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Identification of High-Affinity Slow Anion Channel Blockers and Evidence for Stomatal Regulation by Slow Anion Channels in Guard Cells

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Slow anion channels in the plasma membrane of guard cells have been suggested to constitute an important control mechanism for long-term ion efflux, which produces stomatal closing. Identification of pharmacological blockers of these slow anion channels is instrumental for understanding plant anion channel function and structure. Patch clamp studies were performed on guard cell protoplasts to identify specific extracellular inhibitors of slow anion channels. Extracellular application of the anion channel blockers NPPB and IAA-94 produced a strong inhibition of slow anion channels in the physiological voltage range with half inhibition constants ($K_{1/2}$) of 7 and 10 μM, respectively. Single slow anion channels that had a high open probability at depolarized potentials were identified. Anion channels had a main conductance state of 33 ± 8 pS and were inhibited by IAA-94. DIDS, which has been shown to be a potent blocker of rapid anion channels in guard cells ($K_{1/2}$ = 0.2 μM), blocked less than 20% of peak slow anion currents at extracellular or cytosolic concentrations of 100 μM. The pharmacological properties of slow anion channels described here differ from those recently described for rapid anion channels in guard cells, fortifying the finding that two highly distinct types or modes of voltage- and second messenger-dependent anion channel currents coexist in the guard cell plasma membrane. Bioassays using anion channel blockers provide evidence that slow anion channel currents play a substantial role in the regulation of stomatal closing. Interestingly, slow anion channels may also function as a negative regulator during stomatal opening under the experimental conditions applied here. The identification of specific blockers of slow anion channels reported here permits detailed studies of cell biological functions, modulation, and structural components of slow anion channels in guard cells and other higher plant cells.

INTRODUCTION

In higher plants, anion channels have been suggested to play key roles in controlling cellular functions, including osmoregulation, stomatal movements, anion transport, and signal transduction (Mullins, 1962; Tazawa et al., 1987; Schroeder and Hedrich, 1989; Tyermann, 1992). Many physiological stimuli in higher plants, including phytohormones, elicitors, light signals, and pathogens, induce depolarization of the plasma membrane potential as an early event in signal transduction (Racusen and Satter, 1975; Davies and Schuster, 1981; Kasamo, 1981; Bates and Goldsmith, 1983; Ishikawa et al., 1983; Davies, 1987; Spalding and Cosgrove, 1988; Scheel et al., 1989; Marten et al., 1991; Mathieu et al., 1991; Ullrich and Novacky, 1991; Ehrhardt et al., 1992; Wildon et al., 1992; Kuchitsu et al., 1993). Depolarization can be achieved by anion or Ca^{2+}-permeable channel activation, K^+ channel modulation, or H^+ pump inhibition (Tazawa et al., 1987; Schroeder and Hedrich, 1989).

Signal-induced depolarizations in plant cells have been shown to be accompanied by anion efflux in several cases (Gaffey and Mullins, 1958; Raschke, 1979; MacRobbie, 1981; Satter and Galston, 1981; Davies, 1987; Scheel et al., 1989; Marten et al., 1991; Ullrich and Novacky, 1991). Activation of anion channels leads to anion efflux due to the physiological gradient of anions across the plasma membrane of higher plant cells. Anion efflux in turn can produce membrane depolarizations positive to the K^+ equilibrium potential (Mullins, 1962; Findlay and Hope, 1976; Tazawa, 1987; Keller et al., 1989; Schroeder and Hagiwara, 1989). These depolarizations may provide a mechanism for signal propagation (Davies, 1987; Wildon et al., 1992) and controlled activation of voltage-dependent enzymes, such as depolarization-activated Ca^{2+} channels (P. Thuleau, J.M. Ward, R. Ranjeva, and J.I. Schroeder, manuscript submitted), which in turn can modulate cellular metabolism and transcriptional regulation during signal transduction in higher plants (Hepler and Wayne, 1985; Leonard and Hepler, 1990).

Furthermore, depolarizations by anion channels can control osmoregulation and ion transport during cellular responses in higher plants, as has been suggested for stomatal movements (Keller et al., 1989; Schroeder and Hagiwara, 1989). Stomatal movements control the diffusion of CO_2 into leaves

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for carbon fixation and the transpiration of H_2O to the atmosphere. In response to darkness, elevated CO_2 levels, or abscisic acid (ABA) during drought, stomata close to minimize water loss of plants. Stomatal closing is induced by sustained anion and K^+ efflux from guard cells, which surround stomatal pores (Raschke, 1979; MacRobbie, 1981).

From previous investigations it has been proposed that anion channels in the plasma membrane of guard cells provide a prominent control mechanism for stomatal closing (Keller et al., 1989; Schroeder and Hagiwara, 1989). The proposed model (Schroeder and Hagiwara, 1989) suggests that anion channel activation and the ensuing anion efflux cause membrane depolarization, thereby driving K^+ efflux through outward-rectifying K^+ channels (Schroeder et al., 1987). Simultaneous opening of anion and K^+ channels results in sustained salt efflux from guard cells and the ensuing turgor and volume reduction, which produces stomatal closing (Raschke, 1979; MacRobbie, 1981, 1989). In the case of K^+ channels in guard cells, the lack of extracellular, low molecular weight, and potent (≥95% inhibition) blockers has rendered detailed bioassay studies difficult for determination of ion channel function during stomatal movements.

Recent studies have suggested that at least two distinct types or modes of second messenger- and voltage-dependent anion channel currents coexist in the plasma membrane of guard cells (Schroeder and Keller, 1992). Both types of anion channel currents are enhanced by increases in the cytosolic Ca^{2+} concentration (Schroeder and Hagiwara, 1989; Hedrich et al., 1990), and both are activated by depolarization (Keller et al., 1989; Schroeder and Hagiwara, 1989). Significant differences have been found in the voltage- and time-dependent regulation of these two anion channel current types. The slow and sustained-type (S-type) anion channel current (Schroeder and Hagiwara, 1989) shows extremely slow depolarization-induced activation, with activation times of 1 to 2 min and activation potentials exceedingly more negative than those of rapid anion currents in guard cells (Linder and Raschke, 1992; Schroeder and Keller, 1992). Slow anion channels do not inactivate (Schroeder and Keller, 1992) and can exhibit sustained activation for 60 min or longer (J.I. Schroeder, unpublished data). The slow and sustained nature of these S-type anion currents has led us to suggest that these anion channels provide a major pathway for long-term anion efflux while driving sustained K^+ efflux, which leads to stomatal closing (Schroeder and Hagiwara, 1989).

