Vanadate Inhibits Blue Light-Stimulated Swelling of *Vicia* Guard Cell Protoplasts¹

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**ABSTRACT**

When supplied under low chloride concentrations, vanadate inhibits the blue light-stimulated swelling of *Vicia faba* L. guard cell protoplasts in a dose-dependent fashion. The volume of guard cell protoplasts incubated in 10 mM K-imino-diaceitic acid, 0.4 M mannitol, and 1 mM CaCl₂ remained essentially constant under 1000 μmol m⁻² s⁻¹ red light, but increased an average of 27% after 8 min of the addition of 50 μmol m⁻² s⁻¹ blue light to the background red light. At 500 μM, vanadate completely inhibits the response to blue light. Vanadate also inhibits the swelling of guard cell protoplasts stimulated by the H⁺-ATPase agonist fusicoccin. The vanadate sensitivity of the blue light-stimulated swelling implicates a proton-pumping ATPase as a component of the sensory transduction of blue light in guard cells.

Electrogenic proton pumping at the guard cell plasma membrane provides a driving force for the ion uptake required during stomatal opening (23). A patch clamp study with GCPs³ has characterized a red light-stimulated outward electrical current that implicates the operation of an electrogenic pump at the guard cell plasma membrane (18). The sensitivity of the pump currents to the protonophore CCCP and to vanadate implicates a H⁺-ATPase.

Blue light-stimulated proton pumping at the guard cell plasma membrane has been characterized as outward electrical currents in patch clamp experiments (1) and as medium acidification with suspensions of GCPs (19). A test of the vanadate sensitivity of medium acidification gave negative results. Although orthovanadate is a highly specific inhibitor of P-type ATPases (10), its inability to inhibit blue light-stimulated medium acidification is hard to interpret because of uncertainties about vanadate uptake by cells.

A recent study on stomatal opening in *Commelina* showed that lowering the chloride concentration of the bathing medium enhanced vanadate uptake (16). This observation may explain the vanadate insensitivity observed under some experimental conditions.

We report here on a reevaluation of the vanadate sensitivity of the blue light-stimulated proton pumping in guard cells in experiments measuring blue light-stimulated swelling of *Vicia faba* guard cell protoplasts incubated in low chloride concentrations. The results show that in these conditions, vanadate inhibits both blue light- and FC-stimulated swelling of GCPs.

**MATERIALS AND METHODS**

Plants of *Vicia faba* L. (cv Long Pod) were grown in a greenhouse under natural light conditions and temperatures ranging between 15 and 29°C. Plants were watered daily and fertilized once a week with a solution of Sponnit (Morrison's Orchard Supply Co., Yuba City, CA). Second and third bifoliate leaflets from 5-week-old plants were used for experimentation.

GCPs were isolated as described previously (6). After purification, protoplasts were resuspended in 0.4 M mannitol and 1 mM CaCl₂, and the suspension was maintained in ice until it was used.

For the swelling experiments, GCPs were incubated in 0.4 M mannitol, 1 mM CaCl₂, and the K⁺ salt of the impermeant anion IDA (13) at a final concentration of 10 mM. GCPs were gently stirred in a temperature-controlled chamber. Final GCP concentration was 0.5 × 10⁴ cells/mL. A stock solution of sodium orthovanadate (Sigma Chemical Co.) was prepared by dissolving it in water. The pH was adjusted with Mes to 6.4. This solution was heated to a boiling point and cooled to minimize polymerization (3). The pH was readjusted if necessary.

Protoplast diameters were measured with an Olympus BH-2 microscope attached to a Javelin JE2362A digitizing CCD camera. Optical images of protoplasts were digitized for subsequent analysis. This procedure made it possible to obtain a digitized optical field showing 10 to 15 protoplasts in less than 30 s, allowing diameter measurements (*n* = 50) at 2-min intervals. Image processing was handled with an IBM PC-based MV-1 Board from Metabyte Corporation and Jandel Scientific's JAVA image analysis software. The volume of each GCP was calculated as \((4/3)\pi(d/2)^3\), where *d* is the diameter, and the results are expressed as mean volume ± SE (*n* = 50). GCP populations having a starting mean volume value of 3000 μm³ were considered suitable for further experimentation. All experiments were repeated at least three times.

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³ Abbreviations: GCP, guard cell protoplast; CCCP, carbonylcyanide m-chlorophenylhydrazone; FC, fusicoccin; IDA, imino-diaceitic acid

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Sample irradiation was done with two slide projectors (Carousel 4000, Kodak) equipped with a 300-W mini-multi-mirror projector lamp (General Electric Co.). One projector was equipped with a No. 2424 blue Plexiglas filter (Roehm and Hass, Hayward, CA) with maximum transmittance at 470 nm and a half band width of 100 nm; the other was equipped with a red filter (Schott RG-610). Blue and red light fluence rates were 50 and 1000 μmol m⁻² s⁻¹, respectively. A bifurcated fiber optic bundle allowed sample irradiation at the same spot by either or both lights. Fluence rates were measured with a Li-Cor quantum sensor. All experiments were performed at 28°C.

RESULTS

GCPs incubated in darkness or in 1000 μmol m⁻² s⁻¹ red light showed no volume changes for up to 80 min (data not shown). On the other hand, blue light pulses (1 min, 500 μmol m⁻² s⁻¹) given in a background of red light stimulated a transient swelling that averaged a 25% increase in volume over a 20-min period, after which a subsequent return to baseline levels was observed. However, both the extent and the kinetics of these responses varied substantially between experiments, making it difficult to use the response to pulses for the analysis of the vanadate effect.

