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In the early phase of programmed cell death in Tobacco Bright Yellow 2 cells the mitochondrial adenine nucleotide translocator, adenylate kinase and nucleoside diphosphate kinase are impaired in a reactive oxygen species-dependent manner

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Abstract

To investigate whether and how mitochondria can change in plant programmed cell death (PCD), we used the non-photosynthetic Tobacco Bright Yellow 2 (TBY-2) cells. These can be synchronized to high levels, stand out in terms of growth rate and homogeneity and undergo PCD as a result of heat shock. Using these cells we investigated the activity of certain mitochondrial proteins that have a role in providing ATP and/or other nucleoside triphosphates (NTPs). We show that, already after 2 h from the heat shock, when cell viability remains unaffected, the rate of ADP/ATP exchange due to adenine nucleotide translocator (ANT) activity, and the rate of the reactions catalysed by adenylate kinase (ADK; EC 2.7.4.3) and nucleoside diphosphate kinase (NDPK; EC 2.7.4.6) are inhibited in a non-competitive-like manner. In all cases, externally added ascorbate partially prevented the inhibition. These effects occurred in spite of minor (for ANT) or no changes in the mitochondrial protein levels as immunologically investigated. Interestingly, a decrease of both the steady state level of the ascorbate pool and of the activity of L-galactono-γ-lactone dehydrogenase (GLDH) (EC 1.3.2.3), the mitochondrial enzyme catalysing the last step of ascorbate biosynthesis, were also found.

Keywords: Mitochondria; Plant programmed cell death; Adenine nucleotide translocator; Adenylate kinase; Nucleoside diphosphate kinase; L-galactono-γ-lactone dehydrogenase

1. Introduction

In plants, programmed cell death (PCD) is an integral part of the life cycle being a major component of normal development, preservation of tissue homeostasis, and elimination of damaged cells in response to various forms of abiotic and biotic stresses [1].

The involvement of mitochondria in plant PCD has been shown in a number of systems and a pivotal role for mitochondria in the execution of PCD also in plants [2], as already shown in mammalian systems [3–5], has been established. In particular, it was suggested that mitochondria

Abbreviations: AA, antimycin; ADP DS, ADP detecting system; ADK, adenylate kinase; ANT, adenine nucleotide translocator; ASC, ascorbate; ATP DS, ATP detecting system; BSA, bovine serum albumin; CAT, carboxyatractyloside; CDP, cytidine diphosphate; CN^-, cyanide; HK, hexokinase; DHA, dehydroascorbate; $\Delta\psi$, membrane potential; GL, L-galactono-γ-lactone; GLDH, L-galactono-γ-lactone dehydrogenase; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; G6P-DH, glucose-6-phosphate dehydrogenase; L-LDH, L-lactate dehydrogenase; mGDH, mitochondrial glutamate dehydrogenase; NDPK, nucleoside diphosphate kinase; NTPs, nucleoside triphosphates; OLIGO, oligomycin; PCD, programmed cell death; PEP, phosphoenolpyruvate; PK, pyruvate kinase; RCI, respiratory control index; ROS, reactive oxygen species; ROT, rotenone; TDP, thymidine diphosphate; TBY-2, Tobacco Bright Yellow-2; UDP, uridine diphosphate

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play a crucial role in integrating cellular stress and regulating PCD [2,6,7]. On the other hand, in plant cells, mitochondria are the major source of reactive oxygen species (ROS), which play a major role in mammalian PCD [8]. Nonetheless, how mitochondria contribute in the progression of events leading to plant PCD remains to be fully established.

We have already shown that as a result of short heat treatment at 55 °C, the non-photosynthetic *Nicotiana tabacum* Bright-Yellow 2 (TBY-2) undergo PCD, as indicated by cytoplasmic shrinkage, chromatin condensation and DNA laddering. Moreover we found that ROS are produced in the early phase of PCD with cytochrome *c* release occurring in a ROS dependent manner; after cytochrome *c* release, caspase-like activation occurs, leading to cytochrome *c* degradation en route to PCD [9,10]. If, as in mammalian systems, ATP acts together with the released cytochrome *c* to activate the caspase [11], then this argues for the involvement of enzymes and translocators participating to ATP and phosphate nucleotide homeostasis in cell PCD. Further, the effects may be ROS-dependent, as argued in [11]. The level of ROS in a cell derives from a balance between ROS-producing and -scavenging systems. Consistently, reduced efficiency of ROS scavenging by metabolites and enzymes is an early step in PCD induction [9,12,13]. In this regard, L-galactono-γ-lactone dehydrogenase (GLDH) an integral protein of the inner mitochondrial membrane could play a major role since it catalyses the final step in the synthesis of ascorbate [14–16], an abundant antioxidant in plant tissues which reacts directly with $O_2^{\bullet-}$, OH and $^1O_2$ [17].

In the light of the above considerations, there is reason to suspect that in plant PCD ROS-dependent damage of mitochondrial proteins can occur, those located in the outer compartments of mitochondria being the primary targets. Thus, we investigate here, by using coupled mitochondria isolated from TBY-2 cells, whether and how the adenine nucleotide translocator (ANT)-dependent export of ATP synthesised via oxidative phosphorylation, the ATP production via ADK and the activity of the nucleoside diphosphate kinase (NDPK) can change during the early phases of PCD in the TBY-2 cell line.

We found that, in spite of minor (in the case of ANT) or no (in the case of ADK and NDPK) changes in the protein levels, the activities of the ANT, ADK and NDPK are already impaired in the early stages of heat-shock induced PCD. This occurs in a non-competitive-like manner possibly due to ROS, as suggested by the partial prevention by the antioxidant ascorbate. Consistently, we found that the activity of GLDH and the level of the ascorbate pool decrease.

2. Materials and methods

2.1. Materials

All reagents and enzymes were from Sigma (St. Louis, MO, USA) with the exception of sucrose, Triton X-100, HEPES and Tris (Baker). All chemicals were of the purest grade available and were used as Tris salts at pH 7.0–7.4 adjusted with Tris or HCl. Rotenone, antimycin, oligomycin and FCCP were dissolved in ethanol.

2.2. Cell culture, growth conditions and heat treatments

The suspension of tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) was routinely propagated and cultured at 27 °C essentially as described in [18].

Heat shock was induced as described in [9]. Briefly, a stationary culture was diluted 4:100 (v/v) and cultured for 4 days. Heat-shock at 55 °C was induced by transferring the cell suspension (100 mL) into a water bath at 55 °C with a heating time of 10 min. After heat shock, the cells were returned to 27 °C. Where indicated, ascorbate (ASC; 0.5 mM), was added to the culture medium 30 min before heat treatment. Cell viability was measured using trypan blue staining as described in [18].

2.3. Isolation of mitochondria and cytosolic fractions from TBY-2 cells

Mitochondria were isolated by protoplast fractionation and lysis, followed by differential centrifugation essentially as described in [10]. Briefly, TBY-2 cells (10 g) were incubated with 2% (w/v) Caylase (Cayla, Toulose, France) and 0.1% Pectolyase (Sigma, Milan, Italy) in pre-plasmolysis buffer consisting of 0.65 M mannitol and 25 mM Tris–Mes pH 5.5. The digestion of the cell wall proceeded for 1 h, in the dark, at 30 °C. The protoplasts were sedimented at 200×*g* for 5 min at 25 °C and washed three times with pre-plasmolysis buffer adjusted to pH 6.5. The protoplasts were then suspended in the ice cold lysis buffer (5 mL/g of cell) consisting of 0.3 M sucrose, 10 mM EDTA, 10 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 2 mM cysteine and 0.2% bovine albumin and lysed on ice using a Potter homogeniser, the pestle of which was connected to a motor-driven grinder. Cell homogenate was obtained by twice centrifuging the suspension at 1500×*g* for 5 min at 4 °C to remove cell debris.

Mitochondria were pelleted at 17,000×*g* for 15 min at 4 °C and gently suspended in 0.1–1 mL of isolation buffer consisting of 0.3 M sucrose, 10 mM KH_2PO_4/K_2HPO_4 (pH 7.4), and 0.2% bovine serum albumin (BSA), depending on the starting cell weight, to obtain about 4 mg of mitochondrial protein/mL. The supernatant after the centrifugation at 10,000×*g* was centrifuged again at 15,000×*g* and the supernatant so obtained, utilised as the cytosolic fraction, was split into aliquots and stored at −80 °C. Assay of cell protein was done according to [19]. The integrity of mitochondrial preparations was assessed by checking the presence of adenylate kinase (ADK), a marker enzyme of the mitochondrial intermembrane space, in the cytosolic fractions (absent in condition of integrity of outer mitochondrial membrane). It was further confirmed by measurements in mitochondrial fractions of both the amount and activity (for details see below) of fumarase a marker enzyme of the mitochondrial matrix.

