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Hodgkin-Huxley Analysis of a GCAC1 Anion Channel in the Plasma Membrane of Guard Cells

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Abstract. A quantitative analysis of the time- and voltage-dependent kinetics of the guard cell anion channel (GCAC1) current in guard cell protoplasts from *Vicia faba* was analyzed using the whole-cell patch clamp technique. The voltage-dependent steady-state activation of GCAC1 current followed a Boltzmann distribution. For the corresponding steady-state value of the activation variable a power of two was derived which yielded suitable fits of the time course of voltage-dependent current activation. The GCAC1 mediated chloride current could successfully be described in terms of the Hodgkin-Huxley equations commonly evoked for the Na channel in nerve. After step depolarizations from a potential in the range of the resting potential to potentials above the equilibrium potential for chloride an activation and also an inactivation could be described. The gating of both processes exhibited an inverse relationship on the polarity of the applied step potentials in the order of milliseconds. Deactivating tail currents decline exponentially. The presented analysis contributes to the understanding of the rising phase of the observed action potentials in guard cells of *V. faba*. Evidence is presented that the voltage-dependent kinetic properties of the GCAC1 current are different from those properties described for the excitable anion currents in the plasmalemma of *Chara corallina* (Beilby & Coster, 1979*a*).

Key words: Anion channel GCAC1 — Guard cells — Patch clamp — Voltage-dependent kinetics — Hodgkin-Huxley equations

Introduction

Anion channels in guard cells provide a central control mechanism for the regulation of stomatal movement. Two types of anion currents with distinct voltage-dependent regulation have been observed in guard cell protoplasts from the broad bean *Vicia faba*, a specialized cell type in the epidermal layer of the leaf. One type of anion channel current, the guard cell anion channel (GCAC1), exhibits a steep voltage dependence (Keller et al., 1989; Hedrich et al., 1990; Marten et al., 1991; Marten et al., 1992). As revealed by whole-cell patch clamp recordings of the current-voltage relationship the corresponding chloride current peaks between –40 and –30 mV. Prolonged stimulation by depolarizing voltages causes a complete inactivation of channel current. The other type of anion channel current, the slow activated anion channel (SLAC), has been described by Linder & Raschke (1992) and Schroeder & Keller (1992). The activation of SLAC by depolarization occurs in the time scale of several seconds. A significant inactivation could not be observed. The slow voltage-dependence yields to an about linear course of the corresponding whole-cell current-voltage relationship.

Previously, Beilby and Coster (1979*a*) applied the Hodgkin and Huxley (H-H) formalism (Hodgkin & Huxley, 1952) to the voltage-activated inward chloride current which they considered as the dominating mechanism for the depolarization in plasma membranes of the freshwater alga *Chara corallina*. In contrast to the H-H equations of the Na transients in nerve, a time delay between the depolarizing stimulus and the onset of excitation of up to seconds had to be introduced. Since the patch clamp method was not known at that time voltage-clamp experiments were performed to determine the H-H parameters describing the excitation of the plasma membrane. As a consequence of the experimental approach, the intracellular electrolyte composition was unknown. The measured membrane current was described by a superposition of a chloride current and a second current,

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probably a calcium influx. The analysis allowed a sufficient modeling of the observed action potential (AP) in *C. corallina* which is slower than that in the nerve by a factor of thousand (Beilby & Coster, 1979*b*). AP-like transient changes in the membrane potential have been recognized in various plant cells, too (*cf.* Tazawa et al., 1987; Blatt, 1991). The kinetics and shape of the AP differ strongly between animals and plants as well as between the well-known excitable plant species like *Dionaea, Mimosa* and *Drosera* on the one hand and crop plants on the other (Sussman, 1992; *cf.* Hedrich & Becker, 1994).

Recently AP-like fluctuations were also observed in guard cells of *V. faba* (Thiel et al., 1992). In the opening stoma the resting potential range of the guard cells, −100 to −280 mV, is dominated by an inward rectifying potassium channel and the proton pump (Blatt, 1991; Lohse & Hedrich, 1992). During stomatal closure a voltage-dependent anion channel and delayed rectifying potassium channel are able to catalyze salt efflux from the guard cells (Schroeder et al., 1987; Schroeder & Hedrich, 1989; *cf.* Hedrich, 1994). Fine-tuning of the final amplitude of these two major states and of transient stomatal action is coordinated through the action of various exogenous stimuli such as hormones, CO_2/malate or calcium and nucleotides through membrane-bound perception sites (Schroeder & Hagiwara, 1989; Marten et al., 1991; Hedrich & Marten, 1993). Gradmann et al. (1993) tried to reconstruct the measured action potential of guard cell protoplasts by a superposition of five noninteracting ion transporters of identical membrane density. The current mediated by GCAC1 was included. For the unknown voltage-sensitivity of the different rate constants a simplified Eyring rate theory was proposed (*see* Discussion). But the described model was too complex to yield reliable predictions of the measured shape of action potentials.

In the present study, we applied the whole-cell patch clamp technique (Marty & Neher, 1983) to single isolated guard cell protoplasts to determine the time constants of fast activation, inactivation and deactivation of GCAC1 as a function of voltage. In a first approximation, the voltage-jump current relaxation experiments were analyzed in terms of the H-H equations for the Na channel in nerve. The corresponding number of hypothetical particles that are responsible for the voltage gate was determined. In addition, the voltage-dependent time constant of deactivation was determined. Comparison of our set of H-H parameters, derived for the GCAC1-mediated chloride current, with that obtained on *C. corallina* (Beilby & Coster, 1979*a*) indicates a distinct nature of the chloride channel currents. The results may help to understand the onset of the action potentials in guard cells.

