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Transport and metabolism of L-lactate occur in mitochondria from cerebellar granule cells and are modified in cells undergoing low potassium dependent apoptosis

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Abstract

Having confirmed that externally added L-lactate can enter cerebellar granule cells, we investigated whether and how L-lactate is metabolized by mitochondria from these cells under normal or apoptotic conditions.

(1) L-lactate enters mitochondria, perhaps via an L-lactate/H^+ symporter, and is oxidized in a manner stimulated by ADP. The existence of an L-lactate dehydrogenase, located in the inner mitochondrial compartment, was shown by immunological analysis. Neither the protein level nor the K_m and V_{max} values changed *en route* to apoptosis.
(2) In both normal and apoptotic cell homogenates, externally added L-lactate caused reduction of the intramitochondrial pyridine cofactors, inhibited by phenylsuccinate. This process mirrored L-lactate uptake by mitochondria and occurred with a hyperbolic dependence on L-lactate concentrations. Pyruvate appeared outside mitochondria as a result of external addition of L-lactate. The rate of the process depended on L-lactate concentration and showed saturation characteristics. This shows the occurrence of an intracellular L-lactate/pyruvate shuttle, whose activity was limited by the putative L-lactate/pyruvate antiporter. Both the carriers were different from the monocarboxylate carrier.
(3) L-lactate transport changed *en route* to apoptosis. Uptake increased in the early phase of apoptosis, but decreased in the late phase with characteristics of a non-competitive like inhibition. In contrast, the putative L-lactate/pyruvate antiport decreased *en route* to apoptosis with characteristics of a competitive like inhibition in early apoptosis, and a mixed non-competitive like inhibition in late apoptosis.

Keywords: mitochondria; L-lactate; L-lactate dehydrogenase; transport; metabolism; apoptosis

1. Introduction

One of the most outstanding problems in brain bioenergetics concerns the nature of the cell fuels (see [1]). As far as neurons are concerned, although evidence that L-lactate (L-LAC) can constitute an adequate energy substrate for brain tissue was provided as early as in the 1950s (for refs see [2]), glucose has long been considered the main substrate for neuronal energy metabolism, L-LAC being considered a metabolic waste product and/or a sign of hypoxia in the central nervous system [3]. Over the years, it was confirmed that L-LAC is efficiently oxidized by brain cells *in vitro*. Since L-LAC does not cross the blood–brain barrier easily, blood-borne L-LAC cannot be a significant source

Abbreviations: ANT, adenine nucleotide translocator; ARS, arsenite; DIV, days *in vitro*; BME, basal medium Eagle; α-CCN, α-cyanocinnamate; CGC, cerebellar granule cells; COX, cytochrome oxidase; D-LAC, D-lactate; L-LAC, L-lactate; cLDH, cytosolic L-lactate dehydrogenase; mLDH, mitochondrial L-lactate dehydrogenase; OUA, ouabain; OXA, oxamate; PBS, phosphate buffer saline medium; Pi, phosphate; PDS, pyruvate detecting system; PheSUC, phenylsuccinate; PYR, pyruvate; ROT, rotenone; S.D., standard deviation; SUCC, succinate; S-K25 cells, control cells; S-K5 cells, apoptotic cells; TX-100, Triton-X-100

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[4]. Thus it was proposed that L-LAC could be produced by astrocytes and released in the extracellular space to form a pool readily available for neurons in case of high energy demands [4–9], although this point has been questioned because neurons express the high-affinity MCT2 that is 60% saturated at the resting L-LAC level [10]. In this case L-LAC transport across the plasma membrane was shown (see [11,12]). Similarly, recent investigation of kinetic parameters and activities of L-lactate dehydrogenase (LDH) isoenzymes supported the use of L-LAC by neurons [13].

