J. Membrane Biol. 153, 105–116 (1996)

The Journal of
Membrane
Biology

Effects of Internal K^+ and ABA on the Voltage- and Time-dependence of the Outward K^+-rectifier in *Vicia* Guard Cells

F. Lemtiri-Chlieh
Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA

Received: 1 December 1995/Revised: 8 May 1996

Abstract. One of the main effects of abscisic acid (ABA) is to induce net loss of potassium salts from guard cells enabling the stomata to close. K^+ is released from the vacuole into the cytosol and then to the extracellular space. The effects of increasing cytosolic K^+ on the voltage- and time-dependence of the outwardly rectifying K^+-current ($I_{K,out}$) in guard cell protoplasts (GCP) was examined in the whole-cell configuration of the patch-clamp technique. The same quantitative analysis was performed in the presence of ABA at different internal K^+ concentrations ($[K^+]_i$). Varying $[K^+]_i$ in the patch pipette from 100 to 270 mM increased the magnitude of $I_{K,out}$ in a nonlinear manner and caused a negative shift in the midpoint ($V_{0.5}$) of its steady-state activation curve. External addition of ABA (10–20 μM) also increased the magnitude of $I_{K,out}$ at all $[K^+]_i$, but caused a shift in $V_{0.5}$ of the steady-state activation curve only in those GCP loaded with 150 mM internal K^+ or less. Indeed, $V_{0.5}$ did not shift upon addition of ABA when the $[K^+]_i$ was above 150 mM and up to 270 mM, i.e., the shift in $V_{0.5}$ caused by ABA depended on the $[K^+]_i$. Both increase in $[K^+]_i$ and external addition of ABA, decreased (by ≈ 20%) the activation time constant (τ_n) of $I_{K,out}$. The small decrease in τ_n, in both cases, was found to be independent of the membrane voltage. The results indicate that ABA mimics the effect of increasing cytoplasmic K^+, and suggest that ABA may increase $I_{K,out}$ and alter $V_{0.5}$ of its steady-state activation curve via an enhancement in cytosolic K^+. This report describes for the first time the effects of $[K^+]_i$ on the voltage- and time-dependence of $I_{K,out}$ in guard cells. It also provides an explanation for the quantitative (total membrane current) and qualitative (current kinetics) differences found between intact guard cells and their protoplasts.

Correspondence to: F. Lemtiri-Chlieh

Key words: Outward K^+ current — Abscisic acid — Internal K^+ concentration — Voltage-dependence — Time-dependence — Guard cell protoplast — *Vicia faba*

Introduction

The plant hormone abscisic acid (ABA), produced during conditions of water stress, plays an important role in regulating stomatal movement. ABA inhibits stomatal opening and promotes stomatal closing, thereby reducing transpirational water loss. The mechanisms by which ABA promotes stomatal closing have been extensively studied during the last decade (for reviews, *see* MacRobbie, 1991; Blatt, 1991; Blatt & Thiel, 1993; Assmann, 1993; Ward, Pei & Schroeder, 1995). It is believed that this effect is achieved through a net loss from the guard cells of osmotically active solutes, mainly in the form of potassium salts.

On the question of the origin of this K^+, earlier tracer flux studies, measuring rate of loss of $^{86}Rb^+$ from isolated guard cells of *Commelina communis*. L., have shown that the amount of tracer lost during the ABA transient is greater than the cytoplasmic content at the time of the change (MacRobbie, 1981). Further flux work (MacRobbie, 1990), has also shown a biphasic transient response of $^{86}Rb^+$ efflux from intact guard cells after short periods of applications of ABA. The second (or slow) component of this biphasic response was attributed to the release of $^{86}Rb^+$ from the vacuole into the cytoplasm, following activation of vacuolar K^+ channels. The evidence for such speculation was the absence of the slow component of the efflux transient in tissue loaded for only a short period of time, with little or no tracer in the vacuole. In a recent report, Ward and Schroeder (1994) showed evidence for the existence in the tonoplast of voltage-independent K^+-selective channels (VK). These VK channels have a conductance of 70 pS

in symmetrical 100 mM KCl and are highly selective for K^+ over NH_4^+ and Rb^+. They are activated by physiological concentrations of free cytoplasmic Ca^{2+} (1 μM) and are downregulated by cytoplasmic alkalinization. An additional mechanism by which K^+ ions are flooded into the cytoplasm is via vacuolar anion channels (known as slow vacuolar or S-type channels) which are less selective for K^+ than the VK channels but have been proposed to contribute to long term K^+ release (Ward & Schroeder, 1994; Ward et al., 1995). During stomatal closure, K^+ flooding from the vacuole, will cross the plasma membrane towards the extracellular space through outwardly rectifying voltage-dependent K^+ channels (Schroeder, Hedrich & Fernandez, 1984; Schroeder, Raschke & Neher, 1987; Schroeder, 1988; Blatt, 1987, 1990; Fairley-Grenot & Assmann, 1992*b*, Thiel, MacRobbie & Blatt, 1992; Lemtiri-Chlieh & MacRobbie, 1994). The outward K^+ current ($I_{K,out}$) is insensitive to changes in free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Schroeder & Hagiwara, 1989; Blatt, Thiel & Trentham, 1990), and its increase following ABA treatment still occurs even in the presence of Ca^{2+} chelators such as EGTA and BAPTA (Lemtiri-Chlieh & MacRobbie, 1994). ABA increases cytoplasmic pH (Irving, Gehring & Parish, 1992). $I_{K,out}$ is potentiated by cytoplasmic alkalinization (Blatt, 1992) and the ABA-evoked increase of this current is blocked when changes of cytoplasmic pH (pH_i) are suppressed (Blatt & Armstrong, 1993).

In addition to the effects on $I_{K,out}$, ABA is also known to increase $[Ca^{2+}]_i$ (McAinsh, Brownlee & Hetherington, 1990, 1992; Irving et al., 1992). Lemtiri-Chlieh & MacRobbie (1994) showed an ABA-induced reduction of the inwardly rectifying K^+ current ($I_{K,in}$) in the complete absence of external Ca^{2+}. Thus, release of Ca^{2+} from internal stores is capable of producing the increase in cytoplasmic Ca^{2+}, but this cannot rule out a concomitant influx of external Ca^{2+} in normal conditions i.e., in the presence of external calcium (Schroeder & Hagiwara, 1990). Further, when caged IP_3 is injected into guard cells, upon photolysis, there is an increase in $[Ca^{2+}]_i$ resulting in stomatal closure (Gilroy, Read & Trewavas, 1990) as well as an inhibition of $I_{K,in}$ (Blatt et al, 1990). These effects of IP_3 are parallel to those of ABA and suggest that IP_3 is involved in ABA-induced stomatal closure. There are recent reviews discussing a possible link between ABA and IP_3 turnover in guard cells (MacRobbie, 1992; Assmann, 1993; Blatt & Thiel, 1993). However, it remains uncertain that calcium release from the vacuole is an essential and obligatory step to close the stomatal pore in response to ABA treatment (Gilroy et al., 1991; Allan et al., 1994).

One singular property of plant outward K^+ channels, which distinguishes them from their animal counterpart, (i.e., the delayed potassium channels), is the ability to sense external K^+ and modify their voltage- and time-dependent properties accordingly (Blatt, 1988, 1990; Schroeder, 1988; Vogelzang & Prins, 1994; White & Lemtiri-Chlieh, 1995; Roberts & Tester, 1995). However, this behavior is not novel to ion channels in the plant kingdom, the anomalous inward potassium rectifier (''I_{K1}'') which exists in most animal cells, exhibits such peculiar and fine molecular regulation (Hagiwara & Yoshii, 1979; Sakmann & Trube, 1984). One of the main roles of I_{K1} in animal cells is to maintain the resting potential near the equilibrium potential for K^+ (E_K). In intact guard cells as well as their protoplasts, increasing external K^+ concentration ($[K^+]_o$) shifts the steady-state current- and conductance-voltage characteristics (G_K-V) with E_K to more positive voltages. This implies that opening of the outward K^+ channel will depend, not only on the membrane voltage and the electrochemical driving force for K^+, but also on the extra information of K^+ availability outside of the cell (*see* Blatt, 1991). Nonetheless, is should be noted that contradictory results were found in mesophyll cell protoplasts isolated from traplobes of *Dionaea muscipula* (Iijima & Hagiwara, 1987). The G_K-V relationship of $I_{K,out}$ in these cells was shown to be insensitive to changes in $[K^+]_o$. By contrast, changes in $[K^+]_i$ was found to affect the outward rectification. The authors concluded that the current-voltage relation of $I_{K,out}$ in these mesophyll cells depended only on the membrane voltage and the $[K^+]_i$ but not on $[K^+]_o$.