Materials and Methods

Protoplast Preparation and Current Recording

Guard cell protoplasts were enzymatically isolated from 2–3-week old leaves of the broad bean, *Vicia faba* (Hedrich et al., 1990). Patch pipettes were sealed against the plasma membrane to study ion fluxes in the whole-cell configuration (Hamill et al., 1981). After guard cell protoplasts were added to the recording chamber and stuck to its glass bottom, the chamber was perfused with the extracellular solution (0.13 ml/min). Repetitive voltage ramps of 1 sec duration from −196 to about +70 mV enabled us to record the activation state and voltage dependence of GCAC1 mediated whole-cell anion current. In parallel single and double voltage pulses were applied to guard cell protoplasts after the Ca^{2+}- and nucleotide-dependent activation process of GCAC1 (Hedrich et al., 1990) had reached a quasi steady state.

Current measurements were performed with either an EPC-7 or EPC-9 patch clamp amplifier (List electronic, Darmstadt, and HEKA Lambrecht, FRG). The current records were low-pass filtered by an eight-pole Bessel filter. Membrane potentials have been corrected for the liquid-junction potential (*see* Neher, 1992 and Barry & Lynch, 1991) and for the series resistance (e.g., Fig. 3; R_{series} = 4.3–4.8 MΩ; $I_{steadstate} \leqslant 2600$ pA; $I_0 \leqslant 8000$ pA; C = 6.6–7.7 pF, *cf.* Lohse & Hedrich, 1992).

Data Analysis

Data were digitized with a sample rate of 0.1 msec (VR10, Instrutech, Elmont, NY, and stored on a hard disc. In Fig. 6, the inactivation kinetic was analyzed with the patch clamp program Review (Instrutech) on an Atari Mega ST4. For further analysis of the time course of activation, inactivation and deactivation, every 0.5 (or 1) msec 5 (or 10) original data points were averaged. In consideration of residual capacities, the first or the first two collected data points were omitted. The remaining data points were used to fit the H-H equations. The fitting was done by the nonlinear least-squares fitting programs NFIT and Mathcad (Mathsoft, Cambridge, MA), the parameters being adjusted to obtain the lowest chi-square value. Weightings of the data points were not used. Doubling of the number of digitized data points from the original continuous time course of whole-cell currents did not significantly change the values of the fitted parameters.

An activation variable to the second power, m^2, of the H-H Eq. (1), which was used to describe the activation kinetics of GCAC1, would ask for a sigmoidal increase in current with the onset of the voltage pulse. The predicted activation kinetics, however, could not be demonstrated properly, since residual capacities (even the use of an automatic patch clamp with capacitance compensation (EPC-9)) prevented the required time resolution of less than 100 μsec. Data were presented as mean ± SEM; *n* denotes the number of independent experiments.

Solutions

Solutions contained (in mM): 40 $CaCl_2$, 2 $MgCl_2$, 10 MES/TRIS pH 5.6 in the bathing medium and 150 TEACl, 2 $MgCl_2$, 10 MgATP and Na_2GTP, 0.1 EGTA, 10 HEPES/TRIS pH 7.2 in the pipette (cytoplasm). Experiments were performed at room temperature.

Results

Voltage-dependent Activation and Inactivation of GCAC1

The voltage-dependent properties of GCAC1 were studied in the whole-cell configuration (Hamill et al., 1981).

