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Anion Selectivity of Slow Anion Channels in the Plasma Membrane of Guard Cells[1]

Large Nitrate Permeability

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Closing of stomatal pores in the leaf epidermis of higher plants is mediated by long-term release of potassium and the anions chloride and malate from guard cells and by parallel metabolism of malate. Previous studies have shown that slowly activating anion channels in the plasma membrane of guard cells can provide a major pathway for anion efflux while also controlling K^+ efflux during stomatal closing: Anion efflux produces depolarization of the guard cell plasma membrane that drives K^+ efflux required for stomatal closing. The patch-clamp technique was applied to *Vicia faba* guard cells to determine the permeability of physiologically significant anions and halides through slow anion channels to assess the contribution of these anion channels to anion efflux during stomatal closing. Permeability ratio measurements showed that all tested anions were permeable with the selectivity sequence relative to Cl^- of $NO_3^- > Br^- > F^- \sim Cl^- \sim I^- >$ malate. Large malate concentrations in the cytosol (150 mM) produced a slow down-regulation of slow anion channel currents. Single anion channel currents were recorded that correlated with whole-cell anion currents. Single slow anion channels confirmed the large permeability ratio for nitrate over chloride ions. Furthermore, single-channel studies support previous indications of multiple conductance states of slow anion channels, suggesting cooperativity among anion channels. Anion conductances showed that slow anion channels can mediate physiological rates of Cl^- and initial malate efflux required for mediation of stomatal closure. The large NO_3^- permeability as well as the significant permeabilities of all anions tested indicates that slow anion channels do not discriminate strongly among anions. Furthermore, these data suggest that slow anion channels can provide an efficient pathway for efflux of physiologically important anions from guard cells and possibly also from other higher plant cells that express slow anion channels.

Anion channels in the plasma membrane of higher plant cells have been suggested to play central roles in a variety of cellular processes such as ion transport, signal transduction, growth control, and volume regulation. Guard cells represent a well-suited model system to study regulation, ion selectivity, and physiological functions of anion channels. Plants optimize their atmospheric CO_2 and water vapor exchange by adjusting the aperture of stomatal pores in the leaf epidermis. Stomatal regulation depends on environmental conditions such as humidity, CO_2 levels, and light intensity. Guard cells surrounding stomata directly control the stomatal aperture. Stomatal opening is mediated by turgor and volume increases of guard cells, which result from ion uptake and internal conversion of starch to osmotically active malate (Raschke, 1979). Efflux of potassium and anions (mainly chloride and to a small degree malate) from guard cells and malate metabolism produce turgor and volume reduction and the ensuing closure of stomatal pores (Raschke, 1979; MacRobbie, 1981; Outlaw et al., 1981).

Anion channels in the plasma membrane of guard cells have been suggested to be central mediators of stomatal closing (Keller et al., 1989; Schroeder and Hagiwara, 1989). Recent studies have shown the presence of at least two different types or modes of anion channel currents in guard cells (Schroeder and Keller, 1992). Both anion channels are likely to be involved in stomatal closing, while showing significantly different activation times, voltage dependencies, and pharmacological properties (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Marten et al., 1992, 1993; Schroeder and Keller, 1992; Schroeder et al., 1993). Both channels are activated by depolarization but differ in their activation potential by up to 100 mV and in their half-activation times by approximately 3 orders of magnitude under identical recording conditions.

The rapidly activating anion channels (R type) activate within approximately 50 ms but reclose during prolonged stimulation ($t_{1/2} < 1$ min) (Hedrich et al., 1990). This voltage-dependent desensitization of R-type anion channels is referred to as inactivation (Hille, 1992). This lies in strong contrast to slow anion channels that do not inactivate (desensitize) and can, therefore, produce long-term depolarization and sustained anion efflux (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992) observed during stomatal closing (MacRobbie, 1981). Anion channel-mediated sustained depolarizations positive to the K^+ equilibrium potential in turn can drive potassium efflux required for stomatal closure via

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Abbreviation: pS, picosiemens.

outward conducting K^+ channels (Schroeder et al., 1987; Schroeder, 1988; Blatt and Armstrong, 1993).

The phytohormone ABA is synthesized in response to drought and can cause stomatal closing. ABA triggers elevations in the cytosolic Ca^{2+} concentration of guard cells (McAinsh et al., 1990; Schroeder and Hagiwara, 1990). Slow anion channels (Schroeder and Hagiwara, 1989) and rapid anion channel currents (Hedrich et al., 1990) are enhanced by an increase in the cytosolic and extracellular Ca concentration, respectively. The findings that ABA causes sustained depolarizations (Kusamo, 1981; Ishikawa et al., 1983; Thiel et al., 1992) support the suggestion that slow anion channels play a major role in ABA-induced stomatal closing. Recent findings show that simultaneous block of S-type and R-type anion channels completely abolished ABA- and malate-induced stomatal closing, whereas complete block of R-type anion channels alone had little effect (Schroeder et al., 1993). These findings corroborate the hypothesis (Schroeder and Hagiwara, 1989) that slow anion channels represent a central rate-limiting control mechanism during closure of stomata (Schroeder et al., 1993).

Malate flux studies have suggested that small quantities of the organic anion malate may be released from guard cells during stomatal closing (Van Kirk and Raschke, 1978). Furthermore, transport of anions such as nitrate is vital to many processes during plant nutrition and growth (Glass, 1988). In the present study we have characterized the anion selectivity of S-type anion channels to determine the degree to which slow anion channels distinguish among different anions and to further elucidate the role of these channels during stomatal closing. Relative permeabilities, cellular anion conductances, and single-channel properties of S-type anion channels in guard cells were characterized.

