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Properties of the Signal Transduction Pathway in the Blue Light Response of Stomatal Guard Cells of *Vicia faba* and *Commelina benghalensis*

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Blue light-dependent proton extrusion in guard cell protoplasts from *Vicia faba* and light-dependent stomatal opening in the epidermis of *Commelina benghalensis* are inhibited by the calmodulin (CaM) antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfononamide (W-7) and the myosin light chain kinase (MLCK) inhibitor, 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-7) [Shimazaki, K., Kinoshita, T. and Nishimura, M. (1992) *Plant Physiol.* 99: 1416]. We now suggest that the inhibition occurs in the blue light signaling pathway without affecting the proton pump. Addition of fusicoccin (FC), an activator of H^+-ATPase, to the protoplasts and the epidermis whose blue light-dependent proton extrusion and light-dependent stomatal opening had been inhibited by W-7 and ML-7, induced both proton extrusion and stomatal opening, respectively. Blue light-dependent proton extrusion was inhibited by K-252a, a wide-range inhibitor of protein kinases, and KT5926, a selective inhibitor of MLCK. FC induced proton extrusion in the presence of K-252a and KT5926. In contrast, phenylmercuric acetate (PMA), carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibited both the proton extrusion and stomatal opening, but FC did not induce the responses. These results suggest that W-7, ML-7, K-252a and KT5926 inhibit the signal transduction process by which the perception of blue light is transduced into activation of the proton pump in guard cells, and that MLCK or MLCK-like protein is involved in the blue light response of stomata. The possibility that calcium-dependent, calmodulin independent protein kinase [Harper, J.F. et al. (1991) *Science* 252: 951] functions rather than MLCK in the blue light response of stomata should be noted, however.

Key words: Blue light response — Fusicoccin — Guard cells — H^+-ATPase — Proton pump — Signal transduction.

Stomata on leaves open in response to blue light, and the opening is mediated by an accumulation of potassium salt in guard cells that surround the stomatal pore (Ogawa et al. 1978, Zeiger 1983, Iino et al. 1985, Sharkey and Raschke 1981, Zeiger et al. 1987). Blue light acts as an activator of the proton pump in the plasma membrane of guard cells, and induces proton extrusion, thereby creating an inside-negative electrochemical proton gradient across the plasma membrane (Assmann et al. 1985, Shimazaki et al. 1986). The hyperpolarized membrane potential induces the opening of voltage-gated potassium channels in the plasma membrane of guard cells, and potassium ions are taken up electrophoretically into the cells (Assmann et al. 1985, Schroeder et al. 1987). However, the molecular mechanism by which the perception of blue light is transduced into activation of the proton pump remains largely unknown.

It is postulated that blue light-dependent proton extru-

Abbreviations: CaM, calmodulin; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; FC, fusicoccin; ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine; MLCK, myosin light chain kinase; PMA, phenylmercuric acetate; W-7, *N*-(6-aminohexyl)5-chloro-1-naphthalenesulfononamide.

sion, a driving force for potassium uptake, is mediated by H^+-ATPase in the plasma membrane of guard cells. Its activity is lost when cytoplasmic ATP is removed from the cells (Assmann et al. 1985). Blue light-stimulated swelling of *Vicia* guard cell protoplasts, which corresponds to stomatal opening, and light-induced stomatal opening in *Commelina* and *Vicia* are inhibited by vanadate, an inhibitor of plasma membrane-type H^+-ATPase (Gepstein et al. 1982, Vara and Serrano 1982, Schwartz et al. 1991, Amodeo et al. 1992). Furthermore, immunocytochemical evidence indicates that H^+-ATPase is most abundant in the plasma membrane of *Zea mays* guard cells (Villalba et al. 1991).

Recent investigations have shown that blue light-dependent proton extrusion in *Vicia* guard cell protoplasts and light-dependent stomatal opening in the epidermis of *Commelina benghalensis* are inhibited by CaM antagonists (e.g. W-7) and MLCK inhibitors (e.g. ML-7), which suggests that CaM and MLCK may be involved in the signal transduction process of the stomatal blue light response (Shimazaki et al. 1992).

In the present investigation, we show that FC, an activator of H^+-ATPase in the plasma membrane, induces proton extrusion and stomatal opening in the presence of W-7 or ML-7. Specific inhibition of the light-signaling pathway by K-252a and KT5926, inhibitors of MLCK, is also reported.