On the other hand, nothing is known about the effects of internal K^+ on the voltage- and time-dependence properties of $I_{K,out}$ in guard cells. This report addresses this complementary, but nonetheless crucial question, of the importance of internal K^+ ions in regulating outward K^+ channel in guard cells. Using the whole-cell patch clamp technique, guard cell protoplasts were loaded with varying internal K^+ concentrations and the effects on the voltage- and time-dependence of $I_{K,out}$ were assessed. Moreover, the effect of ABA at different $[K^+]_i$ on the voltage- and time-dependent properties of $I_{K,out}$ has been also studied and the results were compared to those obtained with varying only $[K^+]_i$.

Materials and Methods

Protoplast Isolation

Vicia faba L. *cv* (Bunyan) Bunyan Exhibition was grown on vermiculite under conditions described previously (Blatt, 1987). Guard cell protoplasts (GCP) were isolated from abaxial epidermal strips of 3 to 4 week old leaves following a procedure described previously (Lemtiri-Chlieh & MacRobbie, 1994). Briefly, epidermal strips were floated on 10-ml medium containing 1.8–2.5% of Cellulase Onozuka RS (Yacult Honsha, Japan), 1.7–2% Cellulysin (Calbiochem, Behring Diagnostics, La Jolla), 0.026 % Pectolyase Y-23, 0.26 % BSA, 1 mM $CaCl_2$ (pH 5.56

and osmolality 360 mOsm.Kg^{-1} adjusted with mannitol). Epidermal strips were incubated in a temperature controlled chamber at 28°C. The incubation medium was gently shaken with a Variomag shaker (Model R100, Luckham, Sussex, UK) at a frequently of 0.5 Hz. After 120 to 150 min, released protoplasts were purified by passage through a 30 μm mesh, kept on ice for 2 to 3 min before being centrifuged at 100 g for 4 min (at room temperature). The pellet consisting of guard cell protoplasts was resuspended and kept on ice in 1 or 2 ml of fresh medium containing 0.42 M mannitol, 10 mM 2-[N-Morpholino] ethanesulfonic acid (Mes), 200 μM $CaCl_2$, 2.5 mM KOH (pH 5.55 and osmolality 466 mOsm.Kg^{-1}).

SOLUTIONS

Protoplasts were placed in a 0.5 ml chamber. Once the protoplasts settled to the bottom of the chamber, a bath solution containing (in mM): 10–12 K^+ glutamate, 0.5–1 $CaCl_2$, 2 $MgCl_2$, 5 Mes, pH 5.5 (adjusted with KOH) and osmolality 480–500 mOsm.Kg^{-1} (adjusted with mannitol) was perfused continuously at a rate of 3–4 chamber volumes per minute. Patch pipettes were filled with varying concentrations of K^+ glutamate (to give final concentrations of 100, 150, 170, 200 and 270 mM K^+), 2 mM ATP (Mg or K salt), 2 mM $MgCl_2$, 10 mM N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH was adjusted to 7.8 (unless otherwise stated) with KOH; osmolality was set to 520–540 mOsm.Kg^{-1} (adjusted with mannitol). Abscisic acid was added externally at concentrations between 10 and 20 μM. All chemicals were from Sigma Chemical, Poole, Dorset, UK.

CURRENT-VOLTAGE RECORDING AND ANALYSIS

Patch pipettes (5–10 MΩ) were pulled from Kimax-51 glass capillaries (Kimble 34500; Kimble, Owens-Illinois) using a two stage puller (Narishige PP-83, Japan). All the experiments were performed at room temperature (20 to 22°C) using the standard whole-cell patch clamp technique (Hamill et al., 1981). To monitor cell currents, a patch clamp amplifier Dagan 3900A Integrating Patch Clamp (Dagan Corporation Minneapolis, MN) was used.

Voltage commands and simultaneous signal recordings and analysis were assessed by a microcomputer (Tandon 386/16) connected to the amplifier via a multipurpose I/O device (LM-125) using Bioclamp software (Biologic, Claix, France). After Giga-Ohm seals were formed, obtaining whole-cell configuration was achieved by breaking the underlying membrane using gentle suction, after which the membrane was immediately clamped to voltages close to the Nernst equilibrium potential for K^+ (E_K). GCP were left for 3–5 min before starting any current measurements. All current traces shown were low-pass filtered at 2 kHz before analog-to-digital conversion and were uncorrected for leakage current or capacitative transients. Membrane potentials were corrected for liquid junction potential (−17 to −20 mV) as described by Neher (1992). Nernst equilibrium potentials were calculated after correction for ionic activities (estimated by GEOCHEM-PC software). All the current-voltage relationships were plotted as the time-dependent current (i.e., the steady-state current minus the instantaneous current) *vs.* test potential. Data are expressed as means ±SEM.

Results

VOLTAGE- AND TIME-DEPENDENCE CHARACTERISTICS OF $I_{K,OUT}$ IN *VICIA* GUARD CELL PROTOPLASTS

As shown in previous reports, stepping the membrane potential of guard cells (intact cells or their protoplasts) to values positive of the Nerstian equilibrium potential for K^+ (E_K), activates time- and voltage-dependent outward K^+ currents (Schroeder et al., 1987; Schroeder, 1988; Blatt, 1988, 1990; Hosoi, Iino & Shimazaki, 1988; Fairley-Grenot & Assmann, 1992b; Thiel et al., 1992; Lemtiri-Chlieh & MacRobbie, 1994).

In Fig. 1*A*, an example of a depolarizing family of voltage-steps to voltages more positive to E_K elicited slowly activating potassium outward-rectifying currents ($I_{K,out}$). The membrane was depolarized from −49 to +61 mV in 5 mV increments using 600 msec long square test pulses. Figure 1*A* (upper side) shows current recording of $I_{K,out}$, obtained in response to the voltage protocol described above from one guard cell protoplast (GCP), bathed in 12 mM K^+ and 0.5 mM Ca^{2+} (pH_o 5.56), with 200 mM K^+ and nominal zero calcium (pH_i 7.71) in the patch pipette. The current traces develop with a slow, sigmoidal time course at low test voltage steps, but they tend to develop more rapidly at higher depolarizing test voltages and eventually reach a steady-state value after 500 to 600 msec.

The activation time constant (τ_n) of the current traces shown in Fig. 1*A* were analyzed using Hodgkin-Huxley (HH) type model (Schroeder, 1989; Fairley-Grenot & Assmann, 1993; Van Duijn, 1993; White & Lemtiri-Chlieh, 1995)

$$y = I_i + I_{ss}\,(1\text{-}\exp\,(-t/\tau_n))^p \qquad (1)$$

I_i and I_{ss} are the instantaneous and steady-state currents respectively, p is the number of independent membrane-bound gating particles (or gates) which control the opening of the channel.

To estimate the value of p, current traces obtained from a large number of different GCP at two voltage steps (+21 and +41 mV) were fitted to Eq. (1). Table 1 shows the results obtained from a total number of 32 data fittings. The current traces shown in Fig. 1*A* were then fitted to the HH model to determine τ_n using a fixed value of $p = 2$. The result of τ_n as a function of the membrane test voltage (from −19 to +61 mV in 5 mM increments) is shown in Fig. 1*B*, which highlights a strong voltage-dependence of τ_n (an example of a current trace recorded at +26 mV to which Eq. (1) was fitted is presented in the insert). The values of τ_n decreased from 185 msec at −19 mV to 65 msec at +61 mV following a single exponential function (Eq. (2)) of the same type as described by Van Duijn (1993):

$$\tau_n = \tau_{n,min} + [\tau_o \times \exp(-V \times k)] \qquad (2)$$

($\tau_{n,min}$ is the minimal τ_n and τ_o is the difference value of ($\tau_n - \tau_{n,min}$) at $V = 0$). The best fit was obtained for $\tau_{n,min} = 24$ msec, $\tau_o = 120$ msec and $k = 0.016$ mV^{-1}.