At present, the role of L-LAC metabolism in neuron activity remains a matter of debate. In particular, under conditions in which glucose is required to provide NADPH for fatty acid synthesis (see [1] and references therein), L-LAC, besides being incorporated into lipids (see [1] and references therein), could be the main energy substrate for neurons [14,15]. In particular, Pellerin et al. [16] proved that the relative contributions of glucose and L-LAC (5.5 mM each) to neuronal oxidative metabolism amounted to 21% and 79% respectively, thus showing the major role for L-LAC as an energy source. Consistently, it was recently proposed that L-LAC is the principal end product of neuronal aerobic glycolysis [17]. Moreover this could also occur during neuronal activity thus making L-LAC a suitable indicator of the coupling between oxygen and glucose consumption [14]. Contrarily, it has been argued that L-LAC does not undergo local oxidative phosphorylation ruling out that it can be a significant source of energy for activated neurones [18]. Interestingly, L-LAC was also found to be a precursor for glutamate and GABA synthesis [19]. A further role is suggested in cerebellar granule cells (CGCs) undergoing apoptosis, where L-LAC production is needed to provide ATP by anaerobic glycolysis, especially when oxidative phosphorylation is impaired [20].

Until recently the widely accepted view of oxidative L-LAC metabolism in mammalian tissues has been that it is converted to PYR in the cytosol, PYR is transferred into mitochondria, via its own carrier (elsewhere also termed the monocarboxylate carrier), where it is metabolized via oxidative decarboxylation and the tricarboxylic acid cycle. In particular, no finding has been reported of any possible L-LAC metabolism by mitochondria. Indeed, the existence of lactate dehydrogenase in mammalian mitochondria (mLDH) was for a long time a matter of debate. However, following the evidence for L-LAC oxidation by brain mitochondria [21], in 1971 Baba and Sharma [22] by using histochemical and electron microscopy techniques first showed the existence of mLDH localized in the mitochondria of rat heart and skeletal muscle. Such a proposal was confirmed later in different laboratories [23–25] even though it was argued that the mitochondria were contaminated with cytosolic LDH (cLDH) [26,27]. The existence of mLDH located in the inner mitochondrial compartments of rat heart and liver was finally confirmed when it was shown that heart [28] and liver [29] mitochondria can oxidize L-LAC. An intracellular L-LAC/PYR shuttle in which various mitochondrial carriers, distinct from the monocarboxylate carrier, play major roles was also shown to exist [28,29]. Interestingly, it was shown that L-LAC oxidation by rat liver mitochondria can itself trigger gluconeogenesis *in vitro* [29].

Whether brain mitochondria contain mLDH remains to be firmly established. Although oxidation of L-LAC by brain mitochondria was first reported in 1959 [21], one report [23] has claimed that brain mitochondrial preparations do not exhibit any mLDH activity in distinction to those from heart, kidney, liver, and lymphocyte. However, we found that the amount of ATP produced in S-K5 cells in the presence of oligomycin, i.e. via substrate level phosphorylation, was higher than expected based on the amount of L-LAC produced [20], this suggesting that the L-LAC could be oxidized by mitochondria. To resolve this issue, having confirmed that L-LAC can enter CGCs in our experimental conditions, we have investigated whether mitochondria from control CGCs and cells undergoing apoptosis can metabolize externally added L-LAC.

We show first the occurrence of an mLDH located inside mitochondria isolated from CGCs and suggest that L-LAC traffic across the mitochondrial membranes takes place mediated by two separate novel translocators different from the monocarboxylate carrier as functionally investigated. In cells *en route* to apoptosis neither the activity nor the protein level of mLDH changes, whereas the transport efficiency of the two putative L-LAC translocators is modulated differently. We conclude that, whatever the detailed mechanism of transport, the *in vitro* mitochondrial metabolism of L-LAC in CGCs is regulated by L-LAC transport into mitochondria.

2. Materials and methods

2.1. Reagents

Tissue culture medium and fetal calf serum were purchased from GIBCO (Grand Island, NY) and tissue culture dishes were from NUNC (Taastrup, Denmark). All enzymes and biochemicals were from Sigma Chemicals Co. (St. Louis, MO, USA). Goat anti-LDH antibody was purchased from Abcam (Cambridge, MA); mouse monoclonal anti-cytochrome oxidase subunit IV antibody was purchased from Molecular Probes (Eugene, OR); mouse monoclonal antibody anti-beta-actin (clone AC40) was purchased from Sigma (St. Louis, MO); horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using Enhanced Chemiluminescence Western Blotting reagents were from Amersham. Protein A/G PLUS-agarose was purchased from Santa Cruz Biotechnology, Santa Cruz, California USA.

