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Communication

NO_3^- Enhances the Kinase Activity for Phosphorylation of Phospho*enol*pyruvate Carboxylase and Sucrose Phosphate Synthase Proteins in Wheat Leaves

Evidence from the Effects of Mannose and Okadaic Acid

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ABSTRACT

The aim of this work was to determine which of the two reactions (*i.e.* phosphorylation or dephosphorylation) involved in the establishment of the phosphorylated status of the wheat leaf phospho*enol*pyruvate carboxylase and sucrose phosphate synthase protein responds *in vivo* to NO_3^- uptake and assimilation. Detached mature leaves of wheat (*Triticum aestivum* L. cv Fidel) were fed with N-free (low-NO_3^- leaves) or 40 mM NO_3^- solution (high-NO_3^- leaves). The specific inhibition of the enzyme-protein kinase or phosphatase activities was obtained *in vivo* by addition of mannose or okadaic acid, respectively, in the uptake solution. Mannose at 50 mM, by blocking the kinase reaction, inhibited the processes of NO_3^--dependent phospho*enol*pyruvate carboxylase activation and sucrose phosphate synthase deactivation. Following the addition of mannose, the deactivation of phospho*enol*pyruvate carboxylase and the activation of sucrose phosphate synthase, both due to the enzyme-protein dephosphorylation, were at the same rate in low-NO_3^- and high-NO_3^- leaves, indicating that NO_3^- had no effect *per se* on the enzyme-protein phosphatase activity. Upon treatment with okadaic acid, the higher increase of phospho*enol*pyruvate carboxylase and decrease of sucrose phosphate synthase activities observed in high NO_3^- compared with low NO_3^- leaves showed evidence that NO_3^- enhanced the protein kinase activity. These results support the concept that NO_3^-, or a product of its metabolism, favors the activation of phospho*enol*pyruvate carboxylase and deactivation of sucrose phosphate synthase in wheat leaves by promoting the light activation of the enzyme-protein kinase(s) without affecting the phosphatase(s).

It is now well known that the changes in SPS[1] activity associated with dark to light transition involve a phosphorylation of the protein. This covalent modification leads to a decrease in the activity of this enzyme (8, 11, 16). Recently, it has been shown that in leaves of wheat (a C_3-type plant), light induces the enhancement of the PEPcase phosphorylation status (14) as it does in C_4 plants (3, 6). This results in an increase of the activity and changes in the properties of PEPcase. Thus, the responses to phosphorylation of the two enzymes are opposite, PEPcase being activated whereas SPS is deactivated. Both effects were shown to be enhanced in high-NO_3^- conditions when low-NO_3^- wheat leaves were shifted to high-NO_3^- nutrition in the light (4, 14). This observation supported the hypothesis that NO_3^- favored the phosphorylated form of both enzymes. The steady-state enzyme activity results from the equilibrium of the phosphorylation/dephosphorylation reactions catalyzed by protein kinases and protein phosphatases, respectively. The light stimulation of PEPcase in C_4 plants was shown to result from a complex regulatory cascade involving net *de novo* synthesis of the PEPcase-protein kinase protein leading to an increase in the phosphorylation status of PEPcase (9).

It is now questioned whether NO_3^- or a derivative stimulates the protein kinase(s) and/or inhibits the protein phosphatase(s), thereby activating PEPcase and deactivating SPS. The aim of the investigation reported here was to answer this question. Experiments were performed *in vivo* with detached wheat leaves kept either on N-free solution or 40 mM NO_3^- and treated with okadaic acid or mannose under light. The purpose of one or the other treatment was to modify the equilibrium between the phosphorylation and dephosphorylation reactions. Okadaic acid, a potent and specific inhibitor of the major protein phosphatases identified in plants (5), was used to allow full expression of the kinase activity. Mannose was supplied to decrease the cytosolic Pi by sequestering Pi as mannose-6-phosphate, thereby inhibiting the synthesis of ATP and the kinase activity without inhibiting the phosphatase reaction (17). The decrease of phosphatase activity in the presence of okadaic acid led to an enhancement of the effect of the protein kinase on PEPcase and SPS activities in the presence of NO_3^-. On the other hand, the effect of the phosphatase activity was identical in the presence of mannose, whether the leaves received NO_3^- or not.