The steady-state *I-V* curve (Fig. 1*C*) has been constructed from the current traces shown in Fig. 1*A*, as the

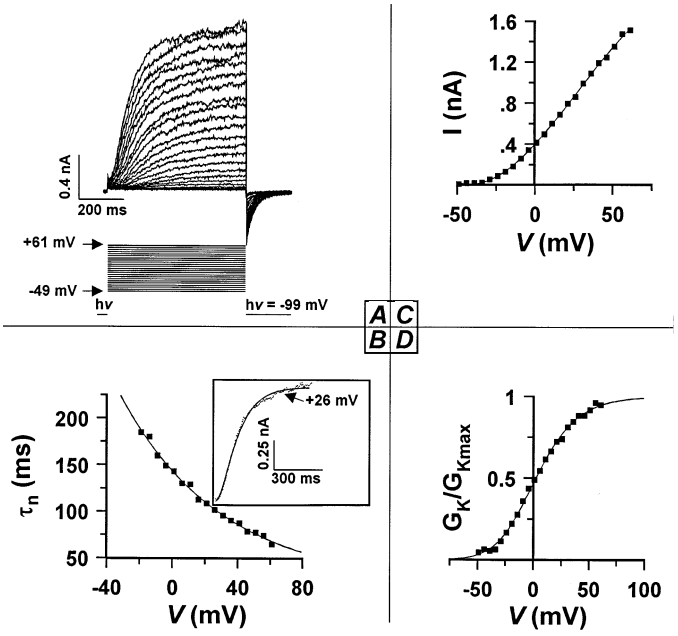

Fig. 1. Electrical properties of $I_{K,out}$. (*A*) A family of outwardly directed voltage-clamp current traces of $I_{K,out}$ (upper section) obtained in response to depolarizing test voltages (lower section) ranging in 5-mV steps from −49 to +61 mV; the holding potential (hν) is −99 mV. Tail currents traces (inwardly directed) in response to repolarization to −99 mV. The patch pipette solution contained 200 mM K^+. (*B*) Activation time constant parameter (τ_n) *vs.* test potential. The data points were obtained by fitting Eq. (1) (with $p = 2$; *see* text for details) to the current traces of $I_{K,out}$, shown in *A*, and for depolarizations ranging in 5 mV from −19 to +61 mV. An example of a current trace (obtained in response to a depolarization step of +26 mV) fitted to Eq. (1) is shown in the insert. Continuous line shows the fitted monoexponential curve (Eq. 2). (*C*) Current-voltage characteristic of the time-dependent component (steady-state minus instantaneous) of the $I_{K,out}$ traces shown in *A*. (*D*) Voltage-dependent steady-state activation (n_∞^2) curve for $I_{K,out}$ conductance. Values of G_K/G_{Kmax} were calculated from the current-voltage relationship shown in *C* (*see* text for further details). Continuous line shows the fitted Boltzmann distribution function (Eq. 3).

Table 1. Estimation of the value of p (*see* Eq. 1) at two membrane voltages

Voltage (mV)	Mean of p ± SEM	Range of p	(n)
+21	2.10 ± 0.10	1.60–2.91	(17)
+41	1.81 ± 0.15	1.03–2.93	(15)

difference between the steady-state current (I_{ss}: measured as a mean value at the end of the test pulse) and the instantaneous current (I_i: measured during the first 10 msec of the test pulse). The *I-V* characteristic shows that the current activates around −30 mV and increases with increasing voltage steps and that the curve is rectifying in the outward direction.

The voltage-dependent steady-state activation parameter (n_∞^p; with $p = 2$) which is shown in Fig. 1*D* was estimated from the steady-state *I-V* curve for the time-dependent currents. The K-conductance or chord conductance (G_K) for each test potential was calculated from the steady-state currents as:

$$G_K = I_K/(V - E_K)$$

I_K is the K^+ current at test potential V after subtracting the instantaneous current and E_K is the calculated Nerstian equilibrium potential. In this experiment E_K was −66.8 mV.

The steady-state value of the activation variable according to Hodgkin-Huxley (1952) model is: $n_\infty^2 = G_K/G_{Kmax}$ (where G_{Kmax} represents the maximum chord conductance) The data of n_∞^2 were then fitted to a Boltzmann distribution function of the type:

$$n_\infty^2 = 1/\{1 + \exp[(V_{0.5} - V)/S]\}^2 \quad (3)$$

where $V_{0.5}$ represents the voltage at which $n_\infty = 0.5$ ($n_\infty^2 = 0.25$) and S is a slope factor equivalent to RT/zF (where z is the valency of the gating charge and R,T and F have their usual meaning). The best fit was obtained for $V_{0.5} = -16.5 \pm 0.5$ mV and $S = 21.5 \pm 0.7$ mV.

The aim of the experiments described in this report is to investigate the effects of changing internal potassium concentration ($[K^+]_i$) on the voltage- and time-dependency of $I_{K,out}$ in *Vicia* guard cell protoplasts. Once established, these effects will be compared with those of ABA.

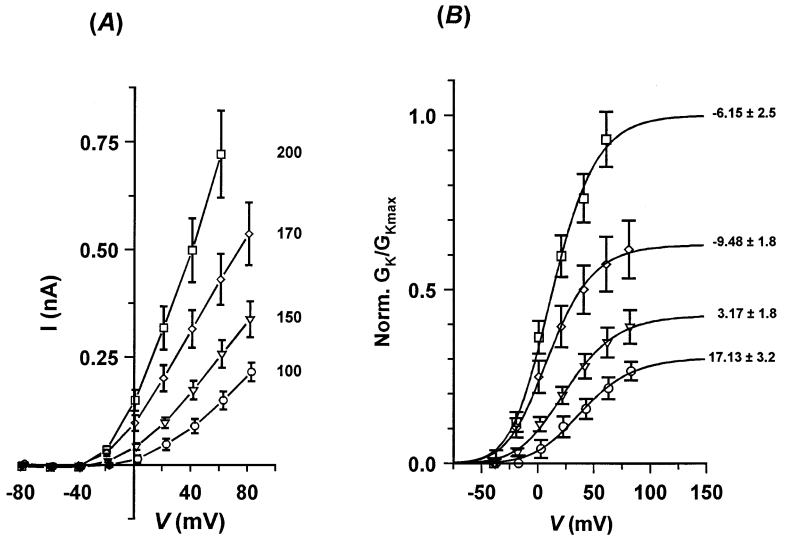

Fig. 2. Effect of internal K^+ on the voltage-dependent steady-state activation of $I_{K,out}$. (*A*) Averaged current-voltage relationships of $I_{K,out}$ obtained from a total number of 46 GCP loaded with four different internal patch pipette media, containing (in mM): 100 (○; $n = 8$), 150 (▽; $n = 12$), 170 (◇; $n = 17$) and 200 (□; $n = 9$) internal K^+. (*B*) Corresponding voltage-dependent steady-state activation curves (same conditions as mentioned in *A*). The curves were fitted to Boltzmann distribution functions (Eq. 3) and normalized to the same maximum conductance value (obtained for $[K^+]_i$ = 200 mM). Next to each curve, a value of the estimated (in mV) $V_{0.5}$ ± SEM, is given.

Effects of Changing the $[K^+]_i$ on the Voltage-dependent Steady-state Activation of $I_{K,out}$

Measurement of $I_{K,out}$ was performed on several GCP using different internal patch pipette media containing the desired concentration of K^+. The averaged results are gathered in Fig. 2*A* which represents four steady-state *I-V* characteristics of $I_{K,out}$ with different $[K^+]_i$ in the range 100–200 mM. The results indicate that the magnitude of $I_{K,out}$ increases steeply with increasing $[K^+]_i$. The increase is nonlinear, i.e., doubling the amount of $[K^+]_i$ from 100 to 200 mM almost quintuples the magnitude of $I_{K,out}$ measured around +60 mV. To assess the voltage-dependence of $I_{K,out}$ in response to increasing $[K^+]_i$, steady-state activation curves as a function of test potential *V* were constructed for each $[K^+]_i$ (Fig. 2*B*). The K-conductance for each test potential was calculated from the steady-state currents as described above. Values of E_K were −50.9, −59.9, −62.9 and −66.8 mV for $[K^+]_i$ of 100, 150, 170 and 200 mM respectively. The steady-state values of the activation variable n_∞^2 were calculated according to HH as described above. The values of $V_{0.5}$ and *S* were estimated by fitting a Boltzmann distribution function (Eq. 2) to the averaged values for each $[K^+]_i$. The values are given in Table 2. Increasing $[K^+]_i$ from 100 to 150 mM shifts $V_{0.5}$ by ≈ 14 mV. A larger shift (≈ 24 mV) is seen when increasing $[K^+]_i$ from 100 to 170–200. Thus, the data show that increasing $[K^+]_i$, and hence increasing the electrochemical driving force for K^+ efflux, will shift $V_{0.5}$ of the steady-state activation n_∞^2 of $I_{K,out}$ towards more negative voltages.