2.4. Fumarase assay

Fumarase (EC 4.2.1.2) activity was measured spectrophotometrically at 240 nm essentially as reported in [20] by following the rate of absorbance increase at 240 nm due to fumarate formation after addition of L-malate addition (50 mM) to isolated mitochondria either in the absence or presence of the detergent Triton X-100 (TX-100, 0.2%). The rate of absorbance increase at 240 nm was measured as tangent to the initial part of the progress curve and expressed as nmol fumarate formed/min × mg protein. The % of integrity of inner membrane was calculated as $1-(v_{\text{o-TX-100}}/v_{\text{o+TX-100 (0.2\%)}})\times 100$. The value of fumarate ε_{240} for fumarate measured under our experimental conditions was 2.44 mM^{-1} cm^{-1}.

2.5. Immunoblot analysis

Immunoblot analysis was performed on mitochondrial extracts from both control and 2h-PCD cell cultures. Mitochondria were incubated with TX-100 (0.2%) on ice, and the samples were centrifuged at 15,000×*g* for 15 min at 4 °C to remove insoluble materials. Supernatants, corresponding to the solubilised mitochondrial fraction were subdivided into aliquots and stored at −80 °C. Mitochondrial proteins (10 μg), were loaded onto a 15% SDS-polyacrylamide gel, separated and transferred to a polyvinylidene difluoride membrane which was probed with the following antibodies: the polyclonal anti-adenine nucleotide translocator (ANT) antibody, the polyclonal anti-ADK

antibody (1:200 dilution; Abgent, San Diego USA), the polyclonal anti-nucleoside diphosphate kinase (NDPK) antibody (1:100 dilution; Abgent, San Diego USA), the polyclonal anti-mitochondrial glutamate dehydrogenase (mGDH) antibody (1:1000 dilution; provided by Dr. F. Rothe, Institut Medizinische Neuobiologie, University of Magdeburg, Magdeburg, Germany). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using enhanced chemiluminescence Western blotting reagents (Amersham, Pharmacia Biotech Italia Srl).

Densitometry values for immunoreactive bands were quantified using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc.); multiple expressions of the same immunoblots in a linear range were performed. Protein levels were calculated as a percentage of the control taken as 100 in arbitrary units after normalization based on the amount of mGDH in each lane on the same filter. The variability of protein levels in the control cells, measured by comparing the percentage of protein level from different control samples on the same filter, was always less than 5%.

2.6. Oxygen uptake measurements

Oxygen uptake measurements were carried out at 25 °C using a Gilson 5/6 oxygraph with a Clark electrode. Mitochondria (0.5 mg protein) were added to 1.5 mL of the respiration medium containing 210 mM mannitol, 70 mM sucrose, 20 mM TRIS–HCl, 5 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 3 mM $MgCl_2$, 5 mg/mL BSA. NADH (1 mM) or succinate (5 mM) was used as a respiratory substrate and either ADP (0.5 mM) or carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 0.125 μM) were added to induce state 3 respiration. When indicated, rotenone (ROT; 2 μg/1.5 mL) and antimycin (AA; 2 μg/1.5 mL) were added to inhibit the respiratory chain complexes. The rate of oxygen uptake was measured as the tangent to the initial part of the progress curve and expressed as nmol O_2/min × mg protein.

2.7. Assay of the safranin response

This was done at 25 °C, essentially as in [9], by measuring absorbance changes at 520 nm of the photometric probe safranin by using a PERKIN-ELMER Lambda-5 spectrophotometer. Mitochondria (0.5 mg protein) were incubated at 25 °C in 2 mL of a standard medium consisting of 0.3 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0) plus 10 μM safranin O in the presence of either NADH (1 mM) or succinate (5 mM).

2.8. Spectroscopic assays

The measurement of ADP/ATP exchange rate was carried out fluorimetrically essentially as reported in [21]. Briefly, TBY-2 mitochondria (0.5 mg protein) were incubated at 25 °C in 2 mL of the standard medium containing the ATP detecting system (ATP DS) consisting of glucose (2.5 mM), hexokinase (HK) (EC 2.7.1.1) (0.2 e.u.), glucose 6-phosphate dehydrogenase (G6P-DH) (EC 1.1.1.49) (0.1 e.u.) and $NADP^+$ (0.25 mM) in the presence of Ap5A (20 μM), used to inhibit the ADK and succinate (5 mM) a respiratory substrate used to energise mitochondria. NADPH formation in the extramitochondrial phase, which reveals ATP appearance due to externally added ADP, was followed fluorimetrically (with 334 nm and 456 nm as excitation and emission wavelengths, respectively). The rate of fluorescence increase due to externally added ADP, was measured as tangent to the initial part of the progress curve and expressed as $NADP^+$ reduced/min × mg protein.

The ADK reaction in isolated mitochondria (0.5 mg protein) was monitored photometrically at 334 nm in the direction of ATP production by using the ATP DS described above in the presence of carboxyatractyloside (CAT; 5 μM) and oligomycin (OLIGO; 2.5 μM) used to block ANT and ATP synthase, respectively. The rate of absorbance increase at 334 nm due to ADP addition was measured as the tangent to the initial part of the progress curve and expressed as nmol $NADP^+$ reduced/min × mg protein.

The NDPK reaction in isolated mitochondria (0.05 mg protein) was monitored photometrically by following the reaction of reversible transfer of the terminal phosphoryl group of ATP to a nucleoside diphosphate acceptor TDP, CDP or UDP, and revealing the appearance of ADP with the ADP detecting system (ADP DS) consisting of the phosphoenolpyruvate (PEP; 1 mM) pyruvate kinase (PK; 0.9 e.u.) NADH (0.2 mM) and of L-lactate dehydrogenase (L-LDH; 1.2 e.u.). CAT (5 μM), OLIGO (2.5 μM) and Ap5A (20 μM) were also present to prevent any ATP production; cyanide (CN^-; 1 mM) was also added to avoid the spontaneous oxidation by mitochondria of externally added NADH. The rate of absorbance decrease at 334 nm observed after the addition of each nucleoside diphosphate to mitochondria in the presence of ADP DS plus ATP (1 mM) was measured as the tangent to the initial part of the progress curve and expressed as nmol ADP formed/min × mg protein. It should be noted that GDP was not used as a substrate for NDPK because it is a substrate for PK included in ADP DS.

The value of ε_{334} for NAD(P)H measured under our experimental conditions was 6.3 mM^{-1} cm^{-1}. Control was made that the heat shock at 55 °C of mitochondria from TBY-2 cells had no effect per se on the activity of any of the proteins investigated.

2.9. Ascorbate pool and GLDH activity

The ascorbate pool was measured in TBY-2 cells as described in [18]. Briefly, this assay is based on the reduction of Fe^{3+} to Fe^{2+} by ASC with the spectrophotometric detection at 525 nm of Fe^{2+} complexed with 2-2′-dypiridyl.

GLDH was assayed as in [22] by following the reduction of cytochrome *c* (cyt *c*) at 550 nm (ε = 21 mM^{-1} cm^{-1}) due to addition of GL (1 mM) to both C-MIT and 2h-PCD-MIT (0.015 mg protein in both cases) incubated at 25 °C in 0.1 mL of medium consisting of 50 mM Tris–HCl (pH 8.0), 60 μM cyt *c*, 1 mM sodium azide, 1 μM FAD, 0.15% (v/v) TX-100. Sodium azide was added to inhibit cytochrome oxidase activity and to avoid an underestimation of GLDH. The change in absorbance not dependent on GL was subtracted from the test values.

3. Results

3.1. The levels of ANT, ADK and NDPK and the mitochondrial coupling in the early phase of TBY-2 PCD

To investigate whether and how mitochondrial ATP-related metabolism can change in the early phase of PCD, we investigated mitochondrial proteins involved in some aspects providing the cell with ATP and/or nucleoside triphosphates (NTPs) other than ATP. Those chosen were the adenine nucleotide translocator (ANT) which causes ATP export from mitochondria in exchange for cytosolic ADP, and ADK and NDPK two enzymes of the mitochondrial intermembrane space which equilibrate either adenylates or other nucleoside di- and triphosphates [23].

To do this, we isolated mitochondria from either TBY-2 control cells (C-MIT) or from the TBY-2 cells 2 h after heat shock at 55 °C (2h-PCD-MIT), at which stage the cell viability is almost unaffected [9]. Mitochondria were intact as shown by measuring the integrity of the mitochondrial inner membrane as in [20], values of about 95% were obtained in both cases. To check for cytosolic contamination, in control experiments, we confirmed the absence of activity of glucose 6 phosphate dehydrogenase, a cytosol marker enzyme, measured as in [24]. Conversely, no activity of ADK, a mitochondrial intermembrane space marker, was found in the cytosolic fraction, thus excluding both the presence of mitochondrial contamination and mitochondrial outer membrane rupture (data not shown).

