Provoked changes in cellular calcium controlled protein phosphorylation and activity of quinate:NAD^+ oxidoreductase in carrot cells

Annick Graziana, Raoul Ranjeva* and Alain M. Boudet

Centre de Physiologie Végétale (L.A. CNRS no.241), Université Paul Sabatier 118, route de Narbonne, 31062 Toulouse Cédex, France

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Quinate:NAD^+ oxidoreductase activity decreased when carrot cell-suspension cultures were supplemented with the Ca^{2+}-ionophore, A-23187 and EGTA. The protein phosphorylation pattern changed as judged by autoradiography. The loss in enzyme activity was correlated with the Ca^{2+} efflux. Addition of Ca^{2+} to protein extracts in combination with calmodulin or not had no effect. Initial quinate:NAD^+ oxidoreductase activity was partially recovered only after preincubation with ATP-Mg^{2+} and Ca^{2+}. The reactivation was abolished by EGTA or fluphenazine. It is concluded that cellular Ca^{2+} controls the enzyme activity by affecting its degree of phosphorylation in vivo.

Calcium *A-23187 Ionophore* *Calmodulin* *Protein phosphorylation* *Protein kinase* *(Carrot)*

1. INTRODUCTION

Different physiological responses involve the amplification of a signal through second messengers such as cAMP and Ca^{2+} as has been established in animal systems [1].

In plants, cAMP, cAMP-binding proteins and adenylate cyclase have been characterized although cAMP has not yet been shown to exert a defined role [2]. In contrast, Ca^{2+} appears at least in vitro as an efficient effector of plant metabolism mainly through the ubiquitous protein modulator: calmodulin. Thus, the Ca^{2+}–CaM complex directly controls different plant enzymes such as NAD-kinase [3,4] or the microsomal Ca^{2+} uptake system [5]. Calcium is also involved in the modulation of protein kinases which phosphorylate histones [6], membrane-bound proteins [7] and specific enzymes [8].

Our work on alicyclic metabolism has shown that the enzyme responsible for the reversible oxidation of quinate, a widely distributed compound in plants [9], is regulated through reversible phosphorylation. Thus, QORase from carrot cell-suspension cultures is activated upon phosphorylation and inactivated when dephosphorylated [8,10]. Moreover, it has been established that the activation is a Ca^{2+}–CaM-dependent process [11] suggesting the possibility of a cascade regulation.

Therefore, it appears that this experimental system may be suitable for studying in greater detail the effects of cellular [Ca^{2+}] on QORase activity and protein phosphorylation. Here, we report the results obtained when Ca^{2+} efflux is provoked by supplementing carrot cell-suspensions with a Ca^{2+}-ionophore.

* To whom reprint requests and correspondence should be addressed

Abbreviations: QORase, quinate:NAD^+ oxidoreductase; CaM, calmodulin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis-(amino ethyl ether)*N,N'*-tetraacetic acid

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2. MATERIALS AND METHODS

2.1. *Materials*

Dehydroquinate was synthesized as in [12]. Carrier-free $H_3{}^{32}PO_4$ was from Amersham (France); A-23187 ionophore from Boehringer (Mannheim). CaM, isolated from bovine brain was a gift from D. Marmé [11]; fluphenazine was a gift from J. Demaille (CNRS, Montpellier). Chelex 100 was purchased from Biorad (France) and EGTA from Sigma (St Louis MO). All other products were of analytical grade.

2.2. *Plant material*

Carrot cell-suspension cultures were grown as in [8]. Cells were harvested aseptically at day 6 and rinsed extensively with sterile 87 mM mannitol solution adjusted to pH 6 as osmoticum (medium A). The solution was pre-treated with Chelex 100 to remove all traces of Ca^{2+}.

2.3. *Efflux experiments*

The cell cake was resuspended aseptically either in medium A (control) or in medium A supplemented with 5 mM EGTA and 15 μM A-23187 ionophore (assay). Usually, 11 g fresh cells were suspended in 250 ml Ehrlenmeyer flasks in 50 ml final vol. Each set of experiments was performed in quadruplicate.

The extracellular Ca^{2+} was measured by atomic absorption spectrophotometry (Varian AA 275 series).

