The Plant Cell, Vol. 11, 1141–1151, June 1999, www.plantcell.org

ATP Binding Cassette Modulators Control Abscisic Acid–Regulated Slow Anion Channels in Guard Cells

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In animal cells, ATP binding cassette (ABC) proteins are a large family of transporters that includes the sulfonylurea receptor and the cystic fibrosis transmembrane conductance regulator (CFTR). These two ABC proteins possess an ion channel activity and bind specific sulfonylureas, such as glibenclamide, but homologs have not been identified in plant cells. We recently have shown that there is an ABC protein in guard cells that is involved in the control of stomatal movements and guard cell outward K^+ current. Because the CFTR, a chloride channel, is sensitive to glibenclamide and able to interact with K^+ channels, we investigated its presence in guard cells. Potent CFTR inhibitors, such as glibenclamide and diphenylamine-2-carboxylic acid, triggered stomatal opening in darkness. The guard cell protoplast slow anion current that was recorded using the whole-cell patch–clamp technique was inhibited rapidly by glibenclamide in a dose-dependent manner; the concentration producing half-maximum inhibition was at 3 μM. Potassium channel openers, which bind to and act through the sulfonylurea receptor in animal cells, completely suppressed the stomatal opening induced by glibenclamide and recovered the glibenclamide-inhibited slow anion current. Abscisic acid is known to regulate slow anion channels and in our study was able to relieve glibenclamide inhibition of slow anion current. Moreover, in epidermal strip bioassays, the stomatal closure triggered by Ca^{2+} or abscisic acid was reversed by glibenclamide. These results suggest that the slow anion channel is an ABC protein or is tightly controlled by such a protein that interacts with the abscisic acid signal transduction pathway in guard cells.

INTRODUCTION

The ATP binding cassette (ABC) superfamily is probably the largest and most diverse family of proteins that mediate ATP-dependent transfer of solutes across membranes in species ranging from *Escherichia coli* to humans (Higgins, 1992). These proteins have become of fundamental interest due to their involvement in numerous pathologies, such as cystic fibrosis, diabetes, or multidrug resistance (Demolombe and Escande, 1996). Among them, both the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptor (SUR) exhibit either an ion channel activity and/or regulate heterologous channels (Higgins, 1995).

The CFTR constitutes a chloride channel inhibited by diphenylamine-2-carboxylic acid (DPC; McCarty et al., 1993), which can control K^+ channels (Valverde et al., 1995; McNicholas et al., 1996; Ishida-Takahashi et al., 1998). The SUR is tightly associated with an inward-rectifying potassium channel (Inagaki et al., 1995) to form the ATP-sensitive K^+ channel. These channels and the CFTR are both receptors for sulfonylureas (Schmid-Antomarchi et al., 1987; Schultz et al., 1996) and are blocked by glibenclamide in numerous tissues (Sheppard and Welsh, 1992; Gopalakrishnan et al., 1993). The SUR is also the receptor for K^+ channel openers (KCOs; Schwanstecher et al., 1998), such as cromakalim, that reverse the inhibitory effect of sulfonylureas (Cook and Quast, 1990; Ashcroft and Ashcroft, 1992).

In contrast to those on animal cells, studies on the intervention of ABC proteins in the control of plant ion channels are in their infancy (Leonhardt et al., 1997). A few ABC protein–encoding sequences have been cloned (Dudler and Hertig, 1992; Smart and Fleming, 1996; Davies et al., 1997; Lu et al., 1997, 1998; Tommasini et al., 1997, 1998; Marin et al., 1998; Sanchez-Fernandez et al., 1998). The functional characterization obtained for some of them indicates their involvement in cellular detoxification (Rea et al., 1998) or in cell elongation (Sidler et al., 1998). Interestingly, the accumulation of an ABC transcript in plants can be correlated to stress conditions, suggesting a hormonal and environmental regulation of these genes (Smart and Fleming, 1996).

A frequently defected early event in plant signal transduction is membrane depolarization induced by an anion efflux along the transmembrane gradient (Marten et al., 1991; Ebel and Cosio, 1994; Ward et al., 1995), and guard cell anion channels are probably the best characterized (Schroeder,

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1995). Essentially, two types of anion channels have been identified at the guard cell plasma membrane (Keller et al., 1989; Schroeder and Hagiwara, 1989), even though the distinction between these two proteins has not been ascertained (Dietrich and Hedrich, 1994), and their identity remains unknown. Rapid anion channels (R type) give rise to transient depolarizations (Schroeder and Keller, 1992), whereas slow anion channels (S type) activate slowly and allow sustained anion efflux necessary for stomatal closure (Schroeder and Keller, 1992).

Because ABC proteins in animal cells are able to control both K^+ and anion currents, we took advantage of the specific pharmacological profile of these proteins to study their putative involvement in the regulation of guard cell anion channels. The intervention of both Ca^{2+} and abscisic acid (ABA), triggering stomatal closure and activation of slow anion channels, also was studied in the presence of ABC protein modulators.

RESULTS

Glibenclamide Prevents Stomatal Closure Induced by External Calcium

Patch–clamp studies with guard cell protoplasts have shown that slow anion currents are activated by external or cytosolic calcium (Schroeder and Hagiwara, 1989; Hedrich et al., 1990) and thus could give rise to the prolonged anion efflux required for stomatal closure. Incubation of *Commelina communis* epidermal strips in the presence of millimolar concentrations of external calcium triggered stomatal closure under white light (Schwartz, 1985). In our study, the addition of 10 μM glibenclamide prevented stomatal closure triggered by external calcium even at millimolar concentrations in fava bean (Figure 1A) or in *C. communis* (Figure 1B), a species highly sensitive to Ca^{2+} (Schwartz, 1985). Conversely, the effect of 3 mM EGTA, which is an impermeant Ca^{2+} chelator able to induce spontaneous opening of stomata in darkness, was impeded by the concomitant application of 20 μM cromakalim (Figure 1C). These results indicate an eventual intervention of a SUR homolog in the control of osmoregulation through Ca^{2+} signaling.