Materials and Methods

Plant materials—Plants of *Vicia faba* L. (Ryosai Issun) and those of *Commelina benghalensis* ssp. were cultured in a greenhouse, and guard cell protoplasts from *Vicia* were isolated enzymatically as described previously (Shimazaki et al. 1992).

Measurements of blue light-dependent proton extrusion—Blue light-dependent proton extrusion by *Vicia* guard cell protoplasts was measured with a glass pH electrode (Beckman 39532) using a dual-beam protocol as described previously (Shimazaki et al. 1992). Light intensity was 100 μmol m^{-2} s^{-1} for the blue light pulse (30 s) and 600 μmol m^{-2} s^{-1} for background red light, respectively. The reaction mixture (1.0 ml) consisted of 0.125 mM MES-NaOH (pH 6.0), 1 mM $CaCl_2$, 0.4 M mannitol, 10 mM KCl and guard cell protoplasts (20–60 μg of soluble protein).

Measurement of light-induced stomatal opening in epidermis—The stomatal aperture of the epidermis in *Commelina benghalensis* was determined using a calibrated ocular micrometer as described previously (Shimazaki et al. 1992), but with a slight modification. Apertures of 80 stomata were measured microscopically within 6 min. The epidermis was peeled from the abaxial side of the leaf with forceps according to the method of Weyers and Travis (1981). Blue light was obtained from a halogen lamp (Sylvania EFR 150W) by passing the light through a blue glass filter (Corning 5–60). Red light was provided by passing the light from a halogen lamp (Sylvania 82V, 300W) through a red glass filter (Corning 2–61). Light intensity was 20 μmol m^{-2} s^{-1} for the blue light and 600 μmol m^{-2} s^{-1} for the red light. The bathing solution (2.5 ml) of the epidermis contained 10 mM MES-KOH (pH 6.1), 30 mM KCl and 0.1 mM $CaCl_2$.

Protein determination—Protein was determined by the method of Bradford (1976).

Chemicals—ML-7, W-7 were purchased from Seikagaku Kogyo (Tokyo). K-252a and KT5926 were from Kyowa Medex (Tokyo).

Results

Effect of FC on proton extrusion in the presence of W-7 and ML-7—When guard cell protoplasts from *Vicia faba* were illuminated with a pulse of blue light, superimposed on the background red light, sustained proton extrusion was induced (Fig. 1a). The magnitude of proton extrusion was 0.510 nmol H^+ per pulse per μg of protein, with a maximum rate of 3.63 nmol H^+ h^{-1} per μg of protein around 1.5 min after the pulse. The proton extrusion ceased 12 to 16 min after the pulse. Fusicoccin (10 μM), an activator of H^+-ATPase in the plasma membrane, elicited sustained proton extrusion without blue light (Fig. 1a). Proton extrusion reached a maximum rate of 2.38 nmol H^+ h^{-1} per μg of protein within 15 min, and showed a constant value for at least 20 min. Blue light-dependent proton extrusion was inhibited strongly by W-7 (200 μM) and ML-7 (100 μM) (Fig. 1b, c), with a magnitude of 0.026 and 0.035 nmol H^+ per pulse per μg of protein, respectively. The maximum rates of proton extrusion were 0.17 and 0.34 nmol H^+ h^{-1} per μg of protein with W-7 and ML-7, respectively. However, after the application of blue light in the presence of inhibitors, further addition of FC (10 μM) to the protoplast suspension induced the proton extrusion (Fig. 1b, c). The rates of FC-induced proton extrusion were 1.50 and 2.39 nmol H^+ h^{-1} per μg of protein with W-7 and ML-7, respectively.

Effect of FC on stomatal opening in the presence of W-7 and ML-7—Support for the above observations was obtained from the effect of inhibitors on stomatal responses in *Commelina benghalensis* epidermis. Stomata opened in response to light, and their apertures ranged from 8 to 12 μm after illumination for 2 h (Fig. 2, 5). Light-dependent stomatal opening was suppressed completely by W-7 and ML-7; however, the stomata opened after further addition of FC in the presence of the inhibitor. Stomatal apertures induced by FC were larger than those induced by light, both in the presence and absence of inhibitor (Fig. 2).

