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Role of Ca^{2+} and EGTA on Stomatal Movements in *Commelina communis* L.[1]

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ABSTRACT

Ca^{2+} (0.1–1.0 millimolar) accelerated dark-induced stomatal closure and reduced stomatal apertures in the light in epidermal peels of *Commelina communis* L. In contrast, ethyleneglycol-bis-(β-aminoethyl ether) N,N'tetraacetic acid (EGTA) (2 millimolar), a Ca^{2+} chelator, prevented closure in the dark and accelerated opening in the light. EGTA did not promote significant opening in the dark. It is therefore concluded that EGTA does not increase ion uptake into guard cells, but rather prevents ion efflux. Addition of EGTA to incubating solutions with 10 millimolar KCl resulted in steady state apertures of 15.6 micrometers, whereas in the absence of EGTA similar apertures required 55 millimolar KCl and 150 millimolar KCl was needed in the presence of 1 millimolar $CaCl_2$. The results demonstrate the importance of Ca^{2+} in the regulation of stomatal closure and point to a role of Ca^{2+} in the regulation of K^+ efflux from stomatal guard cells.

Stomata continuously regulate gas exchange in leaves, in response to environmental changes. Stomatal opening is the result of solute accumulation in the guard cells with K^+ the main cation involved (11, 17). It is commonly held that light increases active efflux of protons, thereby generating an electrical potential. K^+ accumulates in guard cells following its electrochemical gradient (28). Ensuing increases in guard cell turgor and stomatal opening result from the lower osmotic potential and water uptake. Mechanisms involved in stomatal closure are less known; however, evidence indicates that stomatal closure does not result only from a cessation of K^+ uptake, but it also involves metabolic mechanisms causing active K^+ efflux (13, 26).

The involvement of Ca^{2+} in the control of K^+ fluxes is known both in animal (1) and plant tisues (21). Through its control of K^+ flux, Ca^{2+} plays an important role in the regulation of cell volume (7, 9). In stomata of *Vicia faba* the presence of Ca^{2+} increases guard cell specificity for K^+, over other monovalent ions (8). At concentrations of 0.1 to 1 mM Ca^{2+} caused inhibition of opening and partial or total closure in stomata from different species (5, 18, 22, 25, 27). Fujino (5) showed that in *Commelina*, Ca^{2+} was the only divalent cation inhibiting opening and that EDTA, a Ca^{2+} chelator, prevented the closure of open stomata held in the dark for at least 1 h.

Much evidence has been recently presented showing the importance of cytoplasmic Ca^{2+} concentration as a regulatory mechanism for different enzymic activities. Cytoplasmic Ca^{2+} concentrations are primarily regulated by the activity of a Ca^{2+} ATPase which modulates Ca^{2+} fluxes. This ATPase activity is likely to be modulated by plant hormones and light. It is generally accepted that a primary mode of action of Ca^{2+} is often by its binding to Calmodulin, a Ca^{2+} binding protein (3).

The work reported in this paper investigates the role of Ca^{2+} in the regulation of stomatal movements. Obtained results demonstrate that stomatal closure is markedly affected by $CaCl_2$ concentrations as well as EGTA, a specific chelator of Ca^{2+}.