2.2. Cell cultures

Primary cultures of CGCs were obtained from dissociated cerebella of 7-day-old Wistar rats as in Levi et al. [30]. Cells were plated in basal medium Eagle (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicin on dishes coated with poly-L-Lysine. Cells were plated at 2×10^6 per 35 mm dish, 6×10^6 per 60 mm dish, or 15×10^6 per 90 mm dish. Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells.

2.3. Induction of apoptosis

Apoptosis was induced as in D'Mello et al. [31]. After 6–7 days *in vitro* (DIV), cells were washed twice and switched to serum-free BME (S-), containing 5 mM KCl and supplemented with 2 mM glutamine and 100 μg/ml gentamicin for the times reported in the figure legends. Control cells were treated identically but maintained in serum-free BME medium supplemented with 25 mM KCl for the indicated times; they are referred to as S-K25 cells.

Apoptotic cells are referred to as S-K5 cells and the different times after apoptosis induction will be referred to as xtime-S-K5.

2.4. Cell suspension, homogenate and mitochondria preparations

Before each experiment, the culture medium was removed and the plated CGCs were washed with phosphate-buffered saline medium (PBS) containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4 pH 7.4, and then collected by gentle scraping into a volume of medium depending on the particular experiment.

Cell suspensions showed full viability, even though they lacked the morphological organisation observed in culture dishes such as cell–cell and cell–substrate contacts, and had no neurites. Cell integrity, which remained essentially constant for 3–5 h, was quantitatively assessed by confirming the inability of cells to oxidize externally added succinate, which cannot enter intact cells [32], by confirming the ability of ouabain to block glucose transport into the cells [33], and by counting dead cells, identified as large phase-bright cell bodies, as in Volontè et al. [34]. The final cell suspension routinely contained 85–95% intact cells and was prepared after 6–7 DIV.

Cell homogenate from a cell suspension was obtained by 10 strokes with a Dounce Potter homogeniser at room temperature. Rupture of cell membranes was checked by confirming the failure of ouabain to inhibit glucose-dependent oxygen consumption (see above) [33].

Mitochondria were isolated from cell homogenates as in [35]. Briefly, the cellular homogenate is centrifuged at 1500×*g* for 10 min. The pellet is resuspended and centrifuged at 1500×*g* for 10 min. The combined supernatants are centrifuged again at 1500×*g* for 10 min. The supernatant obtained is centrifuged at 17,000×*g* for 11 min. The pellet represents the mitochondrial fraction. All the operations are made on ice and the centrifugations at 4 °C.

Mitochondria, incubated in PBS, were checked both for their coupling by measuring the respiratory control index ratio, i.e. (oxygen uptake rate after ADP addition)/(oxygen uptake rate before ADP addition) which reflects the ability of mitochondria to produce ATP, and for their intactness by measuring in the post-mitochondrial supernatant the activities of adenylate kinase (ADK, E.C.2.7.4.3) and glutamate dehydrogenase (GDH, E.C.1.4.1.3) [36] which are marker enzymes of the mitochondrial intermembrane space and matrix, respectively.

Protein content was determined according to [37] with bovine serum albumin used as a standard.

2.5. Immunoblot analysis

Purified mitochondria from either control or apoptotic CGCs were solubilized in 1% TX-100, 500 mM NaCl, 8 mM MOPS, 0.04 mM EDTA pH 7.2 for 30 min on ice. Solubilized mitochondrial proteins (4 μg) were loaded onto a Tricine-SDS polyacrylamide gel [38], separated, and transferred to a PVDF membrane which was probed with goat anti-LDH antibody. Monoclonal anti-COX and monoclonal anti-beta-actin antibodies were used as a loading control for mitochondrial and cytosolic fractions respectively. Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using Enhanced Chemiluminescence Western Blotting reagents. Relative optical densities and areas of bands were quantified using a GS-700 Imaging Densitometer implemented with Molecular Analyst Software (Bio Rad Laboratories).