Continuous blue light added after a 30-min incubation in red light stimulated a rapid and reproducible swelling of the GCPs (27% increase in volume in 8 min, Fig. 1). This swelling is consistent with the reported stimulation of proton pumping by blue light in a background of red light irradiation (1, 19).

Addition of 500 μm vanadate to the incubation medium at the onset of the red light irradiation completely inhibited the blue-stimulated swelling response (Fig. 1).

The vanadate sensitivity of the swelling response in a low chloride medium was also tested in experiments with the fungal toxin FC. FC, a powerful activator of the H⁺-ATPase (7), has been shown to stimulate stomatal opening in epidermal strips (5, 23) and swelling of GCPs (15). Addition of 15 μM FC to GCPs incubated in darkness in a medium containing 0.4 mM mannitol, 1 mM CaCl₂, 10 mM K-IDA, and 10 mM Mes/NaOH, pH 6.4, caused a rapid and pronounced swelling (Fig. 2), which was nearly completely inhibited by a preincubation in 500 μM vanadate for 30 min. Microscopic examination showed that GCPs treated with FC in the presence of vanadate failed to show the enlarged vacuoles seen in FC-treated cells, and had a more homogeneous distribution of organelles throughout the cytosol (Fig. 3).

A dose-response curve for the vanadate inhibition was obtained by incubating GCPs in different vanadate concentrations for 30 min under 1000 μmol m⁻² s⁻¹ red light. Volume measurements were obtained at that time and then again after addition of 50 μmol m⁻² s⁻¹ of blue light for 12 min (Fig. 4). The results obtained showed that vanadate inhibited the blue light-stimulated swelling in a dose-dependent fashion, with 50% inhibition obtained at 150 μM vanadate. It is of interest that the sensitivity to vanadate decreased at concentrations higher than 500 μM (Fig. 4), probably because of a decrease in the concentration of the active form of vanadate in the suspension medium caused by polymerization reactions (11, 14). These results indicate that some of the studies reporting a vanadate insensitivity at vanadate concentrations >500 μM might need reinterpretation.
DISCUSSION

The characterization of a blue light-induced swelling of GCPs (25) has provided evidence for the physiological competence of GCPs and for an intrinsic light response of guard cells. Subsequent studies have shown that blue light stimulates proton pumping at the guard cell plasma membrane (1, 19). The K⁺ requirement of the swelling (25) implies a relationship between proton pumping, ion uptake, and an increase in guard cell osmotic potentials and swelling.

Proton extrusion at the guard cell plasma membrane could ensue from two alternative pumping mechanisms: a H⁺-ATPase or a redox system analogous to the charge-separating electron transport chains in chloroplasts and mitochondria (12). Indirect evidence for each mechanism has been provided by the characterization of ATPase activity in crude fraction of guard cell membranes (2, 8, 20) and the demonstration of a capacity of guard cells to reduce tetrazolium (22) and to oxidize exogenous NADH (4, 9).

Orthovanadate, a highly specific inhibitor of P-type ATPases (10, 11), provides an experimental tool to distinguish between the two proton-extruding mechanisms. Red light-stimulated proton pumping is inhibited by vanadate supplied intracellularly via the pipette in patch clamp experiments (18), indicating that the observed proton pumping is mediated by a H⁺-ATPase. On the other hand, the blue-light stimulated medium acidification by GCPs was found to be vanadate-insensitive (19), and this observation has been interpreted as evidence for the operation of a proton-extruding redox system in guard cells (12). However, a conclusive interpretation of these vanadate results requires elucidation of the extent of vanadate uptake by the cells.

The importance of vanadate uptake in experiments testing vanadate sensitivity has been elegantly demonstrated in a
recent study showing that chloride and vanadate uptake seem to compete for the same uptake mechanism (16). This competition appears to prevent vanadate uptake in the presence of high Cl⁻ concentrations. Inhibition of vanadate uptake can be important in experiments with guard cells in epidermal peels and GCPs, which commonly include high Cl⁻ concentrations in the incubation medium.

In the present study, we reevaluated the vanadate sensitivity of the blue light response of guard cells under low Cl⁻ concentrations and found that under these conditions, vanadate inhibits the blue light-induced swelling of GCPs. These results indicate that the previously reported vanadate insensitivity of the blue light-stimulated medium acidification by GCPs, measured in the presence of 10 mM KCl, was probably a result of insufficient vanadate uptake. The vanadate sensitivity of the blue light-stimulated swelling implicates a H⁺-ATPase as the underlying mechanism extruding protons and leading to swelling. This conclusion is supported by the similarity between the vanadate sensitivity of the blue light-stimulated swelling and that stimulated by FC, which is a well-characterized H⁺-ATPase-dependent response (7), and by the ATP requirement of the blue light-stimulated outward electrical currents (1).

The implication of a H⁺-ATPase in blue light-stimulated proton pumping provides a unifying link between the guard cell responses to blue and red (PAR) light. Stomatal responses to photosynthetically active radiation are mediated by the guard cell chloroplast (17, 24), which supplies ATP to fuel the H⁺-ATPase, and a hitherto unidentified pump regulator (18). The guard cell chloroplast also has a specific response to blue light (21). It is therefore possible that the stimulation of the H⁺ ATPase by blue light is also mediated by the guard cell chloroplast via the same pump regulator stimulating the pump in response to red light. Studies on the sensory transduction and physiological roles of these guard cell photoresponses should prove rewarding.

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LITERATURE CITED