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Induction of Stomatal Closure by Vanadate or a Light/Dark Transition Involves Ca^{2+}-Calmodulin-Dependent Protein Phosphorylations

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Previous studies indicate that a continual source of adenosine 5′-triphosphate is required for both opening and closing of stomata. However, vanadate (Na_3VO_4 at 500 μM) as well as a light/dark transition induced stomatal closing in epidermal peels of *Commelina communis* L., showing that the stoppage or even the decrease of the activity of the plasma membrane H^+-adenosine 5′-triphosphatase is sufficient to induce stomatal closure. Furthermore, stomatal closing in response to Na_3VO_4 or a light/dark transition was suppressed by inhibitors of metabolism (10 μM carbonyl cyanide *m*-chlorophenylhydrazone) and of protein kinases (20 μM 1-[5-iodonaphthalene-1-sulfonyl]-1H-hexa-hydro-1,4-diazepine), calmodulin antagonists (20 μM *N*-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide), and the anion channel blocker 5-nitro-2,3-phenylpropyllamino benzoic acid (50 μM). These data suggest that the slow, outward rectifying anion channel, whose opening would be related to the membrane potential, and at least one step requiring a protein phosphorylation by a Ca^{2+}-calmodulin-dependent protein kinase of the myosin light chain kinase type might be implicated in the induction of stomatal closing by vanadate or a light/dark transition.

Transpiration and CO_2 assimilation are regulated by stomatal movements resulting from volume changes of guard cells, which depend on ion fluxes (Zeiger, 1983). Stomatal opening is triggered by proton efflux against the transmembrane electrochemical gradient. Therefore, it is an active process that requires metabolic energy. By contrast, stomatal closing is related to depolarization of the plasma membrane, which can result from ion diffusion according to the transmembrane electrochemical gradient. Such a process might be passive. However, the stomatal closing responses to darkness (Karlsson and Schwartz, 1988) and ABA (Weyers et al., 1982) are partially suppressed by hypoxia and metabolic inhibitors, some of which may directly inhibit ATP synthesis in the guard cells. These observations suggest that a continual source of ATP would be required for both opening and closing of stomata.

This working hypothesis has been investigated further by using Na_3VO_4 (sodium orthovanadate), which specifically inhibits the H^+-ATPase of the plasma membrane (Pedersen and Carafoli, 1987). Schwartz et al. (1991) previously showed that vanadate inhibited stomatal opening in the light. These data suggest that the H^+-ATPase of the guard cell plasma membrane was implicated in the light response of closed stomata. Moreover, stomatal opening would require ATP as a fuel for the H^+-ATPase activity.

In an attempt to elucidate the mechanism that requires ATP in stomatal closing, we studied the effect of Na_3VO_4 on the movement of opened stomata. In this paper, we report experiments that establish the induction by vanadate of stomatal closing in epidermal peels of *Commelina communis*. This finding shows that stomatal closure does not require the functioning of the guard cell plasma membrane H^+-ATPase. Furthermore, the stomatal closing response to vanadate was studied in relation to (a) inhibitors of metabolism and of protein kinases, (b) CaM antagonists, and (c) anion channel blockers. Our data suggest that cytosolic ATP might be required for the vanadate-induced stomatal closure by participating in Ca^{2+}-CaM-dependent protein phosphorylations. Moreover, the stomatal closing effect of vanadate would require the opening of the slow anion channel (Schroeder and Keller, 1992). As shown by a comparative study, the same hypothesis has been established in the case of a physiological response of stomata, i.e. the stomatal closing response to a light/dark transition.

MATERIALS AND METHODS

Commelina communis L. seeds were germinated on moistened cellulose tissue for 2 weeks. The seedlings were then transferred to pots containing coarse sand in a growth chamber with a 14-h photoperiod (25°C, RH 60%) followed by 10 h of darkness (20°C, RH 80%). Light (250 μmol m^{-2} s^{-1} PPFD) was supplied by 150-W mercury lamps (HQI-TS; Osram, Germany). The pots were watered three times a day with half-strength Hoagland solution.