MATERIALS AND METHODS

Protoplast Isolation

Guard cell protoplasts were isolated from *Vicia faba* leaves by enzymatic digestion. Two to three leaves were blended three times for 15 s as described elsewhere (Kruse et al., 1989). Purified leaf epidermal layers were subsequently incubated in 1.6% Cellulase Onozuka R-S (Yakult Honsha Co., Tokyo, Japan), 0.016% Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), Gamborg's medium B5, 1 mM $CaCl_2$, 1 mM spermidine (Sigma), and 0.2% BSA (Sigma) in a final volume of 10 mL for approximately 1 h at 15 to 18°C on a linear shaker (2 Hz) (Schroeder, 1988). The osmolality was adjusted to 300 mosmol kg^{-1} with 185 mM D-mannitol, and the pH was adjusted to 5.6 by addition of HCl.

After enzyme treatment, guard cell protoplasts were filtered through a 30-μm nylon net. Protoplasts were washed by adding 40 mL of washing solution (0.1 mM KCl, 0.1 mM $CaCl_2$ adjusted with Tris to pH 5.6 and with D-mannitol to 500 mosmol kg^{-1}) to the incubation medium and were collected by centrifugation at 150*g* for 10 min. The protoplasts (in 1 mL of washing solution) were layered over a gradient of 10 and 20% Ficoll prepared in washing solution. After centrifugation at 150*g* for 10 min, purified protoplasts were collected at the 10 to 20% interface.

Solutions

Pipette solutions, which perfuse the cytosol of guard cells, contained 3.35 mM CaMes (or $CaCl_2$), 7.6 mM EGTA-$(Tris)_2$, 2 mM $MgCl_2$, 4 mM MgATP, 0.2 mM GTP-γ-S, 10 mM Hepes, and 150 mM anions as cesium salt (CsCl, Cs_2-malate, $CsNO_3$, CsI, CsF, CsBr) and were adjusted to pH 7.1 with Tris. Osmolalities were adjusted to 510 mosmol kg^{-1} by adding D-mannitol and verified by a vapor pressure osmometer (Wescor, Logan, UT). Guard cells were extracellularly perfused with solutions containing 30 mM or 150 mM CsCl (see text), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH 5.6, and adjusted to 490 mosmol kg^{-1} by the addition of D-mannitol. Liquid junction potentials that occur at the tip of the patch electrode were corrected as described previously (Schroeder, 1988; Neher, 1992). The liquid junction potential with 150 mM Cs_2-malate in the pipette was −11 mV. Membrane potentials were corrected using the equation $V_m = V_{experiment}$ + liquid junction potential, where V_m is the corrected membrane potential and $V_{experiment}$ is the uncorrected membrane potential recorded during experiments. To determine the permeabilities of halides, a platinum electrode was used to ensure electrode stability.

Data Acquisition and Analysis

Patch-clamp recordings were performed and low-pass filtered with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). On-line data acquisition, voltage protocols, and subsequent data analysis were performed using a TL-125 interface (Axon Instruments) and a 33-MHz 486 AT-compatible personal computer. Quasi-steady-state current-voltage curves were determined by measuring currents at the end of voltage pulses. The temperature was 24°C.

Ionic activities in solution were accounted for using the program Geochem (Parker et al., 1994). Permeability ratios for monovalent anions were calculated using the Goldman equation (Hille, 1992). The permeability ratio for $malate^{2-}$ with respect to Cl^- ($P_{malate2-}/P_{Cl-}$) was derived from the Goldman-Hodgkin-Katz equation for ion fluxes in equilibrium (Fatt and Ginsborg, 1958).

RESULTS

Chloride Selectivity of Slow Anion Channels

Slow anion channels in the plasma membrane of guard cell protoplasts of *V. faba* are activated by long-lasting depolarizations and are closed (deactivated) by hyperpolarization (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). Anion efflux currents through S-type anion channels were analyzed by application of long-term depolarizations, followed by hyperpolarizing voltage pulses to −120 mV using the whole-cell patch-clamp configuration ($n \geq 128$ guard cells; Fig. 1). This hyperpolarization produces a slow decay (deactivation) of slow anion channel currents (Fig. 1). Half-deactivation times of 10 s at −120 mV and incomplete deactivation corresponded to previously demonstrated characteristics of S-type anion currents (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). Significant contributions of rapid anion currents to whole-cell currents were excluded

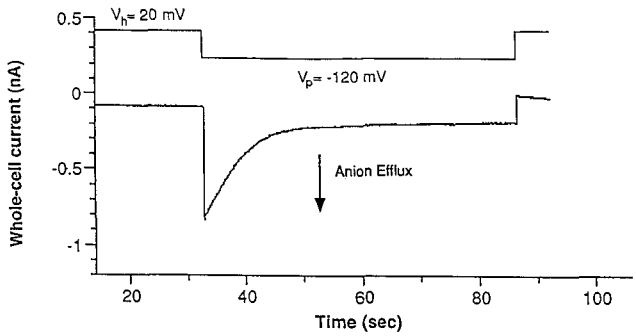

Figure 1. Large deactivating anion efflux currents were recorded by hyperpolarization of the membrane potential from +20 to −120 mV. Slow anion currents were recorded with 150 mM CsCl in the cytosol and 30 mM CsCl in the external medium. Negative currents represent anion efflux in all experiments (arrow). V_h, Holding potential; V_p, pulse potential.

in all experiments by application of rapid depolarizing voltage ramps as described previously (Schroeder and Keller, 1992; Schroeder et al., 1993).

