Plant Physiol. (1994) 105: 999–1006

A Cationic Channel in the Guard Cell Tonoplast of *Allium cepa*[1]

Gabriela Amodeo[2], Ariel Escobar, and Eduardo Zeiger*

Department of Biology (G.A., E.Z.), and Department of Physiology (A.E.), University of California, Los Angeles, California 90024

Stomatal movements depend on an osmoregulation process in which swelling or shrinking of the guard cells opens or closes the stomatal pore. Ions and water fluxes are an essential aspect of guard cell osmoregulation. Thus far, studies of these fluxes have focused on the guard cell plasma membrane. Guard cells, however, are a multi-compartment system that includes a prominent vacuole, which has a primary role in turgor regulation. This study reports on a detailed characterization of an ion channel at the guard cell tonoplast of *Allium cepa* (onion). We used patch-clamp methodology with isolated tonoplast patches to study conduction and gating at the single channel level. A voltage-dependent outward-rectifying cationic channel (210 picosiemens) was the dominant conductance. In symmetrical solutions the channel displayed an ohmic behavior in its current-voltage relationship. It also showed a very large rectification in the open probability. The channel was predominantly cationic and its sequence of ionic selectivity was weak ($Na^+ > K^+ > Rb^+ > Cs^+$). The channel conductance was not affected by intravacuolar pH. Analysis of membrane patches with multiple channels showed that the probability of a channel to open was independent of the opening of the other channels present in the patch and that there was a conservation of the open probability for different channels. Ensemble records generated using a pulse protocol showed slow activation and deactivation kinetics. A first-latency analysis of single-channel records in response to protocols with different prepulse duration indicated that this channel has more than one closed state.

Stomatal movements regulate leaf CO_2 uptake and water loss in response to environmental signals. Volume changes in guard cells mediated by ion fluxes, and carbohydrate and organic acid biosynthesis cause changes in stomatal apertures that modulate leaf gas exchange (Zeiger, 1983; MacRobbie, 1988; Tallmann, 1992). Understanding of the ion fluxes involved in this process should enhance our knowledge of the dynamics of stomatal movements and their regulation. Previous studies of ion fluxes in guard cells have focused on electrogenic pumps and ion channels at the plasma membrane. Several K^+ channels have been characterized in detail (Schroeder et al., 1984, 1987; Schroeder, 1988). Anion and stretch-regulated channels have also been reported (Hedrich et al., 1990; Cosgrove and Hedrich, 1991; Marten et al., 1992; Schroeder and Keller, 1992). On the other hand, very little is known about the regulation of ion fluxes at the guard cell tonoplast (Penny and Bowling, 1974; MacRobbie, 1987, 1988). Vacuoles play an important role in osmoregulation (Matile, 1978; Wink, 1993), and accumulated osmotica in the guard cells are kept in the vacuole (Schnabl and Kottmeier, 1984).

Although there is an initial report on a 240-pS channel at the guard cell tonoplast of *Vicia faba* (Hedrich et al., 1988), no additional studies have been published. The present work reports on a detailed characterization of an ion channel at the guard cell tonoplast in onion (*Allium cepa*). Onion stomata are of interest because of the lack of starch in their chloroplasts, which excludes a role of malate as a K^+ counterion (Schnabl and Ziegler, 1977; Schnabl and Raschke, 1980), and because of the blue light-induced intrinsic green fluorescence of their vacuoles (Zeiger and Hepler, 1979). The recent characterization of quercetins as the fluorescence-emitting chromophore (Urushibara et al., 1992) makes it possible to use this intrinsic fluorescence as a vacuolar pH indicator.

Several studies have reported tonoplast channels from different plant cell types. They show a high unitary conductance (g) with a slow activation mode (about 180 pS in 200 mM KCl) and less selectivity than plasma membrane K^+ channels (Hedrich et al., 1986; Coyaud et al., 1987; Hedrich and Neher, 1987). This low selectivity seems to vary with tissues. A K^+/Cl^- selectivity of 6:1 has been reported in sugar beet (Hedrich and Neher, 1987), whereas others found a lack of selectivity for cations over anions (Hedrich and Kurkdjian, 1988). Fast-activated channels with lower conductances have also been described (Pantoja et al., 1992; Gambale et al., 1993), as has a K^+/proton co-transport process involving a vacuolar H^+ pyrophosphatase (Davies et al., 1992).

MATERIALS AND METHODS

GCPs

Epidermal peels from 12- to 14-d-old cotyledons of *Allium cepa* (Zeiger and Hepler, 1976) were used for the isolation of GCPs. GCPs were isolated according to Gotow et al. (1984) with the following modifications: peels of approximately 50 cotyledons were pre-plasmolyzed in a solution containing 125 mM mannitol plus 1 mM $CaCl_2$ for 10 min and then

[1] This work was supported by grants from the National Science Foundation and the Department of Energy to E.Z.

[2] Permanent address: Departamento de Agronomía y Cerzos, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina.

* Corresponding author; fax 1–310–825–9433.

Abbreviations: GCP, guard cell protoplast; I-V, current-voltage; pS, picosiemens.

transferred to 250 mM mannitol plus 1 mM $CaCl_2$ for another 10 min. These pretreated peels were cut into small pieces and incubated at 28°C with shaking (110 rpm) for 12 min in 0.55% Cellulase RS (Yakult, Tokyo, Japan), 0.005% Pectolyase Y-23 (Seishin, Tokyo, Japan), 0.5% BSA, 250 mM mannitol, and 1 mM $CaCl_2$. The pH was adjusted to 5.5 with HCl. The digested peels were collected in a 225-μm nylon mesh (Spectrum, Houston, TX), thoroughly washed with 450 mM mannitol plus 1 mM $CaCl_2$, and transferred to a solution containing 1% Cellulase RS, 0.01% Pectolyase Y-23, 0.5% BSA, 450 mM mannitol, and 1 mM $CaCl_2$ for 15 min at 28°C with gentle shaking (50 rpm). The released protoplasts were collected using a 30-μm nylon mesh (Spectrum) and washed twice by centrifugation at 110*g* for 6 min with 450 mM mannitol plus 1 mM $CaCl_2$. Purified GCPs were kept on ice in the dark before use.