The other anion channel current is activated rapidly, within 50 msec, by depolarizations positive to −80 mV (Hedrich et al., 1990) and, therefore, should also contribute to anion efflux during stomatal closing (Keller et al., 1989). This rapid-type (R-type) anion channel, however, recloses (inactivates) during prolonged stimulation ($t_{1/2}$ is ~10 to 12 sec; Hedrich et al., 1990), and hence may provide a reduced contribution to long-term anion efflux. The existence of two types of second messenger- and depolarization-activated anion channel currents in guard cells has raised fundamental questions such as:

(1) Which anion currents contribute to stomatal movements and which are essential for stomatal movements?
(2) Do different physiological signals activate either slow or rapid or both anion currents during stomatal regulation?
(3) Do S-type and R-type anion channels contribute to cellular functions in other higher plant cells, which show signal-induced sustained or transient depolarizations accompanied by anion efflux?
(4) Do the two anion channel currents have distinct structural components?

In this study, effects of different classes of anion channel blockers on slow anion channels in guard cells were analyzed. The description of high-affinity blockers of S-type anion channels in this report provides a direct approach for gaining insight into the cellular functions of anion channels.

RESULTS

To analyze the effects of blockers on slow anion channels in guard cells, the patch clamp technique was applied to guard cell protoplasts isolated from broad bean leaves. The use of cesium as the major cation in the bathing and cytosolic solutions abolished K^+ channel currents. Cs^+ is known to be largely impermeant to guard cell K^+ channels (Schroeder, 1988). Elevation in the cytosolic Ca^{2+} concentration enhances slow anion channel currents in guard cells (Schroeder and Hagiwara, 1989). Large, transient S-type anion efflux currents can be recorded by 3-min depolarized holding potentials, which fully activate slow anion channels, followed by hyperpolarization that produces slow closing (deactivation) of S-type anion channels, as shown in Figure 1A (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). At the same time, this protocol abolished rapid anion currents due to a combination of R-type channel inactivation during prolonged depolarization and reduction of activity during whole-cell recordings under the imposed conditions (see Hedrich et al., 1990; Schroeder and Keller, 1992). Abolition of rapid anion currents was verified in all whole-cell experiments by applying rapid linear changes in the membrane potential (voltage ramps) to guard cells as described previously (see Keller et al., 1989; Schroeder and Keller, 1992). This protocol allowed an accurate pharmacological description of slow anion channels in the present study.

Voltage clamp recordings in the range from +40 to −160 mV showed that Cl^- fluxes across the guard cell plasma membrane were the predominant electrogenic currents under the imposed recording conditions. Potentials negative to the equilibrium (Nernst) potential for Cl^- of +32 mV produced anion efflux currents from guard cells (Figure 1A). Negative currents (downward deflections of traces) correspond to anion efflux from guard cells in all figures (see Figure 1A). Potentials positive to ~+32 mV produced anion influx currents, showing that currents were carried by Cl^- (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992).

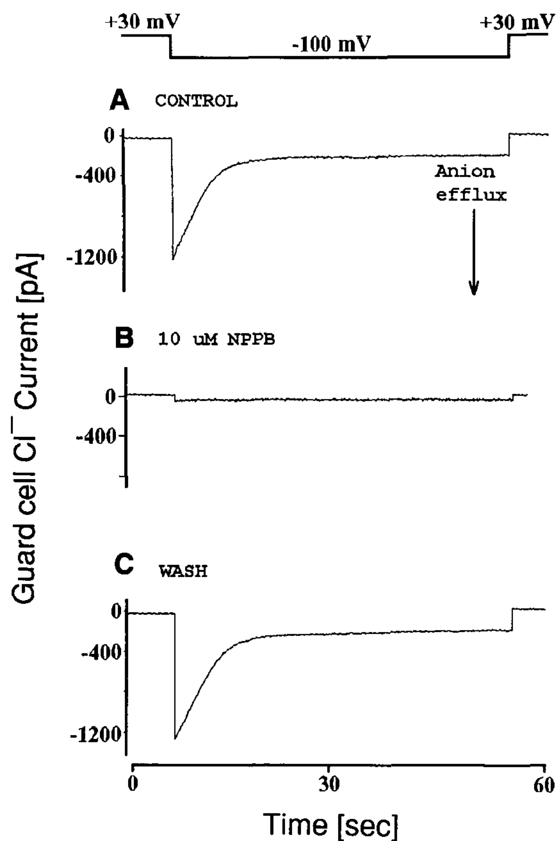

Figure 1. Reversible Block of Slow Anion Channels in Guard Cells by Extracellular Exposure to 10 μM NPPB.

(A) Large deactivating anion efflux currents were recorded by hyperpolarization of the membrane potential from +30 to −100 mV. The top insert shows the voltage-pulse protocol.
(B) Extracellular perfusion of the guard cell with 10 μM NPPB led to a dramatic reduction in slow anion channel currents.
(C) Removal of extracellular NPPB by washing the external medium with 6 mL (30 bath volumes) of the control bathing medium led to a recovery of slow anion channel currents. Negative currents correspond to anion efflux from guard cells in all figures as indicated in **(A)**.

Potent Blockers of Slow Anion Channels

Large deactivating anion efflux currents were produced by stepping the membrane potential from +30 to −100 mV (Figure 1A). When 10 μM of the Cl^- channel blocker 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB) was perfused into the bathing solution, a dramatic inhibition of anion efflux currents was observed (Figure 1B; n = 11). The zero-current potential shifted from +30 mV to negative membrane potentials after perfusion with 10 μM NPPB, displaying blockage of anion channels. Removal of NPPB by extracellular perfusion of guard cells resulted in full recovery of slow anion channel currents (Figure 1C). Furthermore, when 30 μM NPPB was loaded into the cytosol of guard cells, slow anion channels were not blocked and the dependence of steady state anion currents on the membrane potential was not affected (n = 3). These data showed that inhibition of slow anion currents by NPPB occurred from the extracellular membrane side.