Effects of Changing the $[K^+]_i$ on the Sigmoid Activation of $I_{K,out}$

The sigmoid activation of $I_{K,out}$ first used by Hodgkin and Huxley (1952) to describe the delayed outward K^+-current of the squid giant axon, has been used successfully to characterize the activation parameter in plant cells (Schroeder, 1989; Fairley-Grenot & Assmann, 1993; Van Dujin, 1993; White & Lemtiri-Chlieh, 1995). In this study, the same approach was used to determine the voltage-dependent activation time constant, in different pre-set conditions where $[K^+]_i$ was varied from 100 to 270 mM.

Table 2. Values of $V_{0.5}$ and *S* (in mV) as estimated by fitting the data in Fig. 2*B* to Eq. (2)

	100 mM K_i	150 mM K_i	170 mM K_i	200 mM K_i
$V_{0.5}$ ± SE	17.13 ± 3.2	3.17 ± 1.8	−9.48 ± 1.8	−6.15 ± 2.5
S ± SE	26.0 ± 4.1	25.8 ± 2.4	22.7 ± 2.2	22.3 ± 3.4
(*n*)	(8)	(12)	(17)	(9)

Equation (1) was used with a fixed value of $p = 2$ (*see* above for details) to fit the current traces obtained at different voltages and in different $[K^+]_i$ inside the patch pipette. The results from a total number of 26 GCP are presented in Fig. 3*A*. The results indicate a small decrease of τ_n only when increasing $[K^+]_i$ from 100 to higher concentrations, but no significant change of τ_n is seen when increasing the $[K^+]_i$ in the range 150–270 mM.

To try to highlight the small difference seen in the activation time course between guard cells loaded with 100 mM K_i and the other guard cells loaded with 150 to 270 mM K_i, ratios were calculated (τ_n, K_i 100–270/τ_n, K_i 100), and plotted against the membrane test voltage. The results are shown in Fig. 3*B*. The left panel of the figure shows the individual averaged data points for each $[K^+]_i$ taken from Fig. 3*A*. The right panel of Fig. 3*B* shows the averaged ratio for all the guard cells loaded with 150 to 270 mM K_i. This figure shows no significant difference at all four membrane test voltages (+20 to +80 mV). From these calculations, a mean decrease value of τ_n

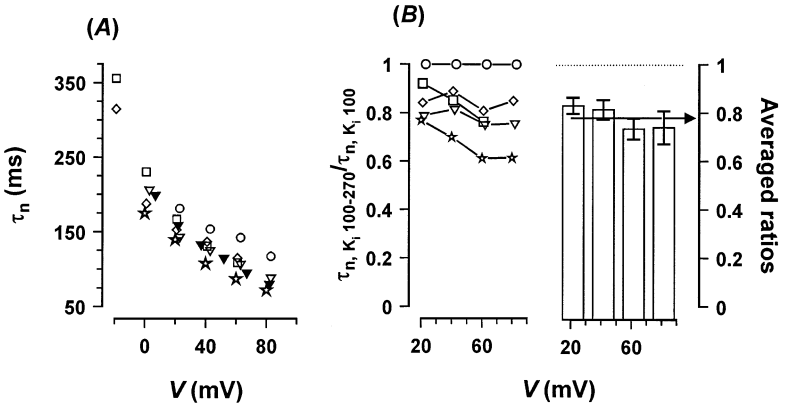

Fig. 3. Effect of internal K^+ on the sigmoidal activation time constant parameter (τ_n) of $I_{K,out}$. (*A*) τ_n as a function of test voltage and $[K^+]_i$. Each data symbol is an average measurement; for clarity SEM bar values have not been represented. Data are as follow (in mM K_i): (○), 100 (n = 7, SEM values are 26, 19.8, 15.4 and 13); (▽), 150 (n = 5, SEM values are 27.2, 17.7, 12.2, 10 and 9.5); (▼), 150 (n = 2, SEM values are 48.2, 47, 22.9, 32.3, 23.5, 20 and 14); (◇) 170 (n = 5, SEM values are 12.3, 11, 14.3, 11.3 and 12.8); (□) 200 (n = 5, SEM values are 27.7, 12.5, 8.6, 6.5 and 8.5) and finally (☆), 270 (n = 2, SEM values are 0.7, 7.68, 15.3, 13.6 and 17.1). (*B*) Left panel: calculated ratios (τ_n, K_i 100–270/τ_n K_i 100) as a function of test voltage and $[K^+]_i$. Symbol code as in *A*. Right panel: Averaged ratios (τ_n, K_i 150–270/τ_n, K_i 100) as a function of test voltage only; the arrow indicate the calculated mean decrease value (≈ 20%) for all test voltages.

came to around 20% when increasing $[K^+]_i$ from 100 mM to 150–270 mM.

Effects of ABA on the Voltage-dependent Steady-state Activation of $I_{K,out}$

To compare the results obtained earlier when varying $[K^+]_i$ with the effects of ABA on guard cells, experiments were performed (12 in total) by adding ABA (10–20 μM) to the bath medium to the same GCP. The *I-V* relationships (from which n_∞^2 was calculated) were measured before and after installation of the effect of ABA. The hormone was added to whole patch-clamped GCP loaded with five different $[K^+]_i$: 100 (n = 3), 150 (n = 2), 170 (n = 3), 200 (n = 2) and 270 mM (n = 2). Figure 4 illustrates an example of a family of current traces obtained by applying 600 msec long square test pulses (−38 to + 82 mV in 20 mV increments) to a GCP loaded with 150 mM K^+ before and after 17 min of adding 10 μM ABA. Steady-state activation curves of $I_{K,out}$ for three different $[K^+]_i$ were constructed and the results are shown in Fig. 5. The estimated $V_{0.5}$ obtained by fitting Boltzmann distributions to each curve are summarized in Table 3. ABA shifts $V_{0.5}$ of the steady-state activation curves by 21 mV when GCP are loaded with 100 mM K_i and to a less extent (13 mV) when they are loaded with 150 mM K_i, but no significant shift was measured when the GCP were loaded with 200 mM K_i. A total of five other GCP loaded respectively with 170 (n = 3) and 270 mM K_i (n = 2) showed no significant shift of $V_{0.5}$ when challenged with ABA (*see* Fig. 8*B*, insert). Thus, the shift in $V_{0.5}$ induced by external ABA application seems to depend strongly on the K^+ content of the GCP before the addition of ABA. The second main effect of ABA was to increase G_K in all K_i concentrations tested but the increase was so variable from cell to cell, and even at any one single $[K^+]_i$ used, that it was difficult

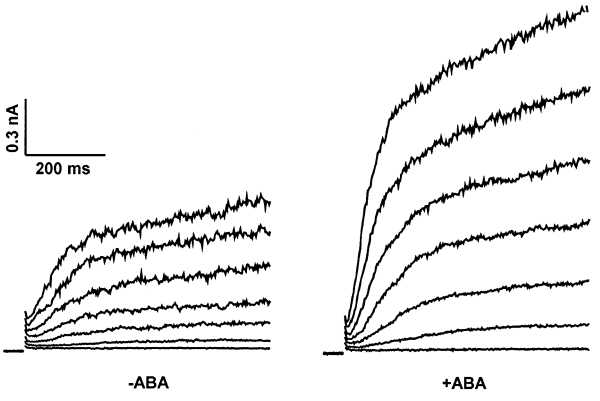

Fig. 4. Effect of ABA on $I_{K,out}$. Left panel: A family of control $I_{K,out}$ current traces obtained in response to depolarizing test voltages ranging in 20 mV steps from −38 to +82 mV; the holding potential (hν) was −68 mV. Right panel: A family of $I_{K,out}$ current traces obtained in response to the same voltage protocol; the current traces were recorded from the same GCP (as in left panel), 17 min after adding 10 μM ABA to the bath medium.

to detect significant differences in small groups of cells (*see* the results in Table 4). However, a clear pattern could be detected when the data were divided into two sub-groups, i.e., low K_i (100–150 mM) and high K_i (170–270 mM). Indeed, in cells loaded with 100–150 mM K_i (n = 5), the mean increase in G_K in the presence of ABA was calculated to be 2.4 ± 0.24 (ranging from 1.6 to 3) while in cells loaded with 170–270 mM K_i (n = 7) the mean increase in G_K turned out to be 1.81 ± 0.17 (ranging from 1.28 to 2.43). This distinction between low and high K_i is also seen in the effects of ABA on $V_{0.5}$ (*see* Fig. 8*B*, insert). Thus, the effects of ABA on the steady-state activation n_∞^2 of $I_{K,out}$ compare well with the effects of varying internal K^+ shown in Fig. 2*B* and Table 2.