[1] Abbreviations: SPS, sucrose phosphate synthase; PEPcase, phospho*enol*pyruvate carboxylase.

MATERIALS AND METHODS

Plant Material

Wheat seeds (*Triticum aestivum* L. cv Fidel) were germinated on moist vermiculite in the greenhouse. After 7 d, plantlets were transferred to pots containing aerated nutrient solution. Nitrogen was given as 0.5 mM KNO_3 added to the N-free solution, which contained O.375 mM KH_2P0_4, 0.125 mM K_2HPO_4, 0.375 mM $MgSO_4$, 0.1 mM NaCl, 1.25 mM $CaSO_4$, 10 mg/L EDTA, and the micronutrients (1), pH 6.

Treatment to Obtain Low NO_3^- and High NO_3^- Mature Leaves

At the age of 20 d, plants had four leaves, the youngest mature leaf was No. 3 counted from the base. Plants were transferred to the N-free solution 48 h before sampling to deplete the NO_3^- content of the tissues. Leaf No. 3 was then detached at the end of a 16-h night period and placed in 1 mL of either N-free nutrient solution (low-NO_3^- leaves) or nutrient solution containing 40 mM KNO_3 for transition from the low-NO_3^- to the high-NO_3^- state (high-NO_3^- leaves) and immediately illuminated, or frozen in liquid nitrogen (control).

Enzyme Extraction

Leaves were sampled at intervals after the start of illumination as indicated in the results. They were immediately frozen in liquid nitrogen and stored at −80°C prior to extraction. Extraction was achieved by grinding frozen leaves one by one (120–150 mg fresh weight) in a mortar chilled to liquid N_2 temperature and resuspending the powder in 1 mL 50 mM Hepes-KOH, pH 7.4; 12 mM $MgCl_2$; 1 mM EGTA; 1 mM EDTA; 1 mM DTT; 10% glycerol; 2 mM benzamidine; 2 mM ϵ-amino-*n*-caproic acid, according to Siegl and Stitt (11). After 10 s of vortex mixing, the suspension was stored at −80°C until the enzymes were assayed (less than 1 d later).

Enzyme Assays

The samples were allowed to thaw to 4°C and the extract was immediately centrifuged (16,000*g* for 2 min). The supernatant was desalted by centrifugal filtration at 5000*g* at 5°C with Sephadex G-25 equilibrated with the extraction buffer supplemented with 0.1% BSA, according to the method of Marques *et al.* (10).

PEPcase was immediately measured spectrophotometrically by coupling the reaction to NADH-oxidation mediated by malate dehydrogenase. The standard assay medium contained in 50 mM Tris-HCl, pH 7.6; 20 μmol $NaHCO_3$; 130 nmol NADH; 10 μmol $MgCl_2$; 5 μmol DTT; 1 unit malate dehydrogenase; 50 μL desalted enzyme solution (1 mL final volume). Reactions, at 30°C, were initiated by the addition of 3.25 μmol phospho*enol*pyruvate. SPS was assayed as in Stitt *et al.* (13), including 2 mM Fru-6-P, 10 mM Glu-6-P, 3 mM UDPG, and 5 mM Pi in the assay to differentiate between the "V_{max} assay" and the "limiting assay" (11).

Treatment of Leaves with Okadaic Acid or Mannose

Low-NO_3^- leaves were detached at the end of a 16-h dark period and placed in 1 mL N-free solution either containing okadaic acid or not. At the end of an additional 4-h dark period, NO_3^- was added at a final concentration of 40 mM to one-half of the okadaic acid-treated leaves and to one-half of the nontreated leaves and light was turned on.