MATERIALS AND METHODS

Plants of *Commelina communis* L. were grown from seeds in the phytotron of the Faculty of Agriculture in Rehovot. Incident sunlight radiation was reduced by about 30% during the summer months (May–November) using shade nets. Temperature was 27°C during the day (0800–1600) and 22°C at night. Natural day length was lengthened to 16 h with incandescent lamps. The plants were grown in trays in a mixture of vermiculite, volcanic tuff, and peat (2:2:1), watered daily with tap water, and fertilized twice a week with a half strength Hoagland nutrient solution. The two top fully open leaves from plants 5 to 7 weeks old were used for the experiments. All experiments were conducted during the morning and were concluded not later than 1500. For the experiments beginning with open stomata, the stomata were induced to open in the intact detached leaves. The detached leaves were immersed in a Petri dish, 14 cm in diameter, containing tap water and held below the water surface with a transparent plastic screen. The bottom of the dish was immersed in a constant temperature water bath kept at 27°C and illuminated by a bank of cool-white WS Gro-Lux fluorescent tubes (200 μmol m^{-2} s^{-1} PAR). These conditions led to stomatal opening and apertures reached 16 to 18 μm after 2 h. Strips of the abaxial epidermis were removed and floated in the light or in the dark for about 30 min on a solution containing 60 mM KCl, 10 mM Mes adjusted to pH 6.1 with Cholin bicarbonate (Fluka AG). The epidermal peels were then transferred to the treatment solutions in Petri dishes, kept at 27°C, and either illuminated with white light (100 μmol m^{-2} s^{-1} PAR), or held in the dark. The treatment solutions contained 10 mM Mes with or without 2 mM EGTA, adjusted to pH 6.1 with Cholin bicarbonate. KCl and $CaCl_2$ concentrations were as indicated in "Results." Each point represents the mean of at least 180 apertures and the standard error did not exceed ±4% of any of the represented values.

RESULTS

Closure rates and the different stomatal apertures were analyzed as a function of $CaCl_2$ concentration or in the presence of 2 mM EGTA in the bathing solutions (Fig. 1, A and B). At the onset of the experiment, average stomatal aperture were 17.6 μm. In the light, 1 mM $CaCl_2$ caused the most marked reduction

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in aperture, 15 μm within 60 min, with 0.1 mM $CaCl_2$ showing a less pronounced effect. Stomata treated with 0.01 mM $CaCl_2$ maintained apertures similar to the controls (Fig. 1A). In the dark, stomata closed faster in the presence of $CaCl_2$ whereas the presence of 2 mM EGTA in the bathing solution, was found to prevent closure (Fig. 1B).

The effect of EGTA upon the rate of stomatal opening and steady state apertures is presented in Figure 2. In the dark, 2 mM EGTA increased stomata apertures above control levels by 2.8 μm within 3 h. In the light, EGTA enhanced both opening rates (13.7 μm in the 1st h as compared with 8.1 μm in the 1st h in the absence of EGTA) and steady state apertures (3.6 μm above controls). When epidermal peels were transferred from light to darkness, stomata reduced their aperture by 8.4 μm within 1 h in the absence of EGTA but remained open for at least 3 h in the presence of the chelator.

Interactions between $CaCl_2$, EGTA, and KCl in the maintenance of steady state apertures were also tested. Epidermal peels with a mean stomatal aperture of 15.6 μm were incubated in solutions of different KCl concentrations, with or without 2 mM EGTA, or with 1 mM $CaCl_2$. Stomatal apertures were then measured after 1 h of incubation in white light 100 μmol m^{-2} s^{-1} (Fig. 3). The results showed that in the presence of 2 mM EGTA, 10 mM KCl was required to prevent stomatal closure while in its absence a concentration of 50 to 60 mM KCl was needed. In the presence of 1 mM $CaCl_2$ more than 150 mM KCl was required to prevent closure. In the presence of EGTA and only 5 mM KCl, average apertures were of 6 μm, with KCl concentrations higher than 10 mM causing further opening above the base line levels. In the absence of EGTA, 20 mM KCl was the lower concentration preventing complete closure while a concentration higher than 60 mM KCl required to cause further opening above the base line.

The possibility that EGTA directly affects membrane permeability rather than by means of chelating the Ca^{2+}, and thus reducing its concentration, was also examined. Epidermal peels with open stomata were incubated in the dark in different $CaCl_2$ concentrations, in the presence of either 1 or 2 mM EGTA (Fig. 4). Without EGTA, stomata had apertures of 3.7 μm and 2.6 μm after 60 min in 0 and 0.01 mM $CaCl_2$, respectively, and were completely closed at higher $CaCl_2$ concentrations. At 1 mM,