The effect of NPPB on slow anion channels was determined at various physiological potentials by applying slow voltage ramps in the membrane potential range from +40 to −160 mV. The steady state current–voltage relationship was recorded in the absence of NPPB, as illustrated in Figure 2. Steady state anion efflux currents showed a voltage dependence with peak current potentials in the range of ∼−70 to −10 mV. The negative slope of the current–voltage relationship at −160 mV (Figure 2) and at −200 mV (Linder and Raschke, 1992; J.I. Schroeder, unpublished data) showed that slow anion channels retained a significant open probability at strongly hyperpolarized potentials under the imposed recording conditions.

Extracellular perfusion with 20 μM NPPB abolished anion currents resulting in a linear low-conductance current–voltage relationship that had a voltage-independent slope (Figure 2). In the presence of greater than 20 μM NPPB, the whole-cell resistance was in the range of the background membrane resistance of guard cells (10 to ⩾20 GΩ) (Schroeder et al., 1987; Schroeder and Fang, 1991). These data show that slow anion channels were blocked to a similar extent at all membrane potentials analyzed at near saturating NPPB concentrations (Figure 2). The block of slow anion channels shown here differs from the block of rapid anion channels in guard cells by 100 μM NPPB, which resulted in a hyperpolarizing shift of peak

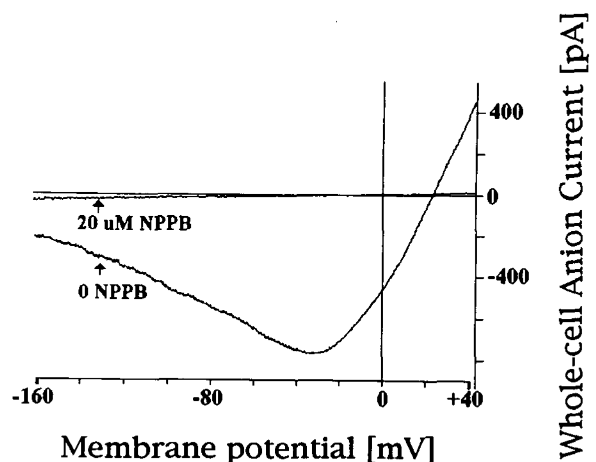

Figure 2. Block of Slow Anion Channels by 20 μM NPPB in the Membrane Potential Range from +40 mV to −160 mV.

The steady state dependence of slow anion channels on the membrane potential was recorded during a slow voltage ramp from +40 to −160 mV (0 NPPB trace). Extracellular perfusion of guard cells with 20 μM NPPB resulted in inhibition of slow anion currents in the entire voltage range analyzed (20 μM NPPB trace).

current potentials by 108 mV (Marten et al., 1992). The dependence of slow anion channel current inhibition on extracellular NPPB concentrations in the range from 0.5 to 50 μM was determined in 14 guard cells, as depicted in Figure 3. NPPB blocked slow anion currents at a half-inhibition concentration ($K_{1/2}$) of 7 μM (Figure 3), showing that NPPB is an effective blocker of S-type anion currents in guard cells.

A family of indanyl-oxyacetic acid derivatives was synthesized by Landry et al. (1989) for use as Cl^- channel blockers (inhibition constant [K_i] ~1 μM) and for labeling, purification, and isolation of mammalian epithelial Cl^- channels. We tested the effects of the indanyl-oxyacetic acid, [6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-indan-5-yl oxy] acetic acid (IAA-94), on slow anion channels in guard cells. Extracellular perfusion of guard cells with 30 μM IAA-94 resulted in inhibition of slow anion currents, as shown in Figure 4. The block of slow anion channels by extracellular IAA-94 was reversible. Perfusion of 30 μM IAA-94 into the cytosol of guard cells did not block slow anion channels (n = 3; data not shown). These data demonstrate that IAA-94 block occurred from the extracellular membrane side. A large portion of the IAA-94–inhibited currents exhibited no time dependence (compare Figures 4A and 4B), supporting previous findings showing large instantaneous anion currents during similar voltage pulses (Schroeder and Hagiwara, 1989; Schroeder and Hagiwara, 1990a; Schroeder and Keller, 1992). Note that S-type channel deactivation is slower in Figure 4 than in Figure 1. Variability in current deactivation within the displayed times (Figures 1 and 4) was observed, suggesting that additional unknown parameters are involved in S-type anion channel regulation.

The physiological effectiveness of the block of slow anion channels by IAA-94 was determined in the potential range from −150 to +50 mV. Figure 5 shows that 30 μM IAA-94 caused an overall reduction in S-type currents in the voltage range analyzed. Peak current potentials of slow anion currents varied from cell to cell (see above). The peak current potentials of S-type anion currents with 5 or 10 μM IAA-94 were within the similar range recorded in the absence of IAA-94 in the same cells. The mechanism of IAA-94 block of S-type anion channels revealed here differs from that of R-type anion channel block, which shows a −58 mV shift in the voltage dependence of R-type channels by 100 μM IAA-94 (Marten et al., 1992). The concentration dependence of IAA-94 inhibition of slow anion currents was analyzed resulting in half-inhibition ($K_{1/2}$) at a concentration of 10 μM IAA-94 (n = 8), as illustrated in Figure 6. IAA-94 at 100 μM produced a physiologically effective inhibition of slow anion channel currents.