2.6. mLDH assay

mLDH assay was performed photometrically by means of a Perkin-Elmer Lambda-5 spectrophotometer at the wavelength 334 nm, at 25 °C, following the absorbance changes reflecting either NADH oxidation or NAD^+ reduction resulting from PYR or L-LAC addition, respectively, as a function of time. For the assays, CGC mitochondria (0.02 mg of mitochondrial protein) were incubated in phosphate buffer 0.1 M pH 7.6 to a final volume of 1 ml with NADH (0.2 mM), in the presence of rotenone (0.2 μg/10μl), to avoid NADH oxidation by complex I when mitochondria are solubilized with TX-100 (0.2%), or NAD^+ (1 mM) plus the detergent TX-100, and the appropriate substrate was then added to start the reaction. The activity was obtained as the tangent to the initial part of the progress curves and expressed as NAD^+ reduced (NADH oxidized)/min × mg of mitochondrial protein ($\varepsilon_{334\ nm}$=6.22 mM^{-1} cm^{-1}).

2.7. Polarographic measurements

O_2 consumption was measured polarographically using a Gilson 5/6 oxygraph with a Clark electrode, as in [36,39]. Either intact cells or cell homogenates in PBS (about 1 mg protein) were incubated in a thermostated (25 °C) water-jacketed glass vessel (final volume 1.5 ml). Since the incubation chamber required continuous stirring to allow O_2 to diffuse freely, the design included a magnetic stirring system. Oxygen uptake was initiated by adding L-LAC either to the suspension or to the cell homogenate. The rate of oxygen uptake was measured from the tangent to the initial part of the progress curve and expressed as natom O/min × mg protein. The sensitivity of the instrument was set so as allow rates of O_2 uptake as low as 0.5 natom O/min × mg protein to be measured.

2.8. Fluorescence Emission Spectra of CGCs

Cell suspension (0.5 mg protein) was incubated at 25 °C in 2 ml of PBS and fluorescence emission spectra (excitation wavelength 334 nm, emission range 400–550 nm) were recorded using an LS50B Luminescence Spectrophotometer (Perkin-Elmer Applied Biosystems).

2.9. Swelling experiments

Swelling of cell homogenate mitochondria was followed at 25 °C using a Perkin-Elmer Lambda-5 spectrophotometer at 546 nm. Cell homogenate in PBS (0.5 mg protein) was rapidly added to isotonic solutions of ammonium salts the pH of which were adjusted to 7.2, and the decrease in the absorbance was continuously recorded (see [40]).

2.10. Fluorimetric and photometric assays

Changes in the redox state of mitochondrial pyridine nucleotides were monitored fluorimetrically, using a Perkin Elmer LS-5 luminometer with excitation and emission wavelengths of 334 nm and 456 nm, respectively. Uptake of L-LAC was monitored essentially as described (see [41]). Cell homogenate (1 mg protein) in 2 ml medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.2) was first incubated in the presence of FCCP (0.12 μM) to oxidize intramitochondrial pyridine nucleotide, and rotenone (0.2 μg/10 μl) was added 2 min later. The substrate L-LAC was then added and reduction of intramitochondrial pyridine nucleotides was measured.

PYR appearance outside mitochondria was monitored by using the PYR detecting system (PDS) (consisting of 0.2 mM NADH plus 1 e.u. LDH) and then following photometrically NADH oxidation caused by externally added L-LAC, which *per se* had no effect on the enzymatic reactions or on the absorbance measured at 334 nm. In this case the $\varepsilon_{334\ nm}$ value measured for NADH under our experimental conditions was found to be 6.5 mM^{-1} cm^{-1}. In each experiment, controls were made of the intactness of the mitochondrial inner membrane by checking the rate of NADH oxidation in the absence of any substrate. When the rate was higher than 5% of that measured following metabolite efflux, mitochondria were not used.

It was confirmed that none of the compounds used affected the enzymes in the metabolite detecting systems. In each experiment NADH was added both in the sample and in the reference cuvette and the rate of NADH oxidation in the sample was calculated as the difference between the rates measured in the two cuvettes. This ensured that the recorded rate of the absorbance change was due only to reactions outside mitochondria (see [29]).

The rates of both fluorescence and absorbance changes were obtained as tangents to the initial parts of the progress curve and expressed as nmol NAD $(P)^+$ reduced (NADH oxidized)/min × mg cell protein, respectively.