Guard Cell Vacuoles

Vacuoles were released by an osmotic shock (Zeiger and Hepler, 1979) given by decreasing the mannitol in the medium to a final osmolarity of 120 mOsm kg^{-1}. Vacuole yield was increased by a pretreatment in 5 mM EGTA for 8 min (adapted from Raschke and Hedrich, 1989) prior to an osmotic shock. A small aliquot of the vacuole suspension was transferred to the patch chamber filled with bath solution adjusted to 600 mOsm kg^{-1}. After 10 min the vacuoles sedimented to the bottom of the chamber. Before starting an experiment, the chamber (500 μL volume) was perfused with fresh filtered bath solution.

Electrophysiology

The patch-clamp technique was used to record ionic currents from isolated inside-out patches (Hamill et al., 1981). Pipettes were pulled from Corning 7052 capillaries (Garner Co., Claremont, CA) using a two-stage vertical puller model PP-83 (Narishige Co., Tokyo, Japan). Pipettes with resistance of 5 to 10 MΩ were coated with a layer of elastomeric silicone (Sylgard, Dow Corning) and heat polished with a microforge built into an Olympus microscope. Single-channel records were obtained with an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA) and were stored either in digital audio tapes (Sony DAT 75-ES) or using an acquisition system (Labmaster, Axon Instruments) connected to an IBM-compatible computer (PC 486, 25 MHz). All experiments were performed at room temperature (20–23°C).

Solutions

The pipette solution (facing the cytoplasmic side of the tonoplast) contained 200 mM KCl, 0.1 mM $CaCl_2$, 10 mM Mops/KOH, pH 7.2, adjusted to 550 mOsm kg^{-1} with mannitol. The bath solution (intravacuolar milieu) contained 200 mM KCl, 1 mM EGTA, 10 mM Mops/KOH, pH 7.2 (or 10 mM Mes/KOH, pH 5.5, as indicated) adjusted to 600 mOsm kg^{-1} with mannitol. The final calculated K^+ concentration was 208 mM. All solutions were freshly made and filtered (Millipore, 0.20 μm). When replacing cations or anions as indicated below, perfusion was carried out by washing 10 times the volume of the chamber after achieving the inside-out configuration. Liquid junction potentials were measured for each intravacuolar solution (bath solution) in a zero-current clamp configuration. No changes in the potential were observed when the cation was replaced in the bath solution (Na^+, Rb^+, and Cs^+). A very small (-0.4 ± 0.2 mV, $n = 5$) junction potential was measured when we established an ionic gradient between the pipette and the bath (200 mM KCl in the pipette and 20 mM KCl in the bath). More important liquid junction potentials appeared when we replaced the chloride in the bath with glutamate or sulfate (see legend for Fig. 2). All the potentials were corrected according to Neher (1992).

Selectivity

Channel selectivity was characterized by replacing monovalent ions (cations or anions) in the bath. Reversal potentials (E_{rev}) were measured from a steady-state I-V relationship and permeability ratios between P_{K+}/P_{cation} and P_{Cl-}/P_{anion} were calculated using an equation derived from a diffusional theory (Goldman, 1943). Under conditions of a KCl gradient between bath and pipette solution (20 and 200 mM, respectively) we used the Goldman, Hodgkin, and Katz equation (Goldman, 1943; Hodgkin and Katz, 1949). The I-V relationships were obtained using mean currents from amplitude histograms of raw data fitted with a Gaussian function. All experiments were repeated at least three times.

Steady-State Analysis

Records of at least 5 min at different potentials were used to determine the voltage dependence of the open probability. Records were filtered at a cut-off frequency of 1 kHz with a four-pole Bessel filter and sampled at 200 μs/point.

When more than one channel was present in the patch, amplitude histograms were generated from the raw data at each potential. These histograms were employed to obtain the probability of having n channels open [$p(n)$] according to:

$$p(n) = \frac{A(n)}{\sum_{n=0}^{n=N} A(n)} \quad (1)$$

where n is the number of open channels, $A(n)$ is the area under the Gaussian function fitted to each peak of the histogram, and N is the number of channels present in the patch. The open probability (P_o) was computed using

$$P_0 = \frac{\sum_{n=0}^{n=N} n \cdot p(n)}{N} \quad (2)$$

Ensemble Records

Pulse protocols given with the computer through the digital-to-analog converter were used for the kinetic analysis of the activation and deactivation of the channel. The sample rate was 2 ms/point. Fifty records of single channels were averaged at different potentials to generate the ensemble profile of the macroscopic current. The data were fitted to obtain activation and deactivation time constants.

First-Latency Analysis

The time needed for the first channel to open in response to a depolarizing pulse was used to generate a cumulative histogram to study the first latency (Aldrich et al., 1983). In records with more than one channel, the obtained times were multiplied by the square root of n channels present in the patch. An n-order independent-state transition Markovian model was fitted to the histograms to determine both the time constants and the number of closed states.

Sign Convention

We have adopted the sign convention for electrical measurements on endomembranes proposed by Bertl et al. (1992). The advantage of this convention is that it rejects the use of electrical ground or amplifier input as points of reference and treats the intracellular space as the electrical reference point. Thus, the potential difference or voltage across the vacuolar membrane (V_m) is calculated as $V_m = V_{cytosol} - V_{vacuole}$. This implies that positive or outward currents across the tonoplast would represent cation flow out of the cytosol (to the vacuolar lumen) and that negative potentials are now referred to as vacuole-positive potentials.