The dependence of steady-state slow anion channel currents on the imposed membrane potential was determined by recording whole-cell currents at membrane potentials in the range from −151 to +89 mV (Fig. 2). The membrane potential at which maximum slow anion efflux currents were observed under the imposed conditions varied in the range from approximately 0 to −80 mV. This variation is in part due to the weak voltage dependence of S-type anion channels. This shift in the peak current potential has been previously reported and suggests that additional parameters contribute to slow anion channel regulation that are not at equilibrium (Schroeder and Keller, 1992; Schroeder et al., 1993). In the present study, reversal potentials of slow anion channels were analyzed that were independent of the peak potential of anion efflux currents. To exclude that other ions than Cl^- contributed to whole-cell currents under the imposed recording conditions, we determined the reversal potential of ion currents in the presence of 36 mM Cl^- in the bath and 154 mM Cl^- in the pipette solution (Fig. 2). The measured reversal potentials of +30 ± 4 mV were close to the Nernst potential for chloride of +32 mV (after correction for ionic activities). The only other ions that had equilibrium potentials more positive than 0 mV were Ca^{2+} and protons. A decrease in the external Ca^{2+} concentration from 40 to 1 and 0.1 mM, while maintaining the Cl^- concentration constant, did not significantly alter the measured reversal potential and the inward and outward currents, indicating no significant contribution of calcium currents to the recorded membrane current in guard cells under the imposed recording conditions (n = 5; data not shown). Furthermore, direct regulation of S-type anion currents by extracellular Ca^{2+} under these conditions was not observed.

Changes in the extracellular pH from 5.5 to 7.2 also did not produce a shift in the reversal potential of whole-cell currents (n = 5; data not shown). When cells were bathed in 154 mM Cl^-, the reversal potential of whole-cell currents shifted to 0 mV (n = 8). These data demonstrated that whole-cell currents were carried by Cl^- ions under the imposed conditions.

Slow anion channels give rise to large instantaneous currents in the membrane potential range from −120 to +100 mV, because of the weak voltage dependence of these anion channels (Fig. 2A; Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). To determine whether other non-anion conductances contributed significantly to whole-cell currents, we applied 30 μM 5-nitro-2,3-phenylpropyllaminobenzoic acid to guard cells, which completely inhibits slow anion channels (≥95% inhibition; Schroeder et al., 1993). In the presence of 5-nitro-2,3-phenylpropyllaminobenzoic acid, membrane conductances of guard cells were low, as reported previously (whole-cell resistances of approximately 5 to >40 GΩ) (Schroeder and Fang, 1991; Schroeder et al., 1993). These data show that zero-current potentials reflect a good measure of the reversal potential of large guard cell anion currents. Single-channel recordings confirmed this conclusion (see below).

Anion Permeability and Conductivity

To determine whether slow anion channels can allow malate efflux, in addition to chloride efflux, during stomatal

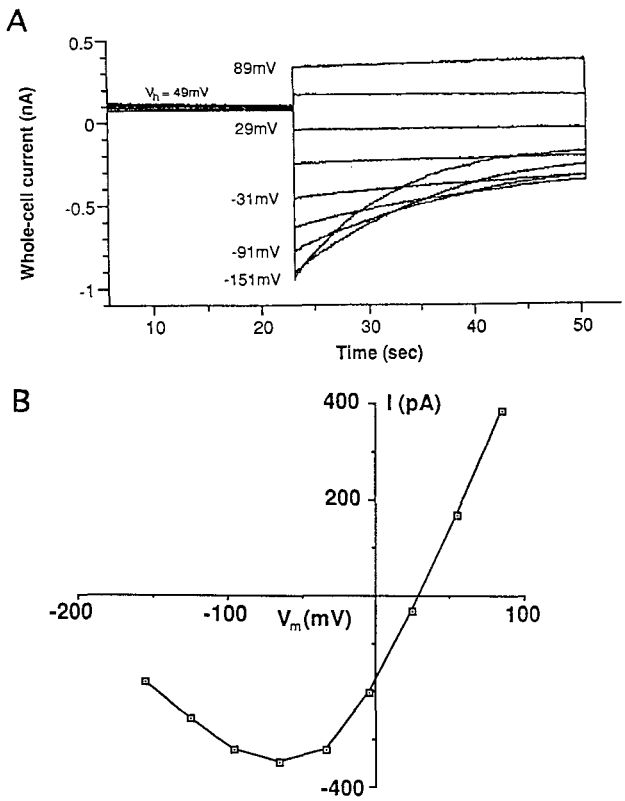

Figure 2. Cl^- selectivity of slow anion channels. A, To obtain quasi-steady-state currents, long voltage pulses were applied to guard cell protoplasts in the membrane potential range from −151 to +89 mV as indicated to the left of recorded current traces. B, Quasi-steady-state currents (I) after deactivation shown in A are plotted as a function of the imposed membrane potential. V_m, Membrane potential.

closing, we analyzed the permeability of malate relative to that of chloride (Fig. 3). In these experiments, guard cells were bathed in 154 mM CsCl. The cytosol was loaded with 150 mM malate, allowing analysis of the ability of slow anion channels to carry malate efflux from guard cells. Recordings of malate efflux under these conditions showed maximum efflux currents in the range of −77 pA at −81 mV (n = 18, Fig. 3A) and reversal potentials at −26 mV (±12 mV SD; n = 18). Malate efflux currents were lower in magnitude than Cl^- efflux currents (cf. Figs. 3A and 4B). The conductance for malate over Cl^- was approximately 0.1 when peak anion

