External pH Effects on the Depolarization-activated K Channels in Guard Cell Protoplasts of *Vicia faba*

NITZA ILAN,* AMNON SCHWARTZ,* and NAVA MORAN‡

From the *Department of Agricultural Botany, Faculty of Agriculture, the Hebrew University of Jerusalem Rehovot 76100; and ‡Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100 Israel

**ABSTRACT** Previous studies reveal that the pH of the apoplastic solution in the guard cell walls may vary between 7.2 and 5.1 in closed and open stomata, respectively. During these aperture and pH changes, massive K⁺ fluxes cross the cellular plasma membrane driving the osmotic turgor and volume changes of guard cells. Therefore, we examined the effect of extracellular pH on the depolarization-activated K⁺ channels (K₀ channels), which constitute the K⁺ efflux pathway, in the plasma membrane of *Vicia faba* guard cell protoplasts. We used patch clamp, both in whole cells as well as in excised outside-out membrane patches.

Approximately 500 K₀ channels, at least, could be activated by depolarization in one protoplast (density: ~0.6 μm⁻²). Acidification from pH 8.1 to 4.4 decreased markedly the whole-cell conductance, Gₓ, of the K₀ channels, shifted its voltage dependence, Gₓ-Eₓ, to the right on the voltage axis, slowed the rate of activation and increased the rate of deactivation, whereas the single channel conductance was not affected significantly. Based on the Gₓ-Eₓ shifts, the estimated average negative surface charge spacing near the K₀ channel is 39 Å. To quantify the effects of protons on the rates of transitions between the hypothesized conformational states of the channels, we fitted the experimental macroscopic steady state conductance-voltage relationship and the voltage dependence of time constants of activation and deactivation, simultaneously, with a sequential three-state model CCO. In terms of this model, protonation affects the voltage-dependent properties via a decrease in localized, rather than homogeneous, surface charge sensed by the gating moieties. In terms of either the CO or CCO model, the protonation of a site with a pKₓ of 4.8 decreases the voltage-independent number of channels, N, that are available for activation by depolarization.

**INTRODUCTION**

The stomata in plants constantly change their aperture controlling leaf gas exchange. Opening of stomata results from uptake of K⁺, Cl⁻, and malate synthesis, followed by osmotic water influx, increase in turgor and guard cells swelling (MacRobbie, 1987;
Outlaw, 1983). The driving force for these fluxes is proton extrusion by the H⁺ pump in the plasma membrane, which hyperpolarizes the cell and acidifies the extracellular milieu (Raschke and Humble, 1973; Spanswick, 1981; Zeiger, 1983; Assmann, Simoncini, and Schroeder, 1985; Shimazaki, Iino, and Zeiger, 1986). Stomatal closure may be initiated by cessation of H⁺ extrusion, followed by loss of ions, mainly Cl⁻ and K⁺, osmotic water efflux, and reduction of guard cell turgor. During this cyclic proton pump operation, apoplastic pH may vary between 7.2 and 5.1, as documented using fluorescence methods and H⁺-sensitive electrodes (Edwards, Smith, and Bowling, 1988). Similar variation of apoplastic pH was determined also in another type of motor cells, pulvinar extensor cells, with H⁺-sensitive electrodes (Lee and Satter, 1989; Starrach and Mayer, 1989).

K channels appear to serve as the pathway for K⁺ movement through the guard cell plasma membrane: K⁺ enters the swelling cell via hyperpolarization-activated K channels (Kᵢ<sub>H</sub>, or Kᵢ<sub>in</sub> channels) and leaves the shrinking cell via depolarization-activated K channel (K<sub>D</sub> or Kᵢ<sub>out</sub> channels) (Schroeder, Raschke, and Neher, 1987; Schroeder, 1988).

Kᵢ<sub>H</sub> and K<sub>D</sub> channels are the most predominant K channel types in the plasma membrane of protoplasts from stomatal guard cells of *Vicia faba* (Hedrich and Schroeder, 1989), and, in fact, in most higher plant cells examined so far (Moran, Ehrenstein, Iwasa, Bare, and Mischke, 1986; Moran, Ehrenstein, Iwasa, Mischke, and Satter, 1988; Moran and Satter, 1989; Schauf and Wilson, 1987; Ketchum, Shrier, and Poole, 1989; see recent reviews by Tester, 1990; Blatt, 1991). The characterization of these channels in plants still lags behind their counterparts in animal cell membranes (see, for example, recent reviews by Sanders, 1990, Hille, 1992) in biophysical as well as in biochemical detail. For example, we are not aware of any information on the density of negative surface charges near the ion channels in plant cell membranes. There are only very few attempts at modeling of channel gating in plants (Bertl, Klieber, and Gradmann, 1988; Sanders, 1990; Schroeder, 1989; Blatt, 1991, 1992; Van Duijn, 1993). When available, gating parameter estimates can serve as a reference for quantitative comparison with data from other systems. Such comparison becomes increasingly important especially now, with the appearance of cloned and reconstituted variants of the K channels from plants (outward rectifier: Cao, Anderova, Crawford, and Schroeder, 1992; inward rectifier: Anderson, Hupriker, Kochian, Lucas, and Gaber, 1992; Schachtman, Schroeder, Lucas, Anderson, and Gaber, 1992; Sentenac, Bonneaud, Minet, Lacroute, Salmon, Gaymard, and Grignon, 1992). Our description of the gating and the external pH effects extends the characterization of K<sub>D</sub> channels in guard cell protoplasts.

In animal cells, cation-selective channels are frequently blocked by extracellular protons (Woodhull, 1973; Hille, 1973; Huang, Catterall, and Ehrenstein, 1978; Hagiwara, Miazaki, Moody, and Patlak, 1978; Begenisich and Danko, 1983), with the apparent pKᵢ<sub>H</sub> of ~5. In contrast, a similar H⁺ concentration activated sodium channels in one reported case (Ueno, Nakaye, and Akaike, 1992), and chloride channels in another (Hanke and Miller, 1983). Since in the case of the guard cell, pH values ranging from 5 to 7.5 are of physiological significance, we set out to examine the effect of extracellular pH on the K<sub>D</sub> channels in guard cell protoplasts isolated from *Vicia faba* leaves. To this end we combined macroscopic steady state and kinetics
data and also single-channel data. The description of gating, based on the macroscopic kinetics of the $K_D$ channels required a minimum of three states: Closed1-Closed2-Open. Therefore, we used the framework of the three-state model and described the effect of pH on the gating of these channels in terms of the Eyring rate theory, i.e., in terms of the transitions between their conformational states. Preliminary reports have appeared in an abstract form (Ilan, Schwartz, and Moran, 1991, 1992).

MATERIALS AND METHODS

Experimental Methods

Plant material. Seeds of *Vicia faba* (supplied by Amir Co., Tel Aviv, Israel) were sown in 12-cm diameter drained pots in a mixture of standard compost and basalt gravel (4:1 by volume) and were watered twice daily with 50% Hogland’s nutrient solution and tap water, alternately. The plants were grown in the Phytotron of the Faculty of Agriculture in Rehovot, in glass-covered growth rooms under natural sunlight (70–75% of outdoor levels) extended to 16-h photoperiod with 75 W incandescent lamps (5 μmol m$^{-2}$ s$^{-1}$ at plant level). Temperature was kept at 22°C during the day (hours 0800–1600) and 17°C at night. Atmospheric humidity was maintained at a constant vapor pressure of 1.2 kPa. The youngest, fully expanded leaves of 3–4 wk-old plants were harvested. Protoplasts were isolated according to procedure of Kruse, Tallman, and Zeiger (1989). We measured the diameters of the spherical protoplasts using a microscope micrometer with a resolution of 0.22 μm (at a magnification of 400). The average diameter of the globular guard cell protoplast was 16.6 ± 1.2 μm (mean ± SD, n = 122), and the calculated average cell surface was 865 ± 125 μm$^2$.

Electrophysiology. The patch-clamp technique is described in detail by Hamill, Marty, Neher, Sakmann, and Sigworth (1981). The application to plant cell protoplasts is described in detail by Moran et al. (1988), and by Moran, Fox, and Satter (1990). Briefly, a drop of the protoplast suspension was added to ~200 μl of the recording solution, the protoplasts were allowed to settle and stick to the glass bottom of the recording chamber and then washed with ~2–3 ml of the recording solution. The patch pipette was then brought into contact with the protoplast and a whole cell configuration was attained, to be followed, in some experiments, by excision of an outside-out patch.