DPC and Glibenclamide, Two Blockers of the CFTR, Induce Stomatal Opening and Inhibit the Slow Anion Current

To investigate a possible interaction between a SUR homolog and guard cell slow anion channels suspected to be targets of Ca^{2+} activation (Schroeder and Hagiwara, 1989), we first checked the specific properties of these channels by using the whole-cell patch–clamp technique. Inward currents obtained under two ionic conditions during a voltage

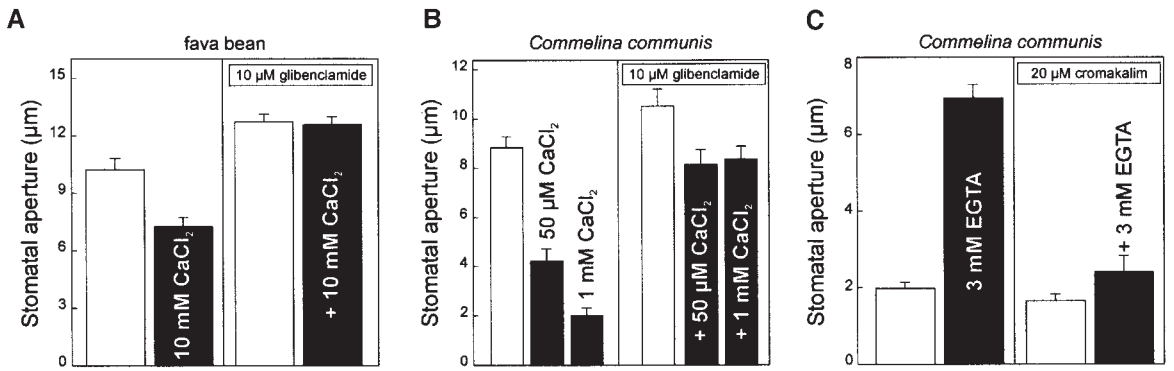

Figure 1. Glibenclamide Inhibits Stomatal Closure Induced by Exogenous Calcium.

(A) and **(B)** A 2.5-hr application of external $CaCl_2$ (solid bars) in fava bean **(A)** or *C. communis* **(B)** triggered stomatal closure in the light, whereas 10 μM glibenclamide promoted stomatal opening. In both species, even millimolar concentrations of external Ca^{2+} were unable to induce stomatal closure in the presence of 10 μM glibenclamide. Stomata were first illuminated for 3 hr, and then Ca^{2+} and/or glibenclamide was added for an additional 2.5-hr period. Open bars represent stomatal aperture under illumination in the absence (open bars at left in **[A]** and **[B]**) or presence (open bars at right in **[A]** and **[B]**) of 10 μM glibenclamide. In the experiment described in **(A)**, the chloride content of each sample was kept constant by adding 20 mM *N*-methylglucamine chloride when necessary.

(C) Stomata from *C. communis* incubated for 3 hr in darkness opened in response to the addition of EGTA, whereas the effect was lost when cromakalim was added. Open bars represent stomatal aperture in darkness in the absence (open bar at left) or presence (open bar at right) of 20 μM cromakalim.

Data are the mean (±SEM) of three independent experiments (100 stomata per condition and per experiment).