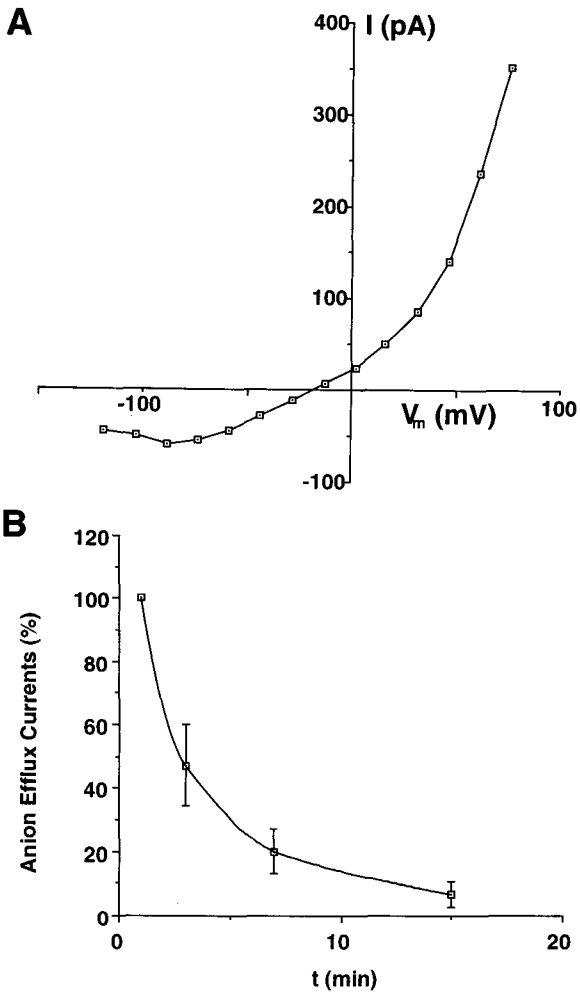

Figure 3. Malate permeability and down-regulation of S-type anion channels by cytosolic malate. A, Long voltage pulses as shown in Figure 2 were applied to protoplasts loaded with 150 mM Cs_2-malate and perfused with bath solutions containing 150 mM CsCl. Steady-state currents after deactivation are plotted as a function of the imposed membrane potentials. The observation of malate efflux currents (negative currents) as well as reversal potentials of −26 mV (n = 18) demonstrated that malate is permeable to slow anion channels (see text). B, Large cytosolic malate concentrations (150 mM) produced a slow down-regulation of slow anion channel currents in guard cells. The magnitude of anion efflux currents at −65 mV is plotted as a function of the duration of cytosolic malate perfusion (error bars are ±SD; n = 4).

efflux current magnitudes were compared, and the permeability ratio for malate over Cl^- was 0.24, as derived using the Goldman-Hodgkin-Katz equation (see "Materials and Methods").

Prolonged exposure of the guard cell cytosol to 150 mM malate, however, led to a slow decline in the magnitude of S-type anion currents in guard cells (Fig. 3B; n = 15). Further investigation is required to determine the mechanism of this negative regulation of slow anion channels by cytosolic malate. During the course of 90% of this decline in slow anion currents (Fig. 3B) the mean reversal potential corrected for the background membrane resistance did not shift significantly from −26 mV. When slow anion currents were reduced by >90% the zero-current potential shifted to 0 mV. These detailed studies further support the conclusion that malate had a small but measurable permeability through slow anion channels. These carefully designed experiments show that S-type anion channels can allow malate efflux from guard cells. However, negative regulation by cytosolic malate can limit anion release.

To elucidate additional anion specificities of slow anion channels, we analyzed permeation of other biophysically or physiologically relevant anions (Table I). In all experiments the anion under investigation was loaded into the cytosol of guard cells at a concentration of 150 mM to determine the ability of S-type anion channels to mediate efflux of these various anions.

When nitrate ions were loaded into the cytosol of guard cells, large anion efflux currents were observed (Fig. 4). The reversal potential of anion currents with 150 mM NO_3^- in the cytosol and 154 mM Cl^- in the bath was +76 mV (±14 mV SD; n = 12). These data indicated a strong selectivity of slow anion channels for nitrate with a relative permeability for nitrate over chloride ($P_{NO_3^-}/P_{Cl^-}$) of 20 ± 11 to 1 (Table I). A high selectivity of anion channels for nitrate has been suggested to indicate a weak binding site for anions in the pore region of anion channels (Wright and Diamond, 1977). Permeability ratio values of multi-ion pores can depend on ion concentrations on both sides of the membrane (Hille, 1992). Comparison of current-voltage relationships of nitrate and Cl^- efflux currents showed that both nitrate and Cl^- can carry large anion efflux currents through slow anion channels (Fig. 4B). Cytosolic anions also influenced the rate of voltage-dependent anion channel closing (deactivation) upon hyperpolarization. For example, with 150 mM nitrate in the cytosol deactivation was on average more rapid than with 150 mM cytosolic Cl^- at a given potential (e.g. cf. Figs. 2A and 4A).

The relative permeabilities of other anions were determined by loading the cytosol of guard cells with 150 mM of the anion under investigation. The resulting permeability sequence indicated a large relative permeability for bromide and only a weak discrimination among iodide, chloride, and fluoride anions. Reversal potential measurements in whole-cell recordings resulted in a selectivity sequence and permeability ratios relative to Cl^- (P_X^-/P_{Cl^-}) of: NO_3^- (20.9 ± 11.2) > Br^- (2.4 ± 1.5) > F^- (1.26 ± 0.4) ~ Cl^- (1 ± 0.04) ~ I^- (0.98 ± 0.16) > malate (0.24 ± 0.19) (Table I).

Single Anion Channel Recordings

The ion selectivity and conductivity of single slow anion channels were determined using the outside-out membrane

patch configuration of the patch-clamp technique (Hamill et al., 1981). Single channels showed reversal potentials at the Cl^- equilibrium potential (n = 10). Rapid linear changes in the membrane potential (ramps) from +120 mV to −100 mV were recorded. With 36 mM Cl^- in the bathing medium and 154 mM Cl^- in the cytosol, reversal potentials of +30 mV were recorded (Fig. 5A). The single-channel conductance was 35 ± 7 pS at very positive potentials (Fig. 5A; n = 10). When the membrane potential was held at various levels for extended durations, steady-state single-channel currents were recorded (Fig. 5B). Single-channel currents included a conductance state of approximately 36 pS and reversed in the range of the anion equilibrium potential (Fig. 5, B and C; n = 8). Changing the external chloride concentration to 154 mM Cl^- resulted in a single-channel conductance of 36 ± 5 pS and current reversal at 0 mV (n = 3). These data confirm the selectivity for Cl^- over cations of single slow anion channels (Linder and Raschke, 1992; Schroeder et al., 1993). Furthermore, blockers of whole-cell slow anion currents also blocked single anion channel currents at the same concentrations (Schroeder et al., 1993).