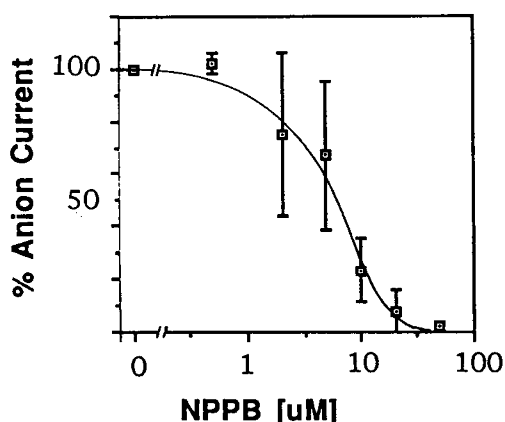

Figure 3. Concentration Dependence of Slow Anion Channel Block by Extracellular NPPB.

Peak anion channel currents were recorded at −80 mV after extracellular perfusion with NPPB and are plotted relative to the control anion current before extracellular perfusion with NPPB (n = 14; error bars = SD).

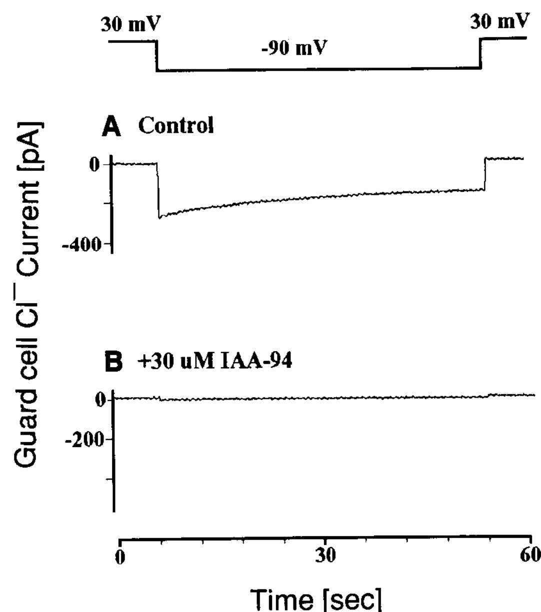

Figure 4. Block of Deactivating Slow Anion Channels by Extracellular Perfusion with 30 μM IAA-94.

(A) Slow anion currents before IAA-94 application.

(B) Slow anion currents after extracellular application of 30 μM IAA-94. The experimental protocol was as described in Figure 1.

Differential Block by DIDS of Slow and Rapid Anion Channels

The most potent blocker of the rapid anion currents in guard cells described to date is 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Extracellular DIDS blocks R-type anion channels with a K_i of 0.2 μM without shifting the voltage dependence (Marten et al., 1993). We tested extracellular DIDS concentrations in the range from 1 to 100 μM. Figure 7 shows that, at 100 μM, DIDS did not block S-type anion currents significantly (n = 9). However, DIDS did increase the rate of deactivation of S-type channels during hyperpolarizations

(compare Figures 7A and 7B). This effect was reversible upon removal of DIDS, suggesting an extracellular site of action.

To determine whether DIDS may block S-type currents from the cytoplasmic membrane side, 100 μM DIDS was loaded into the cytosol of guard cells by intracellular dialysis with patch clamp electrodes. Equilibrium of the pipette solution with the cytoplasm of guard cells did not abolish slow anion currents (*n* = 3; Figure 7C), showing that DIDS produces no potent block of S-type anion channels from either the cytoplasmic or the extracellular membrane side. Another Cl^- channel blocker, $ZnCl_2$, which does not block R-type anion channels (Marten et al., 1993), also did not produce a physiologically significant block of slow anion channels at concentrations of 100 μM and 1 mM (data not shown; *n* = 7).

Identification and Block of Single Anion Channels

Block of slow anion currents by IAA-94 was used to identify and study single S-type anion channels in outside-out membrane patches from guard cells. Under the imposed recording conditions single-channel currents were observed that showed long open times, as illustrated in Figure 8. The reversal potential of single-channel currents was determined during rapid (50 msec) linear changes in membrane potential and lay in the range of +35 mV (*n* = 9) showing Cl^- selectivity (Figure 8A). The single-channel conductance was 33 ± 8 pS (*n* = 10).

Continuous recordings of slow anion channels showed activation by depolarization and extremely long open times lasting up to several seconds (Figures 8A inset and 8B). The addition of 30 μM IAA-94 to the extracellular membrane side blocked

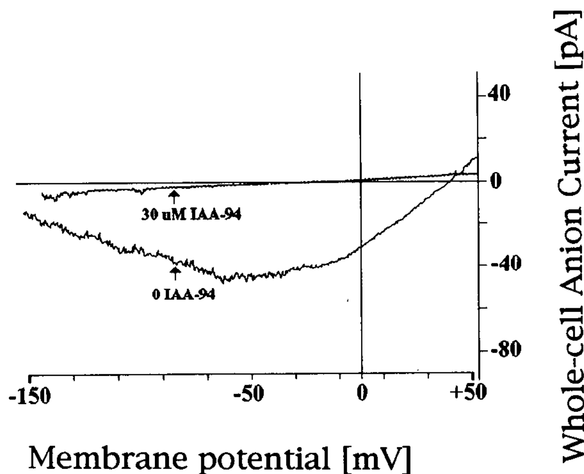

Figure 5. Block of Slow Anion Channels by 30 μM IAA-94 in the Potential Range from +50 to −150 mV.

The steady state voltage dependence of slow anion channel currents was recorded as described in Figure 2 (0 IAA-94 trace). Extracellular perfusion of patch clamped guard cells with 30 μM IAA-94 blocked slow anion channels in the voltage range analyzed.

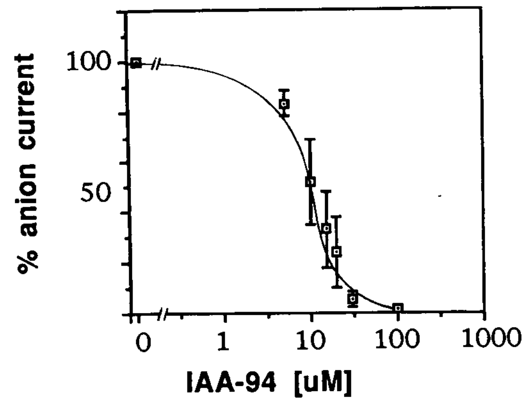

Figure 6. Concentration Dependence of Slow Anion Channel Block by Extracellular IAA-94.

