Effect of Vanadate on Bean Leaf Movement, Stomatal Conductance, Barley Leaf Unrolling, Respiration, and Phosphatase Activity

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ABSTRACT

Vanadate (Na₃VO₄) inhibits leaf movement and stomatal conductance of Phaseolus vulgaris L. cv. Carlos Favorit in light-dark cycles as well as photomorphogenetic leaf unrolling of Hordeum vulgare L. cv. Rupal. Inhibition was 50% by 10 to 100 micromolar vanadate and 100% by millimolar vanadate. Leaf unrolling was also inhibited by oligomycin and diethylstilbestrol.

Millimolar vanadate is required to affect respiration of bean plants and barley leaves, while even this concentration has no effect on respiration of isolated pulvinal tissue.

A nonspecific phosphatase activity extracted from both bean pulvini and barley leaves was found in all of four fractions of a differential centrifugation procedure (i.e. cell walls + nuclei, mitochondria, microsomes, supernatant), but more than 90% of the specific and total activity was located in the supernatant. The phosphatase activities of all fractions were inhibited by concentrations of vanadate similar to those which inhibited leaf movement, stomatal conductance of bean, and unrolling of barley leaves.

Leaf movement in nyctinastic plants as well as leaf unrolling in cereals are phytochrome regulated events. While leaf movements, such as those of Albizzia, Pithecolobium and bean plants are relatively rapid, reversible, light-sensitized and turgor dependent reactions, cereal leaf unrolling is a slow, irreversible, photomorphogenetic process brought about by differential growth. Closing of bean leaves takes 30 to 40 min, while dark-grown primary leaves of barley take about 24 h after light stimulation to complete unrolling. Stomatal movements are as rapid as leaf movements but do not seem to involve phytochrome regulation.

Turgor is a key component in all of the three mentioned types of movement, though it has not yet been established whether or not an asymmetric turgor variation is driving irreversible leaf unrolling as is the case for reversible leaf and stomatal movements. In pulvini, as in stomates, the asymmetric turgor is caused by massive ion fluxes. The mechanism of this ion movement may be elucidated by in vivo and in vitro studies employing specific effectors and inhibitors.

ever since vanadate was found as an impurity in muscle ATP obtained from Sigma (14), research on this compound has expanded immensely. However, relatively few studies concentrate on higher plants. Vanadate, even in µM concentration, is a potent inhibitor of (Na⁺, K⁺)ATPase of animal cells (1, 2, 6, 7), dynein ATPase from cilia, flagella and spindle mechanism (5, 15, 18), sarcoplasmic reticulum (Ca²⁺)ATPase (19, 24), plasmamembrane ATPase of Neurospora (3), and ion stimulated plant root ATPases (8, 11). Mitochondrial ATPase is unaffected by µM vanadate (3, 14), but mM concentration inhibits both mitochondrial and glycolytic energy metabolism (4, 9, 23) as well as myosin ATPase (12, 15). In addition, vanadate has been reported to affect a range of other enzymes (21); thereby, vanadate is clearly not a specific inhibitor of (Na⁺, K⁺)ATPase as originally believed (19). However, in a given system and at a defined low concentration, vanadate may be a selective inhibitor of specific ion stimulated ATPases, thus being a true biological probe (3, 8, 15).

Involvement of ATPases in leaf movements has been suggested for Mimosa pudica (17, 22). In vivo effects of vanadate on a higher plant were first reported in 1979 (20); rhythmic leaflet movement in Albizia julibrissin was inhibited by mM vanadate.

We now report on in vivo effects of vanadate on three turgor driven systems (leaf movement, stomatal movement, and leaf unrolling) that correlate with in vitro effects of vanadate on phosphatase activity of bean pulvini and barley leaves but not with respiration of either.

MATERIALS AND METHODS

Plant material. Bean, Phaseolus vulgaris L. cv. Carlos Favorit, was grown in vermiculite under light-dark cycles (12:12) at 25 C, 70% RH. Fourteen- to sixteen-day-old plants with only two primary leaves were used in all experiments.

Barley, Hordeum vulgare L. cv. Rupal, was grown in a Perlite-soil mixture at 25 C in darkness. Six- to seven-day-old etiolated seedlings, 10 to 12 cm tall, were used in all experiments. A dim green safelight was used whenever plant material was handled during a dark period.