Slow anion channel currents were recorded at potentials that completely closed rapid anion channels (e.g. Fig. 5B, −100 mV trace). Single-channel currents had long open times (Fig. 5B). However, time intervals were observed during which single-channel currents opened and closed in rapid succession ("flicker"; see +80 mV trace in Fig. 5B). The single-channel conductance and reversal potentials of these flickering channels were indistinguishable from the long channel openings in Cl^- solutions, suggesting putative mode transitions (Hille, 1992) of anion channel proteins. These mode shifts may be related to the simultaneous occurrence of both slow and rapid anion currents revealed in guard cells (Schroeder and Keller, 1992).

Chloride (150 mM) in the cytosolic solution was substituted by 150 mM nitrate to analyze nitrate permeation through single anion channels. One single-channel conductance state of slow anion channels was 32 pS ± 5 pS at the reversal potential (Fig. 6). Under these recording conditions, currents reversed in the range +40 mV to +73 mV (Fig. 6), as determined by extrapolation of current-voltage curves resulting in a relative permeability of nitrate over Cl^- of 4.9–19 to 1. These data correlate with permeability ratios determined for slow anion currents in whole-cell recordings (Fig. 4), showing the strong nitrate permeability of S-type anion currents recorded in whole guard cells.

In addition, during voltage ramps a second type of ion channel was observed that had a single-channel conductance

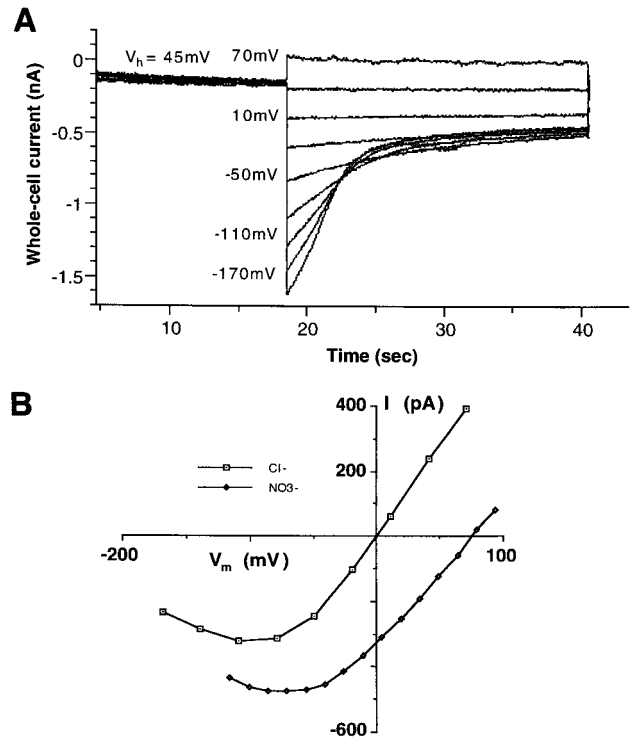

Figure 4. Nitrate permeability of S-type anion channels. A, Whole-cell currents were recorded in guard cell protoplasts with 150 mM $CsNO_3$ in the cytosol of guard cells and 150 mM CsCl in the extracellular medium. Large negative currents represent nitrate efflux. B, The current-voltage relationship with 150 mM NO_3^- in the cytosol (filled symbols) was derived from data shown in A as described in Figure 2. The reversal potential demonstrated large nitrate permeability of slow anion channels in guard cells. For comparison, a typical current-voltage curve is shown for a guard cell containing 150 mM CsCl in the cytosol and 150 mM CsCl in the bath (open symbols).

of approximately 40 pS, reversed at +27 ± 8 mV (n = 7), and showed rapid open-closed transitions. Whether these channels represent rapid anion channel states or another class of ion channel was not further analyzed.

In addition, as reported previously (Schroeder and Keller, 1992; Schroeder et al., 1993), slow anion-selective channel currents with other well-defined single-channel conductances were generally observed (Fig. 7; n > 30 membrane patches). Direct transitions among different channel conductance levels were frequently observed, indicating that

Table I. *Permeability ratios and peak current magnitudes for malate, nitrate, and halides over chloride*

Current reversal potentials for the anions shown were recorded under bi-ionic conditions (see "Materials and Methods") in the whole-cell patch-clamp configuration. Averaged values of reversal potentials were used to calculate the permeability of these anions relative to chloride. Recordings under conditions with 150 mM CsCl in the cytosol and 150 mM CsCl in the extracellular medium served as control showing current reversal at approximately 0 mV.

	Cl^- (n = 3)	$Malate^{2-}$ (n = 18)	NO_3^- (n = 12)	I^- (n = 4)	Br^- (n = 9)	F^- (n = 4)
P_X/P_{Cl}	1 ± 0.04	0.24 ± 0.19	20.9 ± 11.2	0.98 ± 0.16	2.4 ± 1.5	1.26 ± 0.4
I_{Peak} (pA)	−231 ± 183	−77 ± 58	−747 ± 378	−146 ± 112	−791 ± 340	−771 ± 361