Experimental protocol was as described in Figure 3.

single anion channels (Figures 8B and 8C). These data indicate that the observed single anion channel currents contribute to the slow anion currents observed in whole-cell recordings. In addition to these anion channel currents, further single-channel current levels were observed; these included transitions with lower amplitudes, which showed Cl^- selectivity and inhibition by IAA-94, indicating that multiple channel conductance states may contribute to slow-sustained anion channel currents.

Anion Channel Blocker Effects on Stomatal Opening

Effects of anion channel blockers on stomatal opening and closing were analyzed in isolated epidermal strips of broad bean. When epidermal strips were exposed to light for 120 min, stomata opened as shown in Figure 9. Stomatal opening was slightly increased in the presence of 300 μM extracellular DIDS, which completely blocks rapid anion channels (Figure 9; *n* = 210 stomata in seven epidermal strips). Exposure of stomata to 200 μM IAA-94 blocks slow anion currents completely (Figures 4 to 6). In the presence of 200 μM IAA-94, stomata consistently opened much wider, with apertures ~38 ± 14% wider than light-treated controls (Figure 9; *n* = 210 stomata in seven epidermal strips). When 200 μM IAA-94 was added in combination with 300 μM DIDS, stomatal opening was also enhanced when compared to untreated control (Figure 9; *n* = 150 stomata in five epidermal strips). Interestingly, these data indicate that slow anion channels may act as a significant negative regulator during stomatal opening under the imposed conditions.

Anion Channel Blocker Effects on Stomatal Closing

In broad bean, ABA causes variable stomatal closure (Raschke, 1979; Schroeder and Hagiwara, 1990b). Extracellular malate

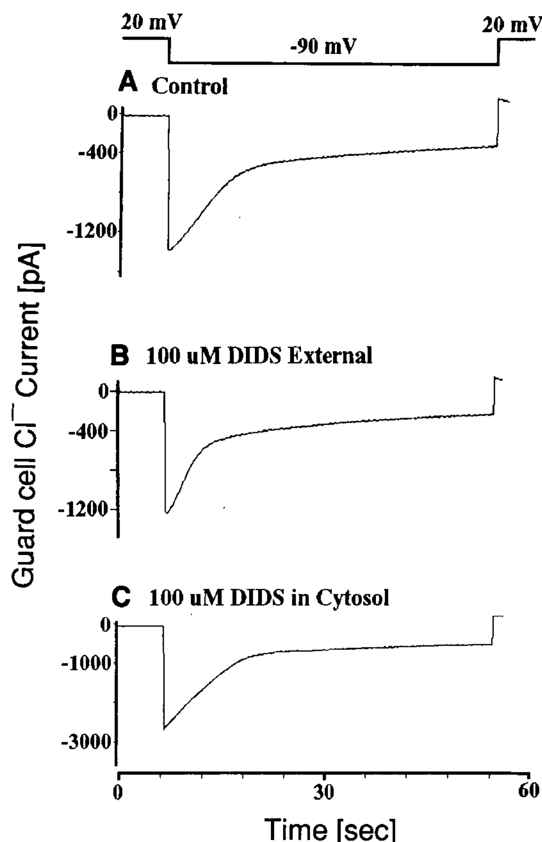

Figure 7. Effect of DIDS on Slow Anion Channels in Guard Cells.

(A) Hyperpolarization of guard cells from +20 to −90 mV produced a large deactivating slow anion efflux current. The top insert shows the voltage pulse protocol.

(B) Extracellular perfusion of the same guard cell with 100 μM DIDS did not cause a physiologically significant block of slow anion currents after 10-min exposure to DIDS.

(C) Cytosolic DIDS (100 μM) also did not induce a physiologically significant block of slow anion channels. DIDS was loaded into the cytosol of guard cells by dialysis from the patch clamp electrode. The illustrated recording was performed 9 min after gaining access to the cytosol (access resistance was 15 MΩ).

has recently been suggested to induce stomatal closure, acting as a CO_2 sensor, in broad bean guard cells (Hedrich and Marten, 1993). Figure 10 illustrates that exposure of open stomata to extracellular ABA in combination with high concentrations of malate (>20 mM required) consistently produced stomatal closure in broad bean (n = 240 stomata in eight epidermal strips). Stomatal closing by coapplication of ABA and malate was stronger than by either stimulus alone. Coapplication of ABA and malate was therefore used as a tool in this initial study to test anion channel blocker effects on stomatal closing. DIDS at 300 μM, which completely blocks rapid anion channels (Marten et al., 1993), inhibited ~20% of ABA- and malate-induced stomatal closing (Figure 10; n = 150 stomata in five epidermal strips).

NPPB effectively blocks slow anion channels at physiological potentials (Figures 1 to 3). When stomata were incubated in 20 μM NPPB, stomatal closing induced by ABA and malate was completely inhibited (Figure 10; n = 150 stomata in five epidermal strips). Together, these data suggest a large contribution of slow anion currents and an additional contribution of rapid anion currents to stomatal closing during coapplication of ABA and malate (Figure 10). The increased opening produced by NPPB (Figure 10, compare first and fourth bars) correlates to enhanced stomatal opening when slow anion channels are blocked (Figure 9). These initial bioassay studies indicate that the pharmacological properties of anion channels

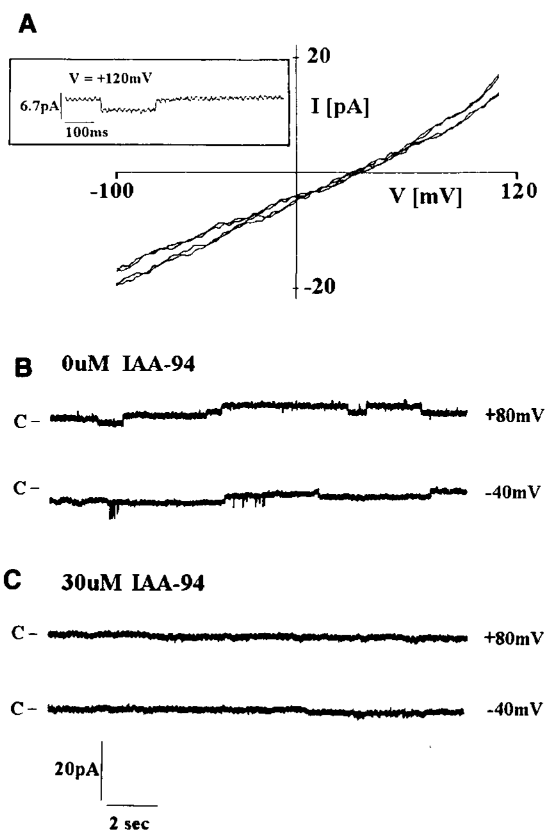

Figure 8. Single-Channel Recordings of Slow Anion Channels in Outside-Out Membrane Patches.

