n_∞) and from the fits of activation and deactivation time constants τ_n. The voltage-dependent rate constants of channel opening α_n and channel closing β_n were fitted by the equations

$$\alpha_n = \alpha_{n^0} \exp[(V_m - V_h)/S\alpha_n] \qquad (8)$$
$$\beta_n = \beta_{n^0} \exp[(V_h - V_m)/S\beta_n] \qquad (9)$$

where α_{n^0} and β_{n^0} are scaling factors, V_h determines the voltage dependence of both rate constants and $S\alpha_n$ and $S\beta_n$ give the steepness of the voltage dependence of the parameters. Curves in Fig. 7 show the fit of these equations with the parameters: $\alpha_{n^0} = 1.9$ sec^{-1}, $\beta_{n^0} = 4.3$ sec^{-1}, $V_h = 0$ mV, $S\alpha_n = 95$ mV and $S\beta_n = 31$ mV. The resulting α_n, β_n curve was highly asymmetrical (Fig. 7, semilog plot).

It should be noted that the three-state kinetic model (Eq. (5)) represents a simplified gating scheme. In single-channel recordings, additional fast flickering closed states were observed, which led to brief closures of open channels for durations in the msec range, as deduced from kinetic analysis of single-channel currents (Schroeder, *unpublished*). Therefore, the kinetic scheme (Eq. (5)) should be regarded as a reduced model, which successfully describes the slow time course of whole-cell outward K^+ currents. The derived model may serve as a basis for a quantitative understanding of macroscopic, $I_{K^+,out}$ currents and their function in plant cells.

Discussion

Use of Guard Cells for the Study of $I_{K^+,out}$

Isolated guard cell protoplasts maintain their physiological functioning such as swelling when exposed

to light and retaining biochemical mechanisms involved in stomatal movements (for review *see* Raschke et al., 1988). Therefore, these cells appear suited for detailed investigation of cell biological and biophysical processes. The well-defined voltage-clamp conditions of whole-cell recordings in this study have permitted a detailed quantitative description of the voltage and time dependence of outward-rectifying K^+ currents.

Using microelectrodes in algae, Findlay and Coleman (1983) have recorded K^+ current activation and deactivation in response to similar voltage-step protocols used here. These microelectrode studies in *Hydrodictyon africanum* show the properties of the time-dependent outward-rectifying K^+ conductance to be very similar to $I_{K^+,out}$ channels studied here. This similarity can be taken as an indication that isolation procedures used in the present study have not significantly altered $I_{K^+,out}$ channel properties. Recently, patch-clamp studies with various plant cells have indicated the existence of time- and voltage-dependent outward-rectifying K^+ currents in all cells investigated (for reviews *see* Tazawa et al., 1987; Hedrich & Schroeder, 1989; *see also* Introduction). Therefore, a quantitative understanding of $I_{K^+,out}$ channel kinetics may be of general significance.

Voltage and Time Dependence of $I_{K^+,out}$ Channels

The steady-state activation curve of $I_{K^+,out}$ (Fig. 6) showed half-maximal activation at -7 mV and saturation for potentials more positive than $+50$ mV. It was observed that $I_{K^+,out}$ exhibited a sigmoidal onset upon depolarization and an exponential decay upon hyperpolarization. The data consistently showed that the sigmoid activation could be accurately modeled when the power of the activation variable p was 2 (Fig. 2). The deactivation of $I_{K^+,out}$ was well described by a single exponential (Figs. 4 and 5). These findings suggest that a single Hodgkin-Huxley conductance underlies the macroscopic $I_{K^+,out}$ and that its kinetics can be described mathematically by a first order reaction with two closed channel states and one open state (Eq. (5)). This was further supported by the previous finding that the slow onset and decay of $I_{K^+,out}$ could be reconstructed by averaging single K^+ channel currents with one open conducting state (Schroeder et al., 1987). A recent study of the large conductance K^+ channels in membrane vesicles from *Acetabularia* resulted in a three-state model (Bertl, Klieber & Gradmann, 1988). It should be noted that in addition to time-dependent activation, an instantaneously rectifying component was found in protoplasts from *Dionaea muscipula* (Iijima & Hagiwara, 1987). In outside-out patches from guard cells, a second outward K^+ channel conductance state was found, which had a very low open probability and a small channel conductance ($\approx$5 pS with 210 mM K^+ internal and 35 mM K^+ external; J. Schroeder, *unpublished*). The contribution of these low conductance channels was estimated to be small in the present study. Recent studies of outward-rectifying K^+ channels in frog atrium showed very similar slow kinetics to those of $I_{K^+,out}$ channels. These atrium K^+ channels were shown to play a major role during action potential repolarization (Hume et al., 1986; Simmons, Creazzo & Hartzell, 1986).