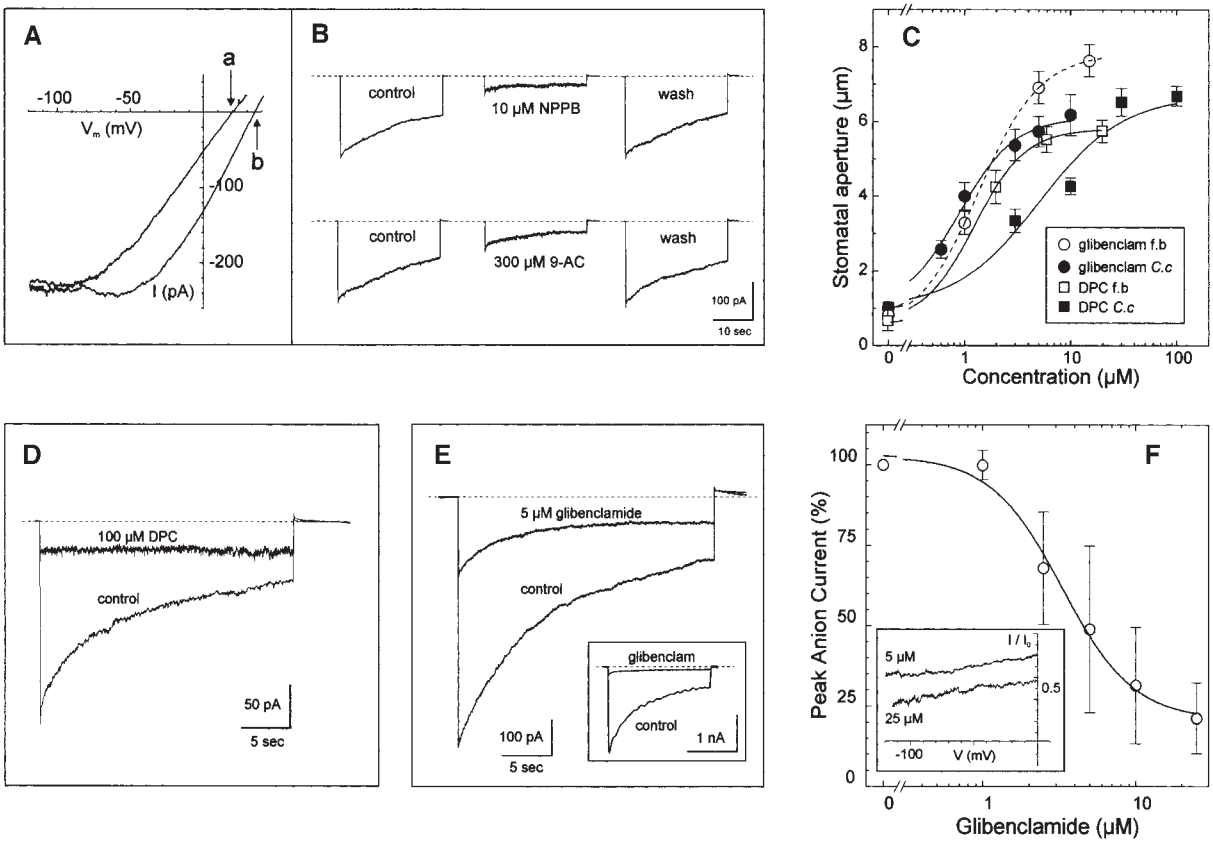

Figure 2. Inhibitors of the CFTR Trigger Dose-Dependent Stomatal Opening in the Dark and Block the Slow Anion Current.

(A) Steady state slow anion currents recorded in fava bean guard cell protoplasts during a voltage ramp in the membrane potential ranging from 40 to −120 mV. The reversal potential was shifted to the right from 19 mV (trace a) to 34 mV (trace b) when the extracellular Cl^- concentration was decreased from 84 to 36 mM. Whole-cell capacitance was 7 pF. Seal resistance was 2 GΩ.

(B) Whole-cell patch–clamp recordings showing a slow and incomplete relaxation of anion currents induced by hyperpolarization of the membrane potential from 0 to −120 mV in fava bean guard cell protoplasts. External application of 10 μM NPPB or 300 μM 9-AC resulted in inhibition of slow anion currents within 2 and 5 min, respectively. After perfusion with the control bath medium, slow anion channel currents were recovered within 13 and 10 min for NPPB and 9-AC, respectively. Dashed lines refer to zero current.

(C) Bioassays illustrating the effects of two inhibitors of CFTR on stomatal movements in *C. communis* (*C.c*; closed symbols) and fava bean (f.b; open symbols). A 2.5-hr application of glibenclamide (glibenclam; circles) or DPC (squares) triggered stomatal opening in the dark in a dose-dependent manner. Data presented are the mean (±SEM) of three independent experiments (100 stomata per condition and per experiment).

(D) Inhibition of slow anion current in fava bean guard cells by the external application of 100 μM DPC. Whole-cell slow anion currents recorded before (control) and 1 min after perfusion with 100 μM DPC are superimposed. Voltage protocol is the same as described for **(B)**. Dashed line refers to zero current. Whole-cell capacitance was 8.4 pF. Seal resistance was 2 GΩ.

(E) Glibenclamide (glibenclam) inhibition of slow anion current in fava bean guard cells. Whole-cell slow anion currents are superimposed and were recorded before (control) and 1 min after perfusion with 5 μM glibenclamide. Whole-cell capacitance was 5.4 pF. Seal resistance was 2 GΩ. In the inset, inhibition by 20 μM glibenclamide of slow anion current in intact guard cell of fava bean was recorded by using the discontinuous single-electrode voltage–clamp technique, as previously described (Forestier et al., 1998a, 1998b). Dashed lines refer to zero current. Horizontal scale is 5 sec.

(F) The amplitude of the peak anion current decreased with glibenclamide concentration in the range of 1 to 25 μM. Data are the mean of 30 independent experiments with guard cells. Error bars represent standard deviation. In the inset, voltage-independent inhibition of slow anion currents by 5 or 25 μM glibenclamide is shown. Current (I) measured after application of the indicated dose of glibenclamide (5 or 25 μM) is normalized relative to control current (I_0) at each tested voltage.

ramp in the range of 40 to −120 mV are presented in Figure 2A. In the presence of 161 mM Cl^- in the cytosol, when the extracellular Cl^- concentration was decreased from 84 (theoretical equilibrium potential for anions = 16 mV) to 36 mM Cl^- (theoretical equilibrium potential = 38 mV), the reversal potential was shifted to the right from 19 (Figure 2A, trace a) to 34 mV (Figure 2A, trace b), attesting to the strong selectivity of these channels for chloride (Figure 2A). Hyperpolarization from 0 to −120 mV, which was applied to the plasma membrane of guard cell protoplasts, elicited large instantaneous currents, which characterized the slow and incomplete relaxation of slow anion channels (control in Figure 2B; Schroeder and Keller, 1992). The steady state current voltage properties and the slow relaxation at hyperpolarized membrane potential were analogous to the properties described for slow anion channels in fava bean guard cells (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992; Schwartz et al., 1995).

To confirm the nature of these currents, we analyzed effects of different potent blockers of slow anion channels (Schroeder et al., 1993; Schwartz et al., 1995). Figure 2B shows that extracellular perfusion of guard cell protoplasts with 10 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) or 300 μM anthracene-9-carboxylic acid (9-AC) inhibited the anion efflux currents by 81 and 51%, respectively. The block of slow anion current was reversed completely after a 10- to 30-min washing (Figure 2B). Based on seven independent guard cells, the mean slow anion channel current inhibition by 10 μM NPPB was 55 ± 32%. The kinetic parameters of these currents, their equilibrium potential, and their sensitivity to anion channel blockers confirm that they were carried by a selective efflux of anions via slow anion channels.

