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**The Role of Calcium in Stomatal Function:
Basic and Applied Implications**

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The role of calcium in stomatal function: basic and applied
implications.

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C. Abstract

The role of Ca^{2+} in stomatal function was investigated. The findings characterized a crucial role of Ca^{2+} in the control of stomatal apertures. Reduction of Ca^{2+} concentration of the incubation medium prevented closing of stomata and increased stomatal opening in response to light and KCl. These studies also showed that EGTA is a useful probe for the analysis of stomatal opening.

A primary aim of the project was the empirical distinction between the opening and closing mechanisms controlling stomatal aperture. All the tested metabolic inhibitors prevented closing and opening to the same extent, rendering them inadequate for the separation of opening from closing. In contrast, EGTA blocks closing without affecting opening. Availability of this treatment made it possible to establish that both opening and closing require metabolic energy. In the absence of Ca^{2+} , exposure of guard cells to hypertonic solution of mannitol caused hydropassive stomatal closure but not leakage of ions. Turgor, therefore, does not appear to be a closing signal.

Turgor, on the other hand, is controlled by the balance of ion influx and efflux in guard cells. Osmoregulation of opening was shown to depend on three different metabolic pathways. Emphasis was placed on the design of a new method for production of guard cell protoplasts. The new technique uses mechanical grinding of leaves, eliminates the labor involved in manual detachment of the epidermis. This new method opens possibility of isolating guard cell protoplasts from important agricultural crops.

Effect of calcium, and calmodulin
inhibitors on ATPase activity
in guard cells

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ABSTRACT

Crude homogenate of Commelina communis L. guard cell protoplasts contains K^+ Mg^{+2} -ATPase activity. The ATPase activity was inhibited by $CaCl_2$ and stimulated by the calmodulin inhibitors, compound 48/80 and chlorpromazine.

INTRODUCTION

Ca^{2+} is a potent inhibitor of stomatal opening (Nejdat 1987, Schwartz 1985, Willmer and Mansfield 1969), while its chelation with EGTA prevents stomatal closure (Schwartz 1985). There are evidence that calcium partially mediates the inhibitory effect of Abscissic acid (ABA) on stomatal opening (De Silva et al. 1985). Hence, calcium seems to play an important role in stomatal regulation, as well as in other developmental and physiological processes, in plants (Hepler and Wayne 1985, Kaus 1987). Studying the mode of its action should contribute to our understanding of stomatal function.

Calmodulin inhibitors stimulates stomatal opening and antagonize exogenous added calcium on closure (Donovan et al. 1985, Nejdat 1987), suggesting calmodulin involvement in guard cell's turgor controle. Recent results indicate that the guard cells has a high plasma membrane H^+ ATPase activity (Fricker and Willmer 1987, Nejdat et al. 1986, Shimazaki and Konodo 1987) and Ca^{2+} was reported to inhibit ATPase activity in protoplasts of Commelina communis L. (Nejdat et al. 1986) and Vicia faba (Shimazaki and Konodo 1987) guard cells.

The involvement of calmodulin in the regulation of ATPase activity in plant systems was reported in the past (Dieter and Marme 1981, Natsomoto et al. 1984, Zocchi 1985).

In this communication we examine the effect of the calmodulin inhibitors chlorpromazine and 48/80 on ATPase activity in crude extract from Commelina communis L. guard cell protoplasts.

MATERIALS & METHODS, RESULTS AND DISCUSSION

For guard cell protoplasts isolation, abaxial epidermis of 50-70 fully expanded leaves of Commelina communis L. plants were peeled on deionized water. The epidermis was floated for 15 min on 400 mM mannitol, 1 mM CaCl_2 and 10 mM MES, pH 5 adjusted with KOH (Medium A). For digestion of the cell walls, the tissues were floated on 3% cellulysin (Calbiochem), 0.05% pectolyase Y-23 and 0.5% BSA dissolved in medium A. After 2.5-3 h in 30°C crude mixture of epidermal and guard cell protoplasts was obtained. The epidermis was washed over a 30 μm nylon mesh net with 60ml of medium A to release adhering protoplasts. The protoplasts were collected by centrifugation at 100 g for 7 min. The pellet was resuspended in 3-4 ml of 400 mM mannitol, 10 mM MES, pH 7 adjusted by KOH (medium B). Uncontaminated guard cell protoplasts were isolated by loading the suspension on 2 ml of 15/90% (v/v), percoll gradient prepared in medium B (Fitzsimons and Weyers 1983) and centrifuged at 400 g for 7 min. Guard cell protoplasts were collected from the 90% interface, diluted 10 fold with medium B, centrifuged at 100 g for 5 min and resuspended in medium B.