All experiments were performed in a voltage-clamp mode, using the Axopatch C-1 amplifier (Axon Instruments, Inc., Foster City, CA), and were under computer control, using a software-hardware system from Axon Instruments (Pclamp program package and the TLI-1-125 Labmaster DMA A/D and D/A peripherals).

Membrane potential was controlled according to a preprogrammed schedule, and the resulting membrane current was filtered at 20–100 Hz (−3 db, 4-pole Bessel filter), sampled at 200–500 Hz and stored for further analysis. The error in voltage clamping of the whole cell membrane, largely due to the access resistance, $R_a$, of the patch pipette was compensated at ~70% by analog circuitry of the Axopatch amplifier. $R_a$ values were ~50 ± 8 MΩ (mean ± SD, n = 27), whereas the resistance of the cells ranged between 4 ± 2 GΩ (mean ± SD, n = 27) at rest (determined from leak current, see below) and 194 ± 46 MΩ (mean ± SD, n = 6) measured at peak activity (based on $G_K$ at 63 mV at pH 8.1). The average capacitance was 8.9 ± 0.2 μF (mean ± SD, n = 25). This permits the calculation of specific capacitance: 1.03 ± 0.03 μF cm$^{-2}$ (mean ± SD, n = 25). The measured membrane potential was corrected for liquid-junction potential determined separately using the experimental solutions and 3 M KCl-filled agar bridges (~17 mV).
Solutions. The intracellular solution in the patch pipette contained (in micromolar): 100 glutamate, 105 K⁺, 6 Mg²⁺, 4 Cl⁻, 0.2 BAPTA (estimated free [Ca²⁺], < 10⁻⁷ M), 4 ATP, 20 HEPES, pH 7.2, and mannitol, to final osmolarity of 520 mOsm. Due to the effectively infinite increase of the cytoplasm volume in the whole-cell configuration and to the fast equilibration of small solutes in this combined pipette-cell compartment (Marty and Neher, 1983), the interior of the cell is clamped at pH 7.2, and the pH changes affect only the external cell surface. The control bath solutions contained 11 K⁺, 1 Ca²⁺, 2 Mg²⁺, 6 Cl⁻, 10 glutamate, 10 MES, pH 5.5. Bath solution for pH 4.4 included 100 MES, rather than 10, or 7 glutamate and 10 MES (we did not observe any differences between the two subgroups); for pH 7 and 8 it included 10 HEPES, rather than MES; and, in addition, 1.25 and 7.5 of N-methylglucamine (NMG), respectively. Solutions with other pH values contained intermediate concentrations of NMG. Final osmolarity of all bath solutions was adjusted with mannitol to 480 mOsm. BAPTA-K₄ was from Molecular Probes, Inc. (Eugene, OR). Mannitol was from Merck. All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Theoretical Considerations

Initial data reduction. First, we obtained the whole-cell steady state and instantaneous IM‧EM (current-voltage) relationships. Due to the delay in channel activation, the instantaneous current (leak) could be determined at the beginning of a depolarizing step from the resting potential. The isochronal (usually: steady state) KE channel current values, IM, were obtained by subtracting this leak current from the current measured at the end of a 5-s voltage pulse. Then, the steady state GK‧EM (chord conductance-membrane potential) relationship was extracted from IM‧EM using Eq. 1:

\[ GK = IM/(EM - E_{rev}) \]  

where \( E_{rev} \) is the reversal potential (Hodgkin and Huxley, 1952) of this current. Whenever tested, the instantaneous IM‧EM relationship was linear over the examined EM range, which justifies the use of Eq. 1 for the calculation of GK.

Analogously, \( E_{rev} \) and the single channel conductance, \( \gamma_s \), were estimated from the single-channel current, I‧EM relationship in outside-out patches. This was obtained either directly, in a response to a ramp-voltage commands, or from the amplitude histograms of steady state current records at various EM.

In addition, we described the kinetics of current activation and of current deactivation in terms of time constants (\( \tau \)'s) vs EM. We obtained these \( \tau \)'s by fitting current relaxations with two (activation) or one (deactivation) exponential term(s).

Modeling: an overview. We used the macroscopic steady state whole-cell data for most of our analyses, examining separately the effect of pH on voltage-independent, and the voltage-dependent properties. The information about kinetics was integrated with that about steady state in the modeling of the voltage-dependent KE channel gating.

We used single-channel data, mainly to obtain directly the single channel conductance and, in addition, for comparison with channel gating in whole cells.

It is important to emphasize that due to the scatter of the pooled data, we did not attempt to compare and choose among different models (except when fitting the activation time courses). We did, however, use several widely accepted general models (see below) and based on these, we extracted estimates of models' parameters. Our only claim is that these parameters are unique.

Boltzmann distribution. The voltage dependence of the steady state \( G_K \) resides in the probability for the KE channel to be open, \( P_o \):

\[ G_K = G_{max} P_o \]
where $G_{\text{max}}$ is the voltage-independent maximum value of $G_K$. Assuming an equilibrium distribution of the $K_D$ channels between a closed and an open state, and replacing the voltage-dependent $P_0$ with a simple Boltzman equation:

$$P_0 = \frac{1}{1 + e^{-zF(EM-E_0)/RT}},$$

where $E_0$ is the half-activation voltage, $z$ is the number of effective charges transferred upon activation, $R$ is the universal gas constant, $F$ is the Faraday constant and $T$ is the absolute temperature, we fitted the individual steady state $G_K$ vs. $EM$ relationships for each cell and pH with the combined equation:

$$G_K = G_{\text{max}}/(1 + e^{-zF(EM-E_0)/RT}).$$

The resulting values of $G_{\text{max}}$ and $E_0$ were used in subsequent analyses.

**Henderson-Hasselbalch relationship for $G_{\text{max}}$.** By definition, $G_{\text{max}}$ is voltage independent. We assumed, instead, that $G_{\text{max}}$ increases as the fraction of the nonprotonated form of the $K_D$ channel, and that only one site undergoes protonation. Therefore, we fitted the mean $G_{\text{max}}$ vs. pH data determined between pH 8.1 and 4.4 with the Henderson-Hasselbalch relationship:

$$G_{\text{max}} = G'/\left(1 + 10^{PK_a-pH}\right),$$

where $G'$ is the maximum conductance in proton absence and $PK_a$ is the pH at which half of the sites are protonated. Because $G_{\text{max}}$ is composed of $N$, the number of available channels in the membrane, and $\gamma_o$, the single channel conductance:

$$G_{\text{max}} = N \cdot \gamma_o,$$

an effect on either one of them could underlie the effect on $G_{\text{max}}$.

**Surface charge.** $E_0$ characterizes the voltage-dependent gating. We assumed that the shift of $E_0$ with acidification results from the titration of negative charges at the external surface of the membrane in the vicinity of the $K_D$ channel (Gilbert and Ehrenstein, 1969, 1970), and fitted the $E_0$ vs. pH relationship with the Grahame equation (Grahame, 1947):

$$\sigma^2 = G^{-2} \sum_i [e^{-z_iF(B-E_0)/RT} - 1]$$

modified to include proton binding (Gilbert and Ehrenstein, 1969, 1970):

$$\sigma^2 = \sigma_o^2 \left(1 + \frac{[H^+]_0}{K_{\text{ac}} e^{-F(B-E_0)/RT}}\right)^{-2},$$

where $\sigma$ is the negative surface charge density, $\sigma_o$ is the total density of the titratable negative charges, $z_i$ are the concentrations of various ions in the medium, $z_i$ are their respective valencies, $K_{\text{ac}}$ is the dissociation constant of the protons from the negative surface charges, $[H^+]_0$ is the proton concentration in the bulk solution, $G$ is a constant equal to 264 (Å/electronic charge) · (mol/liter)$^{1/2}$ at 23°C, and $B$ is the surface potential at the pH (5.5) at which the shift of $E_0$ is defined as 0 mV. The actual parameters fitted were: $\sigma_o$ ($\sigma_o^{-2}$), $PK_{\text{ac}}$ ($=-\log (K_{\text{ac}})$) and $B$.