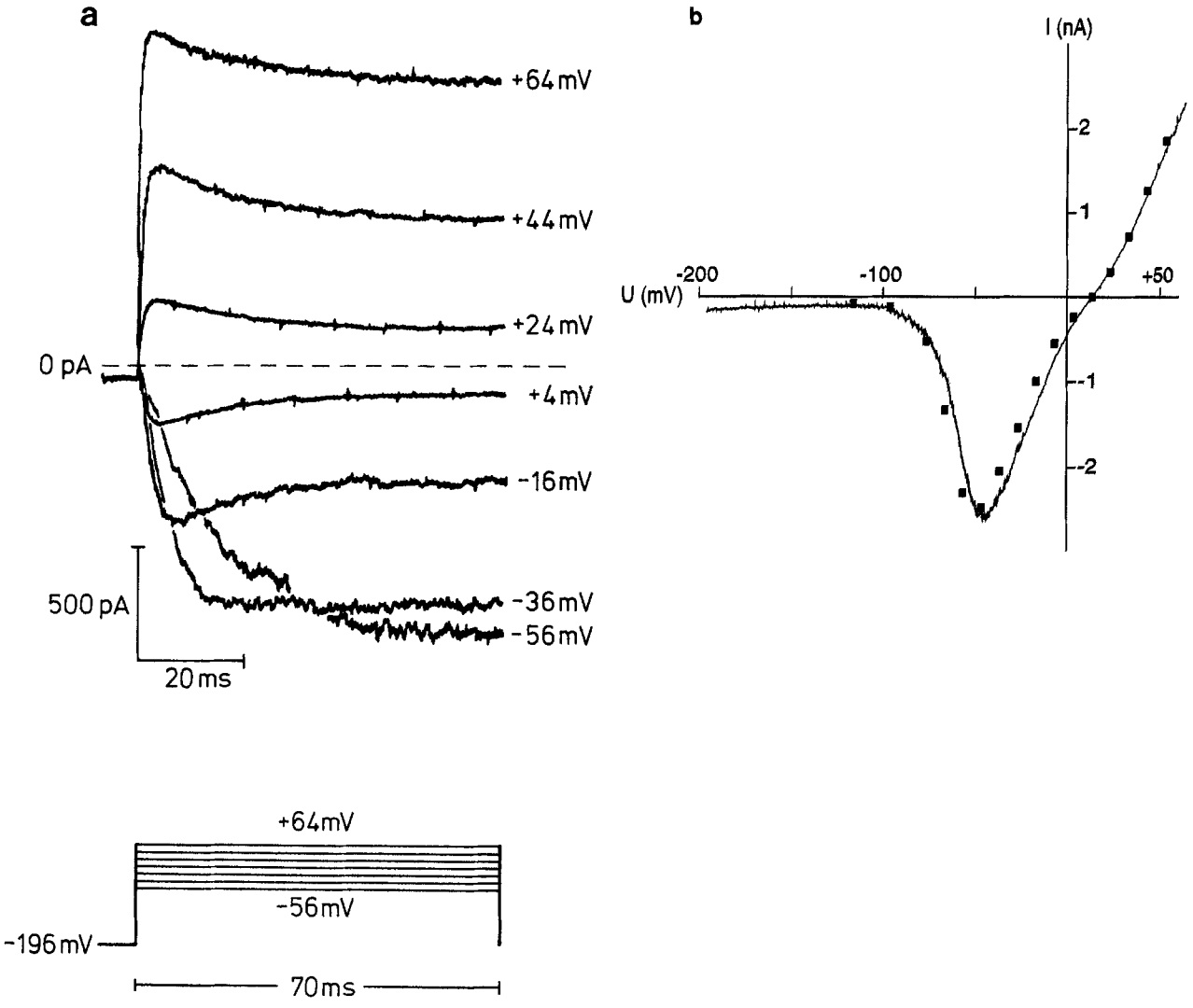

Fig. 1. Voltage- and time-dependent activity of GCAC1 in one protoplast from *V. faba.* (*a*), chloride currents (inward and outward current; upper panel) evoked by consecutively applied voltage pulses of 70 msec duration to −56, −36, −16, 4, 24, 44, and 64 mV (lower panel) from a holding potential of −196 mV. The holding potential was applied for 60 msec, respectively. (*b*), current-voltage relationship elicited by a voltage ramp from −196 mV to +64 mV at 0.25 mV/msec. Closed squares represent the corresponding mean values of the current after the onset of the voltage-jump current relaxations as obtained in the range of 60–70 msec of the data given in *a.*

Previously we presented evidence that the activation of GCAC1 mediated current depends on extracellular calcium as well as on the presence of nucleotides (Hedrich et al., 1990). To achieve reproducible results 40 mM $CaCl_2$ was added to the bath and 10 mM MgATP to the pipette filling solution throughout the experiments. To study quantitatively the voltage-dependent kinetics of the transitions between the open and closed states of GCAC1, a series of single and double potential-step sequences were applied to guard cell protoplasts.

GCAC1-mediated current is characterized by a threshold potential for activation positive to −100 mV (*cf.* resting potential of −100 to −250 mV; Blatt, 1991; Lohse & Hedrich, 1992), a rapid activation, slower inactivation and deactivation (Hedrich et al., 1990). Therefore, when the membrane potential was clamped to −196 mV, a value which corresponds to the resting level of this cell type, only leak-related inward currents (I_{leak}) of −116.6 ± 43.9 pA (n = 8) were observed, indicating that GCAC1 was closed (Figs. 1 and 2). After a depolarizing voltage pulse of 70 msec duration, a rapid activation of the chlorid current ($I_{Cl}(t)$) in the order of milliseconds became visible which was superimposed by a slower inactivation (Fig. 1*a*). As the figure indicates, the latter process became visible at potentials positive to about −50 mV, but a quantitative analysis of fast inacti-

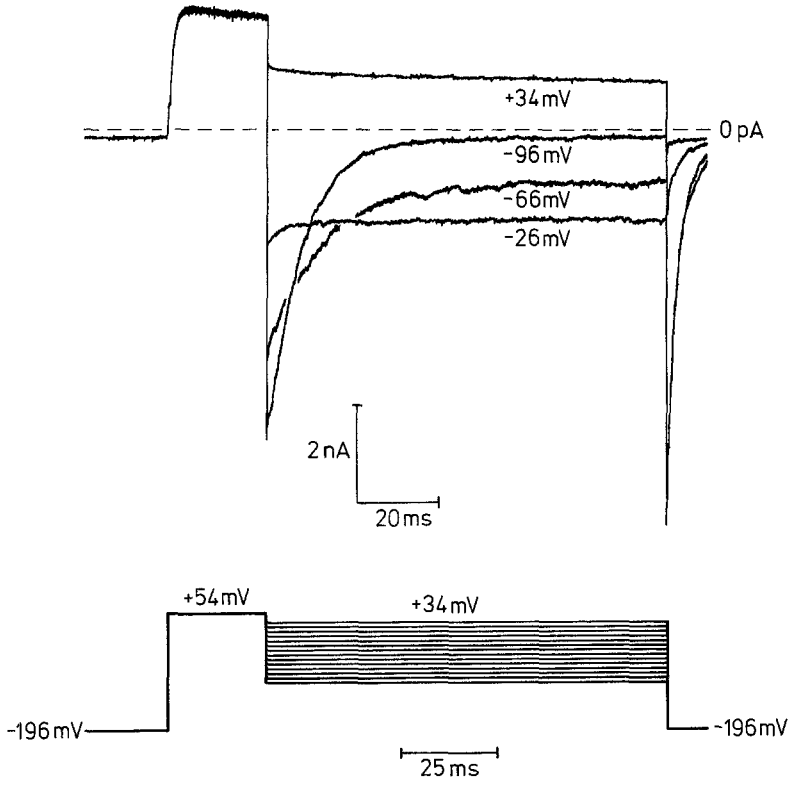

Fig. 2. Whole-cell chloride currents during a double voltage-pulse sequence. The membrane potential was stepped from a holding potential of −196 mV for 25 msec to a prepulse level of +54 mV and then for 100 msec to less depolarized potentials (lower panel). Four current traces are superimposed (upper panel). During the test pulse the macroscopic currents declined with voltage-dependent time courses.

vation (*see below*) could only be performed for depolarizing voltages above about −20 mV.

For an analysis of the underlying gating mechanism of GCAC1, we applied the Hodgkin-Huxley formalism (Hodgkin & Huxley, 1952) which is based on the assumption of a simultaneous and independent movement of activation and inactivation gates/particles. As an analogy to this model, the time- and voltage-dependent anion currents are presented by the empirical relation:

$$I_{Cl}(t) = m(t)^z h(t)\ g_{max}\ (U - E_{Cl}) \qquad (1)$$

wherein z has been considered as the number of independent membrane-bound m particles controlling the rapid activation and an h particle responsible for the inactivation process. g_{max} denotes the maximal anion conductance and E_{Cl} the corresponding reversal potential.