For ATPase activity measurements, pelleted guard cell protoplasts were resuspended in homogenizing medium containing 250 mM sucrose, 1 mM EDTA, 1 mM DTT and 10 mM MES-Tris, pH 7.2. The protoplasts were allowed to swell for 2 min and for complete lysis the protoplasts were forced to pass through 10 μm nylon mesh net. The homogenate was centrifuged at 500 g for 5 min to discard intact protoplasts and chloroplasts. The supernatant was used to measure ATPase activity.

All manipulations were done in ice cold water. The basic reaction mixture for ATPase activity contained 2 mM Tris-ATP, 50 mM Mes-Tris, pH 6.5, 50 μ l extract containing 3-6 μ g protein and other materials as indicated in the legend of each figur. Fifty μ l of extract adds 0.1 and 0.2 mM of DTT and EDTA, respectively, to the reaction mixture. The reaction was conducted for 30 min in 30°C and terminated by the addition of 1 ml Ames reagent (Ames 1966), which also determines the released inorganic phosphate. The colour was developed for 15 min and its intensity was measured at 820 nm. The assays were conducted in triplicates and every experiment was repeated at least twice with different protoplast preparation. Compound 48/80 was found to interfere with colour development and this factor considered when ATPase activity was calculated. Protein was assayed according to Bradford (Bradford 1976).

The guard cell protoplasts crude extract contains high phosphatase activity, inhibited by molybdate (fig. 1) a phosphatase inhibitor (Callagher and Leonard 1982). The residual activity is stimulated by Mg^{2+} indicating ATPase activity (Sze 1984). The Mg-ATPase slightly inhibited by sodium azide indicating minor contribution of mitochondrial ATPase to the measured activity (Gallagher and Leonard 1982). The Mg-ATPase activity in the presence of molybdate and azide is further stimulated by KCl (fig. 1) indicating Mg^{2+} dependent, K^{+} stimulated ATPase activity which are characteristic to plasma membrane ATPase (Sze 1984). These results are consistent with those reported earlier by Nejdat et al. (1986) and Fricker and Willmer (1987). The Mg^{2+} dependent, K^{+} stimulated ATPase were clearly inhibited by $CaCl_2$ (fig. 2). This property was

also reported by Nejdat et al. (1986) and Shimazaki and Konodo (198). The compound 48/80 which was shown to increase stomatal opening (Donovan et al. 1985) stimulates the ATPase activity and was saturated at concentration of 10 ppm (fig. 2). Compound 48/80 partially negate the CaCl_2 effect which may suggest calmodulin involvement. The effect of compound 48/80 is evident at the concentration of 1 ppm (fig. 3A) supporting the specificity of its action. chlorpromazine a calmodulin inhibitor which was also reported to increase stomatal opening (Nejdat 1987) stimulates the ATPase activity in the presence of CaCl_2 (fig. 3A) supporting the suggestion of calmodulin involvement. Calmodulin effect on ATPase activity was reported to be through ATPase phosphorylation due to kinase activity, a calmodulin regulated enzyme (Anderson and Cormier 1978). Mg^{2+} is an important factor for kinase activityt and was reported to increase the phsophorylation of membrane proteins (Veluthambi and Poovaiah 1984). Raising the Mg^{+2} concentration in the reaction mixture to 4 mM increased the ATPase activity but did not affect the inhibitors action (fig. 3a) indicating that 2 mM is saturable as found for protein phosphorylation. Although the effect of the compound 48/80 and chlorpromazine suggests calmodulin involvement in regulating the ATPase activity we failed to get any significant effect of calmodulin on ATPase activity up to 10 $\mu\text{g/ml}$ (fig. 4) using low batches of calmodulin. This result may be due to several reasons. Saturation of the system with endogenous calmodulin may mask the exogenous one. This phenomenon was reported in animal systems where washing out the endogenous calmodulin was done to show significant effect of exogenous calmodulin (Campbell and MacIenann