In animal cells, both the SUR and the CFTR are receptors for sulfonylureas, such as glibenclamide (Schmid-Antomarchi et al., 1987; Schultz et al., 1996). In our study, we investigated the effects on anion channels of glibenclamide and DPC, which are two potent inhibitors of the CFTR (Sheppard and Welsh, 1992; McCarty et al., 1993, respectively). Bioassays conducted with fava bean or *C. communis* epidermal strips demonstrated that both DPC and glibenclamide triggered stomatal opening in the dark in a dose-dependent manner (Figure 2C). Fitting of the dose–response curves gave a half-maximum concentration for the induction of stomatal opening of 5.0 and 0.9 μM for DPC and glibenclamide, respectively, in *C. communis.* When 100 μM DPC was externally perfused, the slow anion current elicited in guard cell protoplasts by hyperpolarization from 0 to −120 mV was blocked by at least 84% (Figure 2D). In five of five guard cells, an inhibition of the slow anion current by 100 μM DPC was observed within 1 min (mean = 78 ± 24%).

To determine whether the effects of sulfonylurea could be correlated with the inhibition of slow anion channels, we in-

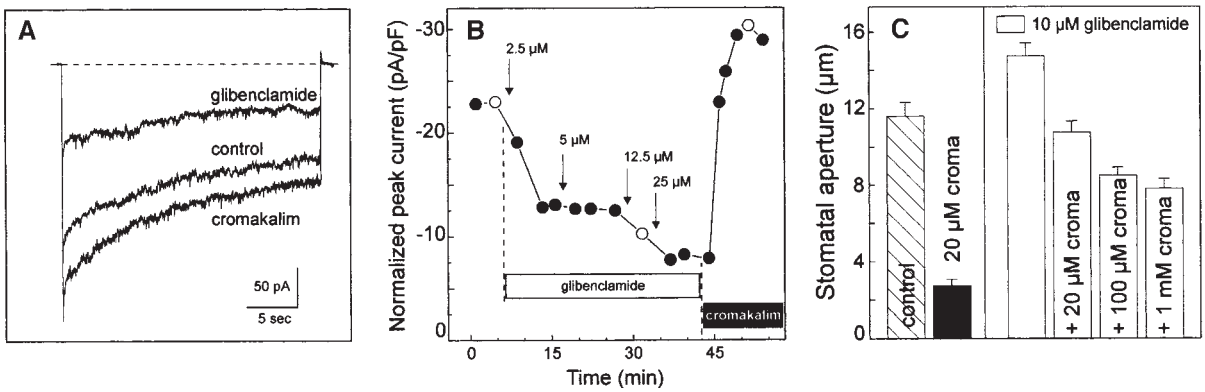

Figure 3. Glibenclamide and Cromakalim Have Antagonistic Effects on Slow Anion Currents.

(A) Inhibition of the slow anion current by 12.5 μM glibenclamide was reversed by 100 μM cromakalim. Recordings of whole-cell slow anion currents were obtained as described in Figure 2. Dashed line refers to zero current. Whole-cell capacitance was 5.1 pF. Seal resistance was 1.2 GΩ.
(B) Time course of the slow anion current from the experiment described in **(A)** at −120 mV. The normalized peak current was plotted versus time before glibenclamide was applied, during the perfusion of increasing glibenclamide concentrations, and after the substitution of glibenclamide for 100 μM cromakalim. Arrows indicate a change in glibenclamide concentration, and the three open circles refer to the three current recordings illustrated in **(A)**.
(C) In *C. communis*, a 2.5-hr application of 20 μM cromakalim (croma; solid bar) triggered stomatal closure in the light, whereas 10 μM glibenclamide promoted stomatal opening. Stomatal opening stimulated by illumination and the application of 10 μM glibenclamide (open bars) was prevented by applying cromakalim in a dose-dependent manner. In this experiment, stomata opened after a 3-hr exposure to light, and then cromakalim and/or glibenclamide was added for an additional 2.5-hr period. Data are the mean (±SEM) of three independent experiments (100 stomata per condition and per experiment).

vestigated the action of glibenclamide on the slow anion current. The relaxation of the slow anion current before and after perfusion of the bath with 5 μM glibenclamide is presented in Figure 2E. In this experiment, a 66% inhibition of the slow anion current was recorded within 3 min. In 10 other independent experiments, extracellular perfusion with 5 μM glibenclamide resulted in a 51 ± 25% inhibition. Slow anion current also was inhibited by glibenclamide in intact guard cells of fava bean (Figure 2E, inset) when the discontinuous single-electrode voltage–clamp technique was used as previously described (Forestier et al., 1998a, 1998b).

In contrast with NPPB and 9-AC and in accordance with previous reports (Findlay, 1992; Leonhardt et al., 1997), even upon prolonged washing with the control bath solution, glibenclamide inhibition was irreversible (data not shown). In experiments with 30 independent cells, inhibition of slow anion current according to extracellular glibenclamide concentration was determined (Figures 2F and 3B). Glibenclamide caused a dose-dependent inhibition of the slow anion current, with half-maximal inhibition at 3.3 ± 0.6 μM. The relative inhibition of slow anion currents by 5 or 25 μM glibenclamide is seldom or never dependent on the membrane potential (Figure 2F, inset). Thus, glibenclamide constitutes an efficient blocker of S-type anion current in fava bean guard cells.