Treatment with mannose was initiated by adding mannose to a final concentration of 50 mM into the uptake solution of low-NO_3^- and high-NO_3^- leaves, 30 or 60 min after the start of illumination.

Chl Determination

Chl of the pellets obtained after centrifugation of the crude enzyme extracts was solubilized in methanol and the absorbance was measured at 652 nm according to the method of Bruinsma (2).

Experiments were repeated at least twice and generally four times. Each leaf was extracted and analyzed separately. Values are means of a minimum of three and a maximum of five leaves. Results are expressed per mg Chl.

RESULTS AND DISCUSSION

Effect of Mannose on PEPcase Activation and SPS Deactivation

The PEPcase activity was increased to 248 and 188% of the activity in the dark in the high-NO_3^- and low-NO_3^- leaves, respectively, within 60 min after the start of the illumination (Fig. 1A). Addition of mannose into the uptake solutions stopped the process of PEPcase light activation of low-NO_3^- leaves and caused deactivation of the previously activated PEPcase of high-NO_3^- leaves to the level of low-NO_3^- activity. By between 120 and 180 min, the decrease of PEPcase activity was the same in high-NO_3^- and low-NO_3^- leaves, indicating that NO_3^- had no effect *per se* on the PEPcase deactivation, that is, on the PEPcase dephosphorylation process. The interpretation is that NO_3^- in the absence of mannose did not affect the protein phosphatase, but it enhanced the protein kinase activity.

NO_3^- caused an initial decrease in SPS activity and within 30 min after detached leaves were illuminated, the SPS V_{max} and SPS limiting activities of high-NO_3^- leaves were 68 and 66%, respectively, of the activities of low-NO_3^- leaves (Fig. 1, B and C). Thereafter, NO_3^- had little effect on the rate of increase of SPS activity in the light. After supplying mannose, there was an increase in the activity of SPS, as in spinach leaves (13). Mannose restored the activity of high-NO_3^- leaves to that of the low-NO_3^- leaves and enhanced the activity of the latter. The SPS activation observed after the addition of mannose is interpreted as resulting from the protein phosphatase activity. The rate of activation was not affected by NO_3^-. The initial decrease of SPS activity in high-NO_3^- leaves upon illumination is interpreted as resulting from the enhancement by NO_3^- of the protein kinase activity.

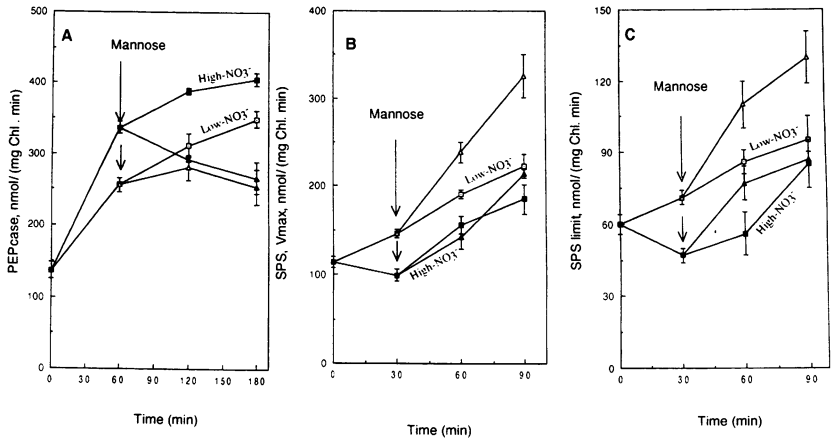

Figure 1. The *in vivo* effect of mannose on the light- and NO_3^--dependent (A) activation of PEPcase, (B) inactivation of SPS, V_{max} assay, and (C) inactivation of SPS, limiting rate assay. Seedlings were kept in the dark for 16 h in the N-free solution before the youngest mature leaves (No. 3, counted from the base) were detached and transferred onto (□) N-free solution (low-NO_3^-leaves) or (■) 40 mM KNO_3 (high-NO_3^- leaves) and illuminated (520 μmol m^{-2} s^{-1}). At time 60 min (A) or 30 min (B, C), mannose was injected at 50 mM final concentration into the (△) N-free and (▲) 40 mM NO_3^- solution. The results are given as the mean ± SE (n = 3).

Effect of Okadaic Acid on PEPcase Activation and SPS Deactivation

Assays revealed that in the leaves that had previously absorbed okadaic acid in the dark, the activity of PEPcase was increased upon illumination, whereas that of SPS was decreased, proportionally to the concentration of okadaic acid in the solution (Fig. 2, A–C). Thus, okadaic acid entered wheat leaf tissue as it did for spinach leaf discs (12). The presence of the phosphatase inhibitor might favor the apparent phosphorylation reaction of the protein kinase/phosphatase equilibrium, hence the phosphorylation of the concerned proteins. The higher increase of PEPcase and decrease of SPS activities in high-NO_3^- leaves compared with low-NO_3^- leaves suggest that the protein kinase activity was higher in high-NO_3^- than in low-NO_3^- leaves.

To estimate the full effect of protein kinase activity *per se* and make an attempt at the quantification of the effect of NO_3^- on this activity, PEPcase activity was measured over a 90-min illumination period in low-NO_3^- and high-NO_3^- leaves pretreated with 2 μM okadaic acid in the dark. Within the first 30 min of illumination, the light activation was 139% of the activity in the dark in low-NO_3^- leaves and 192% in high-NO_3^- leaves (Fig. 3). Inhibition of phosphatases by 2 μM okadaic acid increased the rate of activation in both kinds of leaves and shortened the time necessary for the PEPcase to reach its maximum activity. By comparison of the PEPcase activity of okadaic acid treated-high-NO_3^- leaves with that of low-NO_3^- leaves, it can be inferred that the effect of NO_3^- was a 145 and 127% stimulation of the light-dependent kinase activity at 30 and 90 min illumination, respectively.

CONCLUSION

The use of mannose, which suppressed the kinase reaction by sequestering cytoplasmic orthophosphate, and okadaic acid, a specific inhibitor of protein phosphatases, allowed the distinction between the effect of NO_3^- on the two enzymes responsible for the relative phosphorylation/dephosphorylation activities controlling the overall phosphorylation state of the proteins. Our interpretations are based on the assumption that the rates of activation of PEPcase and deactivation of SPS (phosphorylation) are the expression of the protein kinase activity, whereas the rates of PEPcase deactivation and SPS reactivation (dephosphorylation) are the expression of the protein phosphatase activity. The data presented here indicate that in wheat leaves, high NO_3^- nutrition enhanced the activity of the light-modulated protein kinase but did not affect the protein phosphatase activity. They support the concept that NO_3^- or a product of NO_3^- metabolism, by promoting the enhancement of the light activation of the protein kinase(s), favors the activation of PEPcase and deactivation of

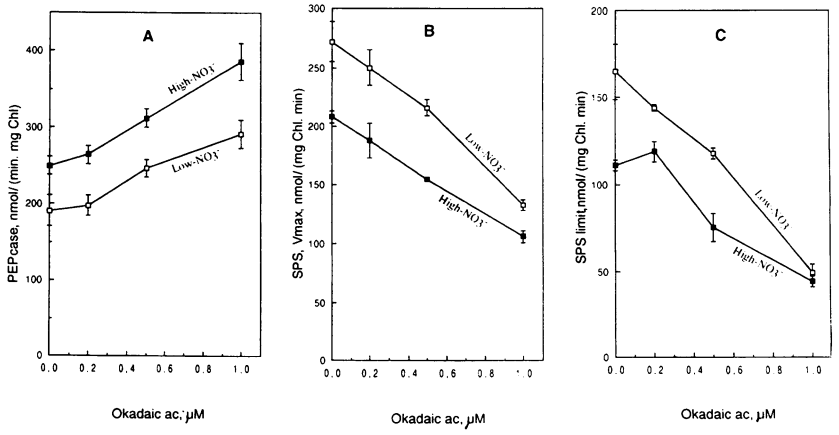

Figure 2. The *in vivo* effect of okadaic acid on the light- and NO_3^--dependent (A) activation of PEPcase, (B) inactivation of SPS, V_{max} assay, and (C) inactivation of SPS, limiting rate assay. Seedlings were kept in the dark for 16 h in the N-free solution before the youngest mature leaves (No. 3, counted from the base) were detached and transferred in dim light to N-free solution containing various concentrations of okadaic acid (0–1 μM). Then they were returned to darkness for 4 h before being illuminated for 60 min (520 μmol m^{-2} s^{-1}), (■) with addition of NO_3^- at 40 mM final concentration in the uptake solution (high-NO_3^- leaves) or (□) without addition of NO_3^- in the uptake solution (low-NO_3^- leaves). The results are given as the mean ± SE (n = 3).

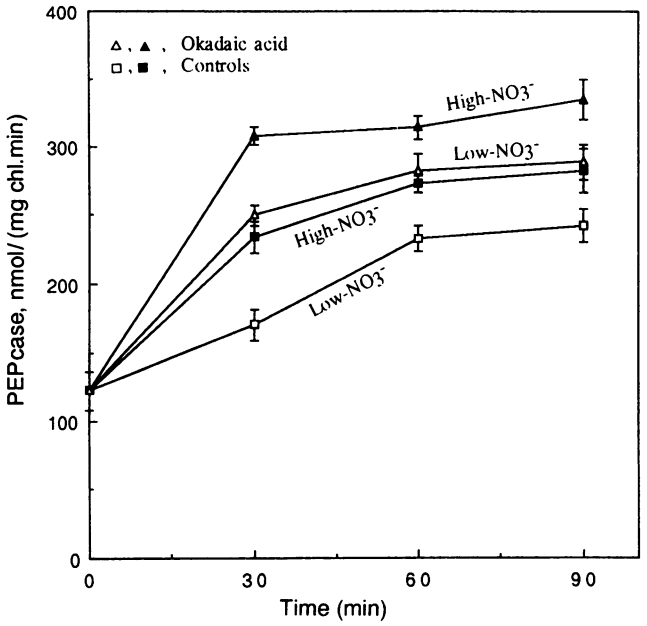

Figure 3. The *in vivo* effect of okadaic acid on the light-dependent and NO_3^--dependent activation of PEPcase. Seedlings were kept in the dark for 16 h in the N-free solution before the youngest mature leaves (No. 3, counted from the base) were detached and transferred in dim light to N-free solution containing 0 (□, ■) or 2 μM okadaic acid (△, ▲). Then they were returned to darkness for 4 h before being illuminated (520 μmol m^{-2} s^{-1}), (■, ▲) with addition of NO_3^- at 40 mM final concentration in the uptake solution (high-NO_3^- leaves) or (□, △) without addition of NO_3^- in the uptake solution (low-NO_3^- leaves). The results are given as the mean ± SE (n = 3).

SPS, and that it is the primary step of the regulatory cascade that controls the carbon flow away from sucrose synthesis and toward amino acid synthesis in leaves exposed to high NO_3^- nutrition (15). The results presented here cannot elucidate the mechanism of NO_3^- enhancement of protein kinase activity, which can be either a response of the light-activated kinase to the metabolic status of the cytoplasm (7) or the stimulation of the light-dependent activation of the kinase. In both cases, the question still to be resolved is whether the *in vivo* light/dark regulation of PEPcase protein and SPS protein kinase activity involves net *de novo* protein synthesis in the light in wheat leaves as it does for PEPcase protein kinase in C_4 leaves (8). These questions are under further investigation.

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