Strips (10 × 5 mm) were prepared from abaxial epidermis of young, fully expanded leaves of 4- to 5-week-old

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Abbreviations: CaM, calmodulin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; IDA, imino-diacetate; ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; MLCK, myosin light-chain kinase; NPPB, 5-nitro-2,3-phenylpropyllamino benzoic acid; $t_{\alpha=0.05}$, T student value for $\alpha = 0.05$; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

plants of *C. communis* L. Leaves were harvested in darkness. Epidermal peels were placed on experimental solutions and illuminated (270 μmol m^{-2} s^{-1} PPFD) for 2 h and 30 min before either the addition of Na_3VO_4 or the light/dark transition. Then, illumination (for testing Na_3VO_4) or darkness (for testing the light/dark transition) continued for 3 h.

It has been shown that decreasing the chloride concentration of the bathing solution enhanced Na_3VO_4 uptake (Schwartz et al., 1991). Therefore, for the test of Na_3VO_4, the experimental solution contained the K^+ salt of the impermeant anion IDA (Raschke and Schnabl, 1978) at a final K^+ of 100 mM (50 mM K_2IDA) instead of KCl. It was buffered at pH 5.8 with 10 mM Mes. Na_3VO_4 was used at 100 to 1000 μM. A stock solution, 100 mM, of Na_3VO_4 (Sigma) was prepared by dissolving it in water. The pH was adjusted with Mes to 6.4. This solution was heated to the boiling point and cooled to minimize polymerization (Gallagher and Leonard, 1982). The pH was readjusted if necessary. For the test of a light/dark transition, the experimental solution contained KCl at 60 mM and was buffered at pH 6.0 with 10 mM Mes.

The different inhibitors used were: 10 μM CCCP (Sigma), the protein kinase inhibitors 20 μM H-7 (Biomol Research Laboratories, Inc., Plymouth Meeting, UK), and 20 μM ML-7 (Biomol Research Laboratories), the CaM antagonists 20 μM W-5 (Biomol Research Laboratories) and 20 μM W-7 (Biomol Research Laboratories), and the anion channel blockers 1 mM DIDS (Sigma) and 50 μM NPPB (Cookson Chemicals, Ltd., Southampton, England). These inhibitors were supplied to the bathing solution 30 min before either the addition of Na_3VO_4 or the beginning of the dark period. The stock solutions of the anion channel blockers were prepared as described by Schwartz et al. (1991) and Schroeder et al. (1993).

Stomatal apertures were measured with an optical microscope (Nikon, Optiphot, Tokyo, Japan) fitted with a camera lucida and a digitizing table (Houston Instrument, Austin, TX) linked to a personal computer (Bull Micral, Paris, France). For all of the treatments of a given experiment, the epidermal strips were obtained from the same abaxial surface of the youngest, fully expanded leaf. This protocol was required because of the variability observed in the response of the control to light for epidermal strips from different plants. For each treatment, five epidermal strips were floated on 10 mL of bathing solution in a Petri dish and 10 stomatal apertures from each epidermal strip were measured. Each data point represented the mean of 50 stomatal apertures, with the confidence limits to the mean for $\alpha = 0.05$ (mean $\pm$ $t_{\alpha=0.05}$ $\times$ SE). For each treatment, the results are means $\pm$ 95% confidence limits is noted in the figures. Each experiment was duplicated at least twice.

RESULTS

Induction of Stomatal Closing by Vanadate or a Light/Dark Transition

Na_3VO_4 at 500 μM induced the strongest closure of the stomata; stomatal aperture decreased by 8.9 μm (Fig. 1). A light/dark transition similarly decreased stomatal aperture by 9.3 μm (Fig. 2C).

Since Na_3VO_4 specifically inhibits the H^+-ATPase of the plasma membrane (Pedersen and Carafoli, 1987), the induction of stomatal closing by Na_3VO_4 (Fig. 1) shows that the functioning of the plasma membrane proton pump is not required for stomatal closing in the light. Moreover, the stoppage of the activity of the guard cell plasma membrane H^+-ATPase is sufficient to induce stomatal closing in the light. Furthermore, it has been shown that a light-to-dark transition, resulting in stomatal closure, triggered depolarization of the plasma membrane of guard cells (Ishikawa et al., 1983). This depolarization of the guard cell plasma membrane in the dark could result from a decrease in the activity of the electrogenic proton pump. These considerations raise the questions whether cytosolic ATP is involved in the induction of stomatal closing either artificially by Na_3VO_4 or physiologically during a light/dark transition and, if so, what process requires ATP. In an attempt to answer these questions, we have investigated whether inhibitors of metabolism and of protein kinases could affect the stomatal closing responses to Na_3VO_4 and to a light/dark transition.