Inhibition by Glibenclamide of the S-Type Anion Current Is Reversed by Cromakalim

In animal cells, glibenclamide and cromakalim produced antagonistic effects on the SUR (Cook and Quast, 1990; Ashcroft and Ashcroft, 1992); therefore, we analyzed the consequence of adding cromakalim after inhibition of the slow anion current by glibenclamide. Cromakalim alone was unable to significantly stimulate the guard cell slow anion current (data not shown). However, glibenclamide inhibition of the slow anion current was reversed by extracellular perfusion of 100 μM cromakalim. The results of a typical experiment are presented in Figure 3A. After perfusion with glibenclamide, the whole-cell anion current was inhibited in a dose-dependent manner (Figure 3B). When glibenclamide was replaced by 100 μM cromakalim, the current was completely restored and even stimulated within minutes. The time course of the normalized peak anion current from this experiment is reported in Figure 3B. With six other guard cells, after a 62 ± 29% mean inhibition of the current by glibenclamide, the remaining current activity was enhanced from 44 up to 265% (mean of 117 ± 94%) by using 100 μM cromakalim. In experiments with fava bean or *C. communis* epidermal strips, KCOs, such as cromakalim, inhibited stomatal opening and triggered stomatal closure under light (Figure 3C). Moreover, cromakalim was able to reverse the effect of glibenclamide in a dose-dependent manner (Figure 3C). These data support the proposed role of an ABC protein in the regulation of the slow anion current in guard cells.

Slow Anion Current Inhibited by NPPB Is Insensitive to Cromakalim

To ensure that reactivation of slow anion channels by cromakalim was not due to the effect of a detergent, we recorded these currents before and after inhibition with 10 μM NPPB (Figure 4A). When 100 μM cromakalim was added in the presence of NPPB, no current reactivation was detected. In bioassays, previously open stomata closed in response to cromakalim (Figure 4B), whereas NPPB promoted stomatal opening under light. The addition of cromakalim in the presence of NPPB did not modify stomatal aperture in a significant manner. Thus, it could be assumed that NPPB and cromakalim interact with a different site and, moreover, that the anion channel blocked by NPPB could no longer be regulated by the ABC protein modulator cromakalim.

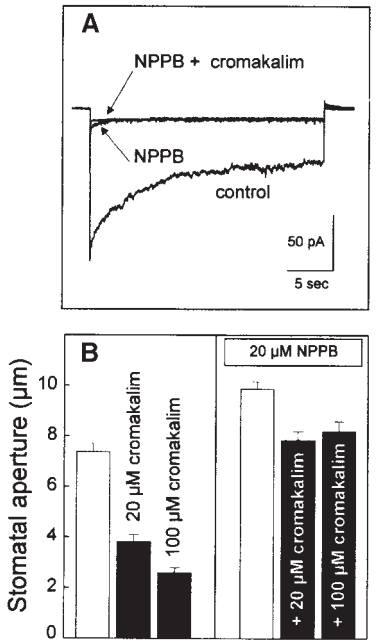

Figure 4. Block of the Slow Anion Current by NPPB Is Insensitive to Cromakalim.

(A) The slow anion current was rapidly inhibited by the addition of 10 μM NPPB. When 100 μM cromakalim was perfused in the presence of the same concentration of NPPB (NPPB + cromakalim), the slow anion current activation was not observed.

(B) In *C. communis*, a 2.5-hr application of 20 or 100 μM cromakalim (solid bars) triggered stomatal closure, whereas 20 μM NPPB promoted stomatal opening. Stimulation of stomatal opening under illumination by 20 μM NPPB (open bars) was weakly affected by a concomitant application of cromakalim. In this experiment, stomata opened after a 3-hr exposure to light, and then cromakalim and/or NPPB was added for an additional 2.5-hr period. Data are the mean (±SEM) of three independent experiments (100 stomata per condition and per experiment).

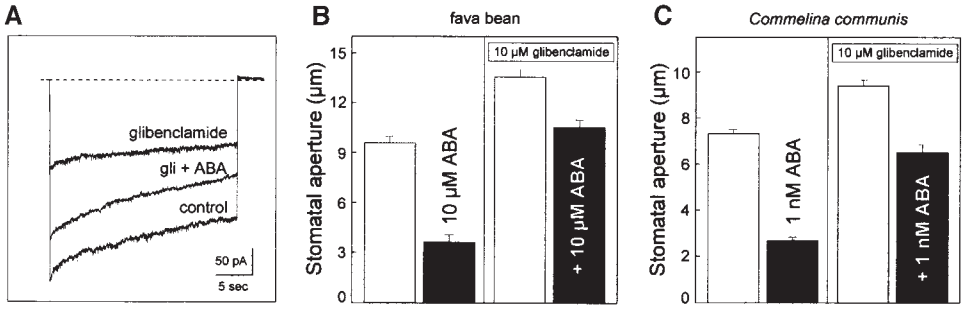

Figure 5. ABA Stimulates Glibenclamide-Inhibited Slow Anion Currents.

(A) The slow anion current was elicited by hyperpolarization of the guard cell plasma membrane from 0 to −120 mV (1) in the absence of any treatment (control), (2) 1 min after perfusion with 5 μM glibenclamide (glibenclamide), and (3) 1 min after perfusion with 5 μM glibenclamide plus 50 μM ABA (gli + ABA). Whole-cell capacitance was 8.4 pF. Seal resistance was 2 GΩ.