**Binomial distribution.** We assumed that there are $N$ available independent and identical channels in a patch, with a voltage-dependent $P_0$ probability to be open. Their product yields the average number of open channels, $n$:

$$n = N \cdot P_0.$$
To extract $P_0$ and $N$ separately, we fitted the binomial distribution:

$$P_r = \frac{N!}{r!(N-r)!} P_0^r (1 - P_0)^{N-r} \quad r = 0, 1, \ldots, N,$$

(10)

to single channel data, where $P_r$ is the probability that $r$ channels will be open simultaneously. Experimentally, $P_r$ is given by the relative weight of the $r$th peak in an amplitude histogram of a single-channel current record (Iwasa, Ehrenstein, Moran, and Jia, 1986).

The CCO model. According to this model, the $K_0$ channel undergoes the following transitions:

$$\text{Closed}_1 \leftrightarrow \text{Closed}_2 \leftrightarrow \text{Open},$$

where $\alpha$, $\beta$, $\gamma$, $\delta$ denote the rate constants of transitions between the neighboring states.

We hypothesized that these rate constants are exponential functions of membrane potential (Hodgkin and Huxley, 1952; Ehrenstein, Blumenthal, Latorre, and Lecar, 1974):

$$\begin{align*}
\alpha &= B_2 e^{-A_2 (EM - E_2) F/RT}, \\
\beta &= B_2 e^{-A_2 (EM - E_2) F/RT}, \\
\gamma &= B_1 e^{A_1 (EM - E_1) F/RT}, \\
\delta &= B_1 e^{-A_1 (EM - E_1) F/RT},
\end{align*}$$

(11)

where $E_M$ is the membrane potential, $A_{21}$ and $A_{12}$, are the effective charges moved (number of elementary charges $\times$ distance of movement) back and forth, respectively, upon transitions $C_1 \leftrightarrow C_2$, $A_{02}$, and $A_{01}$ are the effective charges moved back and forth upon transitions $C_2 \leftrightarrow O$, $E_1$, and $E_2$ are membrane potentials at which the rates of transitions ($C_1 \leftrightarrow C_2$ and $C_2 \leftrightarrow O$, respectively) in opposite directions between the neighboring states are equal and $B_1$ and $B_2$ are the values of rates at $E_1$ or $E_2$, respectively. In a three-state model, the time constants of current relaxations upon step changes of membrane potential, are complicated combinations of the transition rate constants (Huang, Moran, and Ehrenstein, 1984):

$$\tau_{1,2} = \frac{2}{\alpha + \beta + \gamma + \delta \pm \sqrt{(\alpha + \beta + \gamma + \delta)^2 - 4 (\alpha \gamma + \beta \gamma + \beta \delta)}}$$

(12)

In steady state conditions, the probability $P_0$ for a three-state channel to be in an open (conducting) state is given by:

$$P_0 = \frac{1}{1 + \beta/\alpha (1 + \delta/\gamma)}$$

(15)

or, with the ratios $\beta/\alpha$ and $\delta/\gamma$ replaced by two Boltzmann distributions governing the population of the three states (Behrens, Oberhauser, Bezanilla and Latorre, 1989):

$$P_0 = \frac{1}{1 + e^{-z_2 (EM - E_2)/RT} (1 + e^{-z_1 (EM - E_1)/RT})}$$

(14)

where $z_1$ and $z_2$ are the effective charges moved upon transitions $C_1 \leftrightarrow C_2$ and $C_2 \leftrightarrow O$, i.e., the respective sums (in pairs) of $A_{21}$ and $A_{12}$, and of $A_{02}$ and $A_{01}$.

Thus, within the framework of the three-state model, eight parameters and a scaling factor (see below) are required for the quantitative description of both the steady state conductance
and the conductance relaxation kinetics, as a function of membrane potential. These parameters can be extracted by a simultaneous fit of equations 11-14, i.e., \( \tau_1, \tau_2 \) and \( P_0 \) vs \( E_M \) to the experimental data.

**Details of analysis and fitting.** Nernst potentials were calculated from ion concentrations using activity coefficients from Appendices 8.9 and 9.10 from Robinson and Stokes (1965). Initial data reduction was performed with the Pclamp software (Axon Instruments, Inc.).

Mean ± SD were used to indicate range of variability. However, when comparison between means was intended, ±SEM was used. When errors of estimated parameters were given, \( n \) signified the number of points used for the fit.

Eq. 4 was fitted to individual \( G_K-E_M \) relationships using the 386-Matlab program subroutines from Mathworks, Inc. (Natick, MA), implementing the Nelder-Mead simplex algorithm for error minimization.

For the fitting of Eq. 5 to mean \( G_{max} \) values, we used NLIN, the nonlinear regression routine of SAS (Statistical Analysis System, ver. 5) from SAS Institute Inc. (Cary, NC) implementing least mean squares minimization.

The same routine of SAS was also used to fit Eq. 10 (with the constraint that \( N \), the number of channels in the patch, be integer) to the observed distribution of probabilities, \( P_n \), of multiple channel openings in a patch, minimizing the \( \chi^2 \) (the sum, over all combinations of multiple openings, of \( \left[ \frac{Pr(\text{observed}) - Pr(\text{calculated})}{Pr(\text{calculated})} \right]^2 \)).

To extract the surface charge model parameters, we combined the Eqs. 7 and 8 into a polynomial, derived the roots from the coefficients of the polynomial for each experimental value of pH, and minimized the difference between the observed \( E_0 \) (mean values) and that calculated from the larger of the two positive solutions of the polynomial (see also Becchetti, Arcangeli, Del Bene, Olivotto, and Wanke, 1992). To find the polynome roots, we used the Fortran subroutine C02AGF from the commercially available NAG library (from the National Algorithm Group, United Kingdom; releases 14 and 15). The fitting routines were Fortran implementation of unconstrained combined Gauss-Newton and modified Newton algorithm for error minimization (Gill and Murray, 1978), subroutine E04FDF.

For the simultaneous fitting of the CCO model (Eqs. 11-14) we used also the Fortran NAG subroutine E04FDF. While the calculations and fits of the CCO model were performed with the pooled, not averaged, data, for presentation in the figures the normalized values of \( \tau \)'s and \( G_K \)'s were averaged over several cells.

To minimize variability among cells, especially important when fitting models to data, \( G_K \) values at all pH's were normalized to \( G_{max} \) at pH 5.5 for the same cell, the values of \( E_0 \) for each cell and pH were expressed as shifts relative to \( E_0 \) at pH 5.5, and the values of the two activation time constants \( (\tau_1 \) and \( \tau_2 \)) and the single deactivation time constant \( (\tau_3) \) were normalized to the maximum value of \( \tau \) for each cell, and at each pH (which, for the CCO model, is equivalent to the scaling of all four rate constants by the same factor; see Eqs. 11 and 12).

When fitting data with models, several starting guesses for the parameters were tried, to avoid entrapment in a local minimum. The weighing factor used in the fit was equal to the inverse of the standard deviation (when fitting means), calculated separately at each value of \( E_M \). To evaluate goodness of fit we calculated \( R' = 1 - S_{res} / S_{tot} \), where \( S_{res} \) is the sum of squared vertical deviations of data from the model predictions, and \( S_{tot} \) is the sum of squared vertical deviations of data points from the mean. \( R' = 1 \) means perfect fit to the model, \( R' = 0 \) means that the model is no better than a straight horizontal line drawn through the mean (SYSTAT, version 1992, from SYSTAT Inc., Evanston, Ill). In the case of binomial distribution, we evaluated the goodness of fit using the standard \( \chi^2 \) table (Draper, Schultze, and Hansen, 1993), and \( N \) (maximum number of synchronous openings used in the calculation of \( \chi^2 \) degrees of freedom). The number of total non-correlated observations was estimated, roughly, as the ratio
of the total length of the record to the longest time constant of gating observed (Draber et al., 1993).