2.4. *Incorporation of labeled phosphate*

The flasks containing cell-suspensions were supplemented with 3.7 MBq neutralized solution of $H_3{}^{32}PO_4$ for the indicated times. Phosphate absorption was monitored by counting the extracellular radioactivity by the Cerenkov effect (Packard model 46 C). Cells (3 g fresh wt) were ground in 10 ml extracting medium containing: 1 g acid-washed sand, 4% SDS, 20% glycerol, 40 mM EDTA, 10% mercaptoethanol, 125 mM Tris–HCl (pH 6.8). The insoluble fraction was spun down by centrifugation (20000 × *g* for 20 min) and the supernatant was heated for 20 min at 90°C to denature the proteins [13].

Aliquots (100 μl) of the denatured fractions were submitted to SDS–PAGE (10% acrylamide) as in [14]. After the run, gel slabs were fixed in 10% trichloroacetic acid (2 h) and then, heated for 20 min at 90°C to remove the acid-labile bound phosphate (essentially nucleic acids) [13].

The extensively rinsed gels were then dried and autoradiographed for 3 days at −20°C using Kodak XO-mat R films and intensifying screens (Trimax from 3 M, France). The autoradiographs were scanned with a chromoscan densitometer (Joyce and Loebl, France).

2.5. *Enzyme extraction and assays*

The harvested cells were frozen in liquid nitrogen and lyophilized. The proteins were extracted and partially purified as in [8,10]; however, all the buffers were treated with Chelex 100 to remove any trace of Ca^{2+}. QORase activity was measured spectrophotometrically [8]. The assays contained: 6 mM dehydroquinate 0.2 mM NADH; 0.5 M Tris–HCl buffer (pH 8.5) and protein extract in 1 ml final vol. The control was done without dehydroquinate. The reactions were run at 30°C and the changes in absorbance at 340 nm were monitored with a double-beam spectrophotometer (Leres S 67).

3. RESULTS

3.1. *Ca^{2+} efflux and protein phosphorylation*

Carrot cells, suspended in a low-calcium medium, released calcium with time (fig.1). Thus,

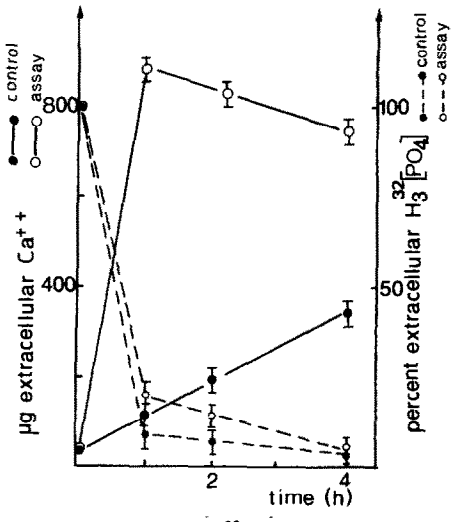

Fig.1. Calcium efflux and $^{32}PO_4$ absorption with time per culture flask. At time 0 the external radioactivity was 3.7 MBq.

the extracellular concentration was 40 μg/flask at time 0 and 320 μg after a 4-h incubation. Addition of A-23187 ionophore led to a 9–10-fold increase in extracellular calcium within 1 h. Then, some Ca^{2+} was reabsorbed by the cells but, in all cases, the intracellular Ca^{2+} was lower in the assays than in the controls.

The Ca^{2+} efflux did not significantly alter the absorption of radioactive phosphate.

When the intracellular calcium decreased, changes in the protein phosphorylation patterns were observed by autoradiography after SDS–PAGE (fig.2). Thus, in the presence of ionophore, at least 3 bands were not labeled. These particular proteins have M_r-values 30000 and 40000. Therefore, it appears that the in vivo phosphorylation pattern depends upon the intracellular [Ca^{2+}]. Such a result strongly suggests that calcium is an in vivo effector in plant protein phosphorylation while it has been shown that Ca^{2+}–CaM controls in vitro phosphorylation [6,7].

3.2. *Ca^{2+}-efflux and QORase activity*

QORase activity was measured in partially purified protein preparations obtained from controls and their corresponding assays sampled at different times. The resulting values were termed extractable activity. QORase-extractable activity dropped with time in both samples (fig.3). The loss was ~30% for the control within 4 h while it was 2-fold higher for the assay. The decrease roughly paralleled the time-dependent calcium efflux which was greater in the presence of ionophore.