(A) Rapid voltage ramps of single-channel currents show reversal at the Cl⁻ equilibrium potential. The inset shows that slow anion channels from the same patch at +120 mV had long open times. The duration of voltage ramps was selected at 50 msec such that open-closed transitions of channels were less likely during the ramps.

(B) Slow anion channels continuously recorded at +80 and −40 mV. C, closed state.

(C) IAA-94 (30 μM) inhibited slow anion channels at +80 and −40 mV. The low-pass filter frequency of continuous single-channel recordings was 4 kHz (10-kHz sampling rate) to analyze rapid transitions between open and closed (C) states of anion channels.

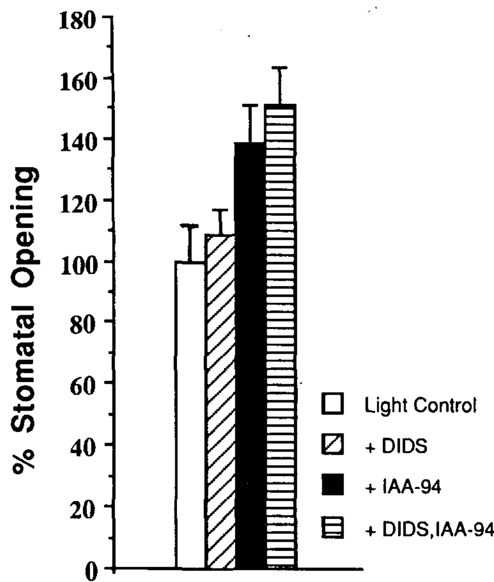

Figure 9. Effects of Anion Channel Blockers on Light-Induced Stomatal Opening.

Stomatal apertures after 2 hr of light (open bar), after 2 hr of light in the presence of 300 μM DIDS (diagonally striped bar), after 2 hr of light in the presence of 200 μM IAA-94 (filled bar), and after 2 hr of light in the presence of both 200 μM IAA-94 and 300 μM DIDS (horizontally striped bar). Error bars = SE.

can be used to study and distinguish physiological functions of slow and rapid anion channels during stomatal regulation.

DISCUSSION

Two Anion Channel Currents in Guard Cells

In guard cells, anion channels have been suggested to be central mediators of stomatal regulation by providing a pathway for anion efflux (Keller et al., 1989; Schroeder and Hagiwara, 1989). In addition, the resulting depolarization drives K^+ efflux through outward-rectifying K^+ channels (Schroeder et al., 1987), thereby producing stomatal closing. Recent data have shown that two distinct types or modes of depolarization-activated anion channel currents coexist in the plasma membrane of guard cells (Schroeder and Keller, 1992). In addition to these depolarization-activated anion channels in guard cells, hyperpolarization-activated anion channels have been described in other higher plant cells (Terry et al., 1991; Tyermann, 1992). The activation of guard cell anion currents at depolarized potentials is essential for the suggested mediation of stomatal closing.

It has been suggested that the slow and sustained anion channel currents provide the central driver for long-term ion efflux required for ABA-induced stomatal closing (Schroeder and Hagiwara, 1989; Linder and Raschke, 1992; Schroeder and Keller, 1992). The findings that ABA mediates sustained depolarizations (Kasamo, 1981; Ishikawa et al., 1983; Thiel et al., 1992), that ABA induces currents with a similar voltage dependence to S-type anion channels (Thiel et al., 1992), as well as the lack of direct ABA effects on R-type anion channels (Marten et al., 1991) all support the hypothesis that the initially described S-type anion channel currents in guard cells are essential in regulation of ABA-induced stomatal closing (Schroeder and Hagiwara, 1989). It is further possible that S-type anion channels play important roles during ion transport processes, movements, transport, osmoregulation, and signal-mediated sustained depolarizations in other higher plant cells (e.g., Racusen and Satter, 1975; Kasamo, 1981; Mathieu et al., 1991; Ullrich and Novacky, 1991; Ehrhardt et al., 1992; Wegner and Raschke, 1992).

A second type of anion channel current was subsequently identified with rapid activation (R-type, also named GCAC1). These rapid channels (Keller et al., 1989) are similar to the Ca^{2+}-dependent, rapid and transient Cl^- channels described in algae (Mullins, 1962; for review, see Tazawa, 1987). The rapid transient activation of R-type anion channel currents suggests a contribution to ion efflux during stomatal closing (Hedrich et al., 1990). Questions regarding respective contributions of both anion currents to stomatal closing and the molecular distinction of anion channels have remained unanswered (see Introduction). The characterization of specific blocker effects on S-type anion channels in this study and the description of

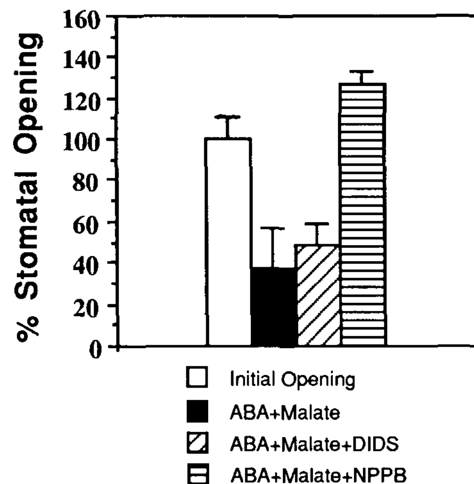

Figure 10. Anion Channel Blockers Inhibit Stomatal Closing.

