Modulation of phospho*enol*pyruvate carboxylase phosphorylation in leaves of *Amaranthus hypochondriacus*, a NAD-ME type of C_4 plant

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Abstract

PEP carboxylase (PEPC) in leaves of C_4 plants is activated by phosphorylation of enzyme by a PEPC-protein kinase (PEPC-PK). We reevaluated the pattern of PEPC phosphorylation in leaf extracts of *Amaranthus hypochondriacus*. It was dependent on Ca^{2+}, the optimum concentration of which for stimulation was 10 mM. The extent of stimulation was inhibited by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a Ca^{2+} chelator. The inhibition by BAPTA was relieved by the addition of Ca^{2+} but not by the addition of Mg^{2+}. The stimulation by Ca^{2+} of PEPC phosphorylation was marginally enhanced by calmodulin (CaM), but not by diacylglycerol (DAG). Phosphorylation was strongly restricted by Ca^{2+} or Ca^{2+}-CaM-dependent protein kinase inhibitors. Thus phosphorylation of PEPC is Ca^{2+}-dependent in leaves of *A. hypochondriacus* and a calcium-dependent protein kinase (CDPK) may modulate PEPC-PK and subsequently the phosphorylation status of PEPC.

Additional key words: calcium; calcium-dependent protein kinase; PEPC-protein kinase.

Introduction

In C_4 plants, phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) undergoes regulatory phosphorylation at a single serine residue near its N-terminus in presence of photosynthetically active radiation (PAR). The phosphorylated form is more active and less sensitive to L-malate (a feed back inhibitor) than the dephosphorylated form. PAR-induced phosphorylation of PEPC involves activation of a complex transduction chain that upregulates the activity of PEPC-protein kinase (PEPC-PK) (Chollet *et al.* 1996, Vidal and Chollet 1997).

Calcium acts as a secondary messenger in a wide variety of signal transduction pathways. Calcium also plays a key role in plant growth and development because changes in cytosolic free Ca^{2+} regulate a large variety of cellular processes through Ca^{2+}-modulated proteins and their targets (Bush 1995). Several types of calcium dependent kinases as well as Ca^{2+}-dependent phosphatases have been identified in plants (Roberts and Harmon 1992, Stone and Walker 1995, Luan 1998, Zielinski 1998).

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Abbreviations: BAPTA - 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; CaM - calmodulin; CDPK - calcium-dependent protein kinase; DAG - diacylglycerol; H_7 - 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; ML_7 - 1-(5-idonaphthalene-1-sulfonyl)-^{1}H-hexahydro-1,4-diazepine; MLCK - myosin light chain kinase; PAR - photosynthetically active radiation; PEPC - phospho*enol*pyruvate carboxylase; PEPC-PK - PEPC-protein kinase; TFP - trifluoperazine; W_7 - N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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There are also reports describing the involvement of Ca^{2+}-independent PEPC-PK in C_4 plants (Jiao and Chollet 1989, 1991, Carter *et al.* 1991, Bakrim *et al.* 1992, Wang and Chollet 1993, Li and Chollet 1994). On the contrary, some authors reported that PEPC phosphorylation was mediated by Ca^{2+}-dependent PEPC-PK (Echevarría *et al.* 1988, Bakrim *et al.* 1992, Ogawa and Izui 1992, Ogawa *et al.* 1992, Pierre *et al.* 1992). It is possible that multiple forms of PEPC-PK (both Ca^{2+}-dependent and Ca^{2+}-independent) are involved in the regulation of PEPC phosphorylation (Bakrim *et al.* 1992, Giglioli-Guivarc'h *et al.* 1996). There are suggestions that cytosolic calcium, pH, and photosynthetic metabolites (such as PGA or pyruvate) may act as secondary messengers in the PAR-activation of C_4 PEPC-PK (Pierre *et al.* 1992, Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996). However, the evidences of involvement of secondary messengers such as calcium, calmodulin, and cAMP during the phosphorylation of PEPC are still ambiguous.

The present article is an attempt to reevaluate the role of calcium on the regulatory phosphorylation of PEPC. The pattern of PEPC phosphorylation was studied by using different kinase inhibitors and activators.

Materials and methods

Plants of *Amaranthus hypochondriacus* L. cv. AG-67 were raised from seeds and were grown in the field (approximate photoperiod of 12 h and temperature of 30-40/25-30 °C day/night). Leaves were harvested from the plant at 09:00 h, approximately 3 h after sunrise. Thirty discs of *ca.* 0.2 cm^2 (cut from leaves under water) were floated on distilled water in a Petri dish and left in darkness for 2 h. These discs were either irradiated at 1000 μmol m^{-2} s^{-1} or kept in darkness for 30 min (Rajagopalan *et al.* 1993).