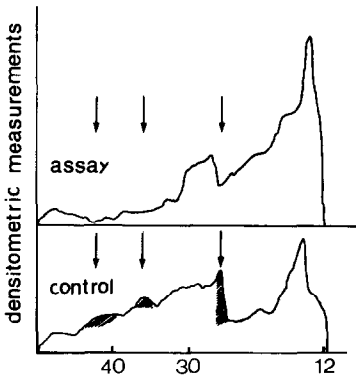

Fig.2. Phosphorylation patterns of proteins in control and assay cultures. The radioscans were obtained for cultures sampled after 2 h efflux. Ordinate in $M_r \times 10^{-3}$.

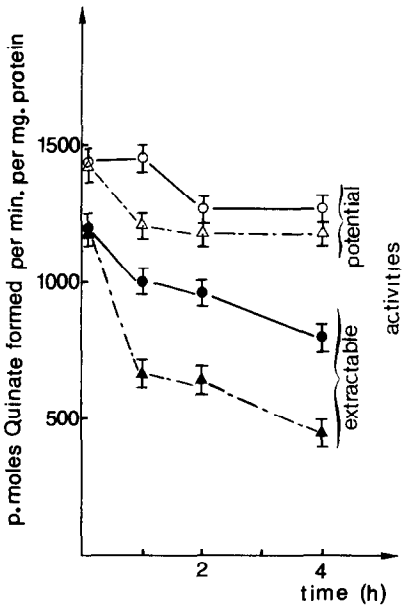

Fig.3. QORase-extractable and potential activity measurements with time. QORase was measured in partially purified extracts before (▲---▲, assay; ●—●, control) and after 20 min incubation at 20°C with 2 mM ATP, 4 mM $MgCl_2$, 1 mM $CaCl_2$ and 50 nM CaM [10] (△---△, assay; ○—○, control) EGTA (3 mM) or fluphenazine (100 μM) inhibited the reactivation process. Since the results were the same as those obtained without incubation, they are not presented for the sake of clarity.

To check if QORase was degraded or only deactivated, each protein preparation was preincubated for 20 min with ATP-Mg^{2+} and Ca^{2+} before assaying the enzyme. Controls were prepared to make sure that neither Ca^{2+} nor CaM exert a direct action on QORase (unpublished).

Under these conditions, the activity measured in a preparation obtained from cells sampled at time zero was raised by 20%. This value was considered to be the potential activity in contrast to the extractable activity. The recovery of the potential activity was by 100% for the control after 1 h efflux and represented only 86%, afterwards. This partial recovery was not due to a limitation in protein kinase nor in CaM since additions of these compounds did not further stimulate the activity.

Concerning the experiments with ionophore, the maximum recovery of the potential activity was

78% regardles of the time. Therefore, the inactivation process appears to be essentially reversible even if side effects do occur probably in relation with the presence of ionophore. Consequently, the cell cultures maintained their ability to reactivate QORase. Experiments with in vivo antagonist (R 24571 from Janssen Chimica) also led to the inactivation of QORase (not shown).

4. DISCUSSION

These results provide evidence that intracellular Ca^{2+} may act in vivo as an effector of plant metabolism through protein phosphorylation. Thus it appears that calcium efflux changes the pattern of protein phosphorylation. Endogenous substrates for CaM-dependent protein kinase are specifically affected (here; unpublished). Likewise, the loss in Ca^{2+} provokes the decrease in QORase activity which may be recovered on incubation with ATP-Mg^{2+} and Ca^{2+}. These data suggest that intracellular calcium controls the ratio of active to inactive forms of QORase via a two-cycle cascade including:

(i) The activation–deactivation of CaM-dependent protein kinase;
(ii) The phosphorylation–dephosphorylation of QORase.

In vivo-provoked cascade regulation has already been observed in different experimental models [1,15]. Thus, in [15] it was shown that glucose or proton ionophore leads to the depolarization of the yeast plasma membrane which results in an increase of the intracellular cAMP level. This, in turn, stimulates cAMP-dependent protein kinase which inactivates fructose biphosphate.

In plants, CaM has been assumed to regulate photosynthesis via NAD-kinase activation [16] while CaM antagonists to prevent hormone responses [17]. These results suggest that Ca^{2+} is a good candidate as second messenger in plant regulatory processes as proposed in [18].

These data are the first to describe a two-cycle cascade triggered by calcium in plants. Efforts are now being made to demonstrate the regulatory effect of natural changes in calcium concentration with biological stimuli and to appreciate rapid changes in QORase activity through in vivo estimation of the enzyme levels.

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