To find out whether heat shock resulting in PCD can cause a change in protein levels of ANT, ADK and NDPK, we used immunoblot analysis with the appropriate antibodies to compare the amount of these mitochondrial proteins in both

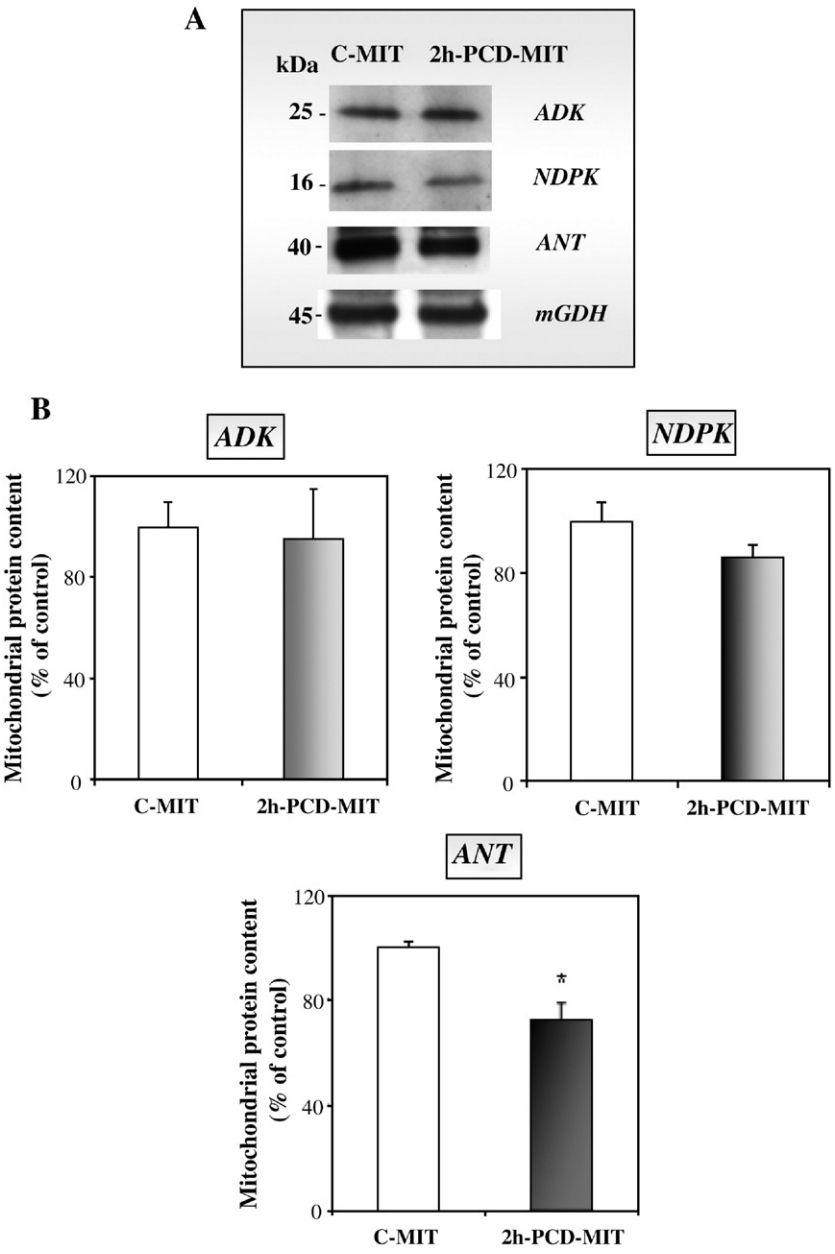

Fig. 1. Immunoblot analysis of levels of ADK, NDPK and ANT in C-MIT and 2h-PCD-MIT. (A) Immunoblotting of mitochondrial fractions obtained from either C-MIT or 2h-PCD-MIT (0.01 mg protein) was performed using anti-ADK, anti-NDPK anti-ANT and anti-mGDH antibodies (see Materials and methods). (B) Densitometric analysis of the ADK, NDPK and ANT contents were expressed, after normalization with mGDH, as a percentage of control. Values represent the mean (±SEM) of three independent measurements. The asterisk (*) indicates a value that is significantly different from the control ($p<0.01$).

C-MIT and 2h-PCD-MIT (Fig. 1A). The level of mGDH, a mitochondrial matrix enzyme, was also determined in the same experiment to normalize the amount of each mitochondrial protein revealed on the same filter. No significant changes in either the molecular weight or the amount of both ADK and NDPK were found in 2h-PCD MIT with respect to C-MIT ($p>0.01$ in three independent experiments). On the other hand, a small reduction in the amount of ANT was found in 2h-PCD-MIT (levels of 75%±6% of the control, $p<0.01$).

Clearly, a stable amount of a mitochondrial protein does not necessarily mean that the catalytic properties or the related metabolic pathways are unaffected, and so we investigated kinetically the reactions catalysed by ANT, ADK and NDPK both in C-MIT and 2h-PCD-MIT.

Since the activities of the investigated proteins are dependent on mitochondrial function, we first checked whether changes in mitochondrial coupling can occur as a result of PCD. In particular, we compared C-MIT and 2h-PCD-MIT (0.5 mg protein, respectively) with respect to their ability to oxidize either NADH (1 mM) or succinate (5 mM) in a manner stimulated by ADP (0.5 mM). The respiratory control index (RCI) (i.e. the ratio between the rate of oxygen uptake in the presence and absence of ADP) was measured as shown in a typical experiment (Fig. 2). In C-MIT, RCIs were equal to 2 and