1982, Chiesi and Caratoli 1982). The limited amount of guard cell protoplasts do not allow to adapt this protocol. On the other hand, the lack of calmodulin effect may stem than the right side - out orientation of the membrane vesicles which prevents the calmodulin to reach the target inside the vesicles. It is also possible that compound 48/80 and chlorpromazine have a non-specific effect, not through calmodulin. However, the activity of the inhibitors at low concentrations (fig. 3) do not support the latter possibility. In conclusion, the results indicate that the calmodulin inhibitors compound 48/80 and chlorpromazine stimulate the K, Mg-ATPase in guard cells and partially abolish the calcium effect. This could be interpreted that calcium effect the ATPase activity partially through calmodulin and through a direct effect on the enzyme.

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Fig. 1 A - ATPase activity in the presence of 2 mM Tris-ATP, 0.2 mM EDTA, 0.1 mM DTT and 50 mM MES-Tris, pH 6.5. B; A + 0.5 mM molybdate. C; B + 2 mM MgCl_2 . D: C + 1 mM sodium azide. E; D + 50 mM KCl.

Fig. 2 Effect of CaCl_2 on ATPase activity. The reaction mixture contained: 2. mM ATP, 2 mM MgCl_2 , 0.2 mM EDTA, 0.1 mM DTT, 0.5 mM ammonium molybdate, 1 mM sodium azide, 50 mM MES-Tris, pH 6.5 in the presence of zero (●—●), 10 (○—○) and 20 ppm (□—□) compound 48/80.

Fig. 3 Effect of chlorpromazine (CP and compound 48/80 on ATPase activity. A - the basic reaction mixture as in Fig. 2 in addition of 0.1 mM CaCl_2 . B - the reaction mixture as in A and 4 mM MgCl_2 .

Fig. 4 Effect of calmodulin on ATPase activity. The basic reaction mixture as in Fig. 2 in addition of 0.1 mM CaCl_2 .

WHOLE-CELL K^+ CURRENTS IN GUARD CELL PROTOPLAST OF VICIA FABA AND
COMMELINA COMMUNIS.

S.M. Assmann, N. Moran, A. Schwartz

Plant Physiol 86: 83.

SUMMARY

1. The whole-cell patch clamping technique was applied to guard cell protoplasts (GCP) of Commelina communis and Vicia faba.
2. GCP from both species exhibited slowly activating outward and inward currents. These currents were identified as K^+ currents on the basis of 1: their reversal potential, 2: their susceptibility to block by TEA and Ba^{2+} . Kinetics of current activation were similar in the two species.
3. Further studies with Vicia faba GCP showed that outward currents inactivated as the recording session proceeded, whereas inward currents did not. Outward and inward currents may be carried by two different types of K^+ channels.
4. Potassium currents such as those recorded here presumably occur in the intact leaf, where fluxes of K^+ across the guard cell membrane mediate stomatal movements.

INTRODUCTION

Stomatal guard cells regulate gaseous exchange between the environment and the leaf interior. In response to appropriate environmental signals such as light, K^+ and Cl^- ions enter the guard cells and, in conjunction with malate synthesis, increase the osmotic content of the guard cell. These processes result in water influx, turgor build-up, guard cell swelling, and stomatal opening (9).

Current data are consistent with a mechanism of K^+ uptake by guard cells in which H^+ extrusion by a H^+ ATPase results in an electrical gradient driving K^+ entry (9). Electrophysiological studies employing the patch clamp technique have demonstrated a blue-light-stimulated outward current from individual guard cell protoplasts (GCP) of Vicia faba (1). The current is observed only in the presence of ATP, is inhibited by the ATPase inhibitor diethylstilbesterol, and occurs against an electrochemical gradient, indicating that it is a pump current (1). The current magnitude is comparable to that of H^+ efflux from guard cells as calculated from measurements of external pH changes (8), suggesting that the current results from the functioning of the H^+ ATPase.

In the present study, we demonstrate that whole-cell patch clamping (2,3) (Fig. 1) can be used to detect the second major ionic event occurring during stomatal responses, namely K^+ fluxes. Potassium currents were observed in two of the species most commonly used for stomatal research, Commelina communis and Vicia faba.