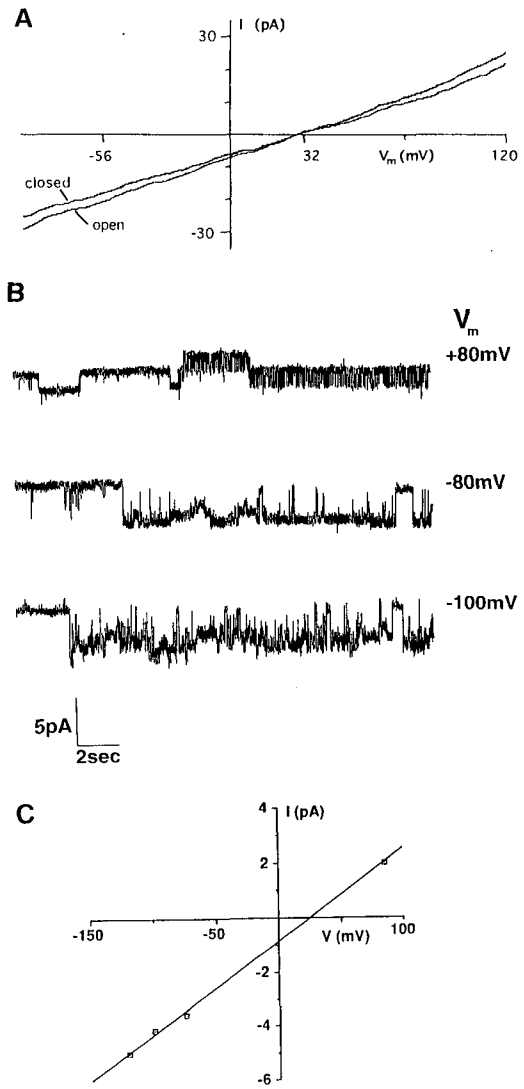

Figure 5. Single-channel recordings of slow anion channels. A, The superposition of single-channel currents is shown, recorded during two voltage ramps ranging from −100 mV to +120 mV. The intersection of single-channel currents for open and closed states at a membrane potential of approximately +30 mV indicates Cl^- selectivity. The duration of voltage ramps was 100 ms to reduce the occurrence of open-closed transitions of anion channels during voltage ramps (Schroeder et al., 1993). Closed and open single-channel current states are indicated. The cytosolic solution included 154 mM Cl^-, and the extracellular medium included 36 mM Cl^-. B, Single slow anion channel currents recorded in outside-out membrane patches. Membrane potentials are indicated to the right of recorded channel currents. C, Current-voltage relationship of a slow anion channel-conducting state B. V_m and V are membrane potential.

cooperativity among anion channel proteins occurs as shown for nitrate (Figs. 6, −80 mV trace, and 7A; $n > 16$ membrane patches). With Cl^- ions in the cytosol, slow anion-selective channel conductances of 18 ± 4 pS ($n = 6$), 36 ± 4 pS, and 52 ± 4 pS ($n = 3$) as well as lower conductances (<10 pS) and larger conductances were recorded (Fig. 7B). These anion channels showed long open times (0.1–40 s), a weak voltage dependence, Cl^- selectivity, and sensitivity to blockers (Schroeder et al., 1993), which are hallmark characteristics of slow anion channels. The occurrence of several well-defined single-channel conductance states and the direct transitions among different conducting states (e.g. Figs. 6A and 7A) suggests that simultaneous and cooperative openings of multiple small unit single channels give rise to the identified 18-, 36-, and 52-pS channel conductance states. These properties of slow anion channels confirm previous observations of multiple conductance states (Schroeder and Keller, 1992; Schroeder et al., 1993), while differing from slow anion channels in another study (Linder and Raschke, 1992) and

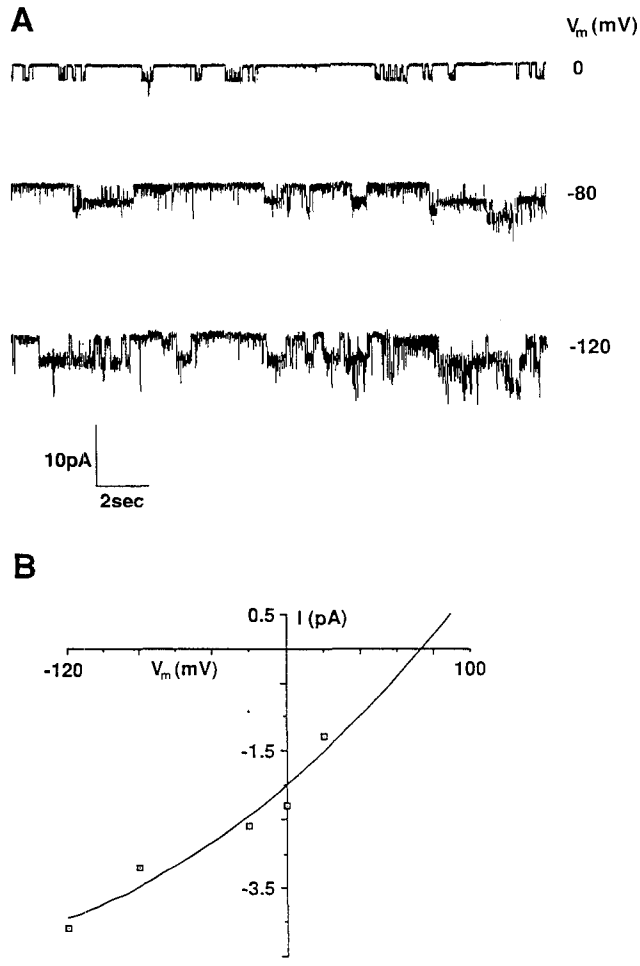

Figure 6. Nitrate permeation through single slow anion channels. A, Single-channel currents recorded with 150 mM $CsNO_3$ on the cytosolic membrane side and 150 mM CsCl in the extracellular solution are shown. Single-channel currents were recorded in the membrane potential range from 0 to −120 mV as indicated to the right of the recorded traces. Downward deflections represent nitrate efflux. B, The amplitude of single-channel currents (I) is plotted as a function of the imposed membrane potential. Regression analysis resulted in a single-channel conductance of 14 pS at very negative voltages and 32 pS in the range of the reversal potential, which was determined by extrapolation and comparison to outward channel currents in other patches.