Figure 6 represents an experiment which was performed with internal K^+ set to 270 mM while 37 mM K^+

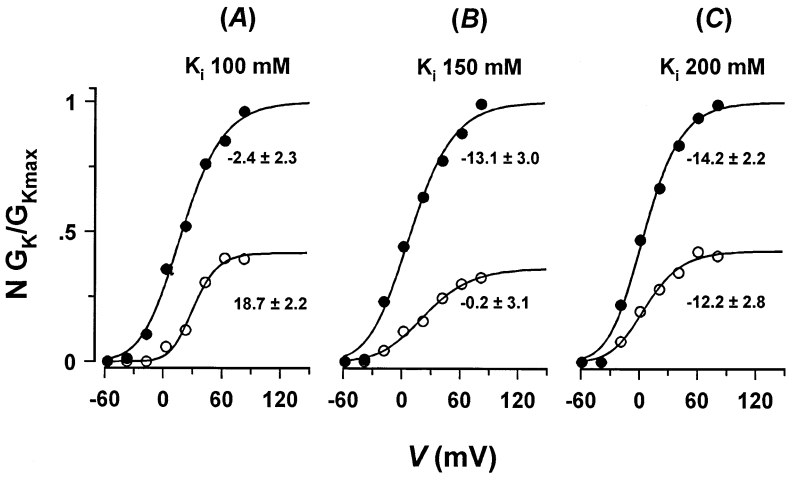

Fig. 5. Effect of ABA on the voltage-dependent steady-state activation of $I_{K,out}$. Normalized G_K/G_{Kmax} are plotted as a function of test voltage before (○) and after (●) adding ABA to the bath medium (10 μM was added in all experiments except for *A* where 15 μM was used); current recordings of $I_{K,out}$ were taken after ABA effect reach steady-state (usually 10 to 20 min). Three GCP loaded with three different $[K^+]_i$ are shown (in mM K^+): (*A*) 100, (*B*) 150 (*see* the original data in Fig. 4) and (*C*) 200.

Table 3. Values of $V_{0.5}$ and S (in mV) before and after ABA as estimated by fitting the data in Fig. 5 to Eq. (2)

	100 mM K_i	150 mM K_i	200 mM K_i
$V_{0.5}$ −ABA	18.7	−0.2	−12.2
$V_{0.5}$ +ABA	−2.4	−13.1	−14.2
S −ABA	14.3	27.8	21.6
S +ABA	24.4	25.5	22.1

was present in the bath medium (instead of the usual 10–12 mM). Current traces before and after ABA are shown and the corresponding voltage-dependent steady-state activation is represented in the insert. The aim of this experiment is to draw attention to the peculiar, yet classic effect, of external K^+ on the voltage-dependency of $I_{K,out}$ (Blatt, 1988, 1990; Schroeder, 1988; White & Lemtiri-Chlieh, 1995; Roberts & Tester, 1995). As shown in Table 5, a positive shift of $V_{0.5}$ (>35 mV) to the right along the voltage axis occurs when increasing external $[K^+]$ from 10 to 37 mM. This table also shows that external K^+ has no influence whatsoever on the effect of ABA on the voltage-dependence of $I_{K,out}$ when GCP are loaded with high $[K^+]_i$. Indeed, no significant shift is seen in both experiments containing respectively 10 and 37 mM external K^+. Therefore, when internal potassium is set to high concentrations (i.e., 270 mM), altering the external potassium concentration has no effect on the ABA response.

Effects of ABA on the Sigmoid Activation of $I_{K,out}$

The HH sigmoidal function was fitted to the current traces (an example is given in Fig. 6) obtained before and after installation of the effect of ABA in GCP loaded with 150 ($n = 3$), 170 ($n = 2$), 200 ($n = 2$) and 270 mM K_i ($n = 2$). The estimated τ_n were variable from cell to cell as indicated by Fig. 7 where ratios ($\tau_{n,+ABA}/\tau_{n,-ABA}$) were calculated. As in Fig. 3*B*, the individual data points for each $[K^+]_i$ before and after the addition of ABA are presented (*see* the upper side of Fig. 7). The lower side of Fig. 7 shows the averaged ratios for all the guard cells loaded with 150 to 270 mM K_i. Again this figure shows no significant difference at all six membrane test voltages and a mean decrease in τ_n when adding ABA was found to be around 20%. This is the same as the decrease in τ_n observed when increasing $[K^+]_i$ from 100 mM to higher concentrations between 150 and 270 mM (*see* Fig 3*B*).

Discussion

Voltage-dependence of the Outward K^+-rectifier is Sensitive to K_i

The voltage-dependence of $I_{K,out}$ upon variation of external potassium concentration is well known in plant cells (Blatt, 1988, 1990; Schroeder, 1988; Vogelzang & Prins, 1994; White & Lemtiri-Chlieh, 1995; Roberts & Tester, 1995; *see also* Table 5 of this manuscript). This behavior is not known in animal cells for the delayed outward rectifier (''I_K''), but is analogous to the behavior of the anomalous potassium rectifier (''I_{K1}'') of most animal cells (Hagiwara & Yoshii, 1979; Sakmann & Trube, 1984). This study shows that the voltage-dependence of $I_{K,out}$ in GCP depend also on internal potassium concentration. Figure 8*A* illustrates this finding (the values of $V_{0.5}$ were taken from Fig. 2*B* and Table 5). Increasing $[K^+]_i$ from 100 to 270 mM induced a steep increase in current magnitude and caused a negative shift in $V_{0.5}$ of the activation curve in accordance with the

Table 4. Ratio of ($G_{Kmax,+ABA}/G_{Kmax,-ABA}$) in all GCP challenged with ABA in the five different $[K^+]_i$ used

	Low K_i			High K_i				
	100 mM K_i (n = 3)		150 mM K_i (n = 2)	170 mM K_i (n = 3)			200 mM K_i (n = 2)	270 mM K_i (n = 2)
Ratio ($G_{Kmax,+ABA}/G_{Kmax,-ABA}$)	2.4	3.0 1.6	2.9 2.8	1.8	1.96 1.5		2.34 1.35	2.4 1.3
Average increase in ABA	2.4 ± 0.24 (n = 5)			1.81 ± 0.17 (n = 7)				

The average increase is calculated by pooling the results obtained in 100 and 150 mM K_i as one group (Low K_i) and by pooling the results obtained in 170, 200 and 270 mM K_i as another group (High K_i).

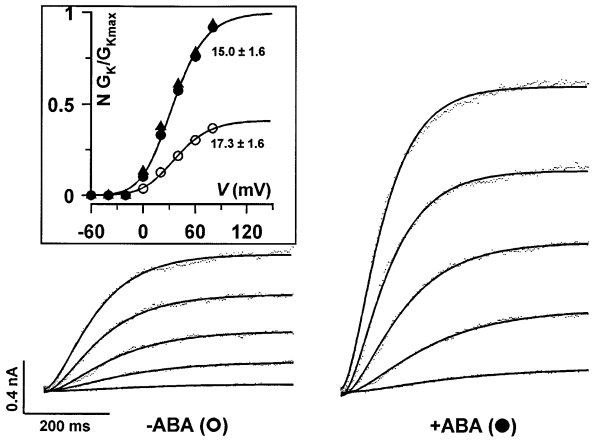

Fig. 6. Effect of ABA on the sigmoidal activation of $I_{K,out}$. Left panel: A family of control $I_{K,out}$ current traces obtained in response to depolarizing test voltages ranging in 20-mV steps from 0 to +80 mV; the holding potential (hν) was −100 mV. Right panel: A family of $I_{K,out}$ current traces obtained in response to the same voltage protocol; the current traces were recorded from the same GCP (as in left panel), 18 min after adding 10 μM ABA to the bath medium. The continuous lines are the fitted current traces according to Eq. 3. Note that this GCP was loaded with 270 mM K^+ and the bath medium contained 37 mM K^+. Insert: corresponding voltage-dependent steady-state activation of $I_{K,out}$ before (○) and after (●) ABA (currents corresponding to voltages from −60 to +80 mV are shown). Another set of data points (▲) is also shown in the insert, which represents the ABA effect after 23 min of perfusing the cell.

Nernst potential for K^+. $V_{0.5}$ shifts with E_K to the left along the voltage axis by about (−) 38 mV. These changes suggest an important role of internal K^+ in gating $I_{K,out}$ (probably by changing the screening of the surface charges at the mouth of the channel) and may contribute to the changes in the electrical properties of guard cell cytoplasmic membrane which occur during stomatal closure.