RESULTS

Guard Cell Vacuoles Have an Outward-Rectifying Cationic Channel

Use of the highly effective cellulolytic enzymes currently available with guard cells of onion cotyledons made it possible to obtain GCPs after 30 min of total digestion time. The GCP preparation was free from mesophyll or epidermal protoplasts. Physiologically competent protoplasts could be kept overnight, although the experiments were usually conducted on the same day. Vacuole diameters ranged between 15 and 20 μm. Vacuole integrity was assessed by observing its intrinsic fluorescence (Zeiger and Hepler, 1979). In inside-out tonoplast patches, the most frequently observed channel showed a high conductance compared with typical plasma membrane channels. A high-conductance tonoplast channel has been reported in the guard cell tonoplast of *V. faba* (Hedrich et al., 1988) and in vacuole membranes of other tissues (Hedrich et al., 1986; Coyaud et al., 1987; Hedrich and Neher, 1987). Figure 1A (closed circles) shows the I-V relationship of the channel, having a slope conductance of 210 ± 17 pS (n = 20) for symmetrical 200 mM KCl. The channel had an ohmic behavior between +100 and −100 mV, both in symmetrical solutions and in the presence of a 20 mM/200 mM KCl ionic gradient (Fig. 1A, closed squares).

The role of cations and anions in the observed fluxes through this channel was investigated with asymmetric KCl solutions. A significant shift in the E_{rev} was observed when the vacuolar side of the tonoplast (bath solution) was perfused with 20 mM KCl, with only a minor change in the single-channel conductance. For this condition, the bath solution was adjusted to 600 mOsm kg^{-1} with mannitol. The final K^+ concentration was 28 mM. The obtained reversal potential was −45 ± 5 mV, indicating that the channel is highly selective for cations (the K^+ Nernstian potential for this condition is −50.4 mV). We also observed a pronounced voltage dependence of the open probability following an outward rectification (Fig. 1B). Up to four single-current levels could be observed at positive potentials (+20 mV), in contrast to a single level recorded at negative potentials (−30 mV).

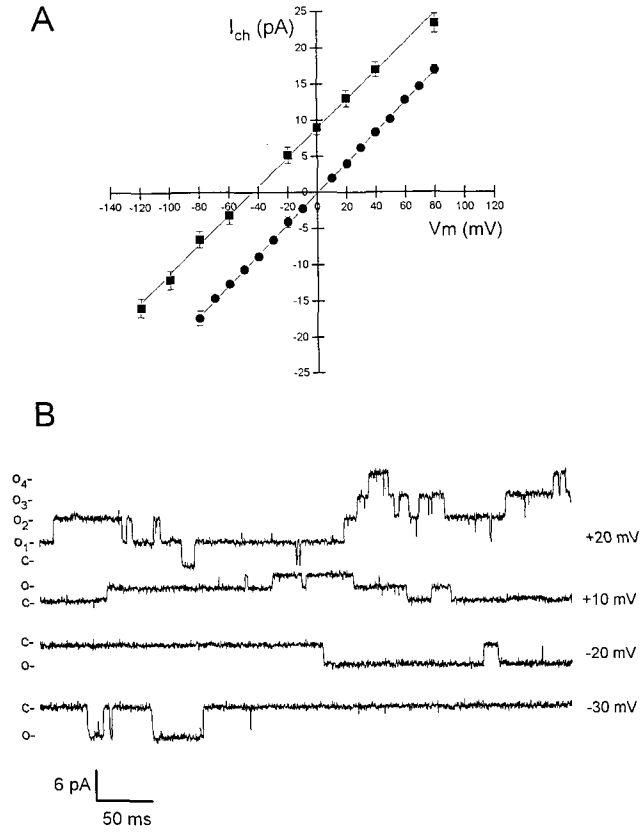

Figure 1. A, I-V relations (I_{ch}-Vm) from single-channel recordings of inside-out patches from onion guard cell tonoplast. The pipette solution contained 200 mM KCl, 0.1 mM $CaCl_2$, and 10 mM Mops/KOH, pH 7.2, adjusted to 550 mOsm kg^{-1} with mannitol. The bath solution contained 1 mM EGTA, 10 mM Mops/KOH, pH 7.2, and 200 mM KCl (●) (n = 20) or 20 mM KCl (■) (n = 3). The bath solution was adjusted to 600 mOsm kg^{-1} with mannitol. Slope conductances were 210 ± 17 and 199 ± 21 pS for the two solutions, respectively. Data points are the mean of the indicated n measurements ± SE. B, Typical single-channel recordings of inside-out patches that were polarized at various potentials, indicated as opening (o) and closing (c) for single channels, and as (o_1, o_2, etc.) for simultaneous events in a same patch.

The Channel Shows a Weak Selectivity for Cations and No pH Sensitivity on the Conductance

The selectivity of the channel was studied using different monovalent cations in the bath solution (Fig. 2A). The sequence of ion permeability was $Na^+ > K^+ > Rb^+ > Cs^+$. The permeability ratios were $P_{K+}/P_{Na+} = 0.62$, $P_{K+}/P_{Rb+} = 1.47$, and $P_{K+}/P_{Cs+} = 2.04$, indicating a weak selectivity for different cations. It is also noteworthy that conductances were almost unchanged and an ohmic behavior was conserved (Fig. 2A).