Effects of Metabolic and Protein Kinases Inhibitors

In the light, the protonophore CCCP at 10 μM reduced stomatal opening by 3.3 μm (Fig. 2A). In the presence of 500 μM VO_4^{3-}, CCCP at 10 μM reduced stomatal closure by 5.1 μm (Fig. 2B). Under a light/dark transition, CCCP at 10 μM reduced stomatal closure by 7.0 μm (Fig. 2C). Thus, the induction of stomatal opening by the light and of stomatal closing by Na_3VO_4 or a light/dark transition was almost completely suppressed by CCCP.

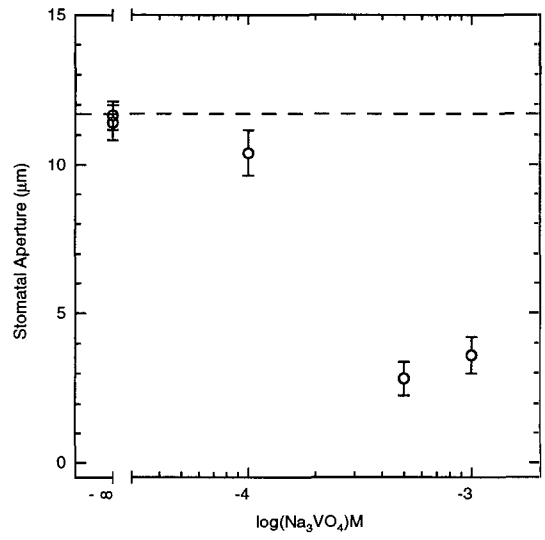

Figure 1. Induction of stomatal closing by Na_3VO_4 in the light (270 μmol m^{-2} s^{-1} PPFD). After 2 h and 30 min of illumination, different concentrations of Na_3VO_4 were added to the buffered solution (50 mM K_2IDA, 10 mM Mes, pH 5.8). Stomatal apertures were measured just before (– – – –) and 3 h after (open symbols) the addition of Na_3VO_4. Results are means $\pm$ $t_{\alpha = 0.05}$ $\times$ SE.

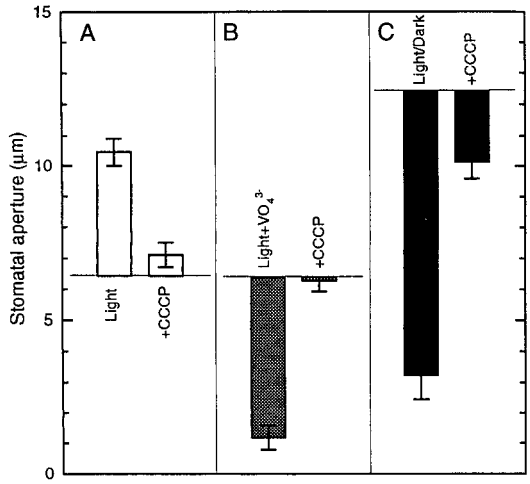

Figure 2. Influence of CCCP on the induction of stomatal closing by Na_3VO_4 or a light/dark transition. After 2 h of illumination (270 μmol m^{-2} s^{-1} PPFD), CCCP (10 μM) was added to the buffered solution. Thirty minutes later, either illumination (A) in the absence of Na_3VO_4 and (B) in the presence of 500 μM Na_3VO_4 or (C) darkness was applied for 3 h. Stomatal apertures were measured just before (base line) and 3 h after (columns) the addition of Na_3VO_4 or the beginning of the dark period. Results are means ± $t_{\alpha = 0.05}$ × SE.