Effects of ABA on the Voltage-dependent Steady-state Activation of the Outward K^+-rectifier Depends on $[K^+]_i$

Figure 8*B* shows the effects of ABA on $V_{0.5}$ parameter of the activation curve of $I_{K,out}$ as a function of the $[K^+]_i$ (the values of $V_{0.5}$ were taken from Fig. 5 and Table 5). The results show that ABA shifts $V_{0.5}$ of the activation curve when the $[K^+]_i$ inside the patch pipette was set to 100 or 150 mM (5 guard cells gave the same result; *see* Fig. 8*B* insert). On the other hand, when the $[K^+]_i$ was set to higher concentrations up to 270 mM, no shift of $V_{0.5}$ occurred after ABA treatment (7 guard cells gave the same result; *see* Fig. 8*B* insert). Moreover, the magnitude of the shift in $V_{0.5}$ seems to be less in guard cells loaded with 150 mM K^+ than those loaded with 100 mM K^+. The effect of ABA on $V_{0.5}$ was clearly dependent on the $[K^+]_i$ of the cells. Examining the effect of ABA on G_K and its dependence on the internal K^+ load was less straightforward, but pooling the results obtained in 100 and 150 mM K_i as one group (low K_i) and the results obtained in 170, 200 and 270 mM K_i as another group (high K_i) showed a clear distinction. The mean increase in G_K was less in GCP loaded with high K_i.

Table 5. Values of $V_{0.5}$ and S (in mV) before and after ABA in two bathing solutions containing 10 and 37 mM K^+

	10 mM K_o	37 mM K_o
$V_{0.5}$ −ABA	−20.9	17.3
$V_{0.5}$ +ABA	−23.0	15.0
S −ABA	16.2	22.8
S +ABA	15.0	21.9

K_i in both experiments was 270 mM. The data were fitted in Eq. (2).

Overall, the results of ABA combined with the effects of $[K^+]_i$ on the voltage-dependent steady-state activation characteristic of $I_{K,out}$ (*see* Fig. 8*A*) could be interpreted as an effect of ABA in inducing K^+ release from the vacuolar pool, thereby (locally and temporarily) increasing cytoplasmic $[K^+]$. In cells loaded with high K^+, the gradient for K^+ to cross the tonoplast decreases comparatively to those loaded with less K^+. This could partly explain why the magnitude of the shift in ABA decreases with increasing K^+ load as well as why the increase of G_K following ABA treatment is relatively less in GCP loaded with high K_i. Another explanation,

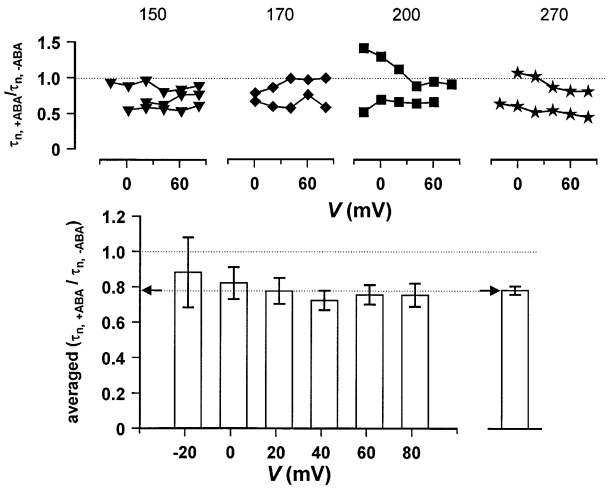

Fig. 7. Effect of ABA on the sigmoidal activation time constant parameter (τ_n) of $I_{K,out}$ in different GCP loaded with varying $[K^+]_i$. Upper panel: calculated ratios ($\tau_{n,\ +ABA}/\tau_{n,\ -ABA}$) as a function of test voltage and $[K^+]_i$. Data are as follow (in mM K^+): (▼) 150; (♦) 170; (■) 200 and finally (★) 270. ABA was added at concentrations between 10 and 20 μM and current recordings of $I_{K,out}$ were usually taken after 10 to 20 min following addition of ABA. Lower panel: averaged ratios ($\tau_{n,\ +ABA}/\tau_{n,\ -ABA}$) as a function of test voltage. The arrow indicates a mean decrease value (≈ 20%) at all test voltages.

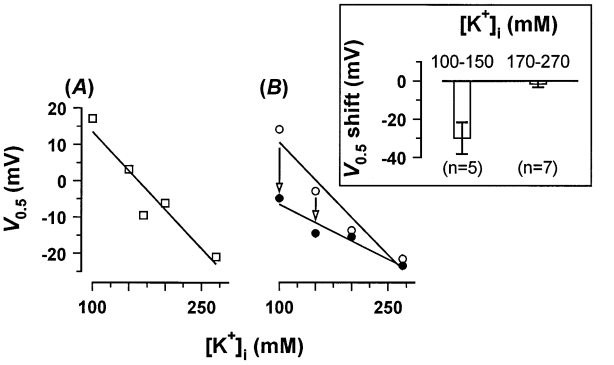

Fig. 8. Comparison between the effect of varying $[K^+]_i$ and ABA on the voltage-dependency of $I_{K,out}$. (*A*) $V_{0.5}$ as a function of $[K^+]_i$. All data points are averaged values (except for the point corresponding to 270 mM) and were taken from Table 2 and Table 3. (*B*) $V_{0.5}$ as a function of $[K^+]_i$ before (○) and after (●) adding ABA to the bath medium (10 μM was added in all experiments, except for the first point corresponding to 100 mM K^+, where 15 μM was used); current recordings of $I_{K,out}$ were usually taken after 10 to 20 min following addition of ABA. Data points were taken from Table 3 and 5. The arrows are proportional to the magnitude of the shift in $V_{0.5}$ after ABA treatment, and indicate its direction. Insert: Averaged $V_{0.5}$ shift as a function of $[K^+]_i$ after ABA treatment. Data were pooled into low K_i (100–150 mM; $n = 5$) and high K_i (170–270 mM; $n = 7$).

which may well play in the same time, could be that the effect of K^+ on $I_{K,out}$ channel saturates beyond 200 mM (*see* Fig. 2*B* and Table 2). In a recent report, Ward and Schroeder (1994) describe the existence of a 70 pS vacuolar K^+ channel (in symmetrical 100 mM KCl) which gives a physical support for ABA-induced vacuolar K^+ release. The mechanism by which ABA could activate this channel, is however obscure for the moment, for the following reasons: this vacuolar K^+ channel has been reported to be activated by (i) internal Ca^{2+} and (ii) acid going pH. Therefore, the regulation of this channel does not fit with the classic findings that the effect of ABA on $I_{K,out}$ in GCP is calcium-independent (Lemtiri-Chlieh & MacRobbie, 1994) and that ABA alkalinizes cytosolic pH (Irving et al., 1992; Blatt & Armstrong, 1993) resulting in an enhancement of $I_{K,out}$ (Blatt & Armstrong, 1993). On the latter point, although the pH effect appears to reduce the vacuolar K^+ release, Ward and Schroeder (1994) have estimated that at pH 8 where K^+ currents recorded from VK channels are only ≈ 25% of those recorded at pH 7, 200 mM K^+ could still be released from the vacuole through VK channels within 30 min. The authors concluded that other mechanism for K^+ release (through SV channels) should contribute to long-term vacuolar K^+ release during stomatal closure. The results obtained in this report in response to ABA with $[K^+]_i$ over 170 mM (which correspond to the usual internal concentration used in experiments involving intact guard cells, *see* next paragraph) are in accordance with those described by Blatt (1990). In intact guard cells, the magnitude of $I_{K,out}$, at any given membrane potential, is strongly enhanced without any effects on the voltage-dependence of channel gating nor on the time course for current sigmoidal activation. However, in conditions where $[K^+]_i$ is low (100–150 mM), the results reported here indicate that addition of ABA could shift the $V_{0.5}$ of the activation curve, and that sigmoidal current activation is slightly affected.

Comparison between the Results Obtained in GCP and Intact Guard Cells

The data obtained in this report, together with some of those obtained in a previous paper (Lemtiri-Chlieh & MacRobbie, 1994) give the answer to some questions which are subject to current debate (Blatt 1988; 1990; 1991; Fairley-Grenot & Assmann, 1993). These questions are related to quantitative (total membrane current) as well as qualitative (voltage- and time-dependent parameters) differences seen between intact guard cells (microelectrode technique) and their protoplasts (patch clamp technique in whole-protoplast mode). Currents are comparatively smaller in GCP and their activation time course is said to be 3- to 10-fold slower with half times slightly dependent on the membrane voltage (Schroeder, 1989; Fairley-Grenot & Assmann, 1993; but *see* Blatt, 1991).