Inhibition of blue light-dependent proton extrusion by protein kinase inhibitors—The effects of K-252a, a wide-

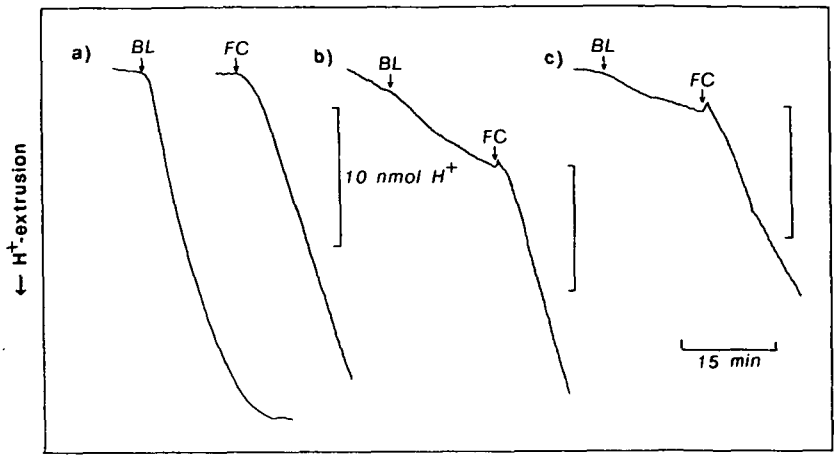

Fig. 1 Blue light-dependent and FC-induced H^+ extrusion in guard cell protoplasts from *Vicia faba* L. Blue light at 100 μmol m^{-2} s^{-1} was given for 30 s, superimposed on the background red light at 600 μmol m^{-2} s^{-1}. Final concentration of FC was 10 μM. (a) FC was added to the protoplast suspension immediately after the blue light-dependent proton extrusion had ceased. Maximum rate of blue light-dependent proton extrusion was 3.68 nmol H^+ h^{-1} per μg of protein, whereas the maximum rate of FC-induced H^+ extrusion was 2.38 nmol H^+ h^{-1} per μg of protein. (b) ML-7 was added to the protoplast suspension at 100 μM, 15 min before the illumination. (c) W-7 was added to the protoplast suspension at 200 μM, 5 min before the illumination with blue light.

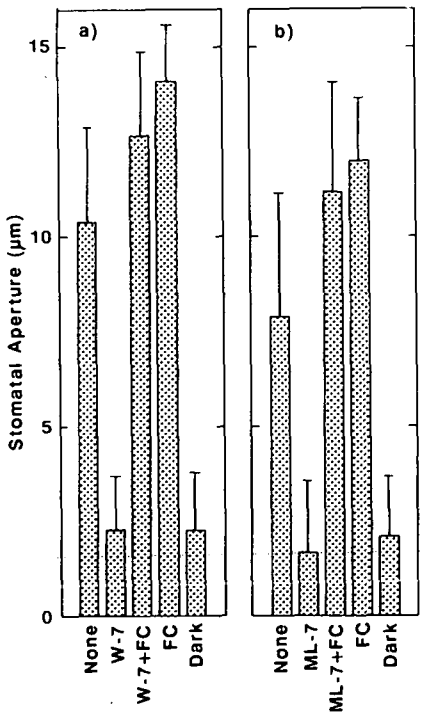

Fig. 2 Induction of stomatal opening in the *Commelina* epidermis by FC in the presence of W-7 or ML-7. W-7 and ML-7 were added to the bathing solution (2.5 ml) 5 min before illumination or addition of FC. Final concentrations of W-7, ML-7 and FC were 200, 100 and 10 μM, respectively. Illumination was performed for 2 h except for "dark" samples.

range inhibitor of protein kinases isolated from *Nocardiopsis* sp., and of KT5926, a selective inhibitor of MLCK which is a derivative of K-252a (Kase et al. 1987, Nakanishi et al. 1988), on blue light-dependent proton extrusion were examined. K-252a inhibited proton extrusion, and the inhibition was concentration-dependent (Table 1). It inhibited blue light-dependent proton extrusion by about 75% at 1 μM, with values of 0.449 and 0.116 nmol H^+ per pulse per μg protein in the control and in the presence of K-252a, respectively (Table 1). KT5926 inhibited the blue light-dependent proton extrusion at the same range of concentration as that for K-252a (Table 1). It inhibited proton extru-

Table 1 Effects of K-252a and KT5926 on blue light-dependent proton extrusion in *Vicia* guard cell protoplasts

Inhibitor	Concentration (μM)	Magnitude of H^+ extrusion (nmol H^+ $pulse^{-1}$ (μg protein)$^{-1}$)
K-252a	0	0.449
	1	0.116
	10	0.047
KT5926	0	0.386
	1	0.078
	10	0.036