The protonophore CCCP (Heytler, 1963) inhibits the plasma membrane H^+-ATPase as well as the cellular ATP synthases in a nonspecific manner, whereas Na_3VO_4 specifically inhibits the plasma membrane H^+-ATPase (Pedersen and Carafoli, 1987). All of the inhibitory properties of CCCP can explain the suppression by CCCP of stomatal opening in the light. However, in the presence of Na_3VO_4, the CCCP-mediated suppression of stomatal closing (Fig. 2B) would only result from the blocking of the cellular ATP synthases. The same assumption may be established for the CCCP-mediated suppression of the stomatal closing response to a light/dark transition, since this response could result from a decrease in the activity of the plasma membrane H^+-ATPase. In our experimental conditions, CCCP can decrease the cytosolic ATP concentration of the guard cells. In this regard, it has been shown (Shimazaki et al., 1983) that CCCP reduced the ATP content of guard cell protoplasts to 30% of the control one. All of these observations suggest that a threshold concentration of cytosolic ATP would be required for the induction of stomatal closing by Na_3VO_4 or a light/dark transition.

As for elucidating which process requires ATP in the stomatal closing responses to Na_3VO_4 or to a light/dark transition, the two inhibitors of protein kinases, H-7 and ML-7, were tested in the light, in the presence of Na_3VO_4, and under a light/dark transition. ML-7 is more specific for the MLCK than H-7; half-inhibition of MLCK is achieved by 0.3 μM ML-7 and 97.0 μM H-7. As for H-7, it inhibits cAMP- and cGMP-dependent protein kinases and protein kinase C in the micromolar range.

ML-7 at 20 μM induced an increase in stomatal opening under light (Fig. 3A) and strongly inhibited stomatal closing triggered by 500 μM VO_4^{3-} or a light/dark transition (Fig. 3, B and C). In contrast, H-7 at 20 μM had no significant effect on the stomatal movements in any of the tested conditions.

Since ML-7 at 20 μM specifically inhibits MLCK, our data suggest that protein phosphorylations mediated by protein kinases of the MLCK type would be involved in the modulation of stomatal opening in the light as well as in the induction of stomatal closing by vanadate or a light/dark transition. In cells of smooth muscle, it has been demonstrated that activation of MLCK depends on the Ca^{2+}-CaM complex (Somlyo and Somlyo, 1994). In an attempt to ascertain the possible involvement of protein kinases of the MLCK type in the induction of stomatal closing by vanadate or a light/dark transition, CaM antagonists were assayed in the light, in the presence of Na_3VO_4, and under a light/dark transition.

Effect of CaM Antagonists

In the light (Fig. 4A), 20 μM CaM antagonist W-7 induced an overopening of stomata of 2.7 μm, whereas 20 μM W-5, a less potent CaM antagonist than W-7, decreased stomatal aperture by 1.2 μm compared to the light control. In the presence of 500 μM VO_4^{3-} (Fig. 4B), W-7 at 20 μM reduced the stomatal closure by 7.8 μm, whereas 20 μM W-5 did not significantly change the stomatal closure. Similarly, under a light/dark transition (Fig. 4C), W-7 at 20 μM reduced the stomatal closure by 9.1 μm, whereas 20 μM W-5 did not significantly change the stomatal closure.

The differential effects of W-7 and W-5 on stomatal aperture show that they do not result from nonspecific inhibitory effects because these two inhibitors have very similar chemical structures. Moreover, W-7 at 20 μM fully inhibited the stomatal closing induced by 125 μM Ca^{2+} (Fig. 5).