MATERIALS AND METHODS

Guard cell protoplasts (GCP) of Vicia faba were isolated as described previously (1). Guard cell protoplasts of Commelina communis were isolated following a procedure modified from (5). Peels from 6-8 leaves of Commelina communis were cut into small pieces and incubated for 1 hr at 29°C in 10 ml of 0.25 M sorbitol containing 2% "Cellulysin" cellulase (Calbiochem) and 0.5 % BSA, pH 5.0. Upon release of epidermal cell protoplasts, peels were transferred to a 0.5 M sorbitol solution containing 2% Cellulysin, 0.5% BSA, 0.5% Driselase, 0.005% Pectolyase Y-23, pH 5.0 and digested for 2 - 4 additional hours at 29°C. The solution, containing released GCP, was filtered through a 30 um nylon mesh, centrifuged at 100g x 8 min. to pellet the protoplasts, and washed once with 0.5 M sorbitol, 1 mM CaCl₂.

Patch clamping was performed using an Axopatch 1B patch clamp (Axon Instruments, Burlingame, CA) on line with a Minta (IBM AT compatible) computer. Data were recorded and analysed using P-Clamp software (Axon Instruments). Unless otherwise specified, solutions for patch clamping were : external (bath) solution = 11 mM KCl, 2 mM MgCl₂, 10 mM MES, 1 mM CaCl₂, pH 5.5, made to 480 milliosmol with D-mannitol, internal (micropipette) solution = 100 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 2 mM EGTA, pH 7.2, made to 530 mosmol with D-mannitol.

RESULTS AND DISCUSSION

Figure 2 depicts guard cell protoplasts (GCP) of Commelina communis. Patch clamping was performed in the whole-cell configuration, in which the currents recorded represent net current across the entire membrane of the cell. The initial seal of the micropipette against the cell membrane which allows for detection of cellular currents was more easily obtainable with Vicia faba than with Commelina communis GCP, presumably because of more complete removal of cell wall material in the former species. In both species, however, slowly activating inward and outward currents were observed (Fig. 3,4). Similar currents have been observed previously in Vicia faba (6). These currents were identified as potassium currents on the basis of their reversal potential, and their sensitivity to inhibition by 2 compounds effective in blocking potassium currents in animal cells (rev. in 3): tetraethylammonium (TEA^+) (data not shown), and barium (Ba^{2+}) (Fig. 5,6). The potassium currents observed presumably are involved in stomatal opening and closure in the intact leaf.

Additional experiments with GCP of Vicia faba revealed that the magnitude of outward potassium currents did not remain constant, but usually exhibited a gradual decay over the recording period (Fig. 7). For seven cells in which the decline was fully documented, disappearance of outward current was complete within 10 min. after initial attainment of the whole cell configuration. Inactivation presumably results from an alteration of the natural cytoplasmic milieu following

equilibration of the cell interior with the solution contained in the patch pipette. Inward currents did not show such inactivation (Fig. 7). The disparate responses of outward and inward K^+ currents suggests that these currents could be carried by two different types of K^+ channels. Such a hypothesis is consistent with recent models of stomatal function that postulate that stomatal opening and stomatal closing are two separate processes (4,7), rather than simply a single process that reverses. Further patch clamping studies will determine whether outward and inward K^+ currents are also affected differentially by biologically relevant stimuli such as light quality and quantity.

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Patch clamping

Fig. 1. Membrane configurations used in patch clamping.

FIGURE 3. WHOLE-CELL CURRENTS FROM A GUARD CELL PROTOPLAST OF *COMMELINA COMMUNIS*. MEMBRANE POTENTIAL WAS CLAMPED AT -60 mV, THE APPROXIMATE EQUILIBRIUM POTENTIAL (E_{K^+}) FOR POTASSIUM. SEVEN SECOND VOLTAGE PULSES (mV) WHICH STEPPED THE MEMBRANE POTENTIAL AWAY FROM E_{K^+} ELICITED OUTWARD (A) OR INWARD (B) K^+ CURRENTS.

FIGURE 4. WHOLE-CELL OUTWARD (A) AND INWARD (B) K^+ CURRENTS FROM A GUARD CELL PROTOPLAST OF VICIA FABA.

FIGURE 5. WHOLE-CELL K^+ CURRENTS IN VICIA FABA. THE CHARGE-CARRIER (K^+) IS IDENTIFIED BY THE REVERSAL OF THE "TAIL" CURRENTS BETWEEN -60 AND -40 mV, AT THE CALCULATED EQUILIBRIUM (NERNST) POTENTIAL FOR POTASSIUM.