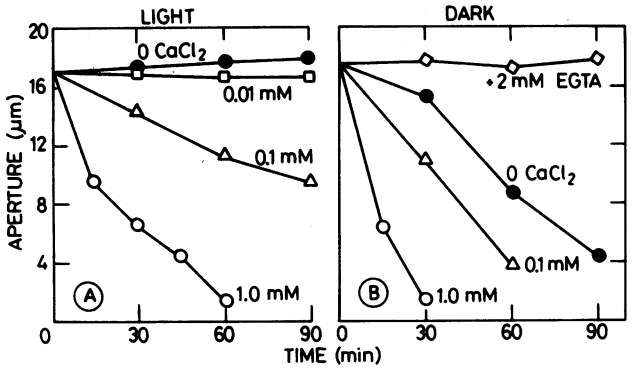

FIG. 1. Effect of $CaCl_2$ on stomatal aperture in epidermal peels. Peels with open stomata were transferred to solutions containing 70 mM KCl, 10 mM Mes together with either $CaCl_2$ or 2 mM EGTA, adjusted to pH 6.1 with cholin bicarbonate, in the light (A) and in the dark (B).

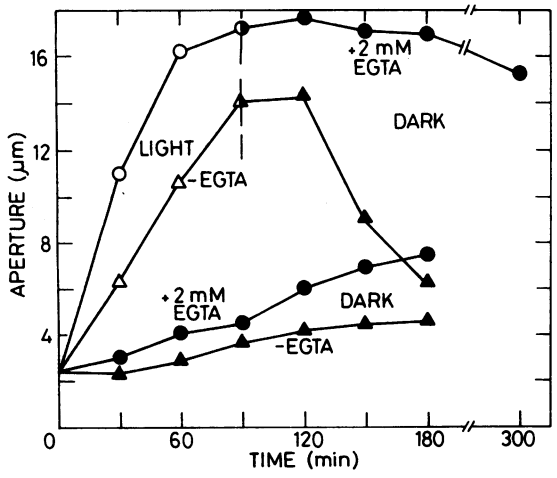

FIG. 2. Stomatal opening in epidermal peels in the light (○, △) and in the dark (●, ▲). The bathing solution contained 70 mM KCl and 10 mM Mes with (●, ○) or without 2 mM EGTA (△, ▲) adjusted to pH 6.1 with Cholin bicarbonate.

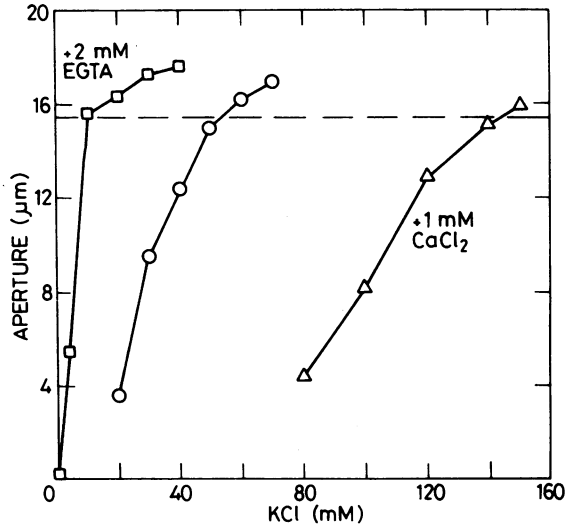

FIG. 3. Effect of KCl concentration on aperture of initially open stomata (- - -). The peels were floated in the light on solutions containing 10 mM Mes alone (○), and with 2 mM EGTA (□), or with 1 mM $CaCl_2$ (△), adjusted to pH 6.1 with Cholin bicarbonates. Apertures were measured after 60 min.