Purification of PEPC: Leaf discs were extracted in chilled mortar and pestle with extraction medium (4 cm^3 for each g) containing 100 mM HEPES-KOH, pH 7.3, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM Na_2EDTA, 10 % (v/v) glycerol, 10 mM 2-mercaptoethanol, 10 mM NaF, and 2 mM PMSF.

PEPC was purified from leaves of *A. hypochondriacus* by a modified procedure of Iglesias *et al.* (1986) and used in most of the experiments (for details see Gayathri *et al.* in press). Protein was estimated by using Bradford's reagent, bovine serum albumin was the standard (Bradford 1976).

***In vitro* phosphorylation of PEPC with $AT^{32}P$** was performed for 60 min at 30 °C according to Jiao and Chollet (1992). The phosphorylation mixture (60 mm^3) contained 0.1 M Tris-HCl (pH 7.5), 20 % (v/v) glycerol, 20 mm^3 of leaf extract (60 mg of protein), 3 μg of purified dark-form PEPC, 10 mM $MgCl_2$, 4 mM phosphocreatine, 10 units creatine phosphokinase, 0.25 mM p^1,p^5-di(adenosine-5') pentaphosphate (AP_5A, from *Sigma Chemical Co.*, USA), 5 mM DTT, 10 mM NaF, 2 mM PMSF, and 100 μM of 555 kBq γ-$AT^{32}P$ (specific activity of 111 kBq mol^{-1}, from *Board of Radiation Isotopes Technology*, Mumbai, India). Various concentrations of Ca^{2+}, BAPTA, or inhibitors were included in the reaction mixture depending on the experiments.

The mixtures were incubated at 30 °C for 60 min. The reaction was stopped by addition of 30 mm^3 of antiserum against PEPC of *Amaranthus* leaves and maintained at 4 °C for overnight. The immunoprecipitates were washed twice with 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl, and 1 % (v/v) *Triton X-100*, and once with 0.1 M Tris-HCl, pH 8.0, and finally resuspended in 10 mm^3 of SDS sample buffer [0.25 M Tris-HCl (pH 6.8), 8 % (m/v) SDS, 50 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol, and 0.04 % (m/v) bromophenol blue]. The mixtures were boiled for 2 min at 100 °C and subjected to SDS-PAGE followed by autoradiography at -80 °C.

Quantification of autoradiograms was made by a computer programme *Image Tools* developed by Don Wilcox, Brent Dove, Doss McDavid, and David Greer at the University of Texas Health Science Center in San Antonio. The area of a band was taken as a relative measure of PEPC phosphorylation.

Results

In vitro phosphorylation assays were performed using rapidly prepared crude extracts of irradiated or dark-adapted *Amaranthus* leaves. In most of these experiments, the assays were supplemented with the purified dark-form of PEPC which acts as the specific substrate for phosphorylation (Nimmo *et al.* 1987, Jiao and Chollet 1988). After the phosphorylation reaction was completed the protein was precipitated with anti-PEPC antiserum and resolved on SDS-PAGE (Fig. 1*A,C*). The autoradiographs illustrate that the phospho-