The number of gating particles z that have to move by the depolarizing voltage to open the channel was estimated from the voltage-dependent open probability of GCAC1. The number is reflected by g/g_{max}, and was derived from double voltage-pulse analysis (*see* Fig. 2). From a holding potential of −196 mV (all channels closed/deactivated) g_{max}, the maximal conductance, was obtained from current relaxations (tail currents, *cf.* Hedrich et al., 1990) and $g = g(U)$, the conductance in the steady-state (t = 100 msec) by steps to various negative and positive voltages U after a prepulse to +54 mV, respectively. This prepulse voltage was selected to maximally activate GCAC1 while minimizing inactivation (Figs. 2 and 3*a*). As expected from an ohmic resistor, instantaneous currents through the open channels (tail amplitudes, Figs. 2 and 3*a*, open circles) exhibited a linear dependence on the potential difference between the reversal potential and the pulse potential U (Fig. 3*a*, open circles). The voltage dependence of steady-state currents (Fig. 3*a*, filled circles) was identical to that obtained from single-pulse experiments (*see* Fig. 1). Anion currents activate positive to −100 mV, peak at −30 to −40 mV and reverse direction at E_{Cl} (*cf.* Hedrich et al., 1990). The fraction of open channels is given by g/g_{max} (Fig. 3*a*, squares) and was derived from the ratio I_{ss}/I_0, steady-state current (Fig. 3*a*, filled circles) to tail current (Fig. 3*a*, open circles). Since during the prepulse a small but noticeable inactivation (Fig. 2), reducing the possible number of the maximally activated channels, could not be avoided, the fraction I_{ss}/I_0 was normalized with respect to the maximal ratio. The corresponding voltage dependence for the probability of the closed-to-open transition ($P(U)$) of GCAC1, can be described in terms of a first-order-reaction represented by the Boltzmann equation:

$$P(U) = \frac{I_{ss}(U)/I_0(U)}{(I_{ss}(U)/I_0(U))_{max}} = \frac{1}{1 + \exp((U - U_{1/2})/\alpha)} \qquad (2)$$

For the experiment shown in Fig. 3*a* (filled squares)

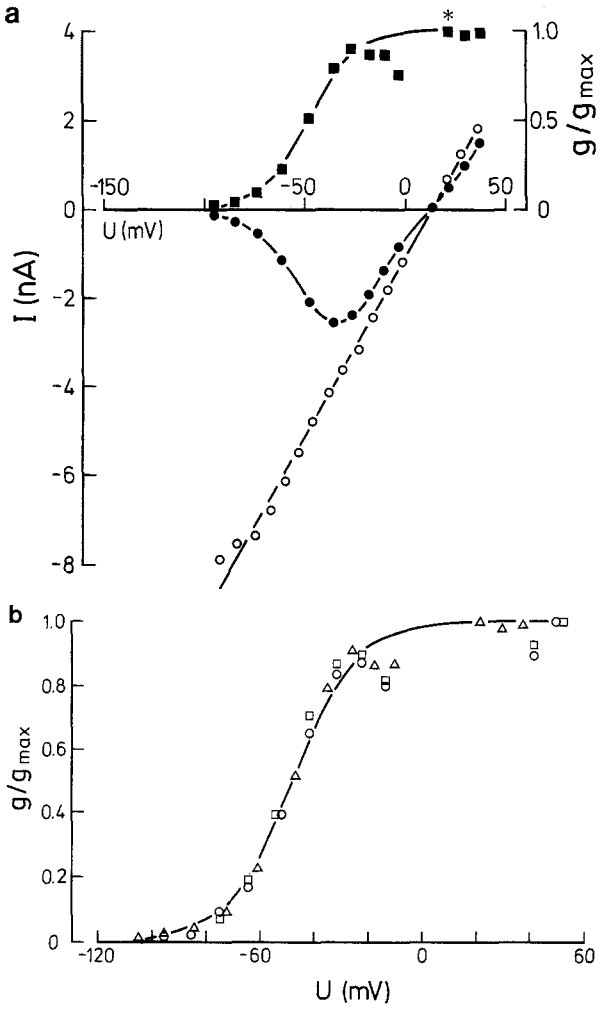

Fig. 3. Voltage dependence of the GCAC1 conductance, instantaneous and steady-state currents deduced from double voltage-pulse experiments in the whole-cell configuration as shown in Fig. 2. (*a*), open circles denote the instantaneous tail current right after application of the test potential and filled circles the corresponding steady-state value at the end of the pulse at 100 msec. Steady-state activation curve for GCAC1, (g/g_{max}, closed squares), the fraction of open channels, was calculated from the ratio of steady-state currents (closed circles) to tail currents (open circles) divided by the maximal value of g/g_{max} (star). g/g_{max} values for a single guard cell protoplast were fitted by a Boltzmann distribution (Eq. (2)) with a slope α = 11.9 mV and a midpoint for activation $U_{1/2}$ = –47.9 mV. (*b*), the data points from three experiments were fitted by a Boltzmann distribution (Eq. (2)) with a slope α = 11.8 mV and a midpoint voltage $U_{1/2}$ = –48.2 mV.

the least-squares fit yielded a midpoint for activation, $U_{1/2}$ (50% of the channels activated), of –47.9 mV and a slope factor α of 11.9 mV. Mean values of these parameters on the basis of three independent cells were $U_{1/2}$ = –48.2 mV and α = 11.8 mV (Fig. 3*b*). From the limiting slope α for an e-fold rise per kT/ze we deduced a value of z = 1.98.