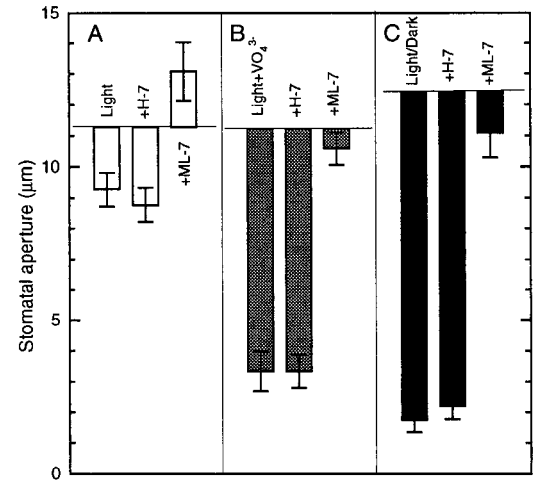

Figure 3. Differential effect of the protein kinases inhibitors H-7 and ML-7 on the induction of stomatal closing by Na_3VO_4 or a light/dark transition. After 2 h of illumination (270 μmol m^{-2} s^{-1} PPFD), H-7 (20 μM) and ML-7 (20 μM) were added to the buffered solution. Thirty minutes later, either illumination (A) in the absence of Na_3VO_4 and (B) in the presence of 500 μM Na_3VO_4 or (C) darkness was applied for 3 h. Stomatal apertures were measured just before (base line) and 3 h after (columns) the addition of Na_3VO_4 or the beginning of the dark period. Results are means ± $t_{\alpha = 0.05}$ × SE.

It has been reported (Hidaka et al., 1980) that the CaM antagonist W-7 binds to CaM after Ca^{2+} has become bound to the protein and, thereby, inhibits the reactions that depend on the Ca^{2+}-CaM complex. Therefore, our last data (Fig. 5) can be explained by the involvement of the Ca^{2+}-CaM complex in the stomatal closing response to Ca^{2+}. Similarly, the specific inhibitory effect of W-7 on the stomatal closing responses to Na_3VO_4 and to a light/dark transition suggests that the Ca^{2+}-CaM complex could participate in these responses.

Data obtained with CCCP (Fig. 2), ML-7 (Fig. 3), and W-7 (Fig. 4) make it most probable that protein phosphorylations would be implicated in the induction of stomatal closing by Na_3VO_4 or a light/dark transition. Such protein phosphorylations could occur at several steps of the transduction pathways induced by vanadate or a light/dark transition. Among these steps, we must consider as equally possible the ones that might be implicated in the activation of channels mediating either K^+ efflux or anion efflux at the tonoplast and the plasma membrane of the guard cells. With regard to the anion channels of the plasma membrane, their putative involvement in the stomatal closing response to Na_3VO_4 or a light/dark transition was investigated by testing blockers of anion channels in the light, in the presence of 500 μM Na_3VO_4, and under a light/dark transition.

Effect of Anion Channel Blockers

Schwartz et al. (1991) have shown that the anion channel blocker DIDS is essentially ineffective in preventing Na_3VO_4 uptake by the guard cells of *C. communis* L. epidermal peels. Likewise, since the Na_3VO_4 induced inhibition of stomatal opening—an inhibitory response requiring penetration of VO_4^{3-} to its intracellular site of action (Schwartz et al., 1991)—was not affected by the anion channel blocker NPPB (Table I), this channel blocker did not prevent Na_3VO_4 uptake by the guard cells. Therefore, DIDS and NPPB were used. In our experimental conditions, NPPB at 50 μM suppressed the stomatal closing responses to VO_4^{3-} (Fig. 6B) and to a light/dark transition (Fig. 6C). Moreover, under light, it induced an increase in the opening of stomata (Fig. 6A). In contrast, DIDS at 1 mM had no significant effect under any of the tested conditions (Fig. 6).

Schroeder et al. (1993) have shown that NPPB, at concentrations ranging from 10 to 50 μM, blocks slow and

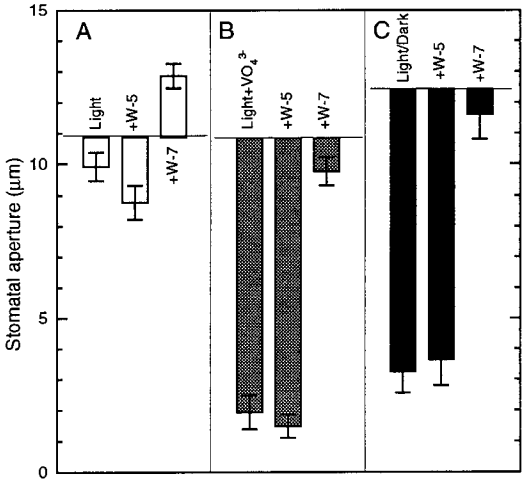

Figure 4. Differential effect of the CaM antagonists W-5 and W-7 on the induction of stomatal closing by Na_3VO_4 or a light/dark transition. After 2 h of illumination (270 μmol m^{-2} s^{-1} PPFD), W-5 (20 μM) and W-7 (20 μM) were added to the buffered solution. Thirty minutes later, either illumination (A) in the absence of Na_3VO_4 and (B) in the presence of 500 μM Na_3VO_4 or (C) darkness was applied for 3 h. Stomatal apertures were measured just before (base line) and 3 h after (columns) the addition of Na_3VO_4 or the beginning of the dark period. Results are means ± $t_{\alpha = 0.05}$ × SE.