A quantitative description of channel opening rates (α_n) and closing rates (β_n) was obtained from the Hodgkin-Huxley model. The resulting voltage dependence of α_n and β_n was highly asymmetrical (Fig. 7). This asymmetry may be explained by gating particles, which sense little of the transmembrane potential drop during activation, while gating particles sense a larger portion of the transmembrane potential during deactivation (Benz & Conti, 1981). Interestingly, in the squid axon Hodgkin and Huxley (1952) found an assymetry in the gating of K^+ currents which was inverse to that found in guard cells.

Physiological Significance of $I_{K^+,out}$

The major physiological function of $I_{K^+,out}$ as a K^+ release pathway for guard cells during closing of gas exchange pores (stomata) in leaves has been studied and discussed in detail in previous reports (Schroeder et al., 1984; Schroeder et al., 1987; Schroeder, 1988). It is well established that conductance changes during action potentials in algae (Cole & Curtis, 1938) are accompanied by an efflux of K^+ ions (Gaffey & Mullins, 1958; Mummert & Gradmann, 1976). K^+ efflux during action potentials has been attributed to an outward-rectifying K^+ conductance, which may play a role in repolarization.

Quantitative knowledge of the kinetics of $I_{K^+,out}$ obtained in this study make it possible to examine its putative role during action potential repolarization. $I_{K^+,out}$ is activated by depolarizations positive to approximately -40 mV and half activated at -7 mV. Hence, $I_{K^+,out}$ channels will open during the depolarization phase of the action potential. Higher plant action potentials remain more depolarized than 0 mV for durations of approximately several hundred milliseconds (*see* Sibaoka, 1966). The time constant for $I_{K^+,out}$ activation at this potential is approximately 350 msec, meaning that a substantial portion of $I_{K^+,out}$ will be activated during each action

potential. $I_{K^+,out}$ activation will initiate repolarization, which in turn will lead to an exponential decay of $I_{K^+,out}$. Unfortunately, action-potential-like depolarizations in guard cells could only be recorded on few occasions (J.I. Schroeder & R. Penner, *unpublished*). However, during current-clamp recordings in *Hydrodictyon africanum*, repolarization times were recorded, which closely resemble $I_{K^+,out}$ kinetics (Findlay & Coleman, 1983).

In conclusion, the existence of an $I_{K^+,out}$ type conductance has been indicated in every plant cell studied to date. The quantitative description of the outward-rectifying K^+ channel currents presented here gives a biophysical basis for $I_{K^+,out}$ channel action and may provide a model for studying plant action potential repolarization. Future voltage-clamp recordings of putative depolarizing inward currents in higher plant cells will be required to understand depolarizing mechanisms with accuracy.

I thank Drs. E. Neher and W. Stühmer for discussions during experiments, which were predominantly performed at the Max Planck Institut für biophysikalische Chemie, Göttingen, FRG. I gratefully acknowledge Dr. S. Hagiwara, Dr. J.A. Umbach, and Mr. J. Deeds for critical reading of the manuscript. J.I.S. was supported by fellowships from the Max Planck Gesellschaft and the Alexander von Humboldt Foundation and National Institute of Health grant No. 5 T32 NSO 7101-10.

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Received 25 July 1988; revised 11 November 1988