No measurable differences were observed in the I-V rela-

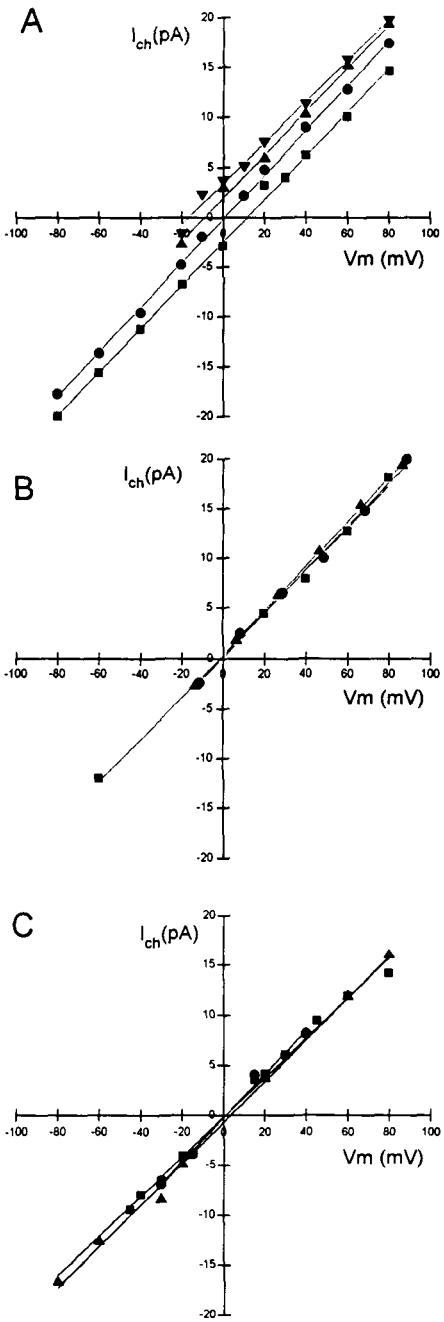

Figure 2. A, Selectivity sequence of the 210-pS channel. Monovalent ions in the bath solution were 200 mM NaCl (■), 200 mM KCl (●), 200 mM RbCl (▲), and 200 mM CsCl (▼). Slope conductances were 216 ± 3.61, 210 ± 7.11, 215 ± 7.23, and 204 ± 6.36 pS, respectively. E_{rev} values were calculated as −12, 0, +9, and +17 mV, respectively (n = 3). B, I-V plot showing effect of changing anions in the bath solution. ●, 200 mM K-glutamate; ▲, 200 mM K_2SO_4. Slope conductances were 217 ± 6.12 and 221 ± 1.99 pS, respectively. Control was symmetrical KCl 200 mM (■). C, Effect of pH on the conductance of the outward-rectifying channel. An initial pH of 7.2 (10 mM Mops/KOH) in both sides of the patch (■) was subsequently changed by perfusion to pH 5.5 (10 mM Mes/KOH) in the vacuolar side of the tonoplast (▲). Vacuoles were also perfused at pH 5.5 before the inside-out configuration was achieved (●). Slope conductances were 210 ± 4.5, 219 ± 11.2, and 207 ± 5.26 pS, respectively. Data shown represent a typical experiment for each case. Each treatment was repeated with at least three different vacuoles with similar results. Liquid junction potentials were corrected and their values were 6.6 ± 0.05 mV for K_2SO_4 and 8.84 ± 0.23 mV for glutamate.

tionship when Cl^- was replaced in the bath by either glutamate or SO_4^{2-} (Fig. 2B). This strengthens the conclusion that the 210-pS channel is highly selective for cations.

The effect of intravacuolar pH on channel conductance was studied by lowering the vacuolar side of the patch to pH 5.5. As shown in Figure 2C, there were no changes in the conductance of the I-V curve in response to the change in pH.

Steady-State Analysis

The voltage dependence of the open probability, and the possible interdependence of the kinetic activity of different channels present in one patch (Fenwick et al., 1982), were investigated in long-time recordings. The open probability of the channel at different voltages was determined using amplitude histograms (Fig. 3A). The histograms clearly show that the populations of n open channels increase as the tonoplast is depolarized (vacuole more negative). The calculated open probabilities (Eq. 2) plotted as function of the membrane potential are shown in Figure 3B.

The independence and conservation of the open probability for each channel was tested by comparing the experimental data with a theoretical prediction for the open probability of the channels at each population level (zero to five channels, see Fig. 3A, insets) generated by a binomial model (Fenwick et al., 1982):

$$p^*(n) = \frac{N!}{n! \cdot (N-n)!} \cdot P_0^n \cdot (1 - P_0)^{N-n} \qquad (3)$$

where $p^*(n)$ is the predicted probability of having n channels open, N is the number of total channels present in the patch, and P_o is the experimental open probability. The insets of Figure 3A show that for five active channels in the patch (n = 5), the predicted values are very close to the experimental data. This indicates that each channel in the patch has the same open probability, and that the kinetic activities of the channels are independent of each other.

Slow Kinetics Characteristics of the Channel Observed in Nonstationary Experiments

The kinetics of the voltage-dependent activation process was studied in pulse experiments. Traces such as those shown in Figure 4B provided information on the time required for n channels in the patch to open in response to a 2.5-s depolarizing pulse. The single-channel "tail" events (Fig. 4E) provided information on the channel deactivation process.

A total of 50 traces from pulse protocols such as those shown in Figure 4B were used to calculate the open probability of the channel as a function of the membrane potential

of the tonoplast. The open probability at each potential [$P_o(t)$] (Horn et al., 1981) is given by:

$$P_0(t) = \frac{\langle n(t) \rangle}{N} \quad (4)$$

This was obtained by finding the maximum total number of channels (N) in all the records and determining the number of open channels [$<n(t)>$] in the last 300 ms of the pulse in each record. These values were used to obtain the mean open probability [$P_o(V_m)$] and SE at each potential (Fig. 4C).

The experimental points were fitted with a Boltzmann function:

$$P_0(V_m) = \frac{P_{0\max}}{1 + e^{[-(V_m - V')/k]}} \quad (5)$$

where $P_{o\ max}$ is the maximum open probability, V_m is the vacuolar membrane potential, V' is the voltage at which the mean open probability is half of the maximum open probability [$P_o(V_m) = P_{o\,max}/2$], and k is the voltage sensitivity of the channel. The obtained values were $V' = +51$ mV and $k = +19$ mV.