It was assumed that at the selected holding potential of –196 mV, the activation gate of the GCAC1 channels is in the closed configuration (m_o = 0) and that the inactivation process is in the off-state (h_o = 1). Equation (1) can then be written as:

$$I_{Cl}(t) = m_{oo}{}^2(1 - \exp^{-t/\tau_m})^2(h_{oo} - (h_{oo} - 1)\exp^{-t/\tau_h})g_{max} (U - E_{Cl}) \quad (3)$$

and the corresponding whole-cell current of the guard cell protoplasts as:

$$I(t) = I_{leak} + I_{Cl}(t) \quad (4)$$

τ_m and τ_h are the time constants of activation and inactivation. m_{oo} and h_{oo} are the steady-state probabilities of GCAC1 to be activated and noninactivated at the end of the test pulse of 70 msec duration, respectively. These four quantities were used as free-running parameters of a least-square fit of Eq. 4 to the measured current records. E_{Cl} and g_{max} were independently determined from records of the corresponding *I–U* relationship (*see* Fig. 1*b*) for the same experiment as of Fig. 1*a*. Figure 1*b* was obtained by application of a voltage ramp from –196 mV to +64 mV. The corresponding whole-cell current was recorded for a voltage change of 0.25 mV/msec. The *I–U* relationship was measured before start of the series of voltage-jump current relaxations and right after it. A comparison exhibited no change within the experimental error. The following data can be read from Fig. 1*b*, E_{Cl} = 13.6 mV and I_{max} (U = –42.6 mV) = 2.6 nA, which yielded g_{max} = 46.3 nS. I_{leak} was determined at –196 mV and assumed to follow an ohmic behavior.

Two representative whole-cell anion current relaxations elicited by single voltage pulses to +4 and +64 mV (Fig. 4), demonstrate that the process of activation and inactivation could be sufficiently described by Eqs. (3) and (4). Obviously, it is possible to partly compensate for the variation in one parameter by a suitable variation in the other parameters. E.g., changes in τ_m can be compensated by changes in m_{oo}, τ_h and h_{oo}. If for example, at a test potential of +64 mV the fitted value of τ_m was changed by about 10% and set constant throughout a new fit, it could be compensated by an increase of τ_h and h_{oo} by about a factor of two while m_{oo} stayed about constant. But the corresponding chi-square value increased by a factor of three and the fit was discarded.

At negative voltages of about –20 mV, the current amplitude due to the activation process is dominating and the inactivation process cannot be analyzed quantitatively because of the small amplitude, while the expected time constant of inactivation would be within the considered range of a few milliseconds (*see* Figs. 1*a* and 5*a*). Therefore, for this range of hyperpolarizing voltages the recorded voltage-jump current relaxations had to be described by the activation process only:

$$I_{Cl}(t) = m_{oo}^2\,(1 - \exp^{-t/\tau_m})^2\; g_{max}\;(U - E_{Cl}) \quad (5)$$

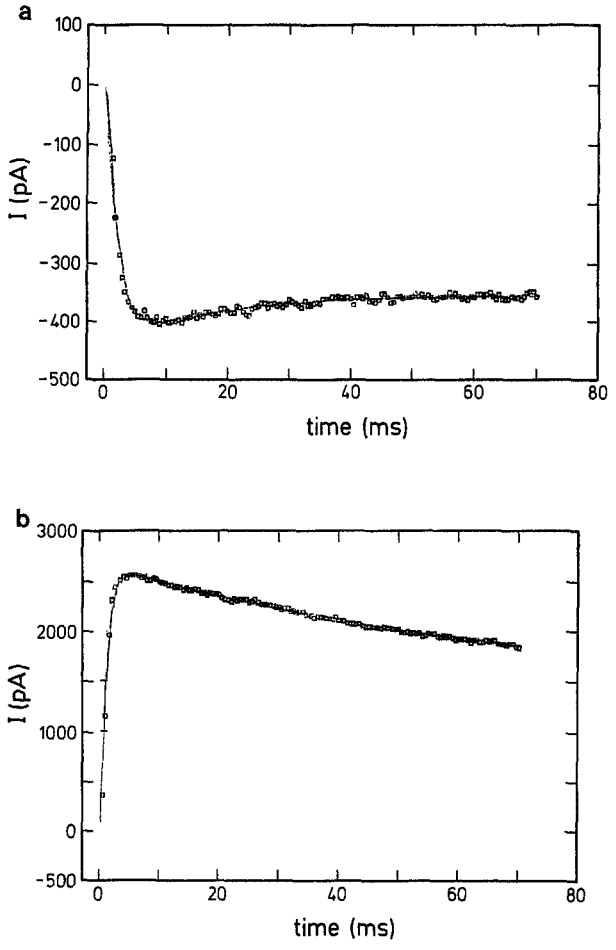

Fig. 4. Nonlinear least-squares fits of the Hodgkin-Huxley equation (4) with z = 2 to whole-cell chloride currents. (*a*), inward current (chloride efflux) elicited by a voltage step from −196 mV to +4 mV. (*b*), outward current (chloride influx) elicited by a voltage step from −196 mV to +64 mV. According to Eq. (4) the derived parameters for the two current relaxations at 64 mV (4 mV) are: I_{Clmax} = 2697 pA (−448 pA), h_{oo} = 0.49 (0.81), τ_m = 0.82 msec (1.54 msec), τ_h = 75.1 msec (19.8 msec).

which yielded a sufficient description of the corresponding current relaxations.

Table 1 summarizes the derived values of m_{oo}, h_{oo}, τ_m *and* τ_h of GCAC1 for the experiment shown in Fig. 1. It shows that τ_m decreases with depolarization while τ_h increases. In addition, the results of three further independently performed experiments are plotted in Fig. 5*a*. Furthermore, m_{oo} increases and h_{oo} decreases with depolarization (Table 1). At +50 mV we were able to deduce a h_{oo} of 0.75 ± 0.06 (n = 4). It should be mentioned however, that the voltage sensitivity of h_{oo} within a population of guard cell protoplasts could adopt different polarities (*see* Discussion).