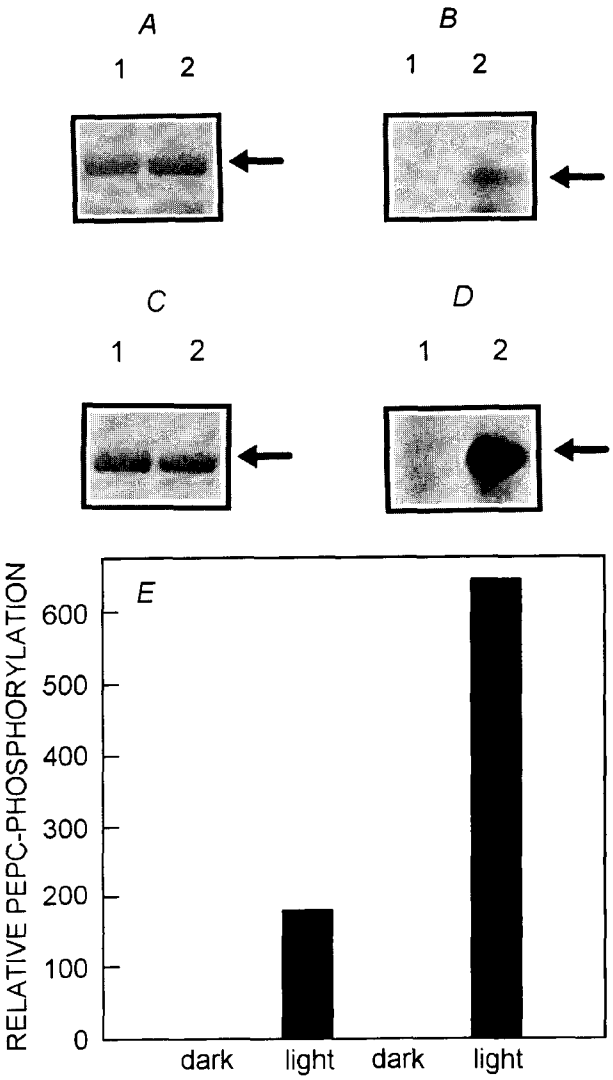

Fig. 1. *In vitro* phosphorylation of PEPC in crude extracts from dark-adapted (*1*) or irradiated (*2*) leaves of *Amaranthus hypochondriacus*. Crude leaf extracts were incubated with phosphorylation mixture containing γ-($AT^{32}P$). Leaves were irradiated for 10 (*A, B*) or 30 (*C, D*) min. *A* and *C* represent Coomassie brilliant blue-stained gels, *B* and *D* autoradiographs after 4 d at -80 °C. The relative degree of PEPC-phosphorylation was quantitated by image analysis (*E*).

rylation of PEPC can be documented only with extracts from irradiated leaves, but not with the corresponding dark-adapted leaves (Figs. 1*B,D,E*).

The phosphorylation of PEPC was dependent on the concentration of Ca^{2+} included in the assay mixture (Fig. 2) and 10 μM $CaCl_2$ was optimal. In contrast, the presence of BAPTA (a Ca^{2+} chelator) suppressed the extent of phosphorylation, otherwise stimulated by Ca^{2+}

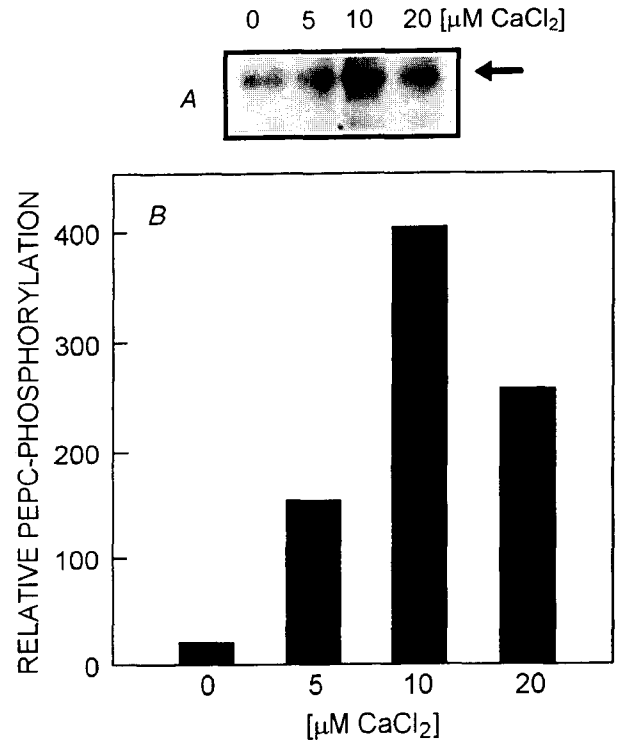

Fig. 2. Effect of Ca^{2+} on *in vitro* phosphorylation of PEPC. Leaves were extracted after irradiation and phosphorylation was carried out in the absence or presence of 0 to 20 μM $CaCl_2$ in the reaction mixture. (*A*) autoradiograph; (*B*) relative degree of PEPC-phosphorylation quantitated by image analysis.