The macroscopic current generated by this population of channels was analyzed in an ensemble record generated from the average of all records at each potential (Horn et al., 1981). Activation and deactivation time constants were obtained by fitting the data to a first-order kinetic process. For an 80-mV depolarizing pulse, the obtained values were 1108 ms for activation (τ_{on}) and 44 ms (τ_{off1}) and 291 ms (τ_{off2}) for deactivation (Fig. 5A). A macroscopic I-V relationship obtained from the ensemble record showed a steep outward rectification (Fig. 5B).

The Channel Has More than One Closed State

The transition between the closed states that leads to the opening of the channel was examined by studying the effect of the length of a depolarizing prepulse (Fig. 6A) on the first latency for single-channel opening. First-latency cumulative histograms for 40- and 100-ms depolarizing prepulses were fitted with an n-order independent-state transition Markovian model (Fig. 6B). Since $n = 2$ in this fitting, it can be inferred that the channel has at least two closed states. There

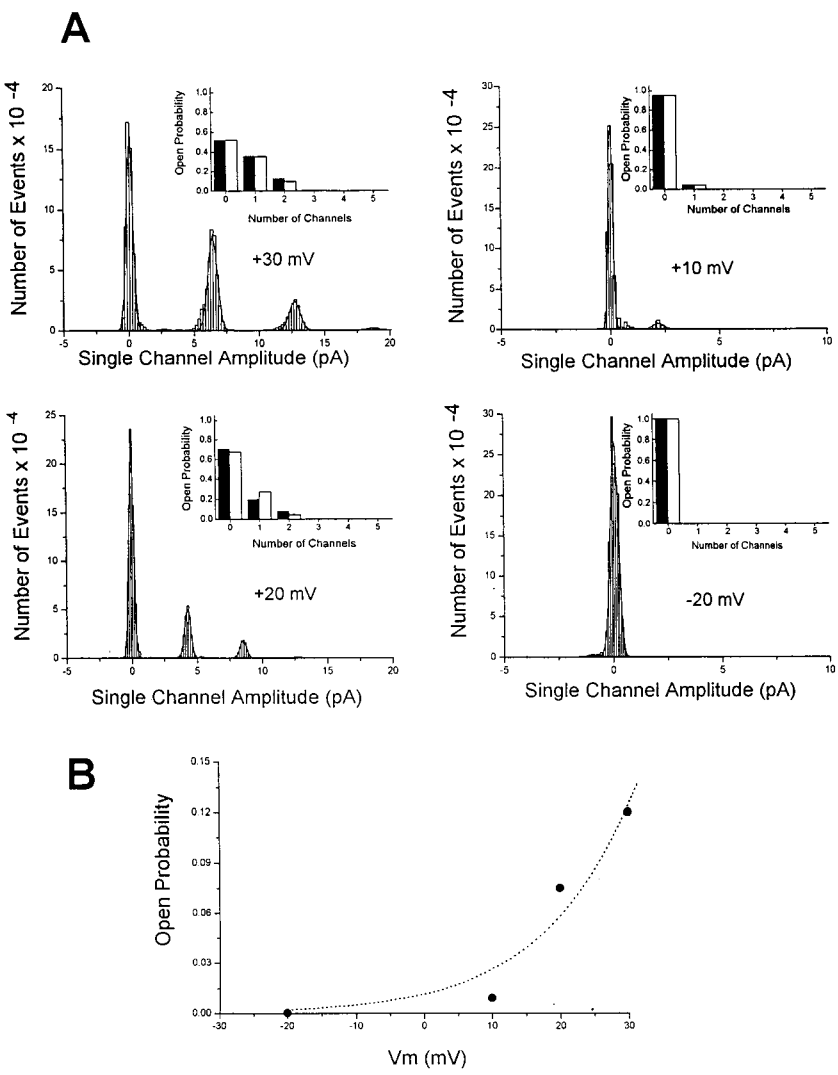

Figure 3. A, Histograms showing single-channel amplitude distribution for different voltages, fitted for multiple Gaussian. The population of n channels increases as the tonoplast is depolarized. Insets, Probability of having n channels open calculated from values obtained by the analysis of amplitude histograms from single-channel recordings (■) and those predicted by a binomial distribution (□). B, Open probabilities of the channel for the voltages indicated in A. Ionic conditions were the same as those for the symmetrical experiments shown in Figure 1.

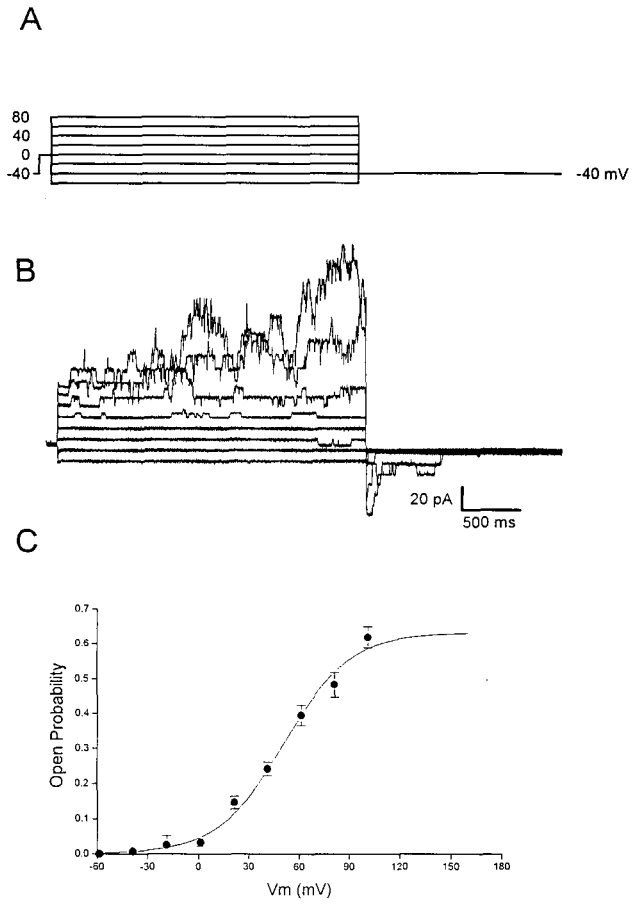

Figure 4. A, Pulse protocol used to generate the ensemble profile of the macroscopic conductance of inside-out patches. A patch was held at 0 mV and stepped from −60 to +80 mV for 2.5 s in increments of +20 mV and then deactivated by changing the voltage to −40 mV. B, Single-channel recording under the pulse protocol shown in A. C, Calculated open probabilities obtained as a function of tonoplast membrane potential. Ionic conditions were the same as those for the symmetrical experiments shown in Figure 1.

was also a clear voltage-dependent shift, resulting in a faster opening of the channel in response to a longer 0-mV depolarizing prepulse. The simplest interpretation of this result is that an increase of the length of the depolarizing prepulse causes an increase in the population of the closed state, which is nearer to the first open state.