Chemicals.

Inhibitors. DES³ (Sigma); oligomycin (Sigma O 4876); ouabain (Sigma O 3125); sodium °-vanadate, Na₃VO₄ (Fisher Scientific Co.); and quercetin (Merck) were used.

Resuspension Buffer. MOPS (25 mM) (Sigma) + 0.25 mM sucrose + 0.1 mM MgCl₂ or MgSO₄ were used, and pH was adjusted to 7.2 with 6 N NaOH.

Homogenizing Buffer. EDTA (3 mM) and 1 mM PMSF (Sigma) were added 12 h before use and 14 mM DTT was added 1 h before use to the resuspension buffer, and pH adjusted to 6.8 with 6 N NaOH.

³ Abbreviations: DES, diethylstilbestrol; MOPS, morpholinopropionate sulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PIPES, 1,4-piperazinediethane sulfonic acid; p-NPP, p-nitrophenyl phosphate; α-NAP, α-naphthyl phosphate.

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2 To whom reprint requests should be sent.
Oxidoreductase Assays. Potassium phosphate buffer, (pH 7.5); digitonin (Merck); Cyt c (Sigma, type VI); NADH (Boehringer-Mannheim, Indianapolis, IN; tetrasodium salt) in the above phosphate buffer; sodium dithionite Na₂S₂O₅ (Merck).

Phosphatase Assay. Buffer for organic phosphates was 25 mM PIPES or MOPS + 1.5 mM MgSO₄, pH adjusted with Tris base. Organic phosphates were always 3 mM; Tris-ATP (Sigma A 0270, vanadate-free); ADP (monopotassium salt, Boehringer-Mannheim); AMP (sodium salt, Sigma type III); p-NPP (disodium salt, Sigma); αNAP (sodium salt, Sigma); pH adjusted with Tris base.

Protein Standard. BSA (Sigma, A 4378).

Leaf Angle. Bean plants were cut just above ground and placed in 20-ml test tubes with 0, 10, 40, and 1000 μM boiled, aqueous or buffered (2.5 mM MOPS, pH 7.1) solution of vanadate and left in the controlled environment with the leaves free to move. The angle between petiole and lamina was measured during consecutive light-dark cycles at regular intervals over a 50-h period. Measurements were made with a protractor, either directly or on projections of 16-mm film from time-lapse photography. When measured directly, plants were damaged and therefore discarded after measuring; each angle, for a given time and vanadate concentration, was the average of 20 to 60 measurements.

Stomatal Conductance. Stomatal conductance was measured with a Li-Cor (Li-Cor, Inc., Lincoln, NE) LI-1600 Steady State porometer at one time only, using 12 bean plants of the above described system in each of two treatments: 0 and 100 μM vanadate, 7 h incubation in light. However, water loss from test tubes with bean plants and 0, 10, 40, and 1000 μM boiled aqueous or buffered solution of vanadate was measured at regular intervals over 24 h in the controlled environment. Each measurement at a given time and vanadate concentration involved at least 10 plants. As evaporation directly from test tubes and transpiration from plants during the dark period was insignificant, water loss from the test tubes was assumed to be solely due to stomatal transpiration. As the plants had no root system, the observed transpiration rates are expressions of stomatal conductance. Stomatal conductance measured with the porometer was equal to those calculated from transpiration rates.

Leaf Unrolling. Ten barley leaf sections per treatment, 1 cm long, taken between the third and fourth cm from the apex of etiolated leaves, were incubated on 2 ml of 0, 1, 10, 100 and 1000 μM buffered (2.5 mM MOPS, pH 7.1) and boiled vanadate solutions, contained in heat sterilized 5-cm Petri dishes. Unrolling was induced by 5 min irradiation with 655 nm red light. After 24 h incubation at 25 C in darkness, the width of the leaf sections was measured with the help of an overhead projector. In addition to vanadate inhibition, the effect of DES, oligomycin, ouabain and quercetin on leaf unrolling was also studied. Further, the time course of vanadate inhibition was studied by incubation on vanadate for 0 to 24 h followed by incubation on water making the total incubation time 24 h in all cases.

All experiments were repeated three or more times and the statistically analyzed data are presented with standard errors of the means.