(B) and **(C)** Illumination of fava bean or *C. communis* epidermal strips for 3 hr and then application of ABA for an additional 2.5-hr period led to stomatal closure (solid bars). In both plants, 10 μM glibenclamide promoted stomatal opening and partially suppressed the stomatal closure triggered by ABA. Open bars represent stomatal aperture under illumination in the absence (open bars at left in **[B]** and **[C]**) or presence (open bars at right in **[B]** and **[C]**) of 10 μM glibenclamide. Note that *C. communis* is more sensitive to ABA than is fava bean. Data are the mean (±SEM) of three independent experiments (100 stomata per condition and per experiment).

ABA Partially Reverses Glibenclamide-Induced Stomatal Opening by Stimulating the Slow Anion Current

ABA synthesized in response to water stress triggers the signaling cascade leading to stomatal closure (Assmann, 1993; Ward et al., 1995). Mediation of stomatal closure is largely under the control of slow anion channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Schroeder et al., 1993). Thus, the key role played by ABA in stomatal regulation was analyzed in relation to putative ABC protein modulation of slow anion channels. As described for Figure 2E, external perfusion of a nonsaturating concentration of glibenclamide led to the partial inhibition of slow anion current (52%; Figure 5A). However, when 50 μM ABA, a dose currently used in patch–clamp recordings (Pei et al., 1997), was added in the presence of glibenclamide, the slow anion current was enhanced by 70% within 1 min. Based on the results with seven independent guard cells, ABA rescued up to 111% (mean of 59 ± 40%) of the anion current previously inhibited by a glibenclamide concentration close to the half-maximum effect. Similarly, in epidermal strip bioassays, the stomatal closure induced by ABA on preopened stomata was impaired largely by a concomitant application of glibenclamide in fava bean (Figure 5B) as well as in *C. communis* (Figure 5C), which is a species that is highly sensitive to ABA.

DISCUSSION

In this study, we demonstrate by using epidermal strip bioassays and patch–clamp experiments that the sulfonylurea glibenclamide and the KCO cromakalim are efficient modulators of the guard cell slow anion channel interfering with the Ca^{2+} and ABA signaling cascades.

Pharmacological Specificity of ABC Protein Modulators

Due to their specificity for the sulfonylurea receptor in humans, glibenclamide and KCOs have been developed for clinical use in patients (see, e.g., Demolombe and Escande, 1996; Lawson, 1996; Lazdunski, 1996; Panten et al., 1996; Luzi and Pozza, 1997). Essentially, the hypoglycemic effect of glibenclamide results in the inhibition of the sulfonylurea receptor, leading to the stimulation of insulin secretion. It is also well established that KCOs are powerful vasodilator agents that directly open ATP-sensitive K^+ channels. Recent electrophysiological and molecular evidence has shown that the SUR is the KCO receptor, with the C-terminal portion of SUR affecting the binding affinity (Forestier et al., 1996; Schwanstecher et al., 1998). Concerning anion channels, glibenclamide is also a potent blocker of the CFTR (Sheppard and Welsh, 1992); however, it only weakly affects the volume-sensitive Cl^- channel $I_{Cl\ swell}$ (20% inhibition at 0.1 mM glibenclamide; Meyer and Korbmacher, 1996). Finally, these compounds are widely used to characterize ABC proteins coupled to ion channels. In plants, the control of stomatal movements by glibenclamide and more especially by KCOs (some of which are more efficient than ABA) is high (Leonhardt et al., 1997). The specificity of these modulators is demonstrated by the fact that KCO concentrations necessary for a half physiological effect in guard cells (0.4 to 0.9 nM; Leonhardt et al., 1997) are even lower than those

described in animal systems for inhibition of the SUR (e.g., Cook and Quast, 1990; Schwanstecher et al., 1998).

Comparison between Guard Cell Anion Channels and Mammalian ABC Proteins

It has been reported previously that guard cell anion channels are possible CFTR homologs (Schmidt et al., 1995; Schulz-Lessdorf et al., 1996). In animal cells, specific anion channel inhibitors are used to distinguish the CFTR from other outward-rectifying chloride channels. The CFTR is blocked by glibenclamide and DPC but is insensitive to 4,4′-diisothiocyanostilbene-2-2′-disulfonic acid (DIDS; see Table 1). In this study, we observed a similar pharmacological profile of inhibition for guard cell slow anion channels, which are blocked by DPC and glibenclamide (Figure 2) but not by DIDS (Schroeder et al., 1993; Forestier et al., 1998b). The CFTR and slow anion channels, in contrast to rapid anion channels, are also very similar in terms of voltage dependence. Moreover, slow anion channels are strongly activated by ATP-dependent phosphorylation, and CFTR is activated by protein kinase A phosphorylation, whereas rapid anion channels can be activated by ATPγS, the nonhydrolyzable analog of ATP (see Table 1). Under our conditions, we never observed a current exhibiting a rapid anion profile. Because the distinction between the rapid and the slow anion channels has not been ascertained, a switch between the two gating modes has been proposed (Dietrich and Hedrich, 1994). We can conclude that the pharmacological compounds tested in this study are at least specific to the S-type anion channels.

However, one discrepancy remains when comparing the slow anion channel and CFTR. Glibenclamide inhibition is reversed by the KCO cromakalim, as shown in Figure 3, whereas other KCOs (diazoxide, minoxidil sulfate, or BRL 38227 [levcromakalim]) are known to block the CFTR (Sheppard and Welsh, 1992). Because NPPB may or may not inhibit the CFTR, depending on the tissue, this blocker cannot be used when comparing these different chloride channels. Taken together, these results suggest that the guard cell slow anion channel is tightly regulated by an ABC protein having at least one sulfonylurea binding site, as described previously for the SUR and the CFTR (Inagaki et al., 1995; McNicholas et al., 1996). To our knowledge, among ABC transporters, neither the AMP-activated chloride conductance EBCR, which recently was suggested to regulate Cl^- channels (van Kuijck et al., 1996), nor the multispecific organic anion transporter CMOAT (Madon et al., 1997) has been shown to be inhibited by glibenclamide.