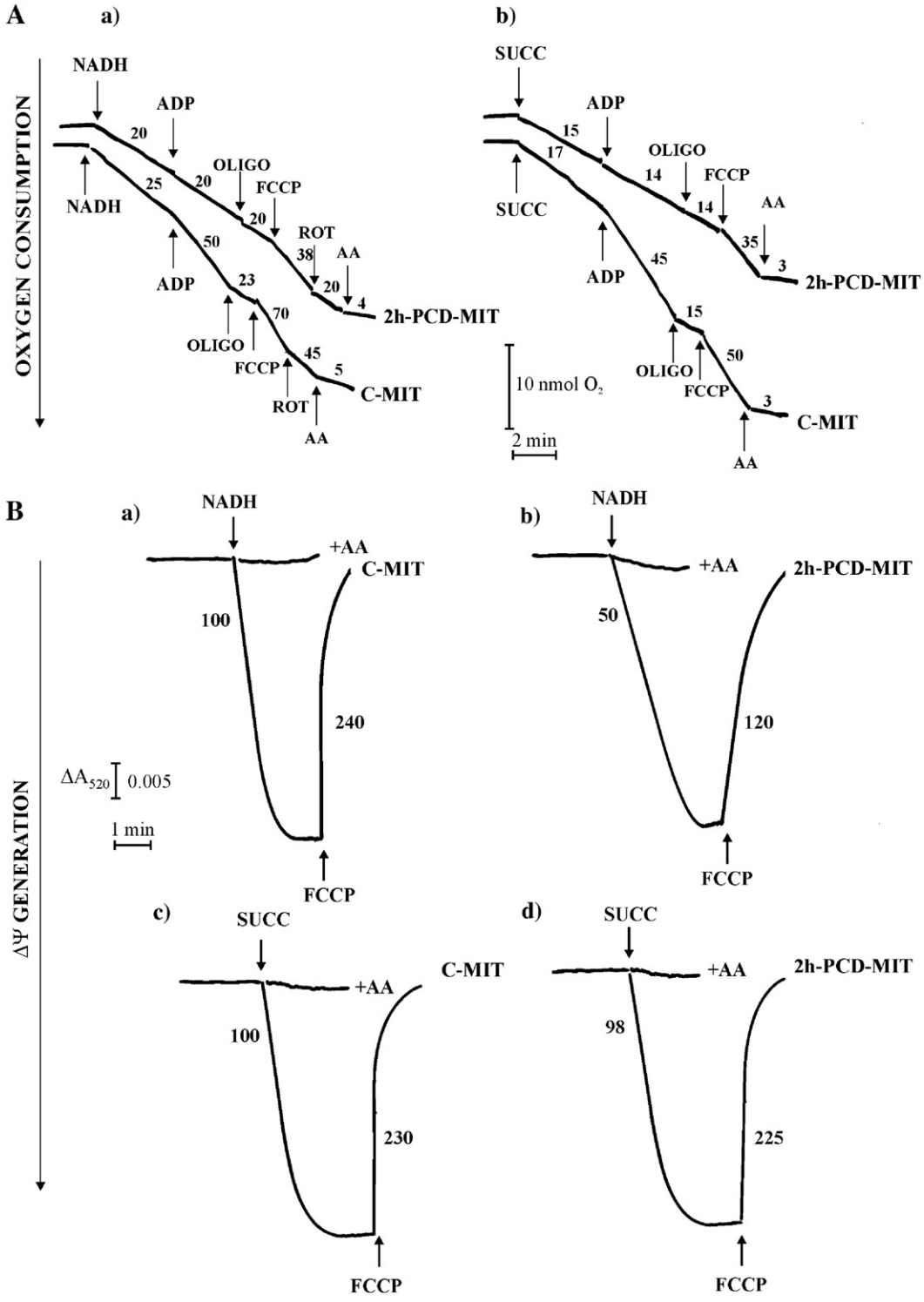

Fig. 2. Oxygen uptake and membrane potential generation by C-MIT and 2h-PCD-MIT arising from externally added NADH or succinate. (A) Both C-MIT and 2h-PCD-MIT (0.5 mg protein) were incubated at 25 °C in 1.5 mL of the respiratory medium (see Materials and methods) and oxygen uptake was measured polarographically. Where indicated, 1 mM NADH (panel a), 5 mM succinate (panel b), 0.5 mM ADP, 2.5 μg OLIGO, 0.125 μM FCCP, 2 μg ROT, and 2 μg AA were added. The rate of oxygen consumption is expressed as nmol O_2/min × mg protein. (B) Both C-MIT and 2h-PCD-MIT (0.5 mg protein) were incubated at 25 °C in 2 mL of the standard medium plus 10 μM of safranin O with continuous measurement of the safranin O absorbance at 520 nm. At the arrows, either 1 mM NADH (panels a and b) or 5 mM succinate (panels c and d) were added. When indicated, 1.25 μM FCCP was added. When present, AA (2 μg/2 mL) was added 1 min before the addition of the substrates. The rate of $\Delta\psi$ generation due to either NADH or succinate addition measured in C-MIT is expressed as a percentage of the control.

2.6 for NADH (Fig. 2A, panel a) and succinate (Fig. 2A, panel b) respectively. In both cases, the ATP synthase inhibitor, oligomycin (OLIGO; 2.5 μM) inhibited oxygen uptake, the rate of oxygen consumption being similar than that measured in state 4. As expected, the oxygen uptake was restored by adding the uncoupler FCCP (0.125 μM) and largely inhibited by

antimycin (AA; 2 μg/1.5 mL). Contrarily, in 2h-PCD-MIT no stimulation by ADP of oxygen uptake was found, whereas FCCP addition still increased the rate of oxygen uptake by up to 300%. However, NADH and succinate were found to differ from one another when used as respiratory substrates: NADH dependent respiration rate measured in the presence of FCCP was found reduced by about 50% in 2h-PCD-MIT with respect to that measured in C-MIT (Fig. 2A, panel a), whereas the rate of oxygen uptake measured with succinate in the presence of FCCP was found reduced by about 30% in 2h-PCD-MIT with respect to C-MIT (Fig. 2A, panel b). The results in Fig. 2A suggest that in the early phase of PCD, mitochondria can still generate an electrochemical proton gradient, as shown by the maintenance of the stimulation of oxygen uptake by the uncoupler FCCP.

These results were confirmed by checking the ability of both NADH and succinate to generate membrane potential ($\Delta\psi$) measured as in [9] (Fig. 2B). Generation of $\Delta\psi$ after addition of either NADH (1 mM) or succinate (5 mM) was revealed by the decrease in absorbance at 520 nm of the photometric probe safranin (see Materials and methods). In C-MIT, generation of $\Delta\psi$ was found to be prevented by AA (2 μg/2 mL) and was abolished by the uncoupler FCCP (0.125 μM) (Fig 2B panels a and c). In 2h-PCD-MIT, a significant decrease in the rate of $\Delta\psi$ generation due to NADH addition was found (50% of the control in this experiment) as well as about 50% of reduction was found in the rate of absorbance increase due to FCCP addition (Fig. 2B, panel b), whereas no difference in the rate of $\Delta\psi$ generation due to succinate addition was found (Fig. 2B, panel d).

3.2. A kinetic study of ANT, ADK and NDPK in the early phase of TBY-2 PCD

In order to find out why ADP fails to stimulate oxygen uptake by 2h-PCD-MIT, we resorted to spectroscopic measurements that allow for the continuous monitoring of ATP efflux from mitochondria incubated with ADP under conditions in which oxidative phosphorylation can occur [25]. To do this, mitochondria were incubated in the presence of the ATP DS consisting of the glucose (2.5 mM), HK (0.2 e.u.) G6P-DH (0.1 e.u.) and $NADP^+$ (0.25 mM) and the appearance of ATP outside mitochondria arising from externally added ADP was monitored as NADPH formation (see the scheme in Fig. 3A).

ATP production by mitochondria incubated with ADP can occur both via ATP synthase activity in oxidative phosphorylation and via ADK which is localised in the intermembrane space; note that in plant mitochondria the ADK-related ATP synthesis was found to exceed the rate of oxidative phosphorylation by two to four times [23].

In a typical experiment, the ATP DS was added to mitochondria in the presence of succinate (5 mM) which was used to energise them. The ATP concentration outside control mitochondria was negligible as no NADPH fluorescence was detected when C-MIT (0.5 mg protein) were incubated in the presence of ATP DS. As a result of addition of 0.05 mM ADP a fast increase in fluorescence occurred showing the presence of ATP outside the mitochondria. As expected, the rate of NADPH formation was not completely inhibited when 5 μM CAT or 20 μM Ap5A, inhibitors of ANT [26] and of ADK [27] respectively, were added separately, but only in the presence of the inhibitor pair (not shown).

Thus, in order to separate the contribution of these two pathways to ATP production outside mitochondria, we used CAT and Ap5A to inhibit ANT and ADK, respectively.

To measure ATP synthesised only via oxidative phosphorylation, both C-MIT and 2h-PCD-MIT (0.5 mg protein each) were incubated with the ATP DS plus Ap5A (20 μM) (Fig. 3B). The addition of ADP (0.05 mM), resulted in an increase in the NADPH fluorescence indicating the appearance of ATP in the extramitochondrial phase (Fig. 3B). The explanation for this is as follows: ADP enters mitochondria in exchange for endogenous ATP; inside the matrix ATP is synthesised via ATP synthase by using the succinate-dependent electrochemical proton gradient, and the newly synthesised ATP exits the mitochondria in exchange with further ADP via ANT (see scheme in Fig. 3A). Interestingly, about 95% inhibition of the rate of ATP appearance was found in 2h-PCD-MIT (Fig. 3B). As a control, we confirmed that the rate of ATP appearance was completely inhibited by adding CAT (5 μM), the ANT inhibitor, in both C-MIT and 2h-PCD-MIT.

When the same experiment was carried out photometrically in presence of CAT (5 μM) and OLIGO (2.5 μM) thus monitoring only the ADK-dependent ATP production, ATP was formed at a rate of 24 nmol/min × mg protein in C-MIT, as a result of 0.05 mM ADP addition; in 2h-PCD-MIT a 40% decrease in the rate of the reaction was found (Fig. 3C). As expected, in both samples, the addition of Ap5A (20 μM) completely blocked the rate of NADPH formation. Taken together, the results of Fig. 3 show a drastic impairment of both the pathways of mitochondrial ATP production already occurring in the early phase of PCD.