The kinetics of the inactivation process is even more complex, since during voltage pulses of 1 min, two additional time constants around 2 and 20 sec (n = 4) were apparent (Fig. 6). Due to the limited stability of the gigaseal and the rundown of channel activity (*see* Discussion), the slow inactivation process could not be analyzed in detail and will not be considered further.

Deactivation

From double voltage-pulse experiments similar to those presented in Fig. 2, we were further able to estimate the voltage dependence of the deactivation process (open → closed transition). In the voltage range presented in Fig. 2 the corresponding tail currents could be described by a single exponential.

$$I(t) = I_{\text{leak}} + I_0 \exp^{-t/\tau_d} \tag{6}$$

τ_d denotes the time constant of deactivation and I_0 the amplitude of the tail current. Figure 5*b* shows the voltage dependence of τ_d as a result of a least-squares fit of Eq. (6) to the corresponding tail currents. $\tau_d(U)$ peaked at −60 to −50 mV and increases with further depolarization.

In terms of the H-H model, in τ_d all time constants are lumped together which correspond to transitions from the conducting, activated state to nonconducting states of GCAC1. Besides the time constants of direct transitions from the open to the closed states (deactivation), τ_d might include transitions through inactivated states. In this respect it should be noted that τ_d (filled triangles) was systematically larger than τ_h (open squares, Fig. 5*b*). Since during double voltage-pulses no additional currents appeared, transitions in the millisecond range from the inactivated to the deactivated state through a conducting state could be excluded.

Discussion

We have analyzed the fast activation and inactivation of the guard cell anion channel (GCAC1) mediated current as a function of voltage. The corresponding rate constants were determined from voltage-jump current-relaxations at whole-cell configuration. An adequate description is based on the Hodgkin-Huxley equations (Hodgkin & Huxley, 1952). To analyze the kinetics of the GCAC1-mediated chloride current, we adjusted the composition of the electrolyte in the bath and in the protoplast to obtain a quasi stationary activation of GCAC1 and to minimize currents in addition to the chloride current. The latter can be read from the finding that the reversal potential (Fig. 1*b*) is close to the Nernst potential for the chloride current. Consequently, a significant calcium influx does not contribute to the voltage-dependent whole-cell current in our experiments. Furthermore, changes in the external free calcium con-

Table 1. Parameters of the Hodgkin-Huxley model (Eq. (3))

U/mV	64	54	44	34	24	4	−6
m_{oo}	0.98	0.99	0.99	0.96	0.96	0.89	0.83
h_{oo}	0.49	0.60	0.69	0.71	0.74	0.81	0.81
τ_h/ms	75.1	57.8	49.0	46.4	36.2	19.8	19.9
τ_m/ms	0.82	0.86	0.86	0.86	0.91	1.54	1.6
χ^2	581	430	324	183	33	39	38
U/mV	−16	−36	−46	−66	−76	−86	
m_{oo}	0.89	0.90	0.89	0.66	0.43	0.30	
τ_m/ms	2.03	4.05	6.1	11.6	10.0	6.6	
χ^2	581	978	1584	2765	3353	1169	

Parameters are derived by least-squares fits of Eq. 4 to the data given in Fig. 1 at different membrane potentials U. χ^2 denotes the corresponding chi-square value. g_{max} = 46.3 nS, E_{Cl} = +13.6 mV and g_{leak} = 30 pS. At U < −6 mV Eq. (5) was fitted to the current relaxations. For further explanations see text.

centration from 40 mM to 1 mM did not alter the magnitude, voltage dependence, kinetics and reversal potential of the described anion current (Lohse & Hedrich, 1995). This observation is in agreement with the situation in the *Characean* (Thiel et al., 1993).

In 1979, Beilby and Coster applied the H-H formalism to chloride currents in plasma membranes of *C. corallina.* At that time the voltage-jump current relaxation experiments were carried out by the voltage clamp method which did not allow control of the electrolyte at the inner phase of the membrane. For both cell systems, chloride channel currents are considered most likely to be the pathway for the large chloride efflux associated with the action potential (Kikuyama et al., 1984; Thiel et al., 1993). Surprisingly, for GCAC1 mediated currents in *V. faba* as well as the anion currents in *C. corallina,* a dependence of channel activity on free cytoplasmic calcium has been reported (Beilby, 1984; Hedrich et al., 1990; Thiel et al., 1993). Therefore it could be assumed that GCAC1 is also present in *C. corallina* which will be discussed below.

On the single channel level, different conductances were derived in cell-free membrane patches, 32–40 pS for the GCAC1 (Keller et al., 1989; Hedrich et al., 1990) and 52, 16 and 4 pS for distinct chloride channels in *C. corallina* (Thiel et al., 1993). But due to the lack of long-term stationarity a detailed analysis of the voltage dependence of kinetics was not possible on the single channel level in guard cell protoplasts of *V. faba.* Also in *C. corallina,* kinetic data about the activation and inactivation of single anion channels are not available (Thiel et al., 1993), therefore their specific contribution to the macroscopic voltage-activated chloride current (Beilby & Coster, 1979*a*) remains unsolved. Yet, on the basis of the commonly applied H-H formalism to the kinetics of the voltage-activated Cl current in *C. corallina* and the GCAC1-mediated current in *V. faba,* a comparison of the corresponding H-H parameters becomes possible.

In *C. corallina,* after depolarization the activation of chloride current starts with a delay in the range of seconds, which was not observed for the chloride current in guard cell protoplasts, of *V. faba* (Fig. 1). Furthermore, the number of independently moving hypothetical gating charges reproducing the activation process has been set to three for *C. corallina* while in *V. faba* we experimentally determined a value of two (*see* Eq. (1) and Fig. 3*b*). But this equivalent charge of about two is a lower limit of the actual number of charges that may move, since charged groups on the channel might move only partly through the membrane potential drop (*see* Hille, 1992). Two gating particles would predict a sigmoidal activation of the current relaxation which probably could not be resolved due to the large membrane RC-time constant of the whole-cell arrangement (*see* Materials and Methods).