K-252a and KT5926 were added to the protoplast suspension about 40 min before illumination with blue light. K-252a and KT5926 were dissolved in DMSO. Final concentration of DMSO in the reaction mixture was 0.5%.

sion by about 80% at 1 μM, with magnitudes of 0.386 and 0.078 nmol H^+ per pulse per μg protein in the control and in the presence of KT5926, respectively. Addition of FC to the protoplast suspensions in the presence of these inhibitors induced rates of proton extrusion similar to those in the controls (Fig. 3). These results suggest that K-252a and KT5926 inhibit the signal transduction pathway in the blue light response of stomata without affecting the proton pump.

Effect of FC on proton extrusion and stomatal opening in the presence of PMA, CCCP and DCCD—Blue light-dependent proton extrusion in guard cell protoplasts

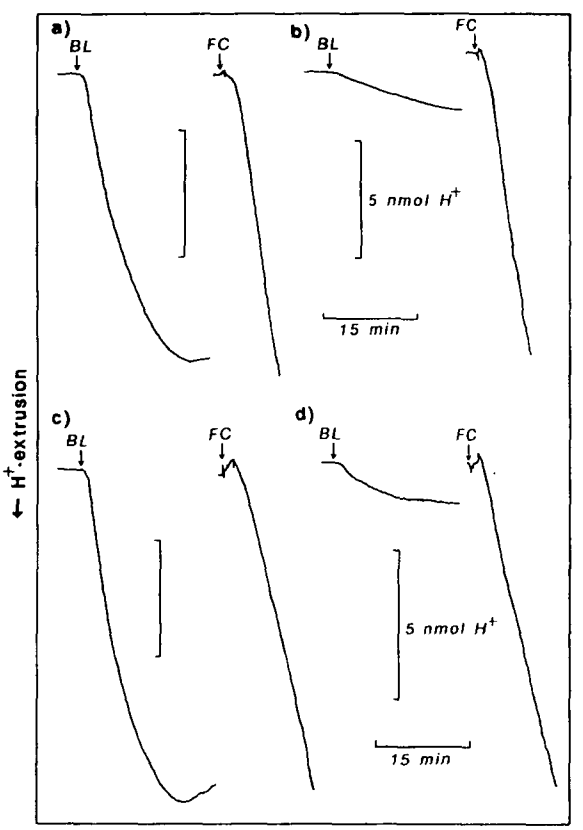

Fig. 3 Inhibition of blue light-dependent proton extrusion in *Vicia* guard cell protoplasts by K-252a and KT5926. (a) Without inhibitor (b) 10 μM K-252a (c) Without inhibitor (d) 10 μM KT5926. K-252a and KT5926 dissolved in DMSO were added at 10 μM, 40 min before blue light illumination. FC dissolved in DMSO was added at 10 μM after the blue light-dependent proton extrusion had ceased. Magnitudes of the blue light-dependent proton extrusion were 0.486, 0.064, 0.349 and 0.031 nmol H^+ per pulse per μg of protein, for (a), (b), (c) and (d), respectively. Maximal rates of FC-induced proton extrusion were 4.96, 5.12, 1.82 and 1.77 nmol H^+ h^{-1} per μg of protein, for (a), (b), (c) and (d), respectively. The reaction mixture (1 ml) contained 23.5 and 41.5 μg of protein as guard cell protoplasts for (a), (b) and for (c), (d), respectively. Final concentration of DMSO was 1% in the presence of FC. Other experimental conditions were the same as in Fig. 1.

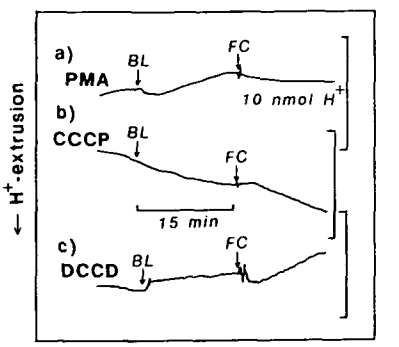

Fig. 4 Inhibition of blue light-dependent proton extrusion in guard cell protoplasts by PMA, CCCP and DCCD. PMA, CCCP and DCCD were added to the suspension 15 min before blue light illumination at final concetrations of 2.5, 5 and 200 μM, respectively. Magnitudes of blue light-dependent proton extrusion were 0.465, 0.016, 0.032 and 0 nmol H^+ per pulse per μg of protein, for control, PMA, CCCP and DCCD, respectively. Maximum rates of proton extrusion induced by FC were 2.63, 0.56 and 0.28 nmol H^+ h^{-1} per μg of protein, for control, PMA and CCCP, respectively. Other experimental conditions were the same as in Fig. 1.