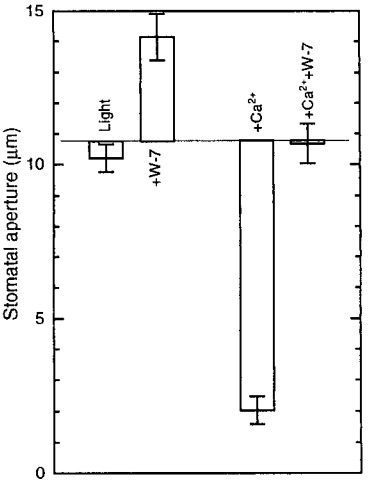

Figure 5. Influence of the CaM antagonist W-7 on the induction of stomatal closing by external Ca^{2+} in the light (270 μmol m^{-2} s^{-1} PPFD). After 2 h of illumination, W-7 (20 μM) was added to the buffered solution. Thirty minutes later, Ca^{2+} (125 μM) was added to the W-7-free and W-7-containing solutions and illumination continued for 3 h. Stomatal apertures were measured just before (base line) and 3 h after (columns) the addition of Ca^{2+}. Error bars correspond to means ± $t_{\alpha = 0.05}$ × SE.

Table I. *The anion channel blocker NPPB does not influence the inhibition of stomatal opening by Na_3VO_4 in epidermal strips of C. communis*

The given values are means ± $t_{\alpha=0.05}$ × SE. To close stomata, epidermal strips were floated on bi-distilled water in darkness for 1 h before transfer to NPPB-containing experimental solutions for 15 min in the dark. Afterward, Na_3VO_4 was added to the bathing solutions in darkness 15 min before illumination (270 μmol m^{-2} s^{-1} PPFD). The stomatal apertures were measured after 2 h and 30 min in the light. ND, Not determined.

Solution	Stomatal Aperture	
	0 μM Na_3VO_4	500 μM Na_3VO_4
	μm	
50 mM K_2IDA	7.68 ± 0.72	0.73 ± 0.38
50 mM K_2IDA + 1 μM NPPB	ND	0.93 ± 0.39
50 mM K_2IDA + 10 μM NPPB	ND	0.93 ± 0.35
50 mM K_2IDA + 50 μM NPPB	ND	0.86 ± 0.34

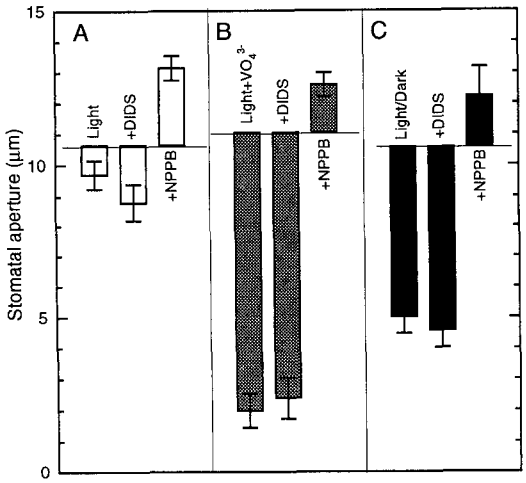

Figure 6. Differential effect of the anion channel blockers DIDS and NPPB on the induction of stomatal closing by Na_3VO_4 or a light/dark transition. After 2 h of illumination (270 μmol m^{-2} s^{-1} PPFD), DIDS (1 mM) and NPPB (50 μM) were added to the buffered solution. Thirty minutes later, either illumination (A) in the absence of Na_3VO_4 and (B) in the presence of 500 μM Na_3VO_4 or (C) darkness was applied for 3 h. Stomatal apertures were measured just before (base line) and 3 h after (columns) the addition of Na_3VO_4 or the beginning of the dark period. Error bars correspond to means ± $t_{\alpha = 0.05}$ × SE.