DISCUSSION

Regulation of ion fluxes across the guard cell plasma membrane and the tonoplast are central to the control of stomatal movements (Zeiger et al., 1978; Raschke et al., 1988; Tallman, 1992). In contrast to the well-characterized electrogenic pumps and ion channels at the plasma membrane (Raschke et al., 1988; Serrano and Zeiger, 1989), the regulation of ion fluxes at the guard cell tonoplast are poorly understood.

The aim of the present study was to characterize ion channels at the tonoplast of onion guard cells. Digestion times needed to isolate GCPs from onion cotyledons are substantially shorter than those required for the isolation of *Vicia* GCPs, the system of choice in previous patch-clamp studies. In addition, K^+ fluxes in onion guard cells are balanced solely by Cl^- fluxes, and the absence of organic anion fluxes typical of other guard cells simplifies the analysis of ion fluxes in the onion system (Schnabl and Ziegler, 1977; Schnabl and Raschke, 1980).

A 210-pS channel was the dominant unitary conductance in isolated tonoplast patches from onion guard cells. This conductance was higher than that of other channels described in root or mesophyll vacuoles (Hedrich et al., 1986). However, it showed a similar voltage dependence and activation mode (slow-vacuolar type) under comparable cytosolic calcium levels (Hedrich et al., 1986; Hedrich and Neher, 1987). The 210-pS channel showed a high selectivity of cations over anions and a low selectivity for monovalent cations. These properties are similar to those reported for a cationic channel in the tonoplast of *Acer* cells (Colombo et al., 1988).

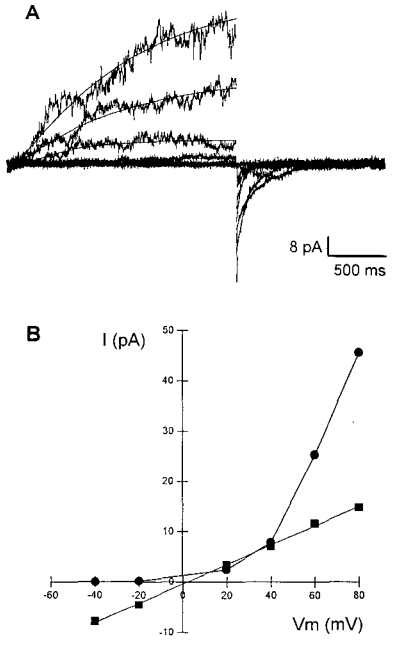

Figure 5. A, Ensemble profile of the macroscopic conductances obtained by averaging 50 records following the pulse protocols shown in Figure 4A. Best fits for activation and deactivation were given by:

$$A \cdot (1 - e^{(-(t-t_0)/\tau_{on})})^n$$

and

$$A_1 \cdot (1 - e^{(-(t-t_0)/\tau_{off1})}) + A_2 \cdot (1 - e^{(-(t-t_0)/\tau_{off2})})$$

respectively, where A, A_1, and A_2 are scaling factors, t is time, τ_{on}, τ_{off1}, and τ_{off2} are activation and deactivation time constants, respectively; and t_o is the time between the beginning of the trace and the application of the pulse. All solutions were as indicated in Figure 1 for symmetrical conditions. B, I-V relationship showing the macroscopic current (●) compared to the single-channel current (■). Experiments were repeated at least four times with similar results.

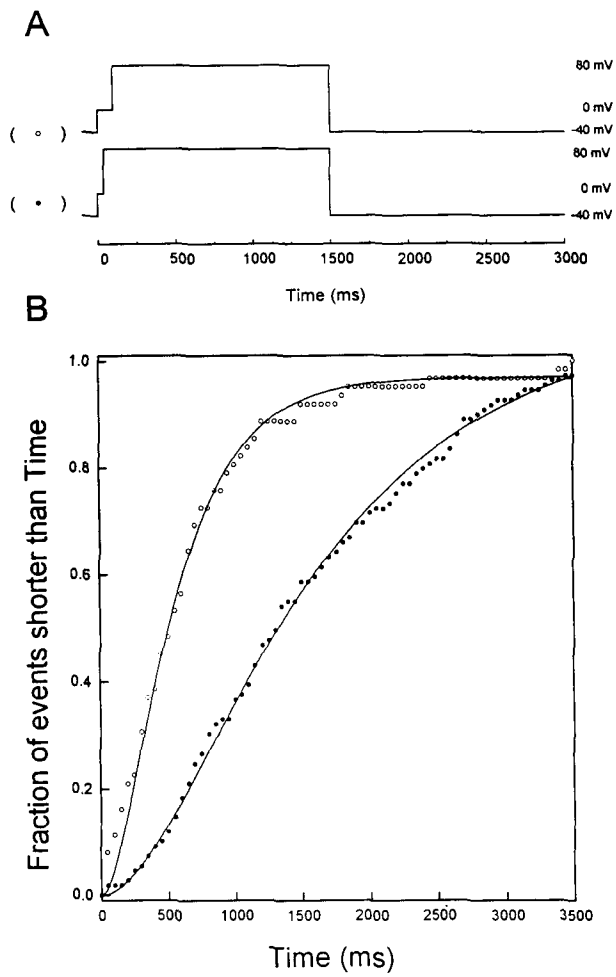

Figure 6. A, Pulse protocol used to study the first latency. A 0-mV prepulse of 100 or 40 ms was applied before activating the channel at +80 mV. B, Cumulative distributions from first latencies (n = 100) for a 100-ms (O) or 40 ms (●) prepulse at 0 mV. Curves were fitted to a function:

$$[1 - e^{(-t/\tau_{on})}]^n.$$

Values for τ were 395 and 1192 ms for a depolarizing prepulse of +40 and +100 ms, respectively. In both cases, $n = 2$.