The absolute values of the time constant of activation (τ_m) as well as their voltage sensitivity differ significantly. In guard cell protoplasts τ_m is about 100-fold faster than in *C. corallina* but still about 20-fold slower than the corresponding time constant for activation of the Na current in squid axon (Hille, 1992). The voltage-dependence of τ_m shows no significant extremes in the voltage range of −66 to +64 mV, but saturation at high and low depolarizing voltages (*see* Fig. 5*b*) cannot be described by the Eyring rate theory as proposed by Gradmann et al. (1993).

At voltages close to and above E_{Cl} the time constant of fast inactivation τ_h could be derived simultaneously. τ_h increases with increasing depolarization as observed for *C. corallina* (Beilby & Coster, 1979*a*), but the reported absolute values are about 10-fold larger and the voltage-sensitivity is significantly smaller. Two observations hindered the determination of τ_h at small depo-

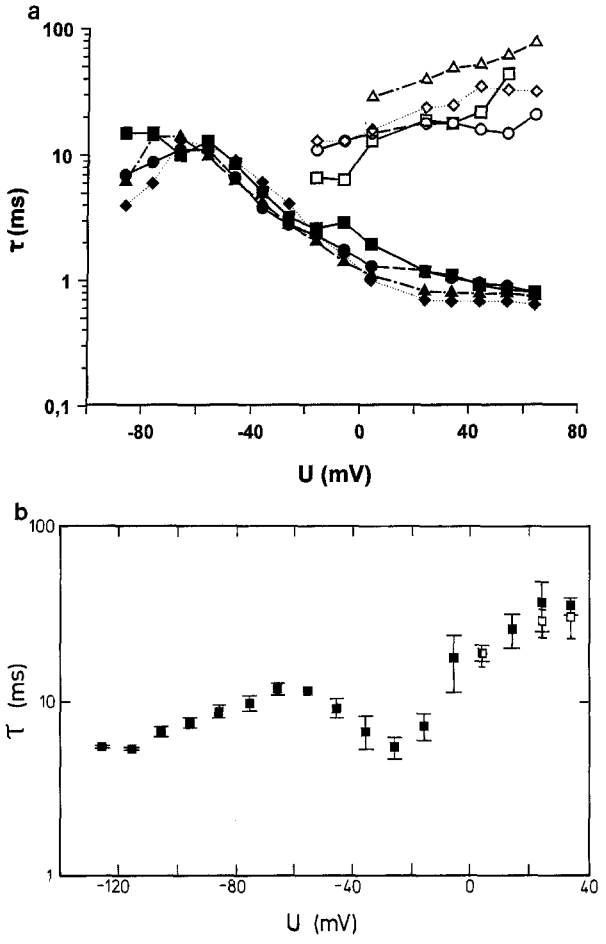

Fig. 5. Semilogarithmic plot of activation, inactivation, and deactivation time constants of GCAC1 as a function of voltage. (*a*), voltage-dependence of activation (filled symbols) and inactivation (corresponding open symbols) deduced by fitting Eq. (4) with z = 2 to the anion currents elicited by single voltage-pulses as shown in Fig. 4. The result of four independent experiments is shown. (*b*), deactivation time constants derived from tail current fits (double-pulse experiments, *see* Fig. 1). At potentials positive to +10 mV the time constants for deactivation (filled squares) and inactivation (open squares) are superimposed (n = 4).

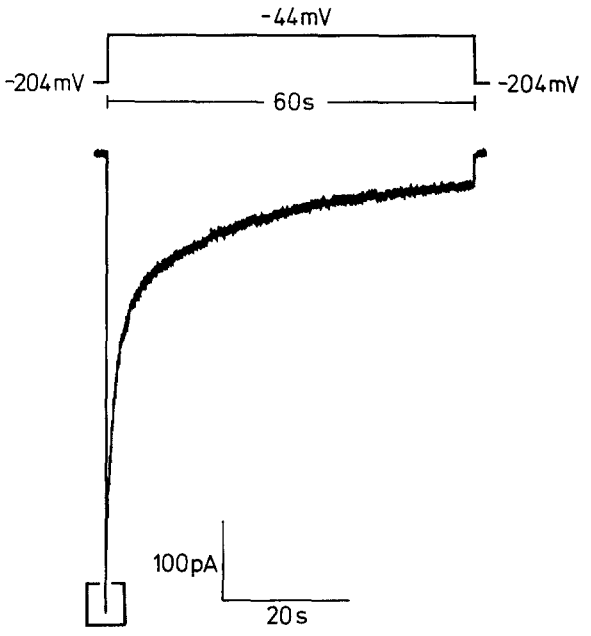

Fig. 6. The inactivation process of GCAC1 includes fast and slow components. Fast (boxed) and slow exponential decay of the inward current following a voltage step from −204 mV to −44 mV with time constants of about 20 msec, 2 sec and 20 sec.

larizing voltages in guard cell protoplasts. First, the total current amplitude and the corresponding contribution of the inactivation process was too small (Fig. 1*a*) and second, the measured values of τ_m and expected for τ_h became comparable (*see* Fig. 5*b*).

It can be shown that the derived time constants of fast activation and inactivation of GCAC1-mediated current are not strongly dependent on the specific theoretical description. As well known, for a simplified sequential three-state model for the channel gating process with a closed, an open and inactivated channel state, the time course of the voltage-jump current relaxation can be described by a sum of two single exponential functions with characteristic time constants (compare Hille, 1978). Application of the latter or of Eq. (4) for fitting the measured current relaxations showed neither a significant difference (*data not shown*) in the description of the recorded time course nor in the corresponding time constants. Since the H-H model is based on the assumption that activation and inactivation are two independent mechanisms, whereas the sequential three state requires that a channel inactivates only after activation, this finding indicates that the observed kinetics of GCAC1 does not allow a clear discrimination whether the activation and inactivation are entirely independent of each other.