Both the failure of added ADP to stimulate the rate of oxygen uptake and the decrease in the rate of ATP efflux in 2h-PCD-MIT could be dependent on impairment of ANT and ATP synthase. Impairment in the electron flow along the respiratory chain can be ruled out given that externally added FCCP can stimulate the succinate-dependent rate of oxygen uptake both in 2h-PCD-MIT and in C-MIT (Fig. 2A, panel b). Thus, in order to ascertain which is the step limiting the rate of appearance of ATP as synthesised via oxidative phosphorylation i.e. to distinguish between the rate of ADP/ATP exchange via ANT and the rate of ATP synthesis via ATP synthase, control flux analysis (the control strength criterion) was applied as in [25,28]. Thus, to find out whether ANT or ATP synthase determined the rate of ATP appearance outside mitochondria, this activity was measured at 0.05 mM ADP in the presence of CAT and OLIGO, respectively. Data are plotted as the reciprocal of the rate of appearance of ATP as a function of the increasing inhibitor concentration (Fig. 4): when CAT was used as inhibitor, a straight line was obtained in the 1/V plot, indicating that the ATP/ADP exchange determines the rate of ATP outside both C-MIT and 2h-PCD-

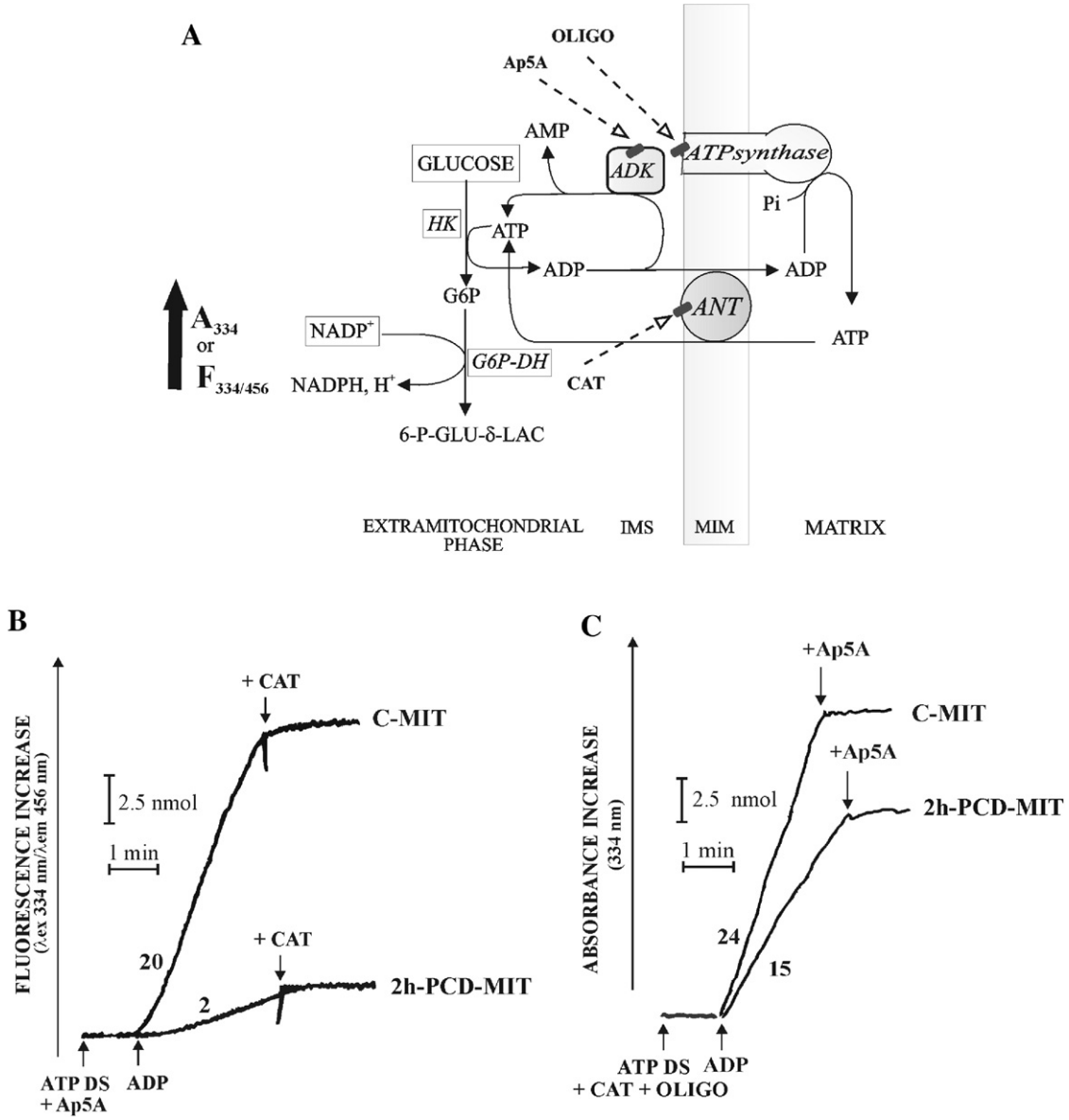

Fig. 3. ATP efflux from mitochondria due to externally added ADP in either C-MIT or 2h-PCD-MIT. (A) Scheme representing the ATP detecting system. For details see text, IMS, inter membrane space; MIM, mitochondrial inner membrane. (B) Inhibition of the rate of ATP appearance in extramitochondrial phase in 2h-PCD-MIT induced by ADP. Both C-MIT and 2h-PCD-MIT (0.5 mg protein) were suspended as reported in Materials and methods. Where indicated, CAT (5 μM) was added. Numbers along curves are rates of change in fluorescence measured as tangents to the initial slope and expressed as nmol $NADP^+$ reduced/min × mg mitochondrial protein. (C) Inhibition of the rate of ADK-related ATP appearance in 2h-PCD-MIT due to externally added ADP. Both C-MIT and 2h-PCD-MIT (0.5 mg protein) were suspended as reported in Materials and Methods. At the arrow, ADP (0.05 mM) was added. Where indicated, Ap5A (20 μM) was added. Numbers along curves are rates of change in absorbance at 334 nm measured as tangents to the initial slope and expressed as nmol $NADP^+$ reduced/min × mg mitochondrial protein.

MIT (Fig. 4A, panel a). On the contrary, with OLIGO, all points are not at the same line (Fig. 4A, panel b): the *y*-axis intercept (equal to zero OLIGO) of the extrapolated lines (obtained between 0.05 and 0.2 μg/mg protein of both C-MIT and 2h-PCD-MIT), differed (1/V lower) from the activity measured at zero OLIGO. Thus, we conclude that in both C-MIT and 2h-PCD-MIT the rate of ATP appearance outside mitochondria is governed by the rate of ADP/ATP exchange via ANT rather than the rate of ATP synthesis via ATP synthase and that already in the early phase of PCD, ANT impairment occurs.

In order to gain further insight into the mechanism by which the rate of ADP/ATP exchange via ANT is impaired in PCD, in a typical experiment we measured the dependence of the rate of ATP efflux on increasing ADP concentrations in both C-MIT and 2h-PCD-MIT and analysed the data using a double reciprocal plot (Fig. 4B). Saturation kinetics were found with mean V_{max} values of 40 ± 5 and 2.5 ± 0.7 nmol/min × mg protein in C-MIT and 2h-PCD-MIT respectively. The mean K_m values were found to be 38 ± 6 μM and 34 ± 8 μM, in C-MIT and 2h-PCD-MIT respectively, as calculated in three experiments carried out with different mitochondrial preparations. The

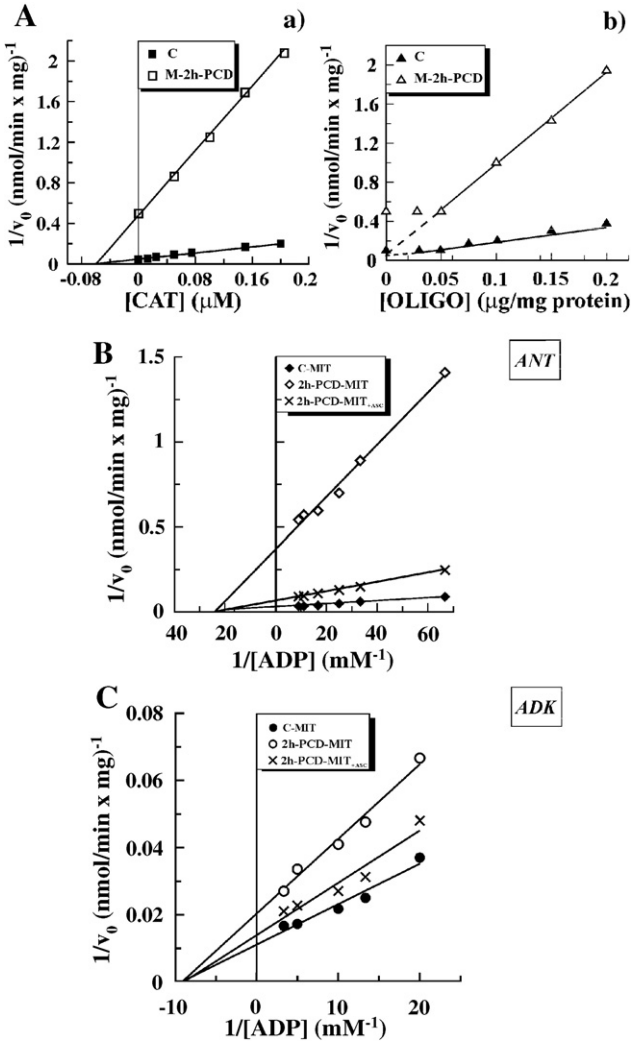

Fig. 4. Inhibition on the rate of ATP appearance induced by ADP via either ANT or ADK in 2h-PCD-MIT. (A) Dixon plot of inhibition by either CAT or OLIGO on the ADP inducing ATP appearance in 2h-PCD-MIT. Both C-MIT and 2h-PCD-MIT (0.5 mg protein) were incubated under condition reported in Materials and methods. ADP (0.05 mM) was added to either C-MIT and 2h-PCD-MIT in the absence or presence of increasing concentrations of either CAT (panel a) or OLIGO (panel b). (B) Dependence of ADP/ATP exchange rate via ANT on increasing ADP concentrations in C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$. ADP was added at the indicated concentrations to C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$ (0.5 mg protein, respectively) and the inverse of the ADP/ATP exchange rate versus the inverse of the ADP concentration measured in C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$, respectively were plotted. (C) Dependence of ADK-related ATP synthesis on increasing ADP concentrations in C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$. ADP was added at the indicated concentrations to C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$ (0.5 mg protein, respectively) and the inverse of the rate of ATP appearance due to ADK reaction was plotted versus the inverse of the ADP concentration measured in C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$, respectively.