RESULTS

Proton Block of Macroscopic \( K_D \) Channel Currents and Conductance

Depolarization of whole-cell membrane above \(-30\) mV elicited outward currents, whose magnitude was proportional to the amplitude of the depolarizing step (Fig. 1). As shown by others (Schroeder et al., 1987; Schroeder, 1988; Assmann, Moran, and Schwartz, 1988), these currents are carried through a ubiquitous type of K channels, the depolarization-activated K channels, which we will term here the \( K_D \) (corresponding to the \( K_{out} \) of Schroeder [1988]). The lower the pH of the external medium, the smaller were the depolarization-activated outward currents. This effect was fully reversible (Fig. 1). The reversal potential (\( E_{rev} \), Hodgkin and Huxley, 1952) of these current was \(-41.8 \pm 0.5\) mV (mean \pm SEM, \( n = 8 \), Fig. 2). For comparison, \( E_{rev} \) for the hyperpolarization-activated K channels (\( K_{in} \), Schroeder [1988]) in the same experiments was \(-52.0 \pm 1.1\) mV (\( n = 10 \), data not shown, but see also Schroeder [1988]). Both \( E_{rev} \) values are close to the calculated Nernst potential of \( K^+ \) in the experimental solutions, \(-54\) mV (the Nernst potentials of the three other permeant ions in the solutions, \( Ca^{2+}, H^+ \) and \( Cl^- \) were, respectively, \( \geq 200\) mV (with internal \( [Ca^{2+}] \) buffered at \(< 10^{-7}\) M), \(+100\) and \(-10\) mV).

While pH changes did not affect the reversal potential (Fig. 2), the whole-cell steady state \( K_D \) channel conductance, \( G_K \) (Eq. 1), was diminished by acidification (Figs. 2 B and 3 A). On average, \( G_K \) decreased by \(~75\%\) with acidification between 8.1 and 4.4 (Fig. 3 A).
Fig. 3B depicts the dual effect of acidification on K_0 channels kinetics: an increase of the t_{1/2} of activation (the half activation time) at 63 mV (full circles), and a decrease of the deactivation time constant, τ_d, at -87 mV (empty circles; the deactivating, tail, currents could be approximated well with a single exponential; data not shown, but see Schroeder [1989]). The slowing down of activation and the speeding up of deactivation are consistent with the blocking effect of protons.

The Effect of Protons on the Steady State Properties of G_K

To learn how the steady state activation of the K_0 channel by depolarization is altered by external protons, we fitted the steady state G_K-E_M relationships at the various pH's with the Boltzmann distribution (Eq. 4; Fig. 3A, solid lines). The voltage-independent maximum conductance, G_{max}, as well as the half-activation voltage, E_0, were both altered by external pH (Fig. 4A and B). The average value of G_{max} at pH 5.5 was 2.6 ± 1.1 (mean ± SD, n = 27). The (normalized; see Materials and Methods) values of G_{max} could be related to pH by the Henderson-Hasselbalch equation (Eq. 5, Fig. 4A, solid line), with a pK_a of 4.8 ± 0.3 (±estimated error, n = 6). If the G_{max} values of pH 8.1 were excluded from the fit, the resulting pK_a was 4.6 ± 0.1 (n = 5; Fig. 4A, dashed line).

The average E_0 at pH 5.5 was 7.7 ± 8.2 mV (mean ± SD, n = 27). Fig. 4B illustrates the shift, relative to pH 5.5, of the average E_0 with pH. Fitting this E_0 shift vs pH with the modified Grahame equation (Eqs. 7 and 8, Fig. 4B, line), yielded the following properties (best fit values) of the assumed negative charges at the external
surface of the membrane in the vicinity of the $K_D$ channel: the average charge separation, $d_l = 39 \pm 12 \text{ Å}$, the pH at which half of the charges are protonated, $pK_w = 4.8 \pm 0.6$ and the surface potential at pH 5.5, $V_s = -20 \pm 13 \text{ mV}$.

In contrast to the change in $E_0$, the average values of $z$ at the different pH's did not vary significantly and their mean was $1.6 \pm 0.4$ (mean ± SD, $n = 54$; Fig. 4 C).

**The Effect of pH on Single $K_D$ Channels**

In all excised outside-out patches, we recorded several, usually identical, channels. Their identification as $K_D$ channels was based (a) on the value of the reversal potential and (b) on the dependence of their activation on depolarization above the reversal potential (Figs. 5, 6), and more specifically, (c) on the same voltage sensitivity as that of macroscopic currents, as reflected in the effective number of gating charges, $z = 2$ (as in Fig. 4 C).

To characterize the voltage dependence of the single $K_D$ channels, as a first approximation a simple Boltzman distribution was fitted to the voltage dependence of the average number of open channels, $\bar{n}$, in one patch at pH 5.5 (Fig. 6 D). The effective number of gating charges was at least 2 (1.7 ± 0.4; ± estimated error). The voltage for half activation was $34 \pm 4 \text{ mV}$ (± estimated error). This is significantly larger than the average $E_0$ value of $7.7 \pm 1.6 \text{ mV}$ (mean ± SEM, $n = 27$) determined in whole cells at pH 5.5 (Fig. 4 B). We were disappointed to find, however, that in 9 out of 11 outside-out patches at pH 5.5 that we examined, $\bar{n}$ fluctuated markedly throughout the experiment (even with records lasting 200 s, with over 1,600 opening
events per record) which precluded the determination of these parameters. A dependence of $\bar{n}$ on depolarization was evident, however, between two ranges of $E_M$: $-17$ to $+3$ mV, vs. $23$ to $43$ mV, when the differences between $\bar{n}$ obtained individually at each $E_M$ were averaged at each $E_M$ range separately. On average, $\bar{n}$ at the higher depolarization range was $0.60 \pm 0.12$ (mean $\pm$ SEM; $n = 10$), which was significantly larger—by $0.21 \pm 0.07$ ($P < 0.01$, single-tailed paired $t$ test)—than $\bar{n}$ at the lower depolarization range.

**Figure 4.** pH dependence of the parameters of steady state $G_K$-$E_M$ relationships. The following are averages ($\pm$SEM) of best-fit parameters extracted from individual $G_K$-$E_M$ relationships at each pH separately, from data of Fig. 3. Where not visible, the error bars are smaller than the symbol sign. (A) The maximum, voltage-independent conductance, $G_{max}$, normalized to $G_{max}$ at pH 5.5 (average $G_{max}$ at pH 5.5 is $2.6 \pm 0.2$ nS ($\pm$SEM, $n = 27$); the error bars at pH 5.5 are SEM of $G_{max}$ before normalization). Continuous line represents a fit, with SD$^{-1}$ as weights, of Eq. 5 in pH range 4.4–8.1, to the mean values of $G_{in}$ with best fit values: $pK_a = 4.81 \pm 0.31$ and $G' = 1.41 \pm 0.19$ (estimated error; $n = 6$, $R' = 0.647$). Dashed line: the data at pH 8.1 excluded from the fit. $pK_a = 4.61 \pm 0.14$ and $G' = 1.19 \pm 0.08$ (estimated error; $n = 5$, $R' = 0.889$). (B) The midpoint of activation, $E_0$, relative to $E_0$ at pH 5.5 (average absolute value of $E_0$ at 5.5: $7.7 \pm 1.6$ mV ($\pm$SEM, $n = 27$; this is indicated by the error bars at pH 5.5). (Line) Fit of Eqs. 4 and 5, with SD$^{-1}$ as weights, to the mean data points, with best fit parameters: average negative surface charge spacing, $(\sigma)^2 = 39 \pm 12$ Å, $pK_w = 4.8 \pm 0.6$, and $B = -20 \pm 13$ mV ($\pm$standard error estimate, $n = 6$; $R' = 0.889$). (C) The effective charge transferred upon channel opening, $z$. Line: linear regression to points, $R' = 0.06$. Note the lack of pH effect on $z$. Average $z = 1.65 \pm 0.10$ ($\pm$SEM, $n = 54$).