Interaction between K^+ and Anion Channels

Several studies indicate that sulfonylurea sensitivity may be conferred or enhanced by the interaction of a channel protein with modulatory subunits. For example, the chloride channel CFTR enhances sulfonylurea sensitivity of ATP-sensitive ROMK2 (for rat renal outer medulla K^+) channels (McNicholas et al., 1996) and of the inward rectifier Kir6.1 and of Kir1.1a (Ishida-Takahashi et al., 1998; Ruknudin et al., 1998, respectively). Such a connection could explain our previous observation that the guard cell outward-rectifying K^+ channel is inhibited by sulfonylureas (Leonhardt et al., 1997). This interaction may constitute a pathway for the regulation of a coordinate efflux via outward K^+ and anion channels during stomatal closure. In this case, this coordinate efflux would mimic the one observed under physiological conditions for the CFTR, the outward-rectifying Cl^-

Table 1. Biophysical and Pharmacological Properties of the Slow and Rapid Guard Cell Anion Channels Compared with the Mammalian Chloride Channel CFTR

Properties	CFTR	Slow Anion Channel	Rapid Anion Channel
Conductance	5 to 10 pS (Tabcharani et al., 1991)	5 pS (Schroeder and Keller, 1992); 36 pS (Schmidt et al., 1995)	>25 pS (Schroeder and Keller, 1992)
Voltage dependent	Weakly (Fischer and Machen, 1994)	Weakly (Schroeder and Keller, 1992)	Highly (Schroeder and Keller, 1992)
Activation	ATP and protein kinase A (Cheng et al., 1991; Bear et al., 1992)	ATP-dependent phosphorylation (Schmidt et al., 1995)	ATP, ATPγS (Hedrich et al., 1990)
Inhibitors			
DPC	Yes (Schwiebert et al., 1994)	Yes[a]	ND[b]
Glibenclamide	Yes (Sheppard and Welsh, 1992)	Yes[a]	ND
Cromakalim	Yes (Sheppard and Welsh, 1992)	No[a]	ND
DIDS	No (Cliff and Frizzell, 1990; Cliff et al., 1992)	No (Schroeder et al., 1993)	Yes (Marten et al., 1993)
NPPB	Yes (Haws et al., 1994)/no (Cunningham et al., 1992)	Yes (Schroeder et al., 1993)	Yes (Marten et al., 1992)

[a] Demonstrated in this article.

[b] ND, not determined.

channels, and the Na^+ channels in epithelial cells (reviewed in Higgins, 1995).

ABC Proteins and Hormonal Signaling

It has been demonstrated that stomatal closure can be induced by elevating cytosolic Ca^{2+} (Schwartz, 1985), resulting in the activation of slow anion channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990). We report here that in the presence of high external Ca^{2+}, glibenclamide still was able to suppress stomatal closure triggered by Ca^{2+} (Figure 1). This result points to the fact that an ABC protein is involved in the induction of stomatal closure by Ca^{2+}. When ABA was used rather than Ca^{2+} to trigger stomatal closure (Figure 5), the addition of glibenclamide partially alleviated the effect of ABA. This is in agreement with the observation that glibenclamide-inhibited currents are reactivated rapidly after application of a large concentration of ABA (Figure 5A), demonstrating an interaction between ABA signaling and ABC protein modulators in the regulation of slow anion channels. An interaction between ABA and ABC protein also has been found in the aquatic plant *Spirodela polyrrhiza.* In this higher eukaryote, the application of ABA results in the accumulation of the transcript encoding the ABC protein TUR2, with this induction being repressed by kinetin (Smart and Fleming, 1996).

In our study, glibenclamide efficiently regulates slow anion channels, resulting in partial block of the current and allowing us to observe a direct and rapid reactivation of the current when ABA was added. This work reports the activation by ABA of slow anion channels in fava bean guard cell protoplasts. Indeed, despite many correlative studies, direct evidence for ABA activation of slow anion channels was lacking for this plant species (Schroeder and Hagiwara, 1989; Schmidt et al., 1995; Schwartz et al., 1995; Schwarz and Schroeder, 1998). One can suppose that the enzymatic treatment used by all groups to obtain guard cell protoplasts leads to complete activation of the slow anion current. Thus, under these conditions, only a partial block of the slow anion current, described here with glibenclamide, allows the subsequent ABA activation of the current (Figure 5).

The demonstration that glibenclamide can antagonize a high calcium concentration (Figure 1) and partially prevents the ABA-induced stomatal closure (Figure 5) confirms that ABA can lead to stomatal closure via a Ca^{2+}-independent pathway, as previously suggested in numerous studies (MacRobbie, 1990; Gilroy et al., 1991; Allan et al., 1994). Even in the absence of water stress, stomatal conductance is regulated by low concentrations of ABA (Koornneef et al., 1984; Cousson and Vavasseur, 1998). Under such normal hydric conditions, the role of this ABC protein could be crucial because its inhibition, mimicked in this study by the application of glibenclamide, inhibits the effect of ABA.