Measurement of relatively large amplitudes of $I_{K,out}$ in whole-protoplast mode is made difficult for two major

reasons: (i) protoplasts are isolated for at least 2 h in the dark. In this condition, the volume of the guard cells and their vacuoles decreases (Fricker & White, 1990) as a result of net K^+ efflux, which leads to the reduction of the stomatal aperture. On the other hand, and apparently for technical reasons, impaling intact guard cells with microelectrodes when they are open (or at least partially open, with stomatal apertures ranging between 6 and 12 μm) is a preferred option (Blatt et al., 1990; Thiel et al., 1992; Blatt & Armstrong, 1993). (ii) a second important reason which adds to the difficulty mentioned above, is the common use of low $[K^+]_i$ (usually 100–120 mM) in the patch pipette filling media used by different patch clamp workers (Schroeder, 1988; 1989; Schroeder & Hagiwara, 1990; Schroeder & Fang, 1991; Fairley-Grenot & Assmann, 1992*a*, 1992*b*; 1993; Li et al., 1994*a*; Luan et al., 1993; Schwartz et al., 1994; Wu & Assmann, 1995); this is also true when using other cell types like mesophyll cells (Kourie & Goldsmith, 1992; Li et al., 1994*a*, 1994*b*), or root cells (Gassmann & Schroeder, 1994; Roberts & Tester, 1995; Wegner & Raschke, 1994; White & Lemtiri-Chlieh, 1995), while in microelectrode experiments using intact guard cells, a typical $[K^+]_i$ of 200 mM is used (Blatt, 1987; 1988; 1990; 1991; Blatt et al., 1990; Thiel & Blatt, 1991; Thiel et al., 1992; Blatt & Armstrong, 1993; Blatt & Thiel, 1994; Obmeyer, Armstrong & Blatt, 1994; Armstrong & Blatt, 1995). Thus the intact guard cells used with the voltage clamp microelectrode technique, are not only open and therefore still possess their cell turgor, but they are also impaled with microelectrodes containing high $[K^+]_i$.

By loading guard cell protoplasts with high $[K^+]_i$ but within the physiological concentration range (for review, *see* MacRobbie, 1987), the data obtained were more comparable to those obtained in the intact guard cells. In 200 mM K_i, currents up to 0.5 ± 0.074 nA (equivalent to ≈ 45 ± 6.7 μA.cm^2, assuming a cell diameter of 18 to 20 μm) have been recorded at +41 mV (n = 9; *see* Fig. 2*A*). In fact, in some experiments, currents up to 1 nA (≈ 90 μA.cm^2) could be recorded at +41 mV (*see* Fig. 1*B*). These values are 3 to 4 times larger than those obtained in *Vicia* GCP in 105 mM K_i and 11–12 mM K_o, for instance Schroeder and Hedrich (1989) show typical current traces of $I_{K,out}$ of about 250 pA (≈ 23 μA.cm^2) at +39 mV and Fairley-Grenot & Assmann (1992*b*) show a mean value around 15 μA.cm^2 at +40 mV (n = 6).

Comparison of the kinetics of activation of $I_{K,out}$ from intact guard cells and their protoplasts, show that in GCP, $I_{K,out}$ exhibit comparatively slow activation kinetics and the sigmoidal activation time course constant τ_n is weakly voltage-dependent (Blatt, 1988; 1990; 1991; Schroeder, 1989; Fairley-Grenot & Assmann, 1993). For instance, a τ_n value of 239 ms at +41 mV is reported by Schroeder (1989) in *Vicia* GCP while in intact guard cells, Blatt (1990) report a typical activation half-time at +50 mV of about 110 msec (equivalent to τ_n ≈ 70 msec). From Fig. 3*A*, a mean time constant τ_n value at all five different $[K^+]_i$ used in this work, was calculated to be around 137 ± 7.46 msec (n = 23) at V = +41 mV. The values are ranging from a minimum of 61 to a maximum of 210 msec (in Fig. 1*C*, an estimated activation time constant τ_n value of ≈ 87 msec at +41 mV is shown). These values are faster, by a factor of two, than the values reported by Schroeder (1989), and are comparable to the values reported by Blatt (1990).

Remarkably, Fig. 1*B* as well as Fig. 3*A* (but to a less extent), show a strong voltage-dependency of τ_n which has never been reported before in *Vicia* GCP (patch-clamped in whole-protoplast mode). It should be noted that Van Duijn (1993) using the patch clamp technique in whole-protoplast mode (tobacco protoplasts), showed also a strong voltage-dependency of τ_n. Thus, the results reported in this study regarding the analysis of the activation kinetics of $I_{K,out}$ (obtained in almost similar conditions as used in intact guard cells i.e., higher $[K^+]_i$) are more comparable to those obtained for the intact guard cells (Blatt, 1988, 1990). As a consequence data obtained from intact guard cells and from guard cell protoplasts should be compared with extreme caution. The results in this paper suggest that these differences may reflect different experimental conditions rather than differences between intact guard cells and their protoplasts (i.e., any consequence of protoplast isolation), or dialysis of the protoplast cytoplasmic constituents, that is, use of patch electrodes with a typical tip diameter of about 1 μm.

In conclusion, the results reported in this paper indicate that the effects of ABA mimics those of increasing $[K^+]_i$. The effects observed upon external perfusion of ABA could be explained as a consequence of an ABA-induced cytosolic K^+-flooding from the vacuole resulting in (i) increasing the driving force for K^+-efflux from guard cells and hence, increasing the magnitude of $I_{K,out}$, (ii) facilitating K^+-efflux by shifting the midpoint of the activation curve towards negative voltages. Furthermore, both MacRobbie's studies (1981, 1990) suggesting that $^{86}Rb^+$ efflux from guard cell vacuoles occurs in ABA-treated guard cells, and the recent characterization of VK channels (Ward & Schroeder, 1994) in the tonoplast of guard cells, give support to the above hypothesis.

I am grateful to Prof. E.A.C. MacRobbie for her continuous and everlasting support. I gratefully acknowledge Dr. M.R. Blatt (Dept. of Biological Sciences, University of London, Wye College, UK) and Dr. P.J. White (Horticulture Research International, Wellsbourne, Warwick, UK) for critical reading of the manuscript, Dr. M. Tester and Dr. S. Roberts (Department of Plant Sciences, University of Cambridge, UK) for helpful discussions. My profound thanks to Dr. Y. Tourneur (Laboratoire de physiologie des éléments excitables, CNRS, URA 180, Villeurbanne, France) and Dr. G. Christé (INSERM U121, Ave du Doyen lepine, Lyon, France) for helpful comments. This work was supported by a grant to Prof. E.A.C. MacRobbie from the Biotechnol-

ogy and Biological Sciences Research Council (Intracellular Signalling Initiative).

References

Allan, A.C., Fricker, M.D., Ward, J.L. Beale, M.H., Trewavas, A.J. 1994. Transduction pathways mediate rapid effects of abscisic-acid in *Commelina* guard-cells. *Plant Cell* **6:**1319–1328

Armstrong, F., Blatt, M.R. 1995. Evidence for K^+ channel control in *Vicia* guard-cells coupled by G proteins to a 7TMS receptor mimetic. *Plant Journal* **8:**187–198

Assmann, S.M. 1993. Signal Transduction in guard cells. *Annu. Rev. Cell Biol.* **9:**345–375

Blatt, M.R. 1987. Electrical characteristics of stomatal guard cells: The ionic basis of the membrane potential and the consequence of potassium chloride leakage from microelectrodes. *Planta* **170:**272–287

Blatt, M.R. 1988. Potassium dependent bipolar gating of K^+ channels in guard cells. *J. Membrane Biol.* **102:**235–246

Blatt, M.R. 1990. Potassium channel currents in intact guard cells: rapid enhancement by abscisic acid. *Planta* **180:**445–455

Blatt, M.R. 1991. Ion channel gating in Plants: physiological implications and integration for stomatal function. *J. Membrane Biol.* **124:**95–112

Blatt, M.R. 1992. K^+ channels of stomatal guard cells: Characteristics of the inward rectifier and its control by pH. *J. Gen. Physiol.* **99:**615–644

Blatt, M.R., Armstrong, F. 1993. K^+ channels of stomatal guard cells: abscisic acid-evoked control of the outward rectifier mediated by cytoplasmic pH. *Planta* **191:**330–341

Blatt, M.R., Thiel, G. 1993. Hormonal control of ion channel gating. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44:**543–567

Blatt, M.R., Thiel, G. 1994. K^+ channels of stomatal guard cells: bimodal control of the K^+ inward-rectifier evoked by auxin. *Plant Journal* **5:**55–68