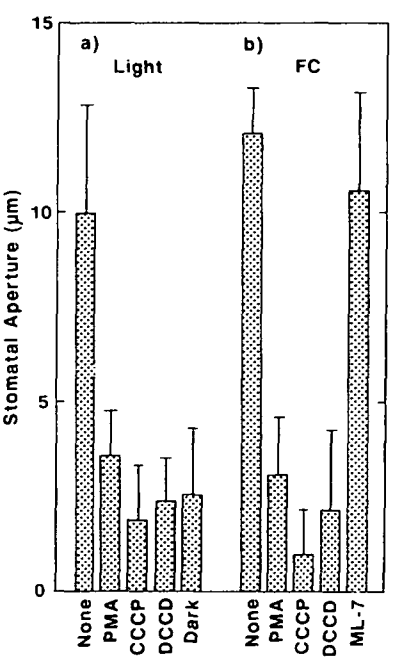

Fig. 5 Blocking of FC-induced stomatal opening in *Commelina* epidermis by PMA, CCCP and DCCD. PMA, CCCP and DCCD were added at 2.5, 5 and 200 μM, respectively, to the bathing solution 5 min before illumination or addition of FC. Other experimental conditions were the same as in Fig. 2.

was inhibited by PMA (2.5 μM), CCCP (5 μM) and DCCD (200 μM) (Fig. 4a, b, c). The values for proton extrusion were 0.016, 0.032 and 0 nmol H^+ per pulse per μg of protein, with PMA, CCCP and DCCD, respectively. Without inhibitor the magnitude was 0.465 nmol H^+ per pulse per μg of protein. In contrast to the inhibitory effects of W-7, ML-7, K-252a and KT5926 (Fig. 1, 3), FC failed to induce proton extrusion with these inhibitors. Light-dependent stomatal opening was inhibited by PMA, CCCP and DCCD (Fig. 5). FC did not induce stomatal opening in the presence of these inhibitors.

These results suggest that W-7, ML-7, K-252a and KT5926 affected neither the energetic status nor the proton pump in guard cells, but inhibited the process(es) by which light signal was transduced into activation of the proton pump in the plasma membrane of the cells.

Discussion

Blue light perceived by photoreceptors leads to activation of the proton pump in the plasma membrane of guard cells through an unknown signal transduction process. The blue light-sensitive pump, a terminal target of the blue light signal, has been suggested to be a H^+-ATPase in the plasma membrane (Assmann et al. 1985, Schwartz et al. 1991, Amodeo et al. 1992), although a redox process in the plasma membrane of guard cells cannot not be excluded (Raghavendra 1990, Pantoja and Willmer 1991, Gautier et al. 1992). A recent study has revealed that blue light-dependent proton extrusion of *Vicia* guard cell protoplasts and light-dependent stomatal opening of *Commelina benghalensis* are inhibited by W-7, a CaM antagonist, and ML-7, a CaM-dependent MLCK inhibitor, suggesting the involvement of CaM and MLCK in the blue light response of stomata (Shimazaki et al. 1992). In the present study, we found that FC, an activator of H^+-ATPase, induced both proton extrusion and stomatal opening that had been inhibited by W-7 or ML-7. This suggests that W-7 and ML-7 inhibit the pathway of light signaling in the processes of blue light response of stomata.