In spite of the fluctuations of $\bar{n}$ (e.g., Fig. 6 D, at pH 4.4), we observed a (reversible!) decrease of channel activity due to acidification. In Fig. 5A, predominantly 1 channel appeared to be active at pH 5.5. In contrast, two channels became clearly active during the voltage ramp at pH 8.1 (Fig. 5 B). A similar tendency during alkalinization from 5.5 to 8.1 was observed also in another patch (not shown). Acidification from pH 5.5 to 4.4 reduced single channel activity in two additional outside-out patches (e.g., Fig. 6). On average (irrespective of $E_M$), in these two
patches, $\bar{n}$ decreased significantly, by $0.66 \pm 0.22$ ($\pm$SEM, $P < 0.01$, 11 determinations), at $E_{rev}$ ranging from $-17$ to $83$ mV. To resolve this decline in channel activity into a separate effect on either $N$ or $P_0$ (Eq. 9), we fitted the distribution of probabilities of the multi channel openings in these patches to the binomial distribution (Eq. 10, illustrated in Figs. 6, E and F). In spite of the fluctuation observed in both $N$ and $P_0$ (not shown), in the above two patches $N$ decreased significantly from $4.4 \pm 0.3$ ($\pm$SEM), by $2.0 \pm 0.3$ ($P < 0.005$), while the average change in $P_0$ was not significantly different from zero ($-0.04 \pm 0.03$; $P < 0.30$, two-sided paired $t$ test). We have not studied systematically the fluctuations in $\bar{n}$ to decide on a possible underlying mechanism, such as shifts between various modes of gating and cooperativity among the channels (e.g., Draber et al., 1993, Iwasa et al., 1986). The elucidation of these phenomena requires additional experiments.

**Figure 5.** The effect of pH between 5.5 and 8.1 on single $K_0$ channels in an outside-out patch. Single channel current (two superimposed traces in each panel) during slow voltage ramps from the holding potential of $-87$ mV to $103$ mV. Upward deflection indicate channel opening and outward current. Dashed lines (fitted by eye) mark the increasing amplitude of the single channel current. Compare the linearity of the single-channel current-voltage relationship with that of the instantaneous $I_{rev}$-$E$ relationship in the whole cell (Fig. 2 B). Note that the $I$-$E$ relationship of the single channel current changed very little during pH change from 5.5 (A) to 8.1 (B). $\gamma_s$ changed from 12.6 to 14.5 to pS and $E_{rev}$ (arrows) remained constant at $-43$ mV. Also note that predominantly one channel was open at pH 5.5 and two channels were frequently open simultaneously at pH 8.1. Data filtered at 20 Hz, and sampled at 100 Hz.

In contrast to the irregularity of $\bar{n}$, the values of $E_{rev}$ and the single channel conductance, $\gamma_s$, were fairly constant at pH 5.5, during each experiment and from patch to patch. $E_{rev}$ of the single-channel currents was identical to that of the whole cell currents ($-42.0 \pm 2.7$ mV (SEM, $n = 7$); two examples are depicted in Figs. 5 and 6). As in whole-cell measurements, pH change did not change $E_{rev}$ significantly (Figs. 5 and 6 C). Neither did it significantly alter $\gamma_s$. The average $\gamma_s$ at pH 5.5 was $10 \pm 2$ pS (SD; $n = 7$). All the following changes were within the 95% confidence interval of the mean of $\gamma_s$ at pH 5.5. In two patches, $\gamma_s$ at pH 5.5 was lower, roughly by 15%, than at pH 8.1 ($12.6$ pS vs $14.5$ pS, respectively; Fig. 5, A and B), or by 8% ($12.6$ pS vs $13.6$; not shown). In two additional patches, acidification from 5.5 to 4.4 increased $\gamma_s$ by $\sim 15\%$, from 9.1 to 10.5 pS (Fig. 6 C), or decreased $\gamma_s$ by 4%, from 12.0 to 11.5 pS (not shown). Thus, if the single channel conductance was similarly pH
dependence of the average level occupancy, $\bar{n}$, calculated by $\bar{n} = \Sigma \bar{W}_i / \Sigma W_i$, where $i$ denotes the level number, and $W_i$ denotes the relative occupancy of the level $i$. (Symbols) $\bar{n}$ at pH 5.5 (○) and 4.4 (○). The line is a simple Boltzmann distribution, equivalent to Eq. 4, $\bar{n} = n_{\text{max}} / \left[ 1 + \exp \left( -\frac{z(E - E_0)F}{RT} \right) \right]$, fitted to data of pH 5.5, with the best-fit parameters $z = 1.7 \pm 0.4$ and $E_0 = 33.9 \pm 4.1$ mV, and the scaling factor, $n_{\text{max}} = 3.3 \pm 0.3$ (± estimated error; $R^2 = 0.9000$). (E) An amplitude histogram of 50 s current record at pH 5.5 at 43 mV, revealing equally spaced peaks (at least five identical channels were observed at this voltage, while maximum of six were observed at 63 mV). The reasonable fit of the Gaussian distribution to the histogram indicates that the filtering at 100 Hz does not affect significantly the estimate of the level probabilities. (F) Fitting binomial distribution (hatched bars) to observed probabilities of opening levels (open bars). With the number of noncorrelated observations estimated roughly as 12 (with the longest $\tau$ of gating of ~ 4 s; see Materials and Methods), the calculated $\chi^2 (0.23)$, indicates the high probability of the applicability of the binomial distribution ($P > 1\%$); and even if the number of noncorrelated observations is increased to 500 (the calculated $\chi^2$ increasing to 9.45), $P > 5\%$. 

Figure 6. The effect of pH between 5.5 and 4.4 on single $K_D$ channels in an outside-out patch. Representative single channel current traces at the indicated membrane potentials, and pH values. Upward deflection indicate channel opening and outward current. 0 current and no openings are indicated by a dash at the left of each record. Note the decrease in activity at the higher depolarizations between pH 5.5 (A) and 4.4 (B). Data filtered at 100 Hz, and sampled at 500 Hz. (C) Current-voltage (I-EM) relationship of the single channels of A and B. (Symbols) Average amplitude of unitary current jumps; ○: pH 4.4, ●: pH 5.5 (the SEM values are smaller than the symbol size). (Line) Linear regression fit to the data; the single channel conductance was 9.1 ± 0.6 at pH 5.5 and 10.5 ± 0.2 at pH 4.4. The respective reversal potentials were: -52.8 ± 8.6 and -45.1 ± 2.3 mV. (D) Voltage
insensitive before the patch excision, it is the decrease in N, the estimated number of available K\textsubscript{D} channels in a cell, and not γ\textsubscript{r} (Eq. 6), that accounts for the decrease in G\textsubscript{max}, the voltage-independent component of the whole-cell conductance.

From the maximum conductance at pH 8.1, 5.3 nS, N in a whole cell was at least \( \sim 500 \). Given the average surface area of the protoplasts, of 865 mm\textsuperscript{2}, the average density of the channels was at least \( \sim 0.6 \text{ mm}^{-2} \). We would like to point out that these estimates are based on the assumption that the maximum probability of opening (\( P_o \)) was 1. An additional process that is voltage-independent in the time scale of our measurements, for example, slow inactivation (although no such inactivation has been observed as yet in V\textit{icia} K\textsubscript{D} channels; Schroeder, 1988), could limit the maximum probability of opening of the channels during depolarization. Thus, the above value for N may be an underestimate of the true number of K\textsubscript{D} channels in the whole cell membrane.

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\text{FIGURE 7. Macroscopic kinetics of activation of K}_D\text{ channels. (Noisy traces)} \text{ Currents elicited by stepping the membrane potential for 4 s from the holding potential of } -87 \text{ mV to 63 mV, at pH 5.0. (Smooth lines) Currents calculated according to (a) C-O model } (A_0 + A_1 e^{-t/\tau}; A_0 = 597, A_1 = -497, \tau = 758 \text{ ms, } R' = .990); (b) classical Hodgkin-Huxley two-identical-independent-particle C-O model } (A_0 + A_1 [1 - e^{-t/\tau}]^2; A_0 = 167, A_1 = 418, \tau = 525 \text{ ms, } R' = .977) \text{ and (c) C-C-O model } (A_0 + A_1 e^{-t/\tau} + A_2 e^{-t/\tau^2}; A_0 = 630, A_1 = -250, A_2 = -353, \tau_1 = 211 \text{ ms, } \tau_2 = 1,295 \text{ ms, } R' = 0.997). 
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\textit{The Three-State Model of the K}_D\text{ Channel Gating}

We were unable to obtain patches with just one K\textsubscript{D} channel, which would enable the direct determination of closing and opening rates. At best, we could estimate the average frequency of channel closing, i.e., \( \beta \), by dividing the number of closing transitions by the time-channel integral (Ehrenstein et al., 1974). For example, in a 50 s record from the multichannel patch of Fig. 6 at pH 5.5 at 3 mV, \( \beta \) was \( \sim 7 \text{ s}^{-1} \). Therefore, and because of the irregularity in the single channel gating in the outside-side-out patches, to gain more insight into the effects of pH on gating, we chose to examine the gating model of the K\textsubscript{D} channels using the kinetics of macroscopic relaxations. Fig. 7 illustrates a comparison of the time course of an activating whole-cell K\textsubscript{D} current with predictions based on three types of models for channel gating: (a) a two-state (C-O) model of a single gating unit; (b) a two-identical-subunits model, in which each subunit independently undergoes C-O transitions, but both of them need to be in the O state for the channel to be open; (c) a sequential three-state model of a single gating subunit. The best fit was obtained with the three-state model.
In fitting the steady state conductance-voltage \((G_K-E_M)\) relationship with the CCO model (Eq. 14), we used \(G_K/G_{max}\) as a measure of \(P_0\) (Eq. 2), where \(G_{max}\) is the previously determined voltage-independent scaling factor of \(G_K\) (Eq. 4).