rapid anion channel currents (Schroeder and Keller, 1992) to a similar extent. Then, the fact that in this concentration range NPPB suppressed the stomatal closing responses to Na_3VO_4 and to a light/dark transition (Fig. 6, B and C, results not shown) does not rule out that both slow and rapid anion channel currents might be involved. In contrast, DIDS, at concentrations ranging from 1 μM to 1 mM, did not affect stomatal closing (Fig. 6, B and C, and results not shown). These last data allow us to conclude that a slow anion channel current is more likely implicated in the stomatal closing responses to vanadate or a light/dark transition, because DIDS specifically blocks the rapid anion channel of the guard cell plasma membrane (Marten et al., 1993). In addition, the fact that an overopening of stomata in the light was obtained with NPPB but not with DIDS (Fig. 6A) supports the hypothesis that the slow anion channel would modulate the stomatal opening response to light (Schroeder et al., 1993).

DISCUSSION

In this study, vanadate was found to induce closing of opened stomata in the light (Fig. 1). Furthermore, some of the characteristics of the stomatal closing responses to vanadate and to a light/dark transition were compared. Both responses were suppressed by the metabolic inhibitor CCCP (Fig. 2), the protein kinases inhibitor ML-7 (Fig. 3), the CaM antagonist W-7 (Fig. 4), and the anion channel blocker NPPB (Fig. 6). These data could indicate that part of the transduction pathway triggered by a light/dark transition might be artificially induced by vanadate.

A common step in these transduction pathways would be depolarization of the guard cell plasma membrane. Indeed, Ishikawa et al. (1983) found that the light/dark transition depolarizes the plasma membrane of *Vicia* guard cells, probably by decreasing the activity of the plasma membrane H^+-ATPase. Moreover, it has been shown in *Vicia* guard cell protoplasts that 10 μM vanadate completely inhibits proton pump currents (Serrano et al., 1988), leading to a depolarization of the plasma membrane. These considerations, together with our data, support the hypothesis that depolarization of the plasma membrane, caused by the stoppage or even the decrease of the electrogenic proton-pumping activity, might be sufficient to induce stomatal closing. Therefore, the stomatal closing responses to vanadate and to the light/dark transition would be related to passive ion fluxes. In this context, one can hypothesize that opening of ion channels, induced by depolarization of the plasma membrane, might be involved in the studied closing responses of stomata.

According to our results (Fig. 2), a threshold concentration of cytosolic ATP would be required for subsequent common steps of stomatal closing induced by vanadate or the dark. Our data (Figs. 3–5) suggest that these steps might be related to protein phosphorylations and that at least some of these protein phosphorylations would be activated by Ca^{2+}-CaM-dependent kinases of the MLCK type.

The formation of the Ca^{2+}-CaM complex and its involvement in the modulation of the activity of protein kinases of the MLCK type would be related to both the availability of CaM and the concentration of cytosolic Ca^{2+}. It has been shown that 500 μM Na_3VO_4 is required for half-inhibition of the plasma membrane Ca^{2+}-ATPase activity from guard cells (Gräf and Weiler, 1989). Also, one can expect that in our experimental conditions, i.e. 500 μM Na_3VO_4 in the bathing medium, vanadate would partly inhibit the activity of the Ca^{2+}-ATPase of the plasma membrane and, thereby, it could induce a small increase in the cytosolic Ca^{2+} concentration.

It is well known that Ca^{2+} strongly inhibits stomatal opening and induces stomatal closing in *C. communis* (Fujino, 1967). In addition, Schwartz (1985) has shown that cytosolic Ca^{2+} participates in the stomatal closing response to a light/dark transition. As for the availability of CaM, its importance would be stressed by the fact that the CaM antagonist W-7 induces a large increase in the free cytosolic Ca^{2+} concentration in *Daucus carota* protoplasts (Gilroy et al., 1987). Indeed, from this last finding, one could expect that W-7 should stimulate stomatal closing through an increase in the cytosolic Ca^{2+} concentration. Nevertheless, W-7 suppressed the stomatal closing responses to vanadate and to a light/dark transition (Fig. 4). The apparent contradiction between these data might be solved by supposing that the promotion of stomatal closure by Ca^{2+} would be obligatorily mediated via the formation of the Ca^{2+}-CaM complex.