strong reduction in the mean V_{max} value of ANT-mediated ADP/ATP transport found in 2h-PCD-MIT with respect to C-MIT resembles the onset of a non-competitive type of inhibition. In the same experiment, the ADP/ATP exchange rate was also measured in mitochondria isolated from cells that had been incubated with 0.5 mM ascorbate before heat treatment (2h-PCD-MIT$_{+ASC}$). The impairment of ANT was prevented by about 40% with mean V_{max} values equal to 16±4 nmol/min×mg protein, as measured in three different experiments. No change in mean K_m value (35±6 μM) with respect to 2h-PCD-MIT was found (Fig. 4B). Control was made that ascorbate itself added in the assay did not affect the measured ANT activity (not shown).

In order to ascertain how the kinetics of ADK are impaired in the early phase of PCD, in a typical experiment we measured the dependence of the rate of ADK-related ATP synthesis on increasing ADP concentrations in both C-MIT and 2h-PCD-MIT (Fig. 4C). With 2h-PCD-MIT we found a reduction in ADK activity with a mean V_{max} value of 48±3 nmol/min×mg protein compared with 80±5 nmol/min×mg protein in C-MIT, as measured in three different experiments, whereas no significant change in mean K_m value (80±15 μM compared with 90±20 μM measured in C-MIT) was found, indicative of the onset of a non-competitive type of inhibition.

In the same experiment, carried out with cells treated with ascorbate, we found a prevention of about 70% of the inhibition of ADK activity with mean V_{max} value of 60±5 nmol/min×mg protein, obtained in three different experiments, but with no change in mean K_m value respect to 2h-PCD-MIT (75±17 μM) (Fig. 4C).

In the light of the above results and since NDPK is attached to outer side of the inner the membrane and appears to interact functionally with ANT [29], thus playing a role in the synthesis of the NTPs other than ATP in channelling of intracellular energy phosphate metabolites [23], we investigated whether and how the activity of this enzyme could change in 2h-PCD-MIT. To achieve this, we measured the reaction of reversible transfer of the terminal phosphoryl group of ATP to a nucleoside diphosphate acceptor (TDP, CDP, UDP) generating the correspondent nucleoside triphosphate (TTP, CTP, UTP) and ADP. Either C-MIT or 2h-PCD-MIT (0.05 mg protein) were incubated in the presence of the ADP DS consisting of PEP (1 mM) PK (1 e.u.) NADH (0.2 mM) and L-LDH (1.2 e.u.) plus CAT (5 μM), OLIGO (2.5 μM) and Ap5A (20 μM), used to prevent any ATP production, and CN^- (1 mM), added to impair electron flow in the respiratory chain, thus preventing a spontaneous oxidation of the externally added NADH (see the scheme in Fig. 5A). When mitochondria were incubated with the ADP DS, the absorbance at 334 nm remained constant both before and after the addition of ATP (1 mM) thus indicating the absence of ADP in the extramitochondrial phase. The addition of 1 mM TDP (Fig. 5B), 1 mM CDP (Fig. 5C) or 1 mM UDP (Fig. 5D), caused a decrease of absorbance at 334 nm indicative of the appearance of ADP resulting from TTP, CTP and UTP, formation respectively. In 2h-PCD-MIT the rates of ADP synthesis via NDPK were found decreased by about 50% for all three nucleoside diphosphates used as substrates.

Fig. 6 shows typical experiments in which the rate of the NDPK reaction was measured as a function of increasing concentrations of TDP (Fig. 6A) CDP (Fig. 6B) and UDP (Fig. 6C) respectively, plotting data according to double reciprocal plot. We found a reduction of about 50% in NDPK activity (V_{max}) in 2h-PCD-MIT compared with C-MIT for all three nucleoside diphosphates used. On the other hand, there was no

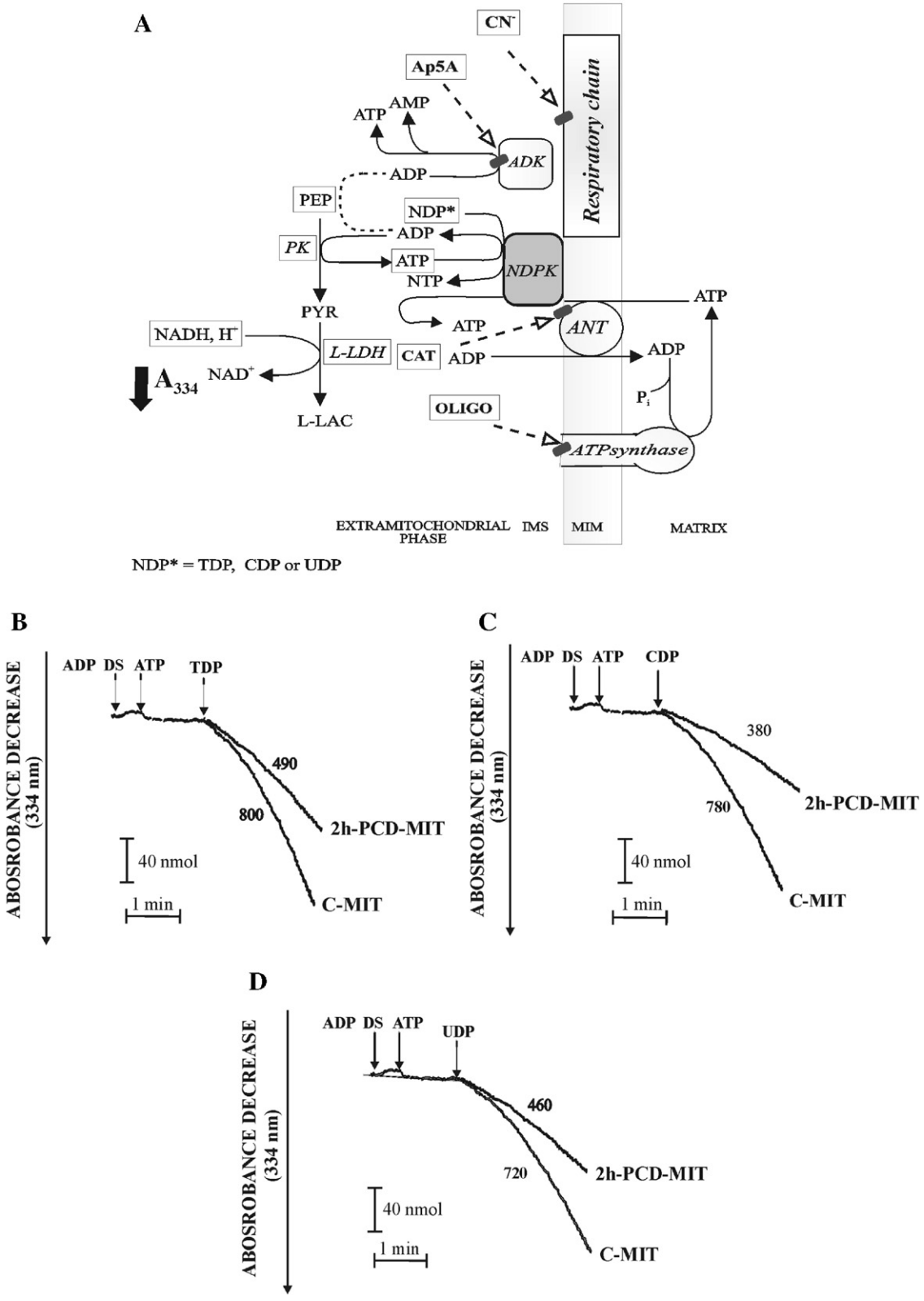

Fig. 5. Inhibition of NDPK-catalysed nucleoside triphosphate synthesis in 2h-PCD-MIT. (A) Scheme representing the ADP detecting system. For details see text. Both C-MIT and 2h-PCD-MIT (0.05 mg protein) were suspended at 25 °C in 2 mL of standard medium in the presence of the ADP DS plus CAT (5 μM), OLIGO (2.5 μM) Ap5A (20 μM) and CN^- (1 mM). After the addition of ATP (1 mM), at the arrows 1 mM TDP (B), 1 mM CDP (C) or 1 mM UDP (D) were added. Numbers along curves are rates of change in absorbance at 334 nm measured as tangents to the initial slope and expressed as nmol NADH oxidized/min × mg mitochondrial protein.