2.11. Statistical analysis and computing

All statistical analyses in this study were performed by using SPSS software. The data were representative of at least three independent neuronal preparations (with comparable results) each one in independent measurements (in each figure legend the number of measurements is reported) and are reported as the mean with the standard deviation (S.D.). Statistical significance of the data was

evaluated using the one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test. $p<0.05$ was considered as significant for all analyses.

Experimental plots were obtained using Grafit (Erithacus software).

3. Results

3.1. CGC mitochondria can metabolize externally added L-LAC

In agreement with [12], we have confirmed that externally added L-LAC can enter CGCs where it is metabolised as an energetic substrate (Fig. 1). To achieve this we first investigated whether addition of 3 mM L-LAC, close enough to L-LAC concentration in the extracellular cerebral fluid (see [11]) to CGCs could result in oxygen consumption. Oxygen uptake was found at a rate of 20 natom O/min × mg cell protein (Fig. 1Aa), and this was completely blocked by oxamate (OXA, 10 mM), a specific inhibitor of L-LDH [42]. In another experiment, the addition of α-cyanocinnamate (α-CCN^-, 1 mM), a powerful inhibitor of monocarboxylate transport in the cell (see [12]), resulted in a decrease of oxygen uptake (9 natom O/min × mg cell protein), whereas this was completely blocked by rotenone (ROT, 0.2 μg/10μl), an inhibitor of Complex I of the mitochondrial respiratory chain (Fig. 1Ab).

In the same experiment, cell emission fluorescence spectra were carried out, as described in [43], using a wavelength pair designed to monitor the intracellular NAD(P)/NAD(P)H ox/redox state (Fig. 1Ac). CGCs were previously incubated with FCCP (0.12 μM) (trace a) and 2 min later with rotenone (trace b): these substances caused the oxidation of the intramitochondrial NAD(P)H, the level of which was very low, and prevented the mitochondrial oxidation of the NAD(P)H newly formed by endogenous substrates, respectively (see Materials and meth-

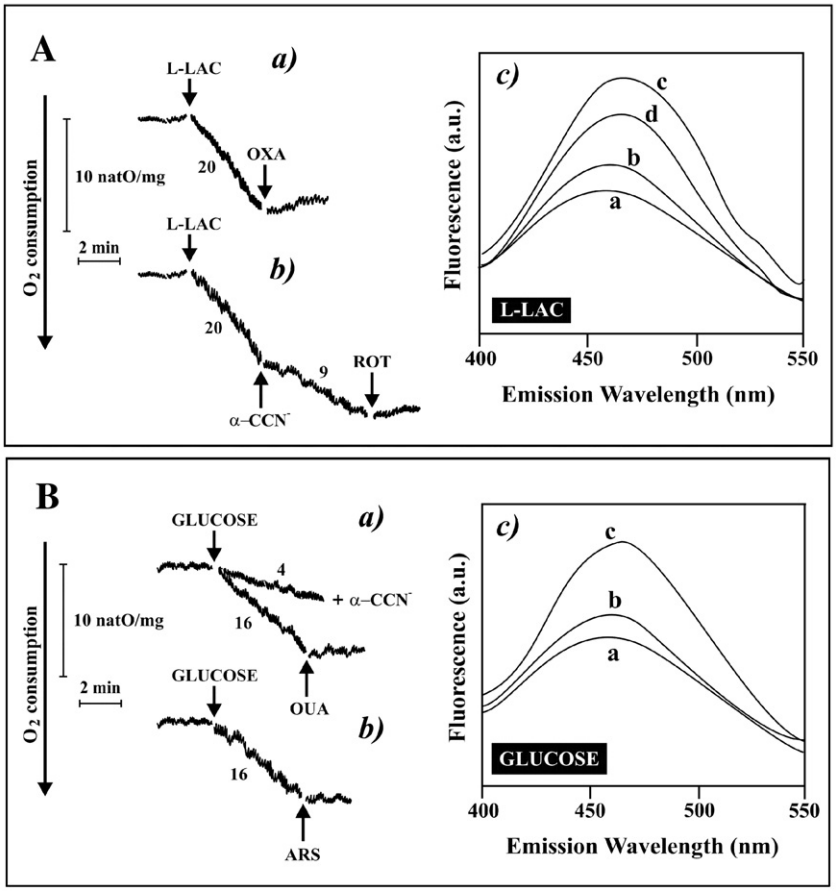