We fitted both \(\tau\)'s of activation and \(\tau\) of deactivation (\(\tau_d\)) vs \(E_M\), simultaneously with \(G_K-E_M\), with the appropriate functions (Eqs. 11–14). \(\tau_d\) was fitted, rather arbitrarily (but see Figs. 8, B and C), along with the shorter time constant, \(\tau_1\). Before fit, to diminish variability, all data were normalized as described in Materials and Methods. The resulting parameters for \(pH\) 5.5 are listed in Table I. To compare with \(\beta\) derived previously from the single channel data from one well-behaved patch, of \(\sim 7\) s\(^{-1}\), we calculated \(\beta\) from the Table I parameters (Eq. 11) for \(E_M\) of 26 mV (taking into account the 26 mV shift of the midpoint of activation between the two sets of data; see above), resulting in the value of \(\sim 4\) s\(^{-1}\).

**pH Effects on the CCO Model Parameters**

Table I summarizes the parameters obtained from simultaneous fitting of the CCO model to \(G_K-E_M\) and \(\tau\)'s-\(E_M\) extracted from experiments conducted at 3 \(pH\) values, 8.1, 5.5, and 4.4, at which data were collected over sufficiently large voltage range.

**TABLE I**

| pH   | \(E_1\) | \(E_2\) | \(z_1\) | \(z_2\) | \(A_{21}\) | \(A_{22}\) | \(B_1\) | \(B_2\) | \(z_1+z_2\) |
|------|--------|--------|--------|--------|---------|---------|--------|--------|-------------|
| 8.1  | 53 ± 25| -42 ± 5| 0.8 ± 0.3| 1.4 ± 0.2| 0.6 ± 0.2| 1.0 ± 0.1| 0.4 ± 0.05| 1.2 ± 0.2| 2.2         |
| 5.5  | -36 ± 16| -2 ± 4 | 1.3 ± 0.9| 1.3 ± 0.04| 1.2 ± 0.9| 0.9 ± 0.04| 0.4 ± 0.02| 1.7 ± 0.01| 2.6         |
| 4.4  | 48 ± 25| -10 ± 4| 0.7 ± 0.5| 1.4 ± 0.3| 0.8 ± 0.2| 1.1 ± 0.2| 0.4 ± 0.02| 1.3 ± 0.2| 2.1         |

\(G_K-E_M\) and \(\tau\)-\(E_M\) relationships, pooled separately at each \(pH\) (from 3, 8, and 4 cells at \(pH\) 8, 5.5, and 4.4, respectively), were fitted simultaneously with Eqs. 6–9. Listed are the best fit parameters (± estimated errors). The values of \(B_1\) and \(B_2\) were rescaled after the fitting using the scaling factors of \(\tau\)'s averaged at the different \(pH\)'s. The "goodness of fit" indicator, \(R'\), for the \(P_0\)-\(E_M\) data was 0.963, 0.973, and 0.919 for \(pH\) 4.4, 8.1, and 5.5, respectively; for the \(\tau\)-\(E_M\) data \(R'\) was 0.807, 0.832, and 0.803, respectively; and for the \(\tau\)-\(E_M\) data \(R'\) was 0.382, 0.314 and 0.077, respectively. See text for further details.

The total number of effective charges moved between the extreme conformations is at least 2, and it is \(pH\) independent (Table I). Irrespective of \(pH\), three out of the four transition rates between the conformational states of the channel are, and remain, voltage dependent (roughly 1 to two effective charges are transferred upon each one of these transitions; Table I), whereas one \(-\gamma\) is voltage independent (the effective charge transferred upon this transition, \(z_1-A_{21}\), is not significantly different from 0 [Table I]).

In the range of \(pH\) 8.1–5.5–4.4, \(pH\) change did not affect significantly any of the other gating parameters, except \(E_2\) and \(B_2\) between \(pH\) 8.1 and 5.5 (with no further significant change between \(pH\) 5.5 and 4.4), and \(E_1\) between \(pH\) 8.1 and 5.5, and again, between 5.5 and 4.4 (Table I). Fig. 8 illustrates the fit of the model to the data at the two extreme \(pH\) values: 4.4 and 8.1. Fig. 8A depicts the logarithmic dependence of the hypothesized transition rate constants \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) on membrane potential at these \(pH\) values. The effect of protons between \(pH\) 8.1 and 4.4 is
reflected only in the simple shift of the characteristic voltage, $E_2$, by 32 ± 10 mV (± sum of error estimates of this parameter at the two pH's). This shift results in an increase of $\beta$, and in less pronounced changes in other rate constants, in particular, at a range of -20 to 80 mV, where the channels are active.

**Figure 8.** Fitting the CCO model simultaneously to $G_k-E_M$ and $\tau - E_M$ relationships of the K_D channel at two pH values, denoted by symbols (data) and lines (calculated) as follows: 8.1: ●●● (n = 3); 4.4: ○○○ - n = 4); data are from a few of the cells of Fig. 3, as well as from a few additional cells. (A) Voltage dependence of transition rate constants, $\alpha$, $\beta$, $\gamma$, $\delta$, at the two pH's, calculated using Eq. 11 with parameters as in Table I. $E_1$, $E_2$: characteristic voltages of the transitions; horizontal arrow marks change upon acidification. (B) $\tau_1 - E_M$ (right) and $\tau_0 - E_M$ (left). Note the alignment of $\tau_1$ and $\tau_0$. (Symbols): Experimental values. (Lines) Eq. 12 with “+” preceding the square-root sign, with $\alpha$, $\beta$, $\gamma$, $\delta$. Note the shift between pH 8.1 and 4.4. (C) $\tau_2 - E_M$. Note the difference in range of values between $\tau_2$ and $\tau_0$. (Symbols) Experimental values. (Lines) Eq. 12 with “−” preceding the square-root sign, with the same $\alpha$, $\beta$, $\gamma$, $\delta$. (D) The pH and voltage dependence of the calculated occupancy, $P$ (lines), of each of the three states, indicated in the Figure by O, C1, and C2. The respective P's were calculated as follows: $P_0$ from Eq. 13, $P_1 = 1/(1 + \gamma/\delta (1 + \alpha/\beta))$; $P_2 = 1 - (P_0 + P_1)$ ($\alpha$, $\beta$, $\gamma$, $\delta$ are those depicted in Fig. 8A). (Symbols) Experimental values of $G_k$ normalized to $G_{max}$ at the two pH values. Note that the calculated shifts of $P_0$ on the voltage axis represent more closely those of $E_2$ than of $E_1$.

These rate constants were used to reconstruct the fit to the data in Fig. 8, B–D. Fig. 8, B and C, depicts the effect of pH on the $\tau$’s of activation and deactivation. The deactivation and the smaller activation time-constant ($\tau_d$ and $\tau_1$) are shifted by roughly 40 mV to more depolarized potentials as pH decreases from 8.1 to 4.4. This
shift explains the opposite effects of pH on \( t_{1/2} \) at 63 mV vs \( \tau_d \) at \(-87\) mV (Fig. 3 B). The shift of the larger time constant (\( \tau_2 \)) does not appear to be significant due to the large scatter of data (Fig. 8 C).