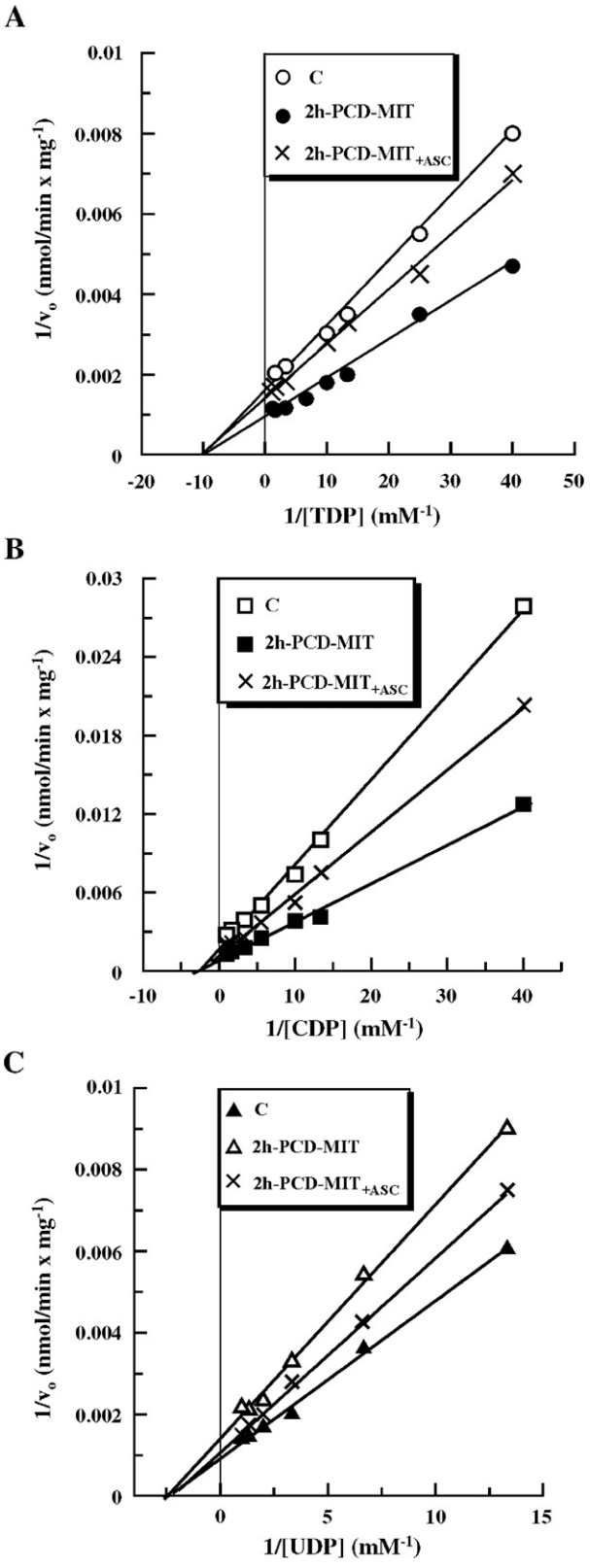

Fig. 6. Dependence of NDPK-catalysed NTP synthesis on increasing TDP, CDP or UDP concentrations in C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$. C-MIT, 2h-PCD-MIT or 2h-PCD-MIT$_{+ASC}$ (0.05 mg protein) were suspended at 25 °C in the same experimental conditions described in Fig. 5. TDP, CDP or UDP were added at the indicated concentrations and the inverse of the rate of ADP appearance was plotted versus the inverse of TDP (A), CDP (B), and UDP (C) concentrations, respectively.

significant change in the affinity (K_m) of NDPK for the substrates, suggestive of the onset of a non-competitive type of inhibition. In the same experiment we found prevention (between 70 and 90%) of the inhibition of NDPK activity in 2h-PCD-MIT pre-treated with ascorbate. The results of three different sets of experiments are reported in Table 1 as means ± SD.

3.3. Ascorbate pool and GLDH activity in the early phase of TBY-2 PCD

In another set of experiments we investigated whether the mitochondrial energy-related impairment found in the early phase of PCD is accompanied by a decrease in the steady state level of ascorbate level measured as in [18]. The levels of cell ascorbate (ASC) and dehydroascorbate (DHA) pool were found to decrease. In particular, a major decrease in levels of ASC in the 2h-PCD cells with respect to the control (C) ($p<0.01$) was observed (Fig. 7A). Since GLDH is a mitochondrial enzyme which catalysed the last step in ascorbate biosynthesis [14–16] we measured the specific activity of GLDH in both C-MIT and 2h-PCD-MIT by following the reduction of cyt *c*, the electron acceptor in the GLDH reaction, after the addition of GL (1 mM) [30]. A remarkable inhibition (up to 60%) of the enzyme activity was found in 2h-PCD-MIT (Fig. 7B).

4. Discussion

In this paper we report initial investigations of the events occurring in the early stages of PCD in plant cells. In particular, we checked whether both the protein level and the activities of ANT, ADK and NDPK were affected in intact mitochondria as a result of induction of PCD. These mitochondrial proteins were chosen because their reactions were connected metabolically, and more importantly because although ATP and ANT were found to play a major role in mammalian apoptosis (for ref. see [11]), similar observations have not been made for plant systems.

We approached this issue by using mitochondria isolated from TBY-2 cells which have been used as a model system for investigations on plant PCD [9,10]. In particular, we investigate coupled mitochondria isolated from TBY-2 cells after 2 h from heat shock; this is the earliest time at which the first analysis can be carried out for technical reasons [10]. At this time cell

Table 1
Kinetic behaviours of NDPK for TDP, CDP and UDP in C-MIT, 2h-PCD-MIT and 2h-PCD-MIT$_{+ASC}$

	V_{max} TDP	V_{max} CDP	V_{max} UDP	K_m TDP	K_m CDP	K_m UDP
	(nmol/min × mg protein)			(μM)		
C-MIT	1014±20	982±20	988±25	80±11	250±20	340±20
2h-PCD-MIT	564±18	446±10	664±24	79±8	230±10	330±20
2h-PCD-MIT$_{+ASC}$	689±16	610±20	960±30	90±6	240±20	335±20

The kinetic parameters of NDPK for TDP, CDP and UDP were measured in the experimental conditions reported in the legend of Fig. 6. Values represent the mean (±SD) of three different experiments.

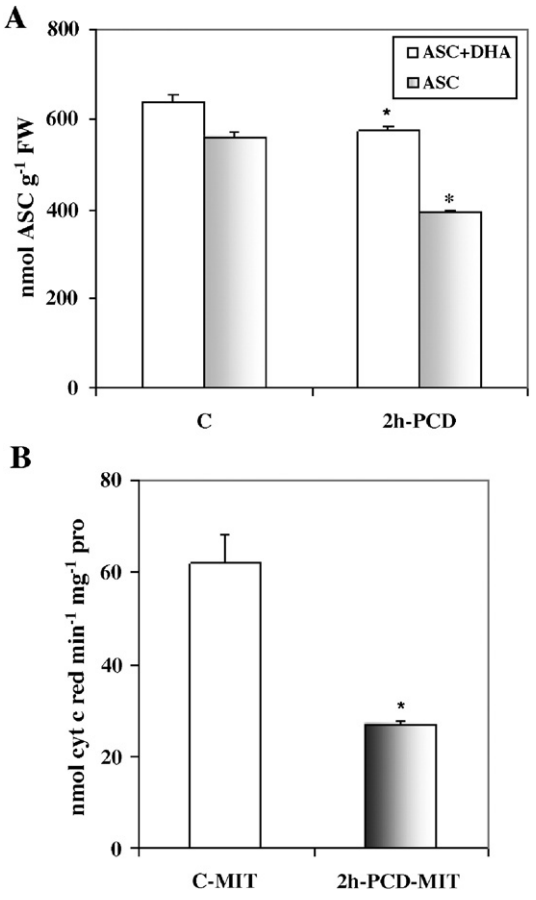

Fig. 7. Cell levels of ASC pool and GLDH activity in C-MIT and 2h-PCD-MIT. (A) Levels of ASC and ASC+DHA were measured in control cells (C) and in cells collected 2 h after heat shock (2h-PCD) as reported in Materials and methods. (B) GLDH activity in both C-MIT and 2h-PCD-MIT (0.05 mg, respectively) was measured as described in Materials and methods. Values represent the mean (±SEM) of three independent measurements. The asterisks (*) indicate values that are significantly different from the control ($p<0.01$).

viability is essentially unaffected, but a strong impairment of ANT, ADK and NDPK activities occurs without a parallel decrease in the protein levels. Because this impairment in activity is prevented by pre-treatment of the cells with the antioxidant ascorbate, we suggest that ROS are somehow involved in the decrease of the enzyme efficiency.