Finally, our data demonstrate that at least one putative ABC protein interacts with an anion channel and hormonal signals in plants. Based on the similarities described above for slow anion channels and the CFTR (Table 1), we could hypothesize that the molecular nature of the guard cell slow anion channel is similar to that of the animal CFTR, because both mediate the sulfonylurea block of the outward K^+ channel and are regulated by DPC and the sulfonylurea glibenclamide (Figure 6). These similarities could be used to design new strategies for the identification of guard cell anion channels. However, the participation of a rather different ABC protein could not be excluded, as demonstrated by the high efficiency of KCOs in plants compared with animals. Despite the fact that a molecular identification of this ABC protein has yet to be obtained, our experiments provide novel insights into slow anion channel regulation and underscore a role for ABC proteins in plant ion channel control and in the Ca^{2+} and ABA signaling cascades.

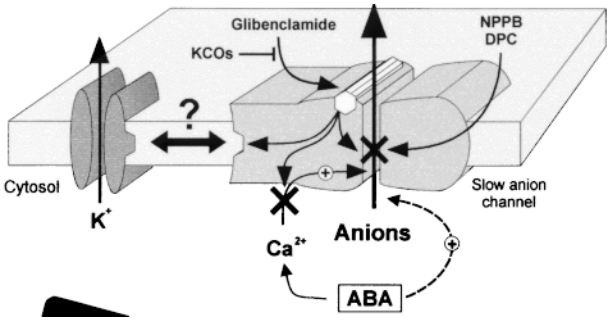

Figure 6. Hypothetical Model for the Coordinate Control of Outward K^+ Channels and Slow Anion Channels in Guard Cells by Sulfonylurea.

Slow anion channels are weakly voltage dependent and known to be activated by ATP-dependent phosphorylation. Here, we have described that these channels are blocked by DPC. Moreover, extracellular application of glibenclamide inhibits the slow anion current and is reversed by cromakalim (KCOs), suggesting that similar to that in animal cells, the receptor for these chemicals could be the same. Glibenclamide also suppresses the Ca^{2+} induction of stomatal closure, impairs the response to ABA, and could favor a protein–protein interaction between the slow anion channel and the outward K^+ channel, conferring to this latter protein a sensitivity to sulfonylurea, as described previously (Leonhardt et al., 1997). X's represent pathways blocked by glibenclamide, NPPB, or DPC. The double-headed arrow indicates the possible interaction between the slow anion and outward K^+ channels.

METHODS

Plant Material and Epidermal Strip Experiments

Plants were grown as described previously (Leonhardt et al., 1997). Bioassays were conducted using *Commelina communis* and fava bean epidermal peels, as reported previously (Leonhardt et al.,

1997). Incubation solutions were 10 mM Mes (pH 6.2) and 60 or 30 mM KCl for *C. communis* or fava bean, respectively.

Guard Cell Isolation and Patch–Clamp Recordings

Guard cell protoplasts were isolated from leaves of 3- to 4-week-old fava bean plants by using enzymatic digestion (Leonhardt et al., 1997) and subsequently were used for patch–clamp experiments. The whole-cell patch–clamp technique was performed to record anion currents specific from slow anion channels in the plasma membrane of fava bean guard cell protoplasts. Whole-cell recordings were obtained using the experimental setup described previously (Leonhardt et al., 1997). After whole-cell configuration (access resistance between 1 and 5 GΩ), the membrane potential was held to 0 mV, and stimulation was applied 5 min later. The voltage protocol consisted of stepping the membrane potential from 0 to −120 mV for 50 sec. To ensure the time stability of the whole-cell anion current, we applied several stimulations for at least 10 min before we applied a modulator. Ion channel modulators were dissolved in the bath solution and perfused by gravity into a 500-μL recording chamber. The bath solution was exchanged (10-fold as much volume as the recording chamber) in <2 min. Inhibition percentage of slow anion current was measured at the peak current. To optimize recordings of Ca^{2+}-activated slow anion channels (Schroeder and Hagiwara, 1989), the bath solution contained 10 mM $CaCl_2$, 30 mM CsCl, 10 mM Mes, and 2 mM $MgCl_2$ and was adjusted to a final osmolality of 460 mmol/kg with sorbitol at pH 5.5. Except as given for Figure 2A, the pipette solution, which equilibrates with the cytosol of guard cells, contained 5 mM ATP–Tris, 3.35 mM $CaCl_2$, 150 mM CsCl, 6.7 mM EGTA, 200 μM GTPγS, 10 mM Hepes, and 2 mM $MgCl_2$ (pH 7.05, osmolality 470 mmol/kg). Under these conditions, the Cl^- equilibrium potential was 27.7 mV. Experiments were performed at room temperature (22 ± 2°C).

Pclamp 6.0.2 software (Axon Instruments, Foster City, CA) was used for voltage pulse, stimulation, and on-line acquisition. Plot and curve fittings were done using Origin 4.0 (MicroCal Software, Northhampton, MA).

Chemicals

Chemicals were purchased from Sigma with the exception of BRL 38227, which was purchased from SmithKline Beecham (Worthing, UK). Abscisic acid (±)-*cis*,*trans* isomer and anion channel blockers, such as 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and anthracene-9-carboxylic acid (9-AC), were dissolved in ethanol. Sulfonylurea and potassium channel opener stock solutions were prepared as previously described (Leonhardt et al., 1997). The final ethanol or dimethyl sulfoxide concentration in the incubation solution never exceeded 0.5% (v/v) and did not affect the anion channel activity.

ACKNOWLEDGMENTS

We thank Dr. Georges Banting (Department of Biochemistry, University of Bristol, UK) and Dr. Susumo Seino (Center for Biomedical Science, Chiba, Japan) for critical reading of the manuscript. This work was supported by the Commissariat à l'Energie Atomique.

Received October 26, 1998; accepted March 29, 1999.

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