It has been suggested that slow-vacuolar-type channels might be involved in malate fluxes across the tonoplast (Hedrich et al., 1986; Coyaud et al., 1987; Hedrich and Neher, 1987). However, this is unlikely to be the case in onion guard cells, which are devoid of malate fluxes (Schnabl and Ziegler, 1977; Schnabl, 1980; Schnabl and Raschke, 1980). Since the selectivity for chloride is negligible, potassium would be expected to be the primary ion permeated by this channel. Potassium is the major monovalent cation in both the cytosol and vacuole of onion guard cells, so the observed low cationic selectivity would lack functional significance. The 210-pS channel conductance was also insensitive to intravacuolar proton concentrations over a range of two pH units.

Further characterization of the channel showed that in patches with more than one channel, each channel opened independently with identical open probability. The data also indicated that in an excised configuration, all the active channels present in a patch were regulated in the same way.

Kinetic analysis of ensemble records showed a slow response time for both the activation and deactivation processes, implying that the channel has a large energy barrier to overcome in both the closed-to-open and the open-to-closed transitions. First-latency experiments indicated that this channel has more than one closed state and that the rate constants for the transitions between the closed states are mostly voltage dependent.

The most interesting characteristic of the channel was its sensitivity to tonoplast depolarization (vacuole negative). The relationship between the membrane potential differences measured at the tonoplast (about −30 mV; Moody and Zeiger, 1978) and the activation curve of the channel (P_o versus V_m; Fig. 4C) indicates that a depolarization (vacuole more negative) of the tonoplast would be required to increase the open probability. Hence, at steady potential differences of approximately −30 mV, the 210-pS channel would remain virtually closed.

Alternatively, the tonoplast membrane could depolarize in response to the dynamics of ion fluxes associated with stomatal movements. If this is the case, the channel will increase its open probability and, given its cationic properties and the ionic environment of onion guard cells, the ensuing conductance will be associated with potassium fluxes. The direction of these fluxes will depend on the K^+ electrochemical gradient. This will lead to K^+ uptake (consider a relatively constant 100 mM K_{cyt} [Penny and Bowling, 1974]; a starting K^+_{vac} of 100 mM when the stomata are closed [Willmer, 1983; MacRobbie, 1988]; and a depolarization of the tonoplast above E_k, 0 mV) or efflux (consider the same K^+_{cyt}; a K^+_{vac} of 300 mM when the stomata are open [Willmer, 1983; MacRobbie, 1988]; and a depolarization of the tonoplast below 28 mV).

The activation of the 210-pS channel might also be regulated by cytosolic calcium concentrations. Other slow-vacuolar-type channels have been shown to be calcium regulated (Hedrich and Neher, 1987), and calcium fluxes associated with stomatal movements are well characterized (Schwartz, 1985; Gilroy et al., 1991). Further studies are needed to determine which physiological conditions of onion guard cells, if any, result in tonoplast depolarization, and the precise role of the 210-pS channel in ion fluxes associated with stomatal movements.

ACKNOWLEDGMENTS

We thank Dr. Julio Vergara for his generous help with laboratory facilities and Dr. Ana María Correa for helpful comments on the manuscript.

Received December 27, 1993; accepted March 22, 1994.

LITERATURE CITED

Aldrich RW, Corey DP, Stevens CF (1983) A reinterpretation of mammalian sodium channel gating based on single channel recording. Nature **306:** 436–441

Bertl A, Blumwald E, Coronado R, Eisenberg R, Findlay G, Gradmann D, Hille B, Köhler K, Holbn H-A, MacRobbie E, Meissner G, Miller C, Neher E, Palade P, Pantoja O, Sanders D, Schroeder J, Slayman C, Spanswick R, Walker A, Williams A (1992) Electrical measurements on endomembranes. Science **258:** 873–874

Colombo R, Cerana R, Lado P, Peres A (1988) Voltage dependent channels permeable to K^+ and Na^+ in the membrane of *Acer pseudoplatanus* vacuoles. J Membr Biol **103:** 227–236

Cosgrove DJ, Hedrich R (1991) Stretch-activated chloride, potassium, and calcium channels coexisting in plasma membranes of guard cells of *Vicia faba* L. Planta **186:** 143–153

Coyaud L, Kurkdjian A, Kado R, Hedrich R (1987) Ion channels and ATP driven pumps involved in ion transport across the tonoplast of sugarbeet vacuoles. Biochim Biophys Acta **902:** 263–268

Davies JM, Poole RJ, Rea PA, Sanders D (1992) Potassium transport into plant vacuoles energized directly by a proton pumping inorganic pyrophosphatase. Proc Natl Acad Sci USA **89:** 11701–11705

Fenwick EM, Marty A, Neher E (1982) Sodium and calcium channels in bovine chromaffin cells. J Physiol **331:** 599–635

Gambale F, Cantu AM, Carpaneto A, Keller BU (1993) Fast and slow activation of voltage dependent ion channels in radish vacuoles. Biophys J **65:** 1837–1843

Gilroy S, Fricker MD, Read ND, Trewavas AJ (1991) Role of calcium in signal transduction of *Commelina* guard cells. Plant Cell **3:** 333–344