Respiration. Five to seven bean plants cut just above ground were placed in 50 ml 0, 10 or 1000 μM buffered (pH 7.1) and boiled vanadate solution and put in a 10-liter cuvette at 25 C. The plants in the cuvette received 0.5 liter of humid air/min and were kept throughout the experiment in alternating darkness and light (Philips HPJ/T 375 w lamp with a 10-cm waterbath heat filter, 1200 μE m⁻² s⁻¹). The CO₂ concentration of the incoming air (Fig. 1A) was measured for several minutes at the beginning of each experiment with an IR gas analyzer (Uras 1; Hartmann & Braun AG, Frankfurt am Main, West Germany) and after 2 and 6 h. The CO₂ concentration of air leaving the cuvette was measured continuously the rest of the time. During the first hour of each experiment the system was left to equilibrate in darkness (Fig. 1). Then vanadate (or buffer) was added, and respiration noted at 60, 90 and 120 min (Rₖ). Subsequently, light was turned on for 1 or 2 h to allow vanadate be taken up by the transpiration stream. Photosynthesis was measured as soon as the reading became stable (Pₛ) and just before the light was turned off (Pₕ). The time of first effect of vanadate on photosynthesis (T) was noted. Respiration was once more noted after 60, 90 and 120 min of darkness (Rₖ). The effect of vanadate on dark respiration and photosynthesis was calculated as the relative difference between Rₛ and Rₖ and between Pₕ and Pₛ corrected for the blind value (when adding buffer only).

Respiration of isolated bean pulvini and barley leaf sections was measured with a Warburg (B. Braun, Melsungen, West Germany) apparatus at 25 C. Bean plants were cut as usual and incubated either 2 or 24 h in 0, 10, 100 or 1000 μM buffered (pH 7.1) and boiled vanadate. O₂ consumption of 10 isolated pulvini in their respective vanadate solutions was subsequently measured every 15 min for 3 h in each of three microcuvettes per treatment. Each experiment was carried out twice.

O₂ consumption of the usual barley leaf sections was measured every 15 min for several hours before and during unrolling within the first 12 h after treatment was commenced (Table 1). Respiration of 10 leaf sections in five experiments was measured in each of two or three microcuvettes in four treatments (pH 7.1): 5 min red light (655 nm), red light + 100 μM vanadate, red light + 1000 μM vanadate and dark control.

Tissue Fractionation. Five g laminal bean pulvini and 5 g petiole reference tissue taken 2 h after the beginning of a light
period or 10 g red light treated, etiolated barley leaves were harvested, homogenized for 8 min at 4 C in homogenizing buffer (1 ml g⁻¹ tissue) with a mortar and pestle, and strained through four layers of cheesecloth. The filtrate was separated into four fractions by differential centrifugation in a Sorval Superspeed RC2-B mounted with a SS 34 rotor. Zero to 1100g (10 min) was designated wall + nucleus fraction (W), the 1100g to 9000g (15 min) was the mitochondrial fraction (OX), the 9000g to 48200g (60 min) was the microsomal fraction (RE) and the supernatant containing soluble enzymes was designated (S). Each pellet was resuspended in 4 ml resuspension buffer, but the mitochondrial fraction only after it was washed, resuspended in 25 ml resuspension buffer and resedimented (9000g, 15 min). The resuspended fractions were further homogenized by sucking them 25 times through the narrow tip of a Pasteur pipette followed by 25 strokes in an Elvehjem homogenizer (B. Braun). All fractions were then frozen (−20 C) until used for assays within 96 h. While frozen fractions retained constant activity for weeks, fractions stored at 4 C were found to degrade in a matter of days.

Enzyme Assays. NADH-Cyt c reductase, NADPH-Cyt c reductase and Cyt c oxidase assays were performed at room temperature (25 C) on a recording Zeiss spectrophotometer according to Hodges and Leonard (13). Relative specific reductase and oxidase activities are taken as indications of relative purity of the microsomal and the mitochondrial fractions, respectively.