Open stomata at time zero (open bar) were reduced in aperture by 2-hr exposure to 50 μM ABA and 40 mM malate in the light (filled bar). Extracellular DIDS (300 μM) partially inhibited stomatal closing (diagonally striped bar). Extracellular NPPB (20 μM) abolished stomatal closing and produced further opening (horizontally striped bar).

rapid anion channel blockers (Marten et al., 1992, 1993) provide tools to approach questions regarding physiological functions, heterogeneity, and structural components of guard cell anion channels.

Physiological Effects of Blockers on Stomatal Movements

Results from studying stomatal closing by coapplication of ABA and malate (Figure 10) provide initial support for the hypotheses that both slow anion currents (Schroeder and Hagiwara, 1989) and, to a lesser degree, rapid anion currents (Keller et al., 1989) contribute to the mediation of stomatal closing under the imposed conditions. It should be noted that 20 μM NPPB blocks slow and rapid anion currents to a similar extent and therefore a simultaneous requirement for both anion currents cannot be ruled out (Figure 10). A further novel role for S-type anion channels is indicated from bioassays (Figure 9). In addition to stimulating stomatal closing, guard cell anion channels may modulate stomatal opening. Data indicate that under the imposed conditions, slow anion channels and, to a lesser degree, rapid anion currents may down-regulate the degree of light-induced stomatal opening (Figure 9). The activation of slow anion currents at potentials more negative than −140 mV may contribute to this negative regulation (Figures 2, 5, and 9; Linder and Raschke, 1992; Schroeder and Keller, 1992). This would require residual slow anion channel activation during stomatal opening. Resting potentials in this range have been recorded in 0.1 mM KCl (Thiel et al., 1992).

R-type anion channel activation has been suggested to be involved in auxin-mediated stomatal opening (Marten et al., 1991). The finding that 300 μM DIDS (Figure 9) and 1 mM DIDS (Schwartz et al., 1991) showed small or no effects on light-induced stomatal opening suggests that R-type anion currents may not be a central regulator of stomatal opening in response to white light.

Data presented here support the hypotheses that S-type anion channels play major roles in stomatal regulation (Schroeder and Hagiwara, 1989) and that slow and rapid anion currents have distinct physiological functions (Schroeder and Keller, 1992). Further, more detailed analysis is necessary to determine whether different physiological signals for stomatal regulation and different experimental conditions may utilize either slow or rapid or both anion channel currents during stomatal movements. The identification of distinct and potent blockers of both S-type and R-type anion currents in guard cells provides the opportunity to determine the requirements of both anion current types during stomatal regulation in response to various signals and experimental conditions. It should be noted, however, that the stomatal bioassay may only be adequately sensitive for testing an absolute requirement of a particular ion channel during stomatal regulation. More subtle involvement may be difficult to analyze in these experiments, particularly because parallel regulation of mechanisms which produce stomatal closing rather than one sequential chain of events has been suggested, based on results from several laboratories (Schroeder, 1992).

Comparison of S-Type and R-Type Anion Channels

The two anion currents in the plasma membrane of guard cells show ~10^3-fold different activation and deactivation times, divergent inactivation characteristics, and greater than 100 mV differences in the activation potential under the same recording conditions (Schroeder and Keller, 1992) (Figure 2).

We have shown that NPPB and IAA-94 are potent blockers of S-type anion channels at physiological potentials (Figures 1 to 6), whereas DIDS (Figure 7) and $ZnCl_2$ showed no physiologically significant reduction in S-type anion currents. In the present study, only marginal physiological block by 100 μM DIDS of slow anion channels was found, although modification of deactivation times indicates possible effects on the voltage dependence (Figure 7; J.I. Schroeder, unpublished data). These results differ markedly from the complete and voltage-independent block of R-type anion channel(s) by DIDS (K_i = 0.2 μM; Marten et al., 1993).

Both NPPB and IAA-94 as well as other anionic compounds, including auxins and ethacrynic acid, shift the activation threshold of R-type anion channels to more negative potentials by different mechanisms (Marten et al., 1991, 1992). This shift in activation to physiological resting potentials implies that these compounds can function as activators of R-type anion channels at nonsaturating concentrations rather than solely as blockers.

Inhibition constants of NPPB (7 μM; Figure 3) and IAA-94 (10 μM; Figure 6) reported here for physiological block of slow anion currents are similar to recently published inhibition constants of NPPB (4 μM) and IAA-94 (7 μM) for rapid anion channels (Marten et al., 1992). Correction for the blocker-induced independent shift in the voltage dependence of R-type anion currents and linear correction for the electrochemical driving force for Cl^- showed that ~36% of R-type anion currents remain unblocked at 30 μM IAA-94 and ~17% remained unblocked at 100 μM IAA-94 (Marten et al., 1992). In contrast, IAA-94 inhibited S-type anion currents similarly at all potentials examined at 30 and 100 μM IAA-94 and produced physiologically significant inhibition (Figures 5 and 6). Complete block of single slow anion channel currents by 30 and 100 μM IAA-94 was observed (Figure 8), whereas single rapid anion channels remained active in the presence of 100 μM IAA-94 at hyperpolarized potentials (Marten et al., 1992). These data suggest that IAA-94 may be a physiologically less effective blocker of R-type anion currents than of S-type anion currents (Figures 4 to 6 and 8).

Slow anion channels had single-channel conductances of 33 ± 8 pS in broad bean (Figure 8) confirming recent recordings in *Xanthium strumarium* and broad bean (Linder and Raschke, 1992), which is very similar to the conductance of rapid anion channels (~36 pS) under comparable conditions (Keller et al., 1989). Steady state variance analysis assumes

a very low open probability of ion channels (Sigworth, 1984). Coarse approximations of single-channel conductance by this method result in minimum estimates (Sigworth, 1984; Schroeder and Keller, 1992), due to the extremely high open probability of slow anion channels (Figure 8; Linder and Raschke, 1992). Furthermore, additional low conductance states of slow anion currents, which were inhibited by IAA-94, decrease variance analysis–derived coarse approximations of single channel conductance.