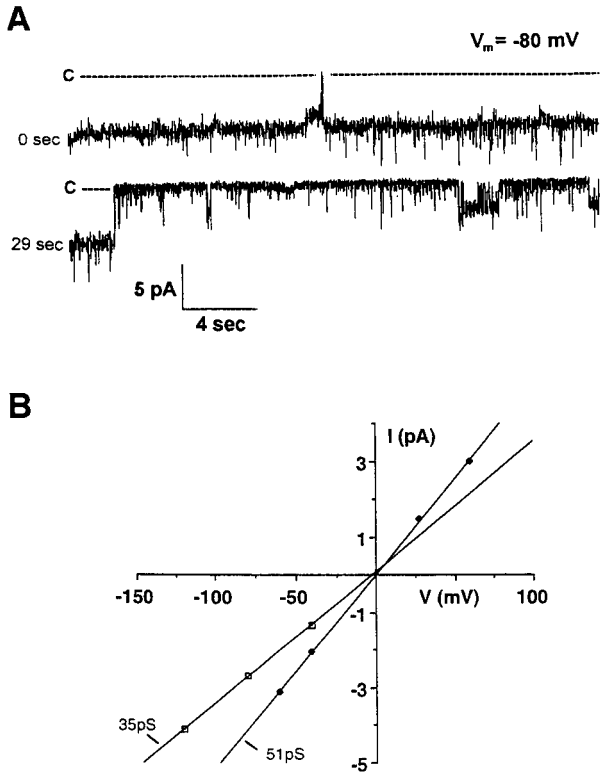

Figure 7. Main conductance states and cooperativity of slow anion channels. A, Single anion channel currents recorded with 150 mM $CsNO_3$ in the cytosolic solution and 150 mM CsCl in the external solution at −80 mV show subconductance states that indicate cooperativity. A continuous recording with a total duration of 58 s is shown. B, Current-voltage curves of two of the multiple anion channel conductance states are shown. In symmetrical 150 mM CsCl these could be described by conductances of 35 and 51 pS.

from rapid anion channels (Hedrich et al., 1990), for which a single conductance state has been reported.

DISCUSSION

Data have supported the hypothesis that slow anion channels provide a molecular mechanism for long-term anion efflux and depolarization, thereby functioning as a driver of K^+ efflux during stomatal closing (Schroeder and Hagiwara, 1989; Linder and Raschke, 1992; Schroeder and Keller, 1992; Schroeder et al., 1993). Simultaneous block of slow and rapid anion channels completely abolished ABA- and malate-induced stomatal closing, whereas rapid anion channel block alone did not significantly influence stomatal closing (Schroeder et al., 1993). These data underline the central role of slow anion channels for stomatal closing, while not excluding a contribution by the rapid mode of anion channels. (It is possible that, in addition to the slow and rapid modes of anion currents, other types of anion channels in guard cells exist). In the present study we have determined the permeability of slow anion channels to anions such as nitrate and malate as well as to the halide anions F^-, Br^-, and I^-. Reversal potential measurements and anion efflux currents showed that slow anion channels in guard cells were permeable to all anions tested.

Cl^- and Malate Efflux during Stomatal Closing

Both Cl^- and malate concentrations are greatly increased in guard cells during stomatal opening (Allaway, 1973; Schnabl and Raschke, 1980). Whether slow anion channels can account for physiological anion efflux rates required to drive stomatal closure can be estimated from anion currents recorded in single guard cells. During stomatal closing average ion efflux rates from guard cells occur that correspond to approximately 30 pmol s^{-1} and per cm^2 of guard cell plasma membrane (Outlaw, 1983). Average peak steady-state Cl^- efflux currents of −231 pA were recorded that correspond to a Cl^- efflux rate of 339 pmol s^{-1} and per cm^2 of guard cell plasma membrane. Average peak malate efflux currents of −77 pA correspond to an initial malate efflux rate of 56.4 pmol cm^{-2} s^{-1} from guard cells. These estimates indicate that slow anion channels can carry physiological rates of both Cl^- and initial malate efflux required for stomatal closing.

With 150 mM malate in the cytosol slow anion channel currents were down-regulated over time (Fig. 3B). If this down-regulation occurs in vivo during stomatal closing, the ability of slow anion channels to release anions would be limited. The finding that slow anion channels were required to produce ABA- and malate-induced stomatal closing (Schroeder et al., 1993) shows that these channels are activated during stomatal closing. Furthermore, high malate concentrations in the cytosol (more than several millimolar) are unlikely during stomatal closing because of the large activity of malic enzyme in guard cells (Outlaw et al., 1981). Conversely, it is possible that down-regulation of anion channels is important during stomatal opening during which time large malate concentrations may accumulate in the cytosol (Michalke and Schnabl, 1987). Because slow anion channels can reduce the degree of stomatal opening (Schroeder et al., 1993), down-regulation of anion channels by malate during stomatal opening could be physiologically important.

Malate flux studies suggest that malate can be released during ABA-induced stomatal closing (Van Kirk and Raschke, 1978). The question remains: Why should guard cells release malate during stomatal closing rather than reconvert all malate to starch for turgor reduction? The requirement of K^+ efflux for stomatal closing via depolarization-activated K^+ channels necessitates a mechanism that depolarizes guard cells (Schroeder et al., 1987). Because of the electrochemical gradient of all ions across the plasma membrane of guard cells, calcium and anions are the main ions able to passively permeate the membrane to create a significant depolarization positive to the equilibrium potential for K^+. However, long-term depolarization produced by sustained and large Ca^{2+} influx currents would result in elevation of the cytosolic Ca^{2+} concentration to levels lethal to cells (Hepler and Wayne, 1985).