Fig. 8 D illustrates the effect of pH on the distribution of the \( K_d \) channels between the three states at pH 4.4 and 8.1. The fit of the CCO model (solid lines) reconstructs the observed shift of \( P_o \) (symbols) by \( \sim 20 \) mV in the depolarized direction as pH decreases from 8.1 to 4.4. The calculated occupancies of states \( C_1 \) and \( C_2 \) shift similarly.

It should be noted here, that in spite of the significant shift of \( E_1 \) between pH 8.1 and 5.5 (by \(-88 \pm 41 \) mV; \( \pm \) sum of error estimates of this parameter at the two pH's), and a concomitant roughly 50-fold decrease in \( \delta \) at pH 5.5, both the \( P_0-E_M \) (implicit in Fig. 3 A) and \( \tau-E_M \) (not shown, but see Fig. 3 B) relationships at pH 5.5 are intermediate between those at pH 8.1 and 4.4, i.e., \( E_1 \) does not affect markedly the measured aspects of channel activation. This may be the source of the relatively large error in estimating this parameter.

DISCUSSION

Our goal is to elucidate the blocking effect of protons on the plant (guard cell) \( K_d \) channel current at the level of the mechanism of \( K_d \) channel activation. Because membrane depolarization is a major activator of this channel, we attempted to quantify the voltage dependence of gating, first by a simple two-state analysis (Boltzmann distribution, Eq. 4), then by a three-state analysis (Eqs. 11–14).

The voltage-independent properties of the \( K_d \) channel in \( Vicia \) protoplasts, i.e., relative ion selectivity, single-channel conductance (with the appropriate corrections for the experimental differences in concentrations), voltage sensitivity (i.e., \( z \)), and even the maximum current in a whole cell, are relatively uniform in the reports from different labs. Noteworthy, the same voltage sensitivity (\( z = 2 \)) has been determined not only for the \( K_d \) channel in the intact cell (Blatt, 1991), but also for the classical delayed rectifier channel in the squid giant axon (Spires and Begenisich, 1992).

In contrast, the voltage-dependent properties of the \( K_d \) channel are not uniform. At comparable conditions of ion concentrations (of \( H^+ \), \( K^+ \) and \( Ca^{2+} \)), the most markedly varying parameter between the different reports about protoplasts is the half-activation voltage, \( E_0 \), including our own determinations at pH 5.5 for the macroscopic currents \( [7.7 \pm 1.6 \text{ mV (mean \pm SEM)}] \) and for the single channel current in an excised patch (\( E_0 = 34 \) mV). From averaged data by Schroeder (1989; solutions identical to ours) which we refitted with Eq. 4, we estimated \( E_0 \) to be \( +11 \) mV, and from those of Fairley-Grenot and Assmann, \( +16 \) mV (1992; we estimated this shift based on the maximum and half-maximum chord conductances extracted from their published averaged data). Interestingly, in the intact cells (Blatt, 1988, 1990, 1991, 1992; \( E_0 \)'s estimated as before) the values of \( E_0 \) appear to vary between preparations, even with similar external solutions, between \( \sim -10 \) and \( \sim -50 \) mV. While in the intact cells, \( E_0 \) shift might reflect the shift in \( E_K \) (Moran, Iwasa, Ehrenstein, Misichke, Bare, and Satter, 1987; also see Blatt, 1991), the internal \([K^+]\) would have to vary roughly fivefold to account for the observed range of \( E_0 \), of \( \sim -40 \) mV. We tend to assume that the intact guard cells used in these experiments were
rather uniform in their turgor, therefore—also in their K⁺ content. Thus, the variability of $E_0$, in protoplasts, as well as in intact cells, is suggestive of a labile cellular process governing the gating of the $K_D$ channel. This may mean that occasionally, one or more of the negatively charged moieties that contribute to the electrical field sensed by the channel gates, is lost, for example, by dephosphorylation. The variability of $E_0$ (Fig. 4 B, for example) accounts, perhaps, for the relatively large scatter of voltage-dependent gating properties, such as the $t's$ and $\tau's$ in our pooled data (Figs. 3 B, 8 B and C; see also Schroeder, 1989, Fig. 3). The fluctuations in $\bar{n}$ in the outside-out patch may be an extreme case of such lability. Interestingly, marked fluctuations in the opening probability were reported in reconstituted Ca-dependent K channels undergoing phosphorylation-dephosphorylation cycles (Chung, Reinhart, Martin, Brautigan, and Levitan, 1991).

The CCO Model Description of $K_D$ Channels in Vicia

The steady state current scaling factor, $G_{max}$, and the total effective charge of gating, $z$, could be approximated relatively closely by a simple Boltzmann analysis (Eq. 4). However, more detailed modeling was required to fit all of our data, including the kinetics of current activation (Fig. 7). Thus, we described the macroscopic activation-deactivation of the $K_D$ channel type in Vicia protoplasts in terms of a sequential, three-state model (CCO), with four voltage-dependent rate constants for transitions between the states. A Hodgkin-Huxley-type 2-independent-subunits (HH) model, has been suggested for the description of this channel, based on analysis of <1 s long current traces (Schroeder, 1989). We found that the CCO model fits 5 s long $K_D$ current traces better. Because the HH model is mathematically equivalent also to a linear three-state CCO model (Armstrong, 1969), in which $\beta = 2\delta$, and $\gamma = 2\alpha$, our approach consists, in fact, of adding four additional independent parameters.

The three-state model allows insight into the intermediate steps of $K_D$ channel gating: it appears that while the transitions into and out of the open state are similarly voltage dependent, most of the voltage dependence between the two closed states resides in the rate constant $\delta$ of the transition back to the far closed state.

It is important to note here that the CCO model for the $K_D$ channel is a reduced model (see also Schroeder, 1989). Single-channel open and closed times, in the range of 20 and 1 ms, respectively (Hosoi, Iino, and, Shimazaki, 1988), reveal another time scale of gating. However, as a larger number of parameters would yield the fitting procedure impractical, in our macroscopic experiments, this fast time scale was filtered out. If, for example, in this reduced CCO model, the O state actually represents a burst of channel openings rather than individual channel openings (i.e., an additional lumped $C_0 \leftrightarrow O_{true}$ transition), then the rate constant $\beta$ should predict the inverse of burst duration. Indeed, the value of such $\beta$ (average frequency of closings determined from one patch, in a record filtered at 20 Hz) was comparable to an average value of $\beta$ calculated for equivalent conditions from whole-cell data. This supports the notion that our analysis of whole cell currents may have relevance to the single channel behavior. Using our reduced model, we aimed to resolve the extracellular pH effects on the transition rates of the channel on a time scale extending roughly between 50 to 5,000 ms.
The Mechanism of pH Blocking Action

In *Vicia* protoplasts, extracellular protons exert a major inhibitory effect on the macroscopic conductance of the *K*\textsubscript{D} channel. We ascribe these effects of protons solely to the external surface of the membrane, due to the efficient buffering of the internal solution (Marty and Neher, 1983). Over most of the voltage range examined (> -20 mV), the steady state whole-cell *K*\textsubscript{D} conductance in protoplasts decreased by ~75% with acidification between 8.1 and 4.4 (Fig. 3). An essentially similar effect (~25% decrease at pH range 7.4–5.5) has been reported for guard cells in situ (Blatt, 1992).

What is the mechanism of these effects on conductance? Do protons change the electrical interactions of the channel and thus the gating (an effect on *P*\textsubscript{0} and on \(\tau\)) by altering: (a) the effective charges of the gating particles (an effect on \(z\)); and (b) the surface charge near the channel (an effect on \(E_0\))?

Do protons affect the voltage-independent parameters of the channels (*G*\textsubscript{max}) by: (a) blocking partially the open channel (an effect on \(\gamma_0\)); and (b) knocking out channels from the membrane or modulating their availability for opening by depolarization (an effect on \(N\))?

Our answers are based on the results from both of our approaches: the two-state and the three-state modeling. Fig. 4 C and Table I reveal that external protons do not alter significantly the voltage sensitivity (i.e., the effective charges) of the *K*\textsubscript{D} channel gating particles.

In contrast, protons do modify the electrical field sensed by the gates, as evident from the shifts of the voltage-dependent properties (*P*\textsubscript{0}, \(\tau\)’s) along the voltage axis (Figs. 4 B, 8 B–D).