Structural Implications

The properties of S-type anion currents show some similarities in one single-channel conductance state and in affinities of NPPB and IAA-94 to R-type anion currents, suggesting possible structural relations between these two anion channels. On the other hand, major differences in the mechanisms of block, voltage, and time dependence are clearly apparent as discussed above. An oxyacetic acid (IAA-23) binding protein with a molecular mass of 60 kD has been identified in guard cell membranes as a putative component of R-type anion channels (Marten et al., 1992). This membrane protein may also be a component of S-type anion channels, based on the potent IAA-94 block of S-type anion currents (Figures 4 to 6 and 8). Two interpretations are most likely for anion channel structure in guard cells. (1) Different protein complexes may form S-type and R-type anion channels, notwithstanding the possibility that some structural motifs or certain subunits can be shared. (2) Changes in protein conformation may result in a switch between gating modes, which results in shifts in the voltage and time dependence, and block mechanisms of DIDS, IAA-94, and NPPB on rapid and slow anion currents. Single-channel recordings show both sustained, long openings (Figure 8) and rapid flickers (C. Schmidt and J.I. Schroeder, unpublished data). Whether this represents functional mode shifts of the identical protein complex from S-type to R-type characteristics remains to be determined and may require molecular biological characterization. Findings reported here, however, provide strong evidence that the cellular functions of slow and rapid anion currents during stomatal regulation can be clearly separated (Figures 9 and 10), supporting previous data suggesting physiologically distinct behavior of both anion currents (Schroeder and Keller, 1992).

In conclusion, the findings in this study strongly support the original hypothesis that slow anion channels are a major mechanism for driving stomatal closing (Schroeder and Hagiwara, 1989) and point to a novel possible role in negative regulation of stomatal opening. The findings of the pharmacological divergence of the two anion current types and the identification of potent blockers of S-type anion channels reported here will be essential for further characterizing structural components and the functions of both anion channels during stomatal regulation in response to various physiological signals. Furthermore, these results may allow analysis of the contribution of specific anion currents to other processes that involve signal-mediated depolarization accompanied by anion efflux in higher plants.

METHODS

Guard Cell Isolation and Patch Clamp Procedures

Guard cell protoplasts were isolated from leaf epidermal strips of 3- to 4-week-old broad bean (*Vicia faba*) plants, as described previously by Schroeder and Fang (1991). Macerozym (1% [w/v]) and Cellulase Onozuka R10 (1.5%; Yakult Honsha, Tokyo, Japan) were used for enzymatic digestion of cell walls during overnight incubations (15 hr at 23°C). Whole-cell and outside-out patch clamp experiments were performed on guard cell protoplasts (Hamill et al., 1981; Schroeder et al., 1987). Before application of all voltage–pulse protocols in this study, the membrane potential was polarized to the holding potential for 3 min or longer to allow stabilization of slow anion channel currents. The duration of slow voltage ramps was 15 min, which is significantly greater than activation/deactivation times, thereby giving rise to steady state current–voltage relationships (Schroeder and Keller, 1992). Patch clamp recordings were performed and low-pass filtered with an amplifier (Axopatch 1D; 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 25-MHz 386 AT-based microcomputer. The temperature was 24°C.

Solutions

The pipette solution that equilibrates with the cytosol of guard cells contained 136 mM CsCl, 1.8 mM $MgCl_2$, 10 mM Hepes, 3.34 mM $CaCl_2$, 7.6 mM EGTA-$(Tris)_2$, (0.29 μM calculated free Ca^{2+}), 4 mM MgATP, 200 μM Li_4-guanosine- 5′-0-(3-thiotriphosphate) (GTPγ S), 4 mM Tris, pH 7.10, in whole-cell experiments. In single-channel studies, no GTPγS was added and the cytosolic Cl^- concentration was increased to 154 mM. Guard cells were extracellularly perfused with a solution containing 30 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Mes, pH 5.7. The Cl^- equilibrium potential in whole cells was +32 mV after correction for ionic activities (Robinson and Stokes, 1958). Osmolalities were adjusted to 490 mmol kg^{-1} by the addition of D-sorbitol and verified by a vapor pressure osmometer (Wescor, Logan, UT). Patch clamped guard cells were exposed to anion channel blockers by peristaltic perfusion (Rainin, Woburn, MA) of the bath (200 μL volume) with 3 to 6 mL of the introduced solution.

Anion Channel Blockers

Fresh solutions of anion channel blockers were prepared at the beginning of experiments and stored in the dark on ice. Stock solutions were prepared by dissolving and sonicating 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) (DIDS; Sigma) in KOH and 6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-indan-5-yl oxy acetic acid (IAA-94) and 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB) (Hoechst, Darmstadt) in ethanol at 5 to 50 mM concentrations. $ZnCl_2$ was added directly to the bath solution.

Stomatal Movement Bioassays

Stomatal opening and closing assays were performed as described elsewhere (Raschke, 1979; Schwartz et al., 1991). Epidermal strips were anchored to the bottom of 3-cm culture dishes and submerged in experimental solutions. For stomatal opening, leaf epidermal strips were exposed to white light (photon fluence rate of 500 μE cm^{-2} sec^{-1}) and CO_2-free air in a solution containing 50 mM KCl, 10 mM Mes, pH 6.15, at 28°C. Stomatal aperture measurements were taken using an ocular micrometer before and after 2-hr exposures to light. The mean aperture of light-induced open stomata in more than or equal to two epidermal strips was defined as 100% on each experimental day. For stomatal closing, epidermal strips were bathed in solutions containing 50 μM abscisic acid, 40 mM malate, 5 mM $CaCl_2$, 10 mM Mes, Tris, pH 6.15. To monitor stomatal closing, stomatal apertures were first opened to 10 to 12 μm. Then the opening solution was replaced by the closing solution for 2 hr under light at 28°C. A second aperture measurement was then taken. Sorbitol solutions adjusted to the same osmolarity did not produce stomatal closure.

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