FIGURE 6. INWARD K^+ CHANNELS IN *Vicia faba* BEFORE (A) AND AFTER (B) BLOCK WITH .6 mM (FINAL CONCENTRATION) $BaCl_2$.

FIGURE 7. DISAPPEARANCE OF OUTWARD K^+ CURRENTS WITH TIME. COMPARE OUTWARD CURRENTS AFTER 4 MIN ($T_E = 4$) WITH THOSE AT THE BEGINNING OF WHOLE-CELL RECORDING ($T_E = 0$, INSET, ONLY THE RESPONSE TO POSITIVE VOLTAGE PULSES SHOWN).

F. Description of Cooperation.

The cooperation between the two groups involved several aspects: The American group has the know-how on gas exchange instrumentation and methodology, which has been valuable for the Rehovot group. Methods of guard cell isolation and handling which were developed in Zeiger's laboratory, including the newly developed method which is part of this report, are currently used by us in patch clamping studies. Also, the cooperation made it possible to exchange newly acquired scientific information and discussion of future directions.

C. G. Objectives and Evaluation of Research Achievements.

The work supported by this grant increased our understanding of the mechanisms that control guard cell's turgor and thus stomatal aperture. Our findings emphasize the crucial role of Ca^{2+} in the control of stomatal aperture through its involvement in the regulation of stomatal closure. The interaction between Ca^{2+} and light, CO_2 , KCl and IAA on stomatal movements were studied. This work showed that mechanisms controlling opening and closing are different and can be regulated independently. The data also point to the usefulness of EGTA for studies of stomatal movements in which influx and efflux processes can be distinguished by the specific effect of Ca^{2+} chelation on K^+ efflux. The presented results show that the unregulated leakage of K^+ from the guard cells is very low

and that reducing metabolism dose not cause K^+ leakage and stomatal closure but, on the contrary, it prevents K^+ efflux and stomatal closure. Furthermore, the results indicate that both K^+ influx and efflux take place through specific K^+ channels and that ATP and/or a membrane potential are needed to keep these channels open. There appears to be coupling between the activity of the plasma membrane proton pump and the membrane conductance for K^+ in the guard cells. Success was also attained in showing that epidermal turgor dose not function in guard cells as a signal for the modulation of apertures. We manipulated the water status of epidermal tissue in the presence and absence of attached mesophyll tissue by altering the external osmotic environment. Ion uptake by guard cells in isolated epidermis was neither inhibited nor stimulated by desiccation, indicating that observed stomatal responses to water deficits and humidity may involve factors external to the epidermis.

A successful characterization of a new method to isolate guard cell protoplasts from mechanically obtained epidermis was also accomplished. This method makes it possible to isolate guard cell protoplasts from agricultural crops. This development will greatly facilitate biochemical investigations in guard cells.

The proposed regulation of transpiration by using Ca^{2+} compounds as anti-transpirants, in post-harvested leafy vegetables and flowers could not be achieved mainly as a result of application problems. However, these goals should be evaluated further with complementary experimental approaches.

H. List of Publications.

Schwartz, A. (1985) The role of Ca^{2+} and EGTA on stomatal movements in Commelina communis L. Plant Physiol. 79, 1003-1005. 3.7

Schwartz, A., N. Illan and D.A. Grantz (1988) Calcium effects on stomatal movements in Commelina communis L. Plant Physiol. 87, 583-587. 3.7

Karlsson, P.E. and A. Schwartz (1988) Characterization of the effects of metabolic inhibitors, ATPase inhibitors and K^+ channel blocker on stomatal opening and closing in isolated epidermis of Commelina communis L. Plant, Cell and Environment 11, 165-172. 1.2

Grantz, D. A. and A. Schwartz. (1988) Guard cells of Commelina communis L. do not respond metabolically to osmotic stress in isolated epidermis: Implication for stomatal responses to drought and humidity. Planta 174, 166-173. 3.1

Tallman, G. and E. Zeiger (1988) Light quality and osmoregulation in Vicia guard cells. Plant Physiol. 88, 887-895. 3.7

Krause, T., G. Tallman and E. Zeiger. (1989) Isolation of guard cell protoplasts from mechanically prepared epidermis of Vicia faba leaves. Plant Physiol. 90, 1382-1386. 3.7