An important information of the H-H parameters is contained in the parameters *m* and *h*. As outlined above, the activation process could be related with two hypothetical gating particles *m*(*t*). Application of Eq. (3) to the current records yielded the voltage dependence of m_{oo}. As observed for *C. corallina* and for the Na current in squid axon m_{oo} was small at hyperpolarization and increased rapidly with depolarization (Table 1). This finding is consistent with the observation that the GCAC1 current amplitude was small at the threshold potential of activation (~ −100 mV) despite the fact that this potential level is far removed from the reversal potential E_{Cl}.

The probability of inactivation is given by *h*. h_{oo} decreased with depolarization (Table 1), but the values are large compared to *Chara,* where the chloride current inactivates completely (Beilby & Coster, 1979*a*). This observation could be based on the contribution of a non-inactivating chloride channel current like SLAC (Schroeder & Keller, 1992). The activation of SLAC by

depolarizing voltage steps occurs in the time scale of several seconds and the inactivation is even slower. The slow voltage dependent gating mechanism is reflected in the linearity of the corresponding *I-U* curves which were recorded during depolarizing voltage ramps of 7 sec. Obviously, this time span is significantly shorter than the time needed for inactivation of SLAC. Therefore, repetitive voltage ramps in a depolarizing direction yielded varying current responses of significant amplitude especially at more hypolarizing voltages ($U < U(I_{Cl,max})$) which reflects this underlying transport mechanism of a slower time scale (Schroeder & Keller, 1992). But four lines of evidence indicate that this type of anion channel of extremely slow voltage-dependent kinetics was not activated in the present experiments. (i), the activation kinetics of the analyzed anion channel, the GCAC1, was faster by more than a factor of a thousand (*see* Fig. 1*a*, Fig. 5*b* and Table 1). (ii), in addition, it showed an inactivation after stimulation by depolarization. (iii), the voltage sensitivity of the time constants of activation of GCAC1 (*see* Fig. 5*a*) is the inverse of that of SLAC (Linder & Raschke, 1992). (iv), as has to be expected for a channel with activation and inactivation kinetics in the millisecond range the corresponding current – voltage curve (Fig. 1*b*) is not linear but exhibits a maximum. Furthermore, a significant current mediated by conducting channels of the GCAC1-type at voltages less than $U(I_{Cl,max})$ was not observed during voltage ramps (*see* Fig. 1*b*). But a comparison of Fig. 1*a* and Fig. 6 indicates that in addition to an activation and inactivation of GCAC1 in the millisecond range, a slow inactivation mechanism is present which occurs in the time range of several seconds. The process of slow inactivation has not been considered in the present analysis which has been focused on the fast kinetics of GCAC1 on the whole-cell level. Experimental conditions that would allow a complete kinetic analysis on the whole-cell as well as on the single channel level could not be achieved in long-term experiments due to lack of stationarity. Therefore we cannot exclude that the derived values of h_{oo} present an upper limit. Furthermore, the observed variability in the voltage sensitivity as well as the absolute values of h_{oo} within a guard cell population might indicate that the voltage-dependent inactivation is under the control of extramembraneous factors (*cf.* Hedrich et al., 1990, Marten & Hedrich, *unpublished results*).

To adapt the H-H model to the GCAC1-mediated current under the various physiological conditions, one would have to take into account modulation of the voltage sensor by growth hormones (Marten et al., 1991), by CO/malate and by external anions (Hedrich & Marten, 1993; Hedrich et al., 1994). It cannot be excluded that GCAC1 and SLAC are not distinct molecular entities (Schroeder & Keller, 1992), but one molecule with distinct transport modes (Hedrich & Dietrich, 1994). Further experiments are necessary to provide evidence on whether or not the variability of h_{oo} reflects transient transitions between different modes of a channel mediated anion transport.

In conclusion, the significant differences in the number of hypothetical activation particles, the delay of activation, the voltage sensitivity of τ_h as well as in the absolute time constants of activation and inactivation give evidence that GCAC1 of guard cell protoplasts does not provide a major transport pathway for the anion current in *C. corallina.* This is in accordance with the observed differences in the corresponding single channel conductances (*see above*).

Finally, we will discuss the physiological significance of GCAC1 in terms of the observed action potential (AP) in guard cells of *V. faba.* The kinetics of an AP can roughly be described by an on phase, a plateau and an off phase. From the reported time course of the AP in guard cells of *V. faba* (Gradmann et al., 1993) the on phase is determined by a depolarization in less than about 10 msec. Both anion channels, the GCAC1 and the SLAC, could be considered as ion pathways involved in the on phase. But SLAC exhibits an activation in a time scale of seconds, which differs by orders of magnitude compared to the former value, and the corresponding time constant even increases with increasing depolarization. In contrast, the activation of GCAC1 occurs within a few milliseconds and becomes faster with increasing depolarization. It is tempting to suggest that the fast activation of GCAC1 is involved in the on phase, while the fast inactivation seems to be reflected in the rapid transition during an AP (Gradmann et al., 1993) from the on phase to the plateau phase. In the latter phase, SLAC might be involved. Likewise, the nature of the ion conductances for complete repolarization is not known in guard cells yet, but could be represented by the voltage-dependent outward-rectifying potassium current which in guard cell protoplasts from *V. faba* has been described by the H-H formalism as well (Schroeder, 1989) and accordingly also in cultured tobacco cells (Van Duijn 1993). But it seems that the variability of the recorded data in plant cell membranes are not well understood. Comparison between outward potassium current from *V. faba* and *Z. mays* revealed some deviations from one another and the application of a simple H-H formalism did not yield a sufficient description of the voltage-dependent gating properties (Fairley-Gernot & Assmann, 1993). With respect to a quantitative theoretical prediction of the time course of the AP, one would need the complete knowledge of the involved electrogenic ion transport systems, their density as well as the corresponding voltage dependent kinetics.

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