Our present data, i.e. W-7-mediated suppression of the stomatal closing responses to vanadate and to a light/dark transition, agree with previous reports showing that CaM antagonists partly inhibited the stomatal closure induced by darkness (Donovan et al., 1985) or by ABA (De Silva et

al., 1985). Shimazaki et al. (1992, 1993) have described that CaM antagonists and inhibitors of protein kinases of the MLCK type block the transduction pathway of blue light. Despite the fact that we did not test precisely the experimental conditions used by Shimazaki et al. (1992, 1993), we never observed any inhibition of stomatal opening under light by CaM antagonists or protein kinases inhibitors. Conversely, in *C. communis*, these inhibitors induced an increase in stomatal aperture under continuous light (Figs. 3A and 4A). An explanation for these paradoxical data would be that both stomatal opening and closing might involve cascades of Ca^{2+}-CaM-dependent protein phosphorylations and might be discriminated by CaM availability and, as suggested by Assmann (1993), by the magnitude, the location, and the temporal pattern of the increase in intracellular free Ca^{2+} concentration.

The anion channel blockers NPPB and DIDS exhibited a differential effect on the stomatal opening response to light and on the stomatal closing responses to vanadate and to a light/dark transition (Fig. 6). These data allow us to propose that the slow anion channel alone would be involved not only in the modulation of the stomatal opening response to light, as suggested by Schroeder et al. (1993), but also in the stomatal closing response to a light/dark transition.

Taken together, our data might suggest that the stomatal closing response to a light/dark transition would result from the activation of the slow anion channel not only by depolarization of the plasma membrane but also via protein phosphorylation processes partly catalyzed by Ca^{2+}-CaM-dependent protein kinases of the MLCK type. This hypothesis is supported by the findings that voltage-dependent anion channels in the plasma membrane of *Vicia* guard cells are regulated by cytosolic triphosphate nucleotides and Ca^{2+} (Hedrich et al., 1990) and that the opening of Cl^--sensitive anion channels in the *Chara* plasma membrane is activated by the Ca^{2+}-CaM complex (Okihara et al., 1993).

However, inhibition of stomatal closing by the protein kinase inhibitor ML-7 might be explained by the blockage of phosphorylation steps involved in processes other than the activation of the slow anion channel. Indeed, the fact that the anion channel blocker NPPB inhibits stomatal closure (Fig. 6) does not demonstrate that ML-7 would block only the opening of anion channels. In particular, ML-7 or other kinase inhibitors might prevent activation of the outward rectifying K^+ channel. With respect to this, the balance between protein phosphorylations and protein dephosphorylations might control partially the activity of the K^+ outward rectifier, as suggested by Thiel and Blatt (1994). Yet, the enhancement of the K^+ outward rectifier would not depend on cytosolic Ca^{2+} (Lemtiri-Chlieh and MacRobbie, 1994), suggesting that, among all of the types of protein phosphorylations, the Ca^{2+}-CaM-dependent protein phosphorylations would not be implicated in the regulation of the K^+ outward rectifier. All of these considerations point out that multiple cascades of different types of protein phosphorylations might be involved in the signal transduction pathways we studied. In this context, it is difficult to identify the phosphorylation steps that are blocked by a given type of protein kinase inhibitors. Therefore, further biochemical and electrophysiological investigations will be needed to identify the substrates of different types of protein kinases, in particular the substrates of the protein kinases of the MLCK type.

It must be emphasized that inhibition by Na_3VO_4 of the proton-pumping activity in the plasma membrane is sufficient to induce stomatal closure in the light. These data indicate that light by itself would regulate stomatal aperture by acting on only the electrical potential of the plasma membrane and not by directly modulating the activities of the different types of ion channels. Moreover, since NPPB, but not DIDS (Fig. 6A), induced an overopening of stomata in the light, activity of the slow anion channel might participate in the regulation of stomatal aperture in the light. Finally, stomatal aperture in the light would be regulated in chief by the balance between the opening activities of the K^+ inward rectifier and of the slow anion channel, such a balance resulting from the level of the plasma membrane polarization and, thereby, from the level of the proton-pumping activity.

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