The Negative Surface Charge Near the *K*\textsubscript{D} Channel

These pH-dependent shifts suggest that protons decrease the negative surface charge density at the external surface of the membrane, in the vicinity of the *K*\textsubscript{D} channel gates.

The slope of \(E_0\)-pH (Fig. 4 B) is indicative (in a complicated way) of the density of such titratable charges (Gilbert and Ehrenstein, 1969, 1970). Thus, it may be of interest to compare such observed shifts and calculated surface charge spacing for the delayed rectifier K channel between various preparations: at pH range 4–6.5, in the axon membrane of the marine worm *Myxicola*, the slope was −9.3 mV/pH unit (Schauf and Davies, 1976), and the average spacing between charges, \(d_0\), was ~13 Å (Begenisich, 1975; Schauf, 1975); in the giant axon of the squid the slope was −18.7 mV/pH unit and \(d_0\) was 8 Å (Carbone, Fioravanti, Prestipino, and Wanke, 1978), in the frog node the slope was −49 mV/pH (Druin and The, 1969) and \(d_0\) was 20 Å (Mozhayeva and Naumov, 1970), and at a pH range 4.4 to 8.1, in *Vicia* protoplasts the slope was −6.2 mV/pH unit (Fig. 4 B). Given the much lower ionic strength of the external medium in our experiments (see Materials and Methods), as compared to the above examples, the small slope in *Vicia* would be consistent with a lower surface charge density (and larger spacing). Indeed, we estimated the average spacing between the negative charges in the vicinity of the *K*\textsubscript{D} channel gates in *Vicia* as 39 Å, considerably larger than in the above examples. (In an interesting contrast to
the animal K channel, recent analysis of external surface charge near a neuronal Ca
channel reveals their large spacing: ~33 Å [Becchetti et al., 1992]). A possible
interpretation of these differences may be that those parts of the channel protein that
contribute to the negative surface charge in the animal K channel (reviewed by Hille,
1992), are different in the K_D channel in the plant cell protoplast.

The above considerations are only rough approximations: the effects of protons on
the gating properties of the K_D channel are only partially consistent with a simple
titration of surface charges. We note a departure from a homogeneous shift of
voltage-dependent properties along the voltage axis (for example: between pH 8.1
and 4.4 P_0 shifts by ~20 mV (Fig. 8 D), while \( \tau_1 \) shifts by ~40 mV, furthermore, \( \tau_1 \)
increases (Fig. 8 C)). This, by itself, does not necessarily contradict the surface charge
theory (Gilbert and Ehrenstein, 1984). However, even the more elementary param-
eters are affected differently: \( E_1 \) and \( E_2 \) do not shift similarly with changes of pH
(Table I). Moreover, the shift of \( E_1 \) is not a monotonic function of pH (Table I).
Therefore, it is unlikely that protons affect simply the external surface charge of the
membrane. Rather, we may interpret this effect as that of protonation of discrete
negative charges, perhaps even more than one class of sites, in the local environment
of the gating subunit, resulting in different electrical fields sensed during the two
conformational transitions (see also Gilbert and Ehrenstein, 1984). If applicable, the
approximate pK_a of this protonation, derived from our data within the framework of
the simple surface charge theory, is 4.8, corresponding to the bulk pH (i.e., apparent
pK_a) of 5.0. Finally, it may be of interest to note that the pK_a of the negative surface
charge site near the neuronal Na channel of the frog was 4.6 (this corresponds
to bulk pH of 5.3; Gilbert and Ehrenstein, 1970), and that near the cardiac Na channel
was 5.16 (Zhang and Siegelbaum, 1991).

**A Separate Effect of pH on G_{max}?**
The shift in P_0-E_{M} accounts for part of the observed decrease of the steady state
conductance with acidification (Fig. 3 A). In addition to this effect on the voltage-
dependent gating parameters, acidification decreases also the voltage-independent
property, G_{max}, i.e., the product N/s (Eq. 6). Because single channel conductance, \( \gamma_0 \),
is hardly affected by pH change, the decrease of macroscopic conductance should be
attributed to the diminished number of available channels, N, due to protonation.
Actually, this is paramount with redefining N as the product of the true number of K_D
channels in the membrane and an additional voltage-independent availability factor,
representing, in this case, the effect of protonation on channel gating. Indeed, the
effect of protonation on N, extracted by binomial analysis of single channel records
from two excised, outside-out patches, is consistent with this conclusion (in spite of
the irregularity of gating). Postulating such a single protonation site modifying the
voltage-independent aspect of K_D channel gating, its pK_a has been estimated as 4.8 ±
0.3 (±estimates of standard error; n = 6). However, if the data at pH 8.1 are
excluded from the fit, pK_a is 4.6 ± 0.1 (n = 5; Fig. 4 A). Indeed, the relatively large
jump in G_{max} between pH 8 and 7.5 suggests an additional protonation site (perhaps
similar to that with pK_a of 9.2 suggested by Mozhayeva and Naumov [1970] for the
frog node K channel), but extension of experiments beyond pH 8 is required to
substantiate this here. Thus the pK_a 4.8 may be interpreted as reflecting a lumped
site rather than a truly single site. For comparison, the delayed rectifier channel in frog myelinated nerve was also blocked by extracellular protons, with a pKₐ of 4.6 (Druin and The, 1969) or 4.4 (Hille, 1973). In Myxicola, the pKₐ for Gₖ inhibition was also 4.4 (Schauf and Davis, 1976), but in the crayfish it was 6.3 (Schrager, 1974). In the Na channels in the frog nerve, the blocking protonation site had a pKₐ of 4.8 (Woodhull, 1973).

In conclusion, in the physiological range of external pH, the plant Kₒ channel availability for activation (N) may be governed by a site similar to that in K channels in animal membranes. This might be a carboxyl side chain group of the channel protein itself, slightly modified by its local environment (Schauf and Davis, 1976). The protonation of such a local discrete negative charge may lead to the compound observed effects of acidification (Gilbert and Ehrenstein, 1984; Zhang and Siegelbaum, 1991). Is then the site that governs N, the voltage-independent channel availability, separate from the site that affects the local electrical fields sensed by the gating moieties? Further experiments are needed to resolve this question.

**Physiological Significance**

The modulatory effects of pH on Kₒ channels are consistent with the physiological requirements for their function. At the apoplastic pH of ~5, prevailing when the stomatal guard cells swell and stomata open (Edwards et al., 1988), acidification might aid the closing of the Kₒ channels by hyperpolarization. While hyperpolarization alone would be sufficient to close the Kₒ channels in this instance, membrane potential could be subject to a separate modification by changes in the relative permeabilities to different ions (for example, depolarization brought about by an increase in permeability to Ca²⁺, or Cl⁻). In such a case, extracellular protons might act as an additional gating element preventing the loss of intracellular K⁺. At the higher apoplastic pH, prevailing when the stomata normally close (Edwards et al., 1988), most of the Kₒ channels will be able to serve as the pathway for K⁺ efflux, when the membrane becomes depolarized. Apoplastic pH changes, together with the plant hormone, Abscisic acid (ABA), have been suggested to act as signals of the decrease in water availability, during which stomata close (Gollan, Schurr, and Schulze, 1999). For example, increased apoplastic pH appears to be correlated with drought stress conditions imposed either by pressure dehydration of detached leaves (Hartung, Radin, and Hendrix, 1988), or by soil dehydration (Gollan et al., 1992). The enhanced activity of the Kₒ channels in guard cells at such conditions would be of beneficial value, since it would promote stomata closure and preserve the plant water potential.

Thanks are due to Ms. A. Ben David for help in protoplast isolation and to Dr. H. Jarosch for help with the SAS and Fortran fitting routines. The authors are grateful to Drs. I. Steinberg, G. Ehrenstein, K. Iwasa, Y. Palti, and S. Assmann for helpful comments on an earlier version of the manuscript and to Dr. Blatt for making his paper available to us before we could see it published.

This research was supported by the US-Israel Binational Science Foundation grant No. 89-00255 to A. Schwartz by the US-Israel Binational Agricultural Research and Development Fund grant No. 91-00293 to N. Moran and the Basic Research Foundation grant No 487/89 from The Israel Academy of Sciences and Humanities to N. Moran.

Original version received 29 September 1992 and accepted version received 9 December 1993.
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