A maximum total cellular malate content as large as 270 fmol per guard cell protoplast has been reported (Michalke and Schnabl, 1987). This corresponds to a large mean cellular malate concentration of approximately 135 mM averaged over various intracellular compartments of guard cells, assuming

a guard cell protoplast volume of approximately 2 pL (Schroeder et al., 1987; Raschke et al., 1988). In the absence of extracellular Cl^-, malate is the major anion accumulating in guard cells during stomatal opening (Allaway, 1973; Raschke and Humble, 1973; Schnabl, 1980). Because of the low Cl^- concentrations in guard cells under these conditions (Schnabl, 1980), chloride efflux alone may limit the degree of depolarization that drives physiological rates of K^+ efflux required for stomatal closing. Malate would appear to be a suitable anion able to contribute to anion efflux required for depolarization of guard cells, particularly when Cl^- is not supplied to guard cells during stomatal opening (Schnabl, 1980; Schnabl and Raschke, 1980). In addition, elevation in the cell wall malate content exerts further activation of rapid anion channels in guard cells (Hedrich and Marten, 1993), and extracellular malate enhances stomatal closing (Hedrich and Marten, 1993; Schroeder et al., 1993). Elevation in extracellular malate has been suggested to function as the CO_2 sensor for guard cells (Hedrich and Marten, 1993), although a positive feedback of effluxing malate on anion channel stimulation appears to be a likely physiological interpretation, particularly because in *V. faba* large concentrations of >25 to 40 mM extracellular malate were required to produce significant stomatal closure without ABA (Schroeder et al., 1993).

To our knowledge, quantitative data for directly comparing the rates of K^+ efflux and malate efflux during stomatal closing are currently limited. In addition, the concentrations of malate in guard cell walls also require further analysis in assessing the contribution of malate efflux to stomatal closing. Note that data showing malate release by guard cells do not exclude reconversion of malate to starch in guard cells for turgor reduction during stomatal closing. High levels of malic enzyme in guard cells suggest that malate is metabolized rapidly during stomatal closing (Outlaw et al., 1981). Considering the central importance of malate and K^+ content for stomatal regulation, it is likely that both malate metabolism and efflux contribute to rapid stomatal closing.

Slow anion channels may contribute to malate efflux and organic anion release in other higher plant cells. For example, putative single S-type anion channels in root cells have been reported (Wegner and Raschke, 1994). Recent data show that malate efflux from root cells contributes to chelation of phytotoxic metals in cell walls (Delhaize et al., 1993). Further investigations will be required to determine whether anion channels in roots show a limited but detectable malate permeability as in guard cells.

Nitrate Selectivity of Slow Anion Channels

Permeability ratios for various anions demonstrated that all tested anions were permeable (Table I). The similarities in anionic permeabilities of the halide anions F^-, Br^-, Cl^-, and I^-, as well as the large permeability of nitrate, suggest a weak binding site for anions in the pore region of these channels (Wright and Diamond, 1977). Nitrate exhibited a strikingly large permeability with respect to Cl^- ions (P_{NO3^-}/P_{Cl^-} approximately 10–20:1). This relative permeability of NO_3^- through slow anion channels differs from the reported relative NO_3^- permeability of rapid anion channels in guard cells (P_{NO3^-}/P_{Cl^-} approximately 4.2; Hedrich and Marten, 1993), indicating a further difference between the two physiologically distinguished modes of guard cell anion channels (Schroeder and Keller, 1992; Schroeder et al., 1993). Interestingly, despite differences in absolute values of relative permeability ratios among different higher plant anion currents, permeation of nitrate appears to be a common characteristic of plant anion channels (Tyerman, 1992; Hedrich and Marten, 1993; Skerett and Tyerman, 1994). Such similarities may be explained by shared structural motifs in the pore region of these plant anion channels.

Nitrate is essential for many vital processes in plant cells (Glass, 1988). Despite the large nitrate permeability, passive nitrate uptake from the cell wall space into guard cells is highly improbable via slow anion channels. At a membrane potential of −120 mV, 20 mM Cl^- in the cytosol, the measured permeability ratio for NO_3^- over Cl^-, and measured cytosolic nitrate in the range of 3 to 5 mM NO_3^- (Zhen et al., 1991; King et al., 1992), passive nitrate uptake would require from 425 to 640 mM NO_3^- in the external medium. Even when nitrate starvation and micromolar nitrate concentrations in the cytosol are assumed, 106 mM external NO_3^- would be required for passive nitrate uptake by S-type anion channels. These estimates show that under common physiological conditions slow anion channels can carry mainly nitrate efflux but cannot contribute to long-term nitrate uptake.

After uptake into higher plant roots, nitrate is transported to other plant tissues. Slow anion channels may contribute to nitrate transport by permitting regulated NO_3^- efflux from specialized plant cells. For example, recent studies have indicated the presence of a low density of putative S-type anion channels in xylem parenchyma cells (Wegner and Raschke, 1992). Xylem loading with nitrate requires nitrate efflux from xylem parenchyma cells. Further research will be required to determine whether slow anion channels in xylem parenchyma cells are also highly permeable to nitrate.

CONCLUSIONS

Slow anion channels were permeable to all anions tested. The specificity of slow anion channels in guard cells for various anions provides a functional basis for allowing physiological anion efflux required for stomatal closure. These data further fortify the hypothesis that slow anion channels play a central role in the mediation and control of stomatal closing. The mechanism of negative regulation of slow anion channels by malate requires further investigation and may play a role during stomatal regulation. A high selectivity for nitrate suggests additional physiological functions of S-type anion channels as a nitrate efflux pathway in higher plant cells. Further investigations of slow anion channels should illuminate the involvement of these anion channels during nutrient transport and signal transduction processes in higher plants.

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