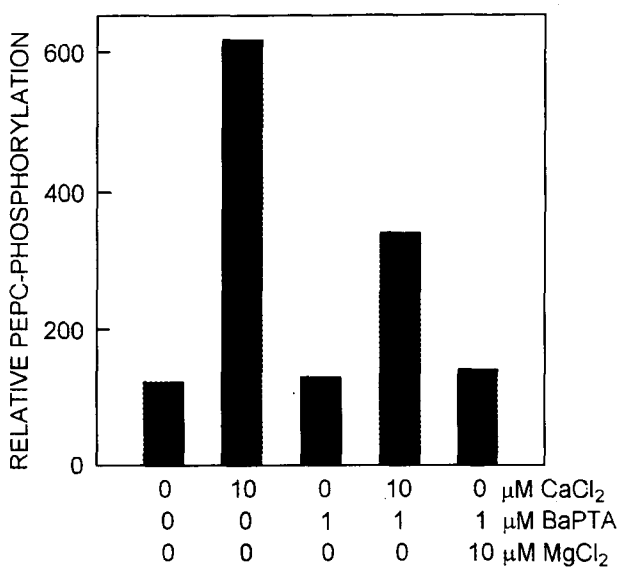

Fig. 3. The suppression of PEPC-phosphorylation by BAPTA (Ca^{2+} chelator) and its reversal by $CaCl_2$. The relative degree of PEPC-phosphorylation was quantitated by image analysis, as indicated in Figs. 1 and 2.

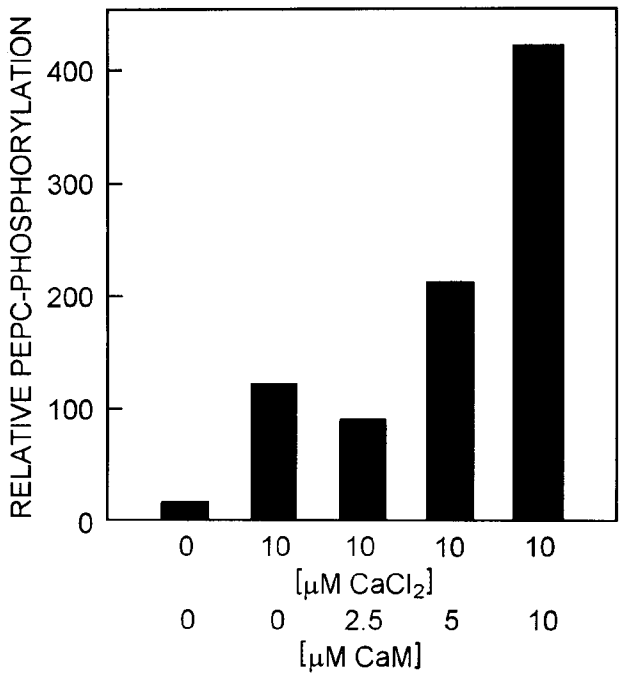

Fig. 4. Further stimulation of PEPC phosphorylation by calmodulin (CaM). The relative degree of PEPC-phosphorylation was quantitated by image analysis.

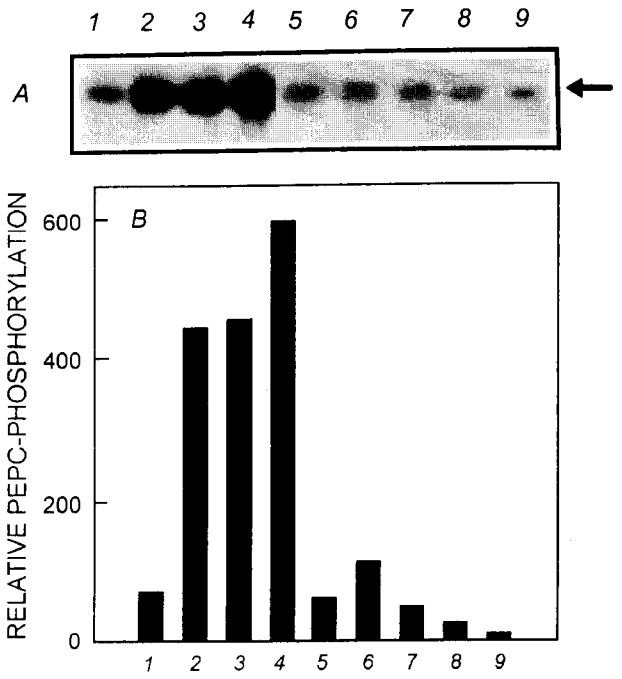

Fig. 5. The sensitivity of PEPC phosphorylation to various activators or inhibitors of protein kinases. *1*: control (no $CaCl_2$); *2*: 10 μM $CaCl_2$; *3*: 10 mM $CaCl_2$ + phosphatidyl serine (10 g m^{-3}) + diacylglycerol (200 μM); *4*: 10 μg CaM + 10 μM $CaCl_2$; *5*: 500 μM H_7; *6*: 50 μM staurosporine; *7*: 500 μM TFP; *8*: 500 μM W_7; *9*: 100 μM ML_7. (*A*) autoradiograph; (*B*) the relative degree of PEPC-phosphorylation quantitated by image analysis.