Goldman ED (1943) Potential, impedance and rectification in membranes. J Gen Physiol **27:** 37–60

Gotow K, Shimazaki K, Kondo N, Syono K (1984) Photosynthesis-dependent volume regulation in guard cell protoplasts from *Vicia faba* L. Plant Cell Physiol **25:** 671–675

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pfluegers Arch **391:** 85–100

Hedrich R, Barbier-Brygoo H, Felle H, Flügge UI, Lüttge U, Maathius FJM, Marx S, Prins HA, Raschke K, Schnabl H, Schroeder JI, Struve I, Taiz L, Ziegler P (1988) General mechanisms for solute transport across the tonoplast of plant vacuoles: a patch-clamp survey of ion channels and proton pumps. Bot Acta **101:** 7–13

Hedrich R, Busch H, Raschke K (1990) Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. EMBO J **9:** 3889–3892

Hedrich R, Flügge UI, Fernandez JM (1986) Patch-clamp studies of ion transport in isolated plant vacuoles. FEBS Lett **204:** 228–232

Hedrich R, Kurkdjian A (1988) Characterization of an anion-permeable channel from sugarbeet vacuoles: effect of inhibitors. EMBO J **7:** 3661–3666

Hedrich R, Neher E (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. Nature **329:** 833–835

Hodgkin AL, Katz B (1949) The effect of sodium ions on the electrical activity of the giant axon of the squid. J Physiol **108:** 37–77

Horn R, Patlak J, Stevens CF (1981) Sodium channels need not open before they inactivate. Nature **291:** 426–427

MacRobbie EAC (1987) Ionic relations of guard cells. *In* E Zeiger, GD Farquhar, IR Cowan, eds, Stomatal Function. Stanford Press, Stanford, CA, pp 125–162

MacRobbie EAC (1988) Stomatal guard cells. *In* DA Baker, JL Hall, eds, Solute Transport in Plant Cells and Tissues. Wiley, New York, pp 453–497

Marten I, Zeilinger C, Redhead C, Landry DW, Al Awqati Q, Hedrich R (1992) Identification and modulation of a voltage-dependent anion channel in the plasma membrane of guard cells by high-affinity ligands. EMBO J **11:** 3569–3575

Matile P (1978) Biochemistry and function of vacuoles. Annu Rev Plant Physiol **29:** 193–213

Moody W, Zeiger E (1978) Electrophysiological properties of onion guard cells. Planta **139:** 159–165

Neher E (1992) Correction for liquid junction potentials in patch clamp experiments. Methods Enzymol **207:** 123–131

Pantoja O, Gelli A, Blumwald E (1992) Characterization of vacuolar malate and K^+ channels under physiological conditions. Plant Physiol **100:** 1137–1141

Penny MG, Bowling DJF (1974) A study of potassium gradients in the epidermis of intact leaves of *Commelina communis* L. in relation to stomatal opening. Planta **119:** 17–25

Raschke K, Hedrich R (1989) Patch clamp measurements on isolated guard cell protoplasts and vacuoles. Methods Enzymol **174:** 312–330

Raschke K, Hedrich R, Reckmann U, Schroeder JI (1988) Exploring biophysical and biochemical components of the motor that drives stomatal movement. Bot Acta **101:** 283–294

Schnabl H (1980) CO_2 and malate metabolism in starch-containing and starch-lacking guard cell protoplasts. Planta **149:** 52–58

Schnabl H, Kottmeier C (1984) Determination of malate levels during the swelling of vacuoles isolated from guard cell protoplasts. Planta **161:** 27–31

Schnabl H, Raschke K (1980) Potassium chloride as stomatal osmoticum in *Allium cepa* L., a species devoid of starch in guard cells. Plant Physiol **65:** 88–93

Schnabl H, Ziegler H (1977) Mechanism of stomatal movement in *Allium cepa* L. Planta **136:** 37–43

Schroeder JI (1988) K^+ transport properties of K^+ channels in the plasma membrane of *Vicia faba* guard cells. J Gen Physiol **192:** 667–683

Schroeder JI, Hedrich R, Fernandez JM (1984) Potassium selective single channels in guard cell protoplasts of *Vicia faba*. Nature **312:** 361–362

Schroeder JI, Keller BU (1992) Two types of anion channel currents in guard cells with distinct voltage regulation. Proc Natl Acad Sci USA **89:** 5025- 5029

Schroeder JI, Rachske K, Neher E (1987) Voltage dependence of K^+ channels in guard cell protoplasts. Proc Natl Acad Sci USA **84:** 4108–4112

Schwartz A (1985) Role of Ca^{2+} and EGTA on stomatal movements in *Commelina communis* L. Plant Physiol **79:** 1003–1005

Serrano EE, Zeiger E (1989) Sensory transduction and electrical signaling in guard cells. Plant Physiol **91:** 795–799

Tallman G (1992) The chemiosmotic model of stomatal opening revisited. Crit Rev Plant Sci **11:** 35–57

Urushibara S, Kitayama Y, Watanabe T, Okuno T, Watarai A, Matsumoto T (1992) New flavonol glycosides, major determinants inducing the green fluorescence in the guard cells of *Allium cepa* L. Tetrahedron Lett **33:** 1213- 1216

Willmer C (1983) Stomata. Longman, London, pp 113–143

Wink M (1993) The plant vacuole: a multifunctional compartment. J Exp Bot **44:** 231–246

Zeiger E (1983) The biology of stomatal guard cells. Annu Rev Plant Physiol **34:** 441–475

Zeiger E, Bloom AJ, Hepler PK (1978) Ion transport in stomatal guard cells: a chemiosmotic hypothesis. What's New in Plant Physiology **9:** 29–31

Zeiger E, Hepler PK (1976) Production of guard cell protoplasts from onion and tobacco. Plant Physiol **58:** 492–498

Zeiger E, Hepler PK (1979) Blue light induced intrinsic vacuolar fluorescence in onion guard cells. J Cell Sci **37:** 1–10