From these results, the inhibition of blue light-dependent proton extrusion by K-252a and KT5926 will be expected. K-252a inhibits MLCK (Nakanishi et al. 1988), and KT5926 selectively inhibits MLCK (Nakanishi et al. 1990). As shown in Table 1, both K-252a and KT5926 at 1 μM suppressed the blue light-dependent proton extrusion by about 80% in *Vicia* guard cell protoplasts, and these inhibitors showed a similar concentration dependency. K-252a and KT5926 inhibit MLCK in a competitive manner in respect with ATP, and have similar inhibition constants (K_i); the values were 0.020 μM and 0.018 μM for K-252a and KT5926, respectively. K-252a has similar K_i values (around 0.02 μM) for protein kinase C, cAMP-dependent protein kinase and cGMP-dependent protein kinase. In contrast, KT5926 has much larger K_i values; the values for protein kinase C, cAMP-dependent protein kinase and cGMP-dependent protein kinase were found to be 0.723, 1.20 and 0.158 μM, respectively (Nakanishi et al. 1990). Thus, the similar concentration dependency for inhibition by K-252a and KT5926 strongly suggests the involvement of MLCK in the blue light response of stomata, although a permeability of the inhibitors across the plasma membrane of guard cells should be considered. This agrees well with our previous results (Shimazaki et al. 1992). It should be noted, however, that the concentration required for 50% inhibition (IC_{50}) was remarkably affected by ATP concentration in the reaction system. For example, the IC_{50} values of KT5926 were 0.072 and 0.39 μM for 0.2 and 1.6 mM ATP, respectively, in the phosphorylation reaction of myosin light chain by chicken gizzard MLCK (Nakanishi 1990). Since ATP concentration in the cytoplasm of guard cells is most likely to be an order of mM, μM range of the kinase inhibitor will be required to inhibit MLCK or MLCK-like proteins in guard cells. Furthermore, the inhibitory action of K-252a and KT5926 seems to be specific to the light-signaling pathway without affecting the proton pump, because FC induced the same rate of proton extrusion in the presence of these inhibitors as those in the controls (Fig. 3).

Specific inhibition of the light signaling pathway by W-7, ML-7, K-252a and KT5926 was supported by the contrasting inhibitory effects of PMA, CCCP and DCCD. These chemical agents suppressed both proton extrusion and the stomatal opening; however, FC failed to restore stomatal responses in the presence of these inhibitors. These inhibitors affected the activity of the proton pump in guard cells. PMA markedly reduces the level of ATP, a fuel for the pump, in guard cell protoplasts (Shimazaki et al. 1983). CCCP acts as a protonophore, which dissipates the proton gradient across the membrane and reduces the ATP level. DCCD acts as an inhibitor of H^+-ATPases in the plasma membrane as well as that of the F-type ATPase of chloroplasts and mitochondria (Sze 1985).

In animal cells, MLCK phosphorylates myosin light chain and this phosphorylation is essential for the contraction of smooth muscle cells (Adelstein and Eisenberg 1980, Ito et al. 1989). MLCK-catalyzed phosphorylation of myosin light chain is also required for the activation of platelets (Saitoh et al. 1986). There are, however, few reports on the functional role of MLCK in plant cells. In Chara cells, it has been suggested that the phosphorylation of myosin causes the cessation of cytoplasmic streaming (Tominaga et al. 1987). Recently, Ca^{2+}-dependent protein kinase, which has a regulatory domain similar to CaM (Harper et al. 1991), has been proposed to inhibit the cytoplasmic streaming in Chara cells, and has been shown to phosphorylate a putative myosin light chain (McCurdy and Harmon 1992). The enzyme is sensitive to W-7 and K-252a, also (Harmon et al. 1987, Li et al. 1991). Thus, the involve-

ment of Ca^{2+}-dependent, calmodulin-independent protein kinase in the blue light response of stomata could not be excluded.

FC activates H^+-ATPase in the plasma membrane of most higher plant cells as well as that of guard cells, and induces the proton extrusion (Marre 1979, Shimazaki et al. 1986, Rasi-Caldogno et al. 1986, Assmann and Schwartz 1992, Johansson et al. 1993). The activation process is initiated by the attachment of FC to the FC-binding protein in the plasma membrane (Aducci et al. 1988). Most recent studies indicate that the signal of FC is transmitted to the C-terminal inhibitory domain of the molecule of H^+-ATPase via this binding protein (Johansson et al. 1993), thereby increasing the activity of H^+-ATPase. Since FC elicited proton extrusion in the presence of W-7, ML-7, K-252a and KT5926, the activation signal of FC could be transmitted to the H^+-ATPase molecule in the presence of these inhibitors. This suggests that the FC-binding protein acts on the site closer to the molecule of H^+-ATPase than the sites of inhibition by antagonist and kinase inhibitors, or acts independently from the signaling pathway of blue light.

Finally, all of the results obtained in the present investigation are consistent with that MLCK or MLCK-like protein is involved in the blue light response of stomata. However, more direct evidence will be required to show this, because the data were solely provided from the use of pharmacological tools that are usually used in animal cells.

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