Phosphatase was assayed with AMP (pH 5.3 to 9.0), ADP (pH 6.8) AMP (pH 6.8, p-NPP (pH 6.0 and 8.5) and α-NAP (pH 6.0). The assay was performed with 20 µl sample protein (or buffer) + 500 µl 3 mM organic phosphate (or buffer) ± inhibitor (or buffer) mixed at 0 C, agitated 30 min in a 30 C waterbath, and 80 µl 50% TCA added to stop enzymatic reaction. Each assay was repeated at least three times. Released Pi was assayed at room temperature (25 C) by the method of Dryer et al. (10). Care was taken to compensate for nonenzymic hydrolysis, sample absorbance, and phosphate content as well as interfering substances (e.g. the slight interference of 1 mm vanadate). Hydrolysis of p-NPP and α-NAP at acid pH was taken as indication of acid phosphatase activity and hydrolysis of p-NPP at basic pH was taken as indication of basic phosphatase activity.

Protein was determined by the method of Lowry et al. (16), care being taken to eliminate or compensate for interfering substances, in particular the homogenizing buffer.

RESULTS

Leaf Angle. Bean plants cut and placed in water or buffer (pH 7.1) continue to move their leaves for several days in light-dark cycles. When incubated with aqueous or buffered (pH 7.1) vanadate solutions, oscillations were induced by increasing concentrations up to 1 mM which proved lethal after only a few hours (Fig. 2B). Final opening in the second and third light periods was inhibited 50% by 15 µM vanadate and final closing in the second dark period was inhibited 50% by 60 µM vanadate (Fig. 2A). As inhibition is found to be progressive, the difference in inhibition of final opening and final closing in light and dark periods is not important, and this is an expression of this process. The effect of vanadate was independent of pH (6.0 to 7.1).

Stomatal Conductance. Stomatal conductance of the excised bean plants during the first light period was inhibited by increasing vanadate concentrations (Fig. 3B), but was not affected measurably during the dark period when transpiration was insignificant, except when a vanadate concentration proved lethal. The (t) indicates time of measurement with a Li-Cor LI-1600 Steady State porometer. Measured stomatal conductance was 0.45 cm s⁻¹ when plants were placed in water and 0.23 cm s⁻¹ when plants were placed in 100 µM vanadate (pH 7.1). These values differ less than 10% from stomatal conductance calculated from the transpiration data given in Figure 3B. Stomatal conductance was inhibited 50% by 100 µM vanadate (Fig. 3A).

Leaf Unrolling. Two of the tested ATPase inhibitors, ouabain and quercetin, in concentrations ranging between 1 µM and 1 mM were without any effect on the red light stimulated unrolling of barley leaves (data not presented). The others, oligomycin, DES and vanadate proved to be powerful inhibitors of the unrolling process, particularly oligomycin (Fig. 4A). Leaf unrolling was inhibited 50% when incubated with 1.5 µM oligomycin, 4 µM DES or 15 µM vanadate, respectively.

Figure 4B shows the time course of barley leaf unrolling in darkness after irradiation for 5 min with 658 nm red light. One curve illustrates simple unrolling for 24 h on water, while the other curve shows unrolling in the same period during which a varying number of the initial hours were spent on 1 mM vanadate. The more or less symmetrically opposing curves with their strong negative correlation (correlation coefficient r = −0.93) are highly suggestive of a quantitative and gradual inhibition. Once the leaves were washed free of the inhibitor and placed on vanadate-free medium, they unrolled partly within 24 h, indicating the reversible nature of the inhibition. None of the effective inhibitors however, nullified the initial phytochrome stimulus which triggers the unrolling process.
EFFECTS OF VANADATE ON MOVEMENT

Respiration. The results of respiration experiments with bean plants and vanadate are given in Table II. While 1 mM vanadate taken up during 2 h of light significantly (t-test, significance level 0.01) increases dark respiration by the average of 20%, 100 μM vanadate does not affect respiration in the same period. As indicated in Table II, 1 mM vanadate may increase respiration even within 1 h. Photosynthesis is reduced 5% by 100 μM vanadate and 20% by 1 mM vanadate (significance level 0.005 or better). Photosynthesis is on the average affected after 92 min and 72 min by the low and high vanadate concentrations, respectively. However, the reduction in photosynthesis is thought to result from reduced leaf area index caused by withering rather than from physiologic inhibition.

The respiration of isolated laminal pulvini from bean plants incubated 2 or 24 h with 0, 10, 100 or 1000 μM vanadate was not, at any level, significantly affected.