For the sake of clarity, the results obtained with each of the three proteins will be discussed separately.

4.1. Impairment of ANT activity in the early phase of TBY-2 PCD

We showed that as an early effect of heat shock, which triggers the cells to PCD, the activity of ANT is decreased. The first indication of this was obtained in polarographic experiments where we found that 2h-PCD-MIT can consume oxygen in a manner not stimulated by externally added ADP, but enhanced by the uncoupler FCCP (Fig. 2). This is confirmed by the experiments in Fig. 4 in which we directly show that ATP is exported from mitochondria following external addition of ADP with a rate depending on the ANT activity and not on that of ATP synthase. We found a strong inhibition in the rate of ADP/ATP exchange via ANT in 2h-PCD-MIT (Fig. 4) with a drastic reduction in transport efficiency in spite of only a minor decrease of the amount of the protein (Fig. 1). Thus, the overall effect is due essentially to a double impairment of ANT function i.e. minor decrease in the protein level and strong decrease in the catalytic efficiency. In contrast, the affinity of ANT for ADP does not change as indicated by the constant value of K_m. The partial prevention of impairment of activity due to addition of ascorbate is consistent with a ROS-dependent effect. In this case, the unchanged K_m implies that the binding site of ANT for ADP is unaffected.

It has been shown that ANT in rat liver mitochondria is impaired due to interaction of ROS with thiol groups in the protein molecule [11]. Unfortunately, this could not be checked in our experimental system. Nonetheless, this should be borne in mind as possible origin of the effect together with the possibility of modification of mitochondrial cardiolipin which is known to be needed for correct ADP/ATP exchange [31].

It could be argued that the strong decrease in V_{max} value for ADP/ATP transport found in 2h-PCD-MIT could arise from alteration of one of the processes leading to ATP production via oxidative phosphorylation, including the electrochemical proton gradient. Such a possibility is ruled out by the evidence that already in the early phase of PCD, as also in C-MIT, the rate limiting-step is the CAT-depend step i.e. the ANT-mediated transport across the mitochondrial membrane (Fig. 4A, panel a). Moreover our results confirm that in the early phase of PCD, mitochondria are still coupled, as found by the maintenance of the stimulation by the uncoupler FCCP (Fig. 2A, panel b) and by the capability of both NADH and succinate to generate a membrane potential (Fig. 2B, panel d).

Interestingly, our results give some insight into the effect of PCD on the ATP synthase activity, as derived from the investigation made of the kinetics of ATP appearance after addition of ADP under conditions in which oxidative phosphorylation can occur. When the rates of ATP appearance were plotted as a function of increasing concentration of the ATP synthase inhibitor oligomycin (Fig. 4A panel b), both in C-MIT and in 2h-PCD-MIT, the intercepts at the y axis of the linear regressions in the region where [oligomycin] >0.05 μg/mg protein represent the reciprocals of the rate of the reaction inhibited by oligomycin, i.e. the ADP phosphorylation via ATP synthase. Given that these values are identical in C-MIT and in 2h-PCD-MIT, we conclude that in the early phase of PCD the ATP synthase reaction is not affected.

We have previously shown [10], cytochrome c release from mitochondria isolated from TBY-2 cells undergoing PCD as a result of heat shock, and speculation was made that, as in cerebellar granule cells [32], the released cytochrome c could act both as ROS scavenger and as electron donor to drive ATP synthesis, perhaps contributing to the transient increase of ATP level [33]. However we show here that already after 2 h from heat shock, when cytochrome c has been already released [10], ATP production outside mitochondria decreases. In this regard plant PCD, as investigated here, appears to differ from the

cerebellar granule cell apoptosis, even though the possibility that the released cytochrome *c* works in this system as in the mammalian one, at least in very short time we cannot rule out that such an event could occur.

4.2. Impairment of ADK and NDPK activities in the early phase of TBY-2 PCD

The impairment of ADK and NDPK in the early phase of PCD is particularly interesting because reduction of the enzyme activity occurs in the absence of any decrease in protein level. This evidence makes it clear that at least in these cases, the induction of PCD is immediately accompanied by stress signalling (probably ROS production). There might subsequently be a reduction in protein level, but we have no information on this regard, this point requiring further investigation. In both cases we found a decrease of V_{max} which depends on a change in the transport efficiency (K_{cat}) rather than on a decrease in the amount of protein (Fig. 1). These results show that some caution must be exercised in using protein expression as a definitive tool for monitoring changes in metabolism.

It has been previously shown that the activity of mitochondrial ADK equilibrated the ATP levels with a rate exceeding the rate of oxidative phosphorylation by two to four times the maximum activity of ATP synthase and only when the ADK reaction approached equilibrium was oxidative phosphorylation the primary mechanism for net ATP synthesis [23]. Whether this applies in our system remains to be established. Nonetheless, by comparing the change in the enzyme efficiency (calculated as the ratio between V_{max} /K_m) we found that as a result of PCD induction the ANT efficiency is reduced about 20 fold, whereas it is reduced by only a half for ADK. Independent of the cytosolic concentration of ADP, we are forced to assume that in the early phase of PCD mitochondrial ATP is primarily formed via ADK. Nonetheless, this proposal is consistent with the requirement for ATP for apoptosome formation and the progressive alteration to become a component of the mitochondrial permeability transition. However such a hypothesis requires further investigation.

In the light of the crucial role of NDPK as a link between the cellular pool of ATP maintained by catabolic metabolism and NTPs utilised for various intracellular anabolic functions [23], we consider that the inhibition of NDPK that occurs in the early phase of PCD will result in impairment of the regulation and the channelling of intracellular energy phosphate metabolites. Moreover, the inhibition of NDPK activity could affect the functional interaction between NDPK and ANT, which is suggested to facilitate a higher rate of ATP export without building up an unfavourable ATP gradient over the membrane [29].

Interestingly, we found that the antioxidant ascorbate can partially prevent the impairment of the investigated reactions during PCD. Since ascorbate does not per se affect the kinetics parameters of the enzymes we are forced to conclude that its activity depends on its antioxidant power, i.e. that ROS production directly affects the investigated proteins.

4.3. Decrease in the ASC pool and impairment of GLDH in the early phase of TBY-2 PCD

Consistently with the effect of ascorbate in preventing inhibition of the mitochondrial proteins investigated, it should be noted that the level of ROS in a cell derives from a balance between ROS-producing and -scavenging systems. As a consequence, reduced efficiency of ROS scavenging by metabolites and enzymes would be expected to play a major role in induction of PCD.

We have previously found that heat shock resulted in an increased oxidation of ascorbate, which started immediately after heat-induced PCD [9]. It is also interesting to note that the most commonly occurring biosynthetic pathway for ascorbate in plants ends in mitochondria, with the conversion of GL into ASC, from where this ROS scavenger molecule is distributed to all the ASC-utilising cellular compartments [34]. Our results show that GLDH, the enzyme catalysing the mitochondrial ASC biosynthetic step [14], is inhibited at an early stage in cells undergoing PCD. It has been suggested that GLDH is an integral part of plant mitochondrial complex I, the redox state of which affect GLDH catalysis [30].

Our results do not allow us to exclude the possibility that GLDH inhibition could be a consequence of Complex I inhibition. Whatever the cause of GLDH inhibition, it results in a decrease in the amount of ASC available to counteract the ROS. It is worth noting that, in spite of the fact that the total ascorbate pool (ASC+DHA) of the whole cells is only moderately decreased in 2-h PCD cells (20%), a decrease which becomes more marked with time [9], the GLDH activity is much more strongly decreased (more than 60%) already after 2 h of heat shock treatment. This probably results in depletion in the ascorbate pool and a decrease in ROS scavenging system more rapidly in mitochondria than in other cellular compartments, thus supporting the hypothesis that mitochondria act as a nodal point in the network of reactions triggered by PCD inducers.

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