Blatt, M.R., Thiel, G., Trentham, D.R. 1990. Reversible inactivation of K^+ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-triphosphate. *Nature* **346:**766–769

Fairley-Grenot, K.A., Assmann, S.M. 1992*a*. Permeation of Ca^{2+} through K^+ channels in the plasma membrane of *Vicia faba* guard cells. *J. Membrane Biol.* **128:**103–113

Fairley-Grenot, K.A., Assmann, S.M. 1992*b*. Whole-cell K^+ current across the plasma membrane of guard cells from a grass: *Zea mays*. *Planta* **186:**282–293

Fairley-Grenot, K.A., Assmann, S.M. 1993. Comparison of K^+-channel activation and deactivation in guard cells from a dicotyledon (*Vicia faba* L.) and a graminaceous monocotyledon (*Zea mays*). *Planta* **189:**410–419

Fricker, M.D., White, N. 1990. Volume measurement of guard cell vacuoles during stomatal movements using confocal microscopy. *Micro* **90:**345–348

Gassmann, W., Schroeder J.I. 1994. Inward-rectifying K^+ channels in root hairs of wheat. A mechanism for aluminium-sensitive low-affinity K^+ uptake and membrane potential control. *Plant Physiol.* **105:**1399–1408

Gilroy, S., Fricker, M.D., Read, N.D., Trewavas, A.J. 1991. Role of calcium in signal transduction of *Commelina* guard cells. *Plant Cell* **3:**333–344

Gilroy, S., Read, N.D., Trewavas, A.J. 1990. Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphates initiates stomatal closure. *Nature* **346:**769–771

Hagiwara, S., Yoshii M. 1979. Effects of internal potassium and sodium on the anomalous rectification of the starfish egg as examined by internal perfusion. *J. Physiol.* **292:**251–265

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch clamp techniques for high-resolution current recording from cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hodgkin, A.L., Huxley, A.F. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117:**500–544

Hosoi, S. Iino, M., Shimazaki, K. 1988. Outward rectifying K^+ channel in stomatal guard cell protoplasts. *Plant Cell Physiol.* **29:**907–911

Iijima, T., Hagiwara, S. 1987. Voltage-dependent K channels in protoplasts of trap-lobe cells of *Dionaea muscipula*. *J. Membrane Biol.* **100:**73–81

Irving, H.R., Gehring, C.A., Parish, R.W. 1992. Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *Proc. Natl. Acad. Sci. USA* **89:**1790–1794

Kourie, J., Goldsmith, M.H. 1992. K^+ channels are responsible for an inwardly rectifying current in the plasma membrane of mesophyll protoplasts of *Avena sativa*. *Plant Physiol.* **98:**1087–1097

Lemtiri-Chlieh, F., MacRobbie, E.A.C. 1994. Role of calcium in the modulation of *Vicia* guard cell potassium channels by abscisic acid: a patch-clamp study. *J. Membrane Biol.* **137:**99–107

Li, W., Luan, S. Schreiber, S.L., Assmann, S.M. 1994*a*. Evidence for protein phosphatase 1 and 2A regulation of K^+ channels in two types of leaf cells. *Plant Physiol.* **106:**963–970

Li, W., Luan, S., Schreiber, S.L., Assmann, S.M. 1994*b*. Cyclic AMP stimulates K^+ channel activity in mesophyll cells of *Vicia faba* L. *Plant Physiol.* **106:**957–961

Laun,S., Li, W., Rusnak, F., Assmann, S.M., Schreiber, S.T. 1993. Immunosuppressants implicate protein phosphatase regulation of K^+ channels in guard cells. *Proc. Natl. Acad. Sci. USA.* **90:**2202–2206

MacRobbie, E.A.C. 1981. Effects of ABA in 'isolated' guard cells of *Commelina communis* L. *J. Exp. Bot.* **32:**563–572

MacRobbie, E.A.C. 1987. Ionic relations of guard cells. *In:* Stomatal Function. E. Zeiger, G.D. Farquhar, R.D. Cowan, editors. pp. 125–162. Stanford University Press, Stanford, CA

MacRobbie, E.A.C. 1990. Calcium-dependent and calcium-independent events in the initiation of stomatal closure by abscisic acid. *Proc. R. Soc. Lond. B* **214:**214–219

MacRobbie, E.A.C. 1991. Effect of ABA on ion transport and stomatal regulation. *In:* Abscisic acid: Physiology and Biochemistry. W.J. Davies and H.G. Jones, editors. pp. 153–168. Oxford: Bio Scientific

MacRobbie, E.A.C. 1992. Calcium and ABA induced stomatal closure. *Phil. Trans. R. Soc. Lond. B* **338:**5–18

McAinsh, M.R., Brownlee, C., Hetherington, A.M. 1990. Abscisic acid-induced elevation of cytosolic free Ca^{2+} precedes stomatal closure. *Nature* **343:**186–188

McAinsh, M.R., Brownlee, C., Hetherington, A.M. 1992. Visualizing changes in cytosolic-free Ca^{2+} during the response of stomatal guard cells to abscisic acid. *Plant Cell* **4:**1113–1122

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

Obmeyer, G., Armstrong, F., Blatt, M.R. 1994. Selective block of the K^+ inward rectifier at the *Vicia* guard cell plasma membrane. *J. Membrane Biol.* **137:**249–259

Roberts, S., Tester, M. 1995. Inward and outward K^+-selective currents in the plama membrane of protoplasts from maize root cortex and stele. *Plant Journal* **8:**811–825

Sakmann, B., Trube, G. 1984. Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart. *J. Physiol.* **347:**641–657

Schroeder, J.I. 1988. K^+ transport properties of K^+ channels in the

plasma membrane of *Vicia faba* guard cells. *J. Gen. Physiol.* **92:**667–683

Schroeder, J.I. 1989. Quantitative analysis of outward rectifying K^+ channel currents in guard cell protoplasts from *Vicia faba*. *J. Membrane Biol.* **107:**229–235

Schroeder, J.I., Fang, H.H. 1991. Inward-rectifying K^+ channels in guard-cells provide a mechanism for low affinity K^+ uptake. *Proc. Natl. Acad. Sci. USA* **88:**11583–11587

Schroeder, J.I., Hagiwara, S. 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338:**427–430

Schroeder, J.I., Hagiwara, S. 1990. Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of nonselective Ca^{2+} permeable channels. *Proc. Natl. Acad. Sci. USA* **87:**9305–9309

Schroeder, J.I., Hedrich, R. 1989. Involvement of ion channels and active transport in osmoregulation and signalling in higher plant cells. *TIBS.* **14:**187–192

Schroeder, J.I., Hedrich, R., Fernandez, J.M. 1984. Potassium-selective single channels in guard cell protoplasts of *Vicia faba. Nature* **312:**361–362

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

Schwartz, A., Wu, W-H., Tucker, E.B., Assmann S.M. 1994. Inhibition of inward K^+ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. *Proc. Natl. Acad. Sci. USA* **91:**4019–4023

Thiel, G., Blatt, M.R. 1991. The mechanism of ion permeation through K^+ channels of stomatal guard cells: voltage-dependent block by Na^+. *J. Plant Physiol.* **138:**326–334

Thiel, G., MacRobbie, E.A.C., Blatt, M.R. 1992. Membrane transport in stomatal guard cells: The importance of voltage control. *J. Membrane Biol.* **126:**1–18

Van Duijn, B. 1993. Hodgkin-Huxley analysis of whole-cell outward rectifying K^+-currents in protoplasts from tobacco cell suspension cultures. *J. Membrane Biol.* **132:**77–85

Vogelzang, S.A., Prins, H.B.A. 1994. Patch clamp analysis of the dominant plasma membrane K^+ channel in root cell protoplasts of *Plantago media* L. Its significance for the P and K state. *J. Membrane Biol.* **141:**113–122

Ward, J.M., Pei, Z-M., Schroeder, J.I. 1995. Roles of ion channels in initiation of signal transduction in higher plants. *The Plant Cell* **7:**833–844

Ward, J.M., Schroeder, J.I. 1994. Calcium-active channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *The Plant Cell* **6:**669–683

Wegner, L., Raschke, K. 1994. Ion channels in the xylem parenchyma of barley roots. *Plant Physiol.* **105:**799–813

White, P.J., Lemtiri-Chlieh, F. 1995. Potassium currents across the plasma membrane of protoplasts derived from rye roots: a patch clamp study. *J. Exp Bot.* **46:**497–511

Wu, W-H., Assmann, S.M. 1995. Is ATP required for K^+ channel activation in *Vicia* guard cells. *Plant Physiol.* **107:**101–109