For any treatment, respiration of barley leaf sections was constant before and during unrolling with 1 to 12 h after treatment was commenced. Average O2 consumption during four treatments is given for five experiments in Table I, along with the standard error of the means. Red light (5 min, 658 nm, 24 μE m⁻² s⁻¹) increased respiration 28% over dark control (significance level better than 0.001). Although 100 μM vanadate did not significantly affect respiration, 1 mM vanadate reduced O2 consumption to the level of the dark control; only red light treated leaf sections incubated with buffer unroll.

Tissue Fractionation and Enzyme Activities. The mitochondrial (OX) and microsomal (RE) fractions were both characterized by containing 70% or more of their respective marker enzymes, Cyt c oxidase and NADH-Cyt c reductase. NADPH-Cyt c reductase was found to be only 10% as active as NADH-Cyt c reductase.

Phosphatase activity was found in all fractions of laminal bean pulvini and petiole as well as in barley leaves, whether or not given 5 min red light. In all cases more than 90% of the specific and total activity was found in the supernatant fraction. There was three times more protein (2.0 as against 0.7 mg g⁻¹ fresh weight) and double the dry weight (8% as against 4%) in the pulvinar compared with petiole tissue.

Figure 5 illustrates the specific activity of the phosphatase from bean pulvini and petiole fractions with five different organic phosphates, relative to the activity of the pulvinar supernatant fraction with ATP as substrate. One hundred per cent activity corresponded to 50 μmol phosphate mg⁻¹ protein min⁻¹. Nonspecif-

Table II. Results of Respiration and Photosynthesis Measurements of Excised, Rootless Bean Plants

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specific ATPase and ADPase activities were found to be slightly more active than acid p-NPPase. Acid α-NAPase proved to be only 10% as active as ATPase. ATPase activity was not affected by the presence of 50 mm KCl or by changes in pH 6.0 through 7.5.

Phosphatase activity of barley leaves was measured only with ATP and acid p-NPP as substrates.

**Vanadate Inhibition of Phosphatase.** The phosphatase activity of all fractions studied was inhibited by similar concentrations of vanadate, though somewhat dependent on the organic substrate. Fifty per cent inhibition was obtained by 15 μM vanadate for pulvinal supernatant with ATP as substrate, 90 μM vanadate for pulvinal supernatant with acid p-NPP as substrate (Fig. 6), and 30 μM vanadate for barley leaf supernatant with ATP as substrate (data not presented). The data of Figure 6 represent the average of 10 experiments. Nonspecific ATPase activity was found to be slightly but significantly stimulated by 0.1 to 1.0 μM vanadate. A t-test proved the probability of stimulation to be 93% and 70%, respectively. We may offer no explanation for the observed stimulation.

**DISCUSSION**

In the described experiments, the use of vanadate was purely as a nonspecific probe. This is obvious because: (a) a specific ATPase was not purified. (b) vanadate inhibited the hydrolysis of ATP as well as acid p-NPP, at approximately the same low concentrations (10 to 100 μM). (c) vanadate inhibited nonspecific ATPase of all fractions at approximately the same concentrations.

Leaf movement is first affected 8 to 12 h after cut off bean plants are supplied with vanadate. However, as even 24 h incubation of plants with vanadate does not affect respiration of isolated laminal pulvini, the effect of vanadate on leaf movement is not mediated by an effect on respiration. Vanadate may work through the observed potent effect on general phosphatase in pulvini (Fig. 6), but as the effect on leaf movement is a long-term one, vanadate may also work through an effect on ABA-level (transpiration is rapidly affected) or protein synthesis.

Transpiration is affected significantly by vanadate concentrations as low as 40 μM within 2 h. Though 1 mM vanadate increases respiration in the same period of incubation, 100 μM does not affect it. Thus the primary (shortest visible) in vivo effect of vanadate on transpiration is not on respiration. Secondary effects (e.g. protein synthesis) are not thought to be of importance in short-term experiments. However, these may be mediated through a general phosphatase.

Unrolling of barley leaf sections is affected by as low concentrations of vanadate as 1 μM. As it takes as much as 1 mM to affect respiration, vanadate does not inhibit unrolling due to an effect on respiration. On the other hand there is a great similarity between the effect of vanadate on barley leaf unrolling and phosphatase activity. We find it likely that vanadate suppresses unrolling by inhibiting a phosphatase, as other inhibitors of phosphatases (ATPases) i.e. DES and oligomycin also suppress unrolling.

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