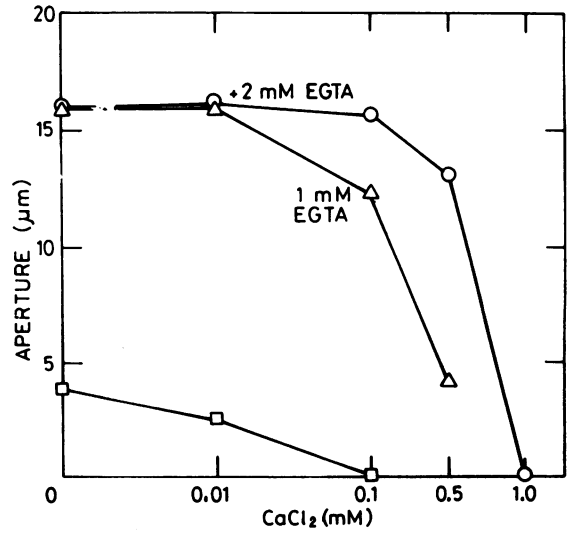

FIG. 4. Effect of $CaCl_2$ concentration on the maintenance of stomatal aperture (initially open of 16 μm). The bathing solution contained 70 mM KCl, 10 mM Mes (□) and 1 mM EGTA (△), or 2 mM EGTA (○) adjusted to pH 6.1 with Cholin bicarbonate. Apertures were measured after 60 min in the dark.

$CaCl_2$ caused complete closure in the presence of EGTA (2 mM). These results indicate that in order to inhibit closure at least all the added Ca^{2+} has to be chelated.

DISCUSSION

Ca^{2+} (0.1–1 mM) accelerates stomatal closure in the dark and causes closure in the light by inducing osmoticum efflux, with K^+ being its main component (Fig. 1, A and B). The prevention of closure in the dark (Figs. 1 and 2) is the sole result of Ca^{2+} chelation, as indicated by the fact that higher $CaCl_2$ concentrations (>0.1 mM), probably above the chelation capacity of the EGTA at these conditions, allowed stomatal closure (Fig. 4). Intracellular and apoplastic Ca^{2+} concentration in guard cells is presently unknown, but apoplastic Ca^{2+} chelation probably reduces its concentration in sites where K^+ efflux is controlled, on the plasmalemma or in the cytoplasm, and stops K^+ efflux. Ca^{2+} concentration in guard cells and the mode of its regulation is presently unknown. The fact that the optimal KCl concentration needed for stomatal opening in *Commelina* is several times higher than that in *Vicia faba* is consistent with the higher sensitivity of *Commelina* to Ca^{2+} (21). *V. faba* guard cells may have more effective means to reduce Ca^{2+} concentration and thus K^+ efflux. It is possible that the relatively high KCl concentration required by *Commelina* compensates for K^+ efflux which takes place simultaneously with its influx during the opening process. Cessation of K^+ efflux can also explain the faster opening rate and wider apertures in the presence of EGTA (Fig. 2).

The observation that in the dark EGTA does not induce significant opening, indicates that it does not affect ion influx directly. The response to EGTA contrasts with that of fusicoccin, which causes rapid opening of stomata (10, 16) even under conditions generally unfavorable for opening (24). Apparently, fusicoccin induces proton extrusion from cells, generating an electrochemical gradient and ion influx (15). Recent evidence showed that K^+ efflux from guard cells is not a passive process (12). Similarly, stomatal closure is not necessarily accompanied by a cessation of K^+ uptake but rather is caused by increased efflux (14). These facts are compatible with earlier findings in connection with the inhibition of stomatal closure by metabolic inhibitors, such as azide, KCN, and DNP (5, 19, 20).

The presence of single ion channels which showed high selectivity for K^+ has been recently demonstrated in the plasmalemma of guard cell protoplasts of *V. faba* (23). Available information does not indicate whether Ca^{2+} is actively involved in K^+ efflux or in the gating of channels through which K^+ is released. A Ca^{2+} requirement for K^+ fluxes is well documented in animal tissues (1, 6, 7) and plant tissues (3) as a component of the mechanism regulating cell volume. On the other hand, Ca^{2+} both reduces K^+ efflux and increases influx (2, 4) in some plant tissues.

Our findings emphasize the crucial role of Ca^{2+} in the control of stomatal aperture through its involvement in the regulation of closure. 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.

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