(Fig. 3). The inhibition by BAPTA was relieved by the addition of Ca^{2+}, but not by the inclusion of Mg^{2+}. The stimulation of PEPC phosphorylation by Ca^{2+} was further enhanced by CaM (Fig. 4).

The effect of several kinase inhibitors and activators on PEPC phosphorylation was studied in order to identify the type of protein kinase involved in PEPC phosphorylation. Fig. 5 illustrates the effects of such kinase inhibitors and activators. Phosphorylation of PEPC was promoted by the addition of Ca^{2+} (Fig. 5). There was only marginal effect by the addition of phosphatidyl serine and diacylglycerol. W_7 and TFP, two CaM antagonists, decreased the extent of phosphorylation of PEPC. H_7 (protein kinase C inhibitor), staurosporine (CaM kinase inhibitor), and ML_7 (an inhibitor of myosin light chain kinase, MLCK) abolished the phosphorylation (Fig. 5).

Discussion

In view of the conflicting reports on the role of Ca^{2+} in the regulation of PEPC-PK activity, our observations on the marked regulation by Ca^{2+} of PEPC activity (Figs. 1 to 3) are important. Most of the earlier experiments were performed on NADP-ME type C_4 plants, while the present report is the first on an NAD-ME type C_4 plant. The kinase responsible for PEPC-phosphorylation in *Amaranthus* leaves can be attributed to the calcium-dependent protein kinases rather than calcium-independent kinases.

The effect of EGTA of different protein kinase inhibitors during phosphorylation assay demonstrated that Ca^{2+}- or Ca^{2+}/CaM-dependent protein kinase was involved in phosphorylation of PEPC in sorghum leaves (Echevarría *et al.* 1988, Vidal *et al.* 1990, Ogawa *et al.* 1992, Ogawa and Izui 1992). On the other hand, there are reports suggesting that PEPC-PK is Ca^{2+}-independent *in vitro* (Chollet *et al.* 1990, Echevarría *et al.* 1990, Jiao and Chollet 1991, McNaughton *et al.* 1991, Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996). Ca^{2+}-dependent and Ca^{2+}-independent protein kinases were isolated from sorghum leaves (Bakrim *et al.* 1992). Recent reports using mesophyll protoplasts of *Digitaria sanguinalis* and *Sorghum* indicated that the stimulation

by Ca^{2+} can be seen only during *in situ* phosphorylation of PEPC (Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996, Nhiri *et al.* 1998).

Thus, the reports on regulation of C_4 PEPC-PK by calcium have been controversial. Suggestions are made that the regulation by calcium occurs at a level up stream of actual PEPC-PK (Chollet *et al.* 1996, Vidal and Chollet 1997). Further experiments are therefore needed to elucidate the nature of the Ca^{2+}-independent PEPC-PK from C_4 leaves.

Our results (Fig. 5) endorse the view that PEPC-phosphorylation occurs in a Ca^{2+} or CaM-dependent manner in crude leaf extracts of *Amaranthus*. The PEPC-PK was inhibited by the CaM antagonist (W_7) and MLCK inhibitor (ML_7), but did not require Ca^{2+}-binding protein (CaM) for activity. The presence of Ca^{2+}-CaM dependent PEPC-PK in sorghum crude leaf extracts was previously reported by Echevarría *et al.* (1988). Ogawa *et al.* (1992) suggested that MLCK type of kinase was involved in the phosphorylation of PEPC in maize leaves. These responses of PEPC-phosphorylation were reminiscent of CDPK, a protein kinase which has an intrinsic, CaM-like, Ca^{2+}-binding regulatory domain (Roberts and Harmon 1992).

We therefore suggest that the regulation by Ca^{2+} or CaM could be at an upstream level of regulation of PEPC-PK. For example, a CDPK-like protein-kinase may modulate the Ca^{2+}-independent PEPC-PK. Recently it has been reported that a multicyclic protein kinase cascade in the cytosol of mesophyll cell may involve a CDPK or a regulatory protein to stimulate in its turn a Ca^{2+}-independent PEPC-PK (Nhiri *et al.* 1998). CDPK from maize leaves has recently been purified and characterized (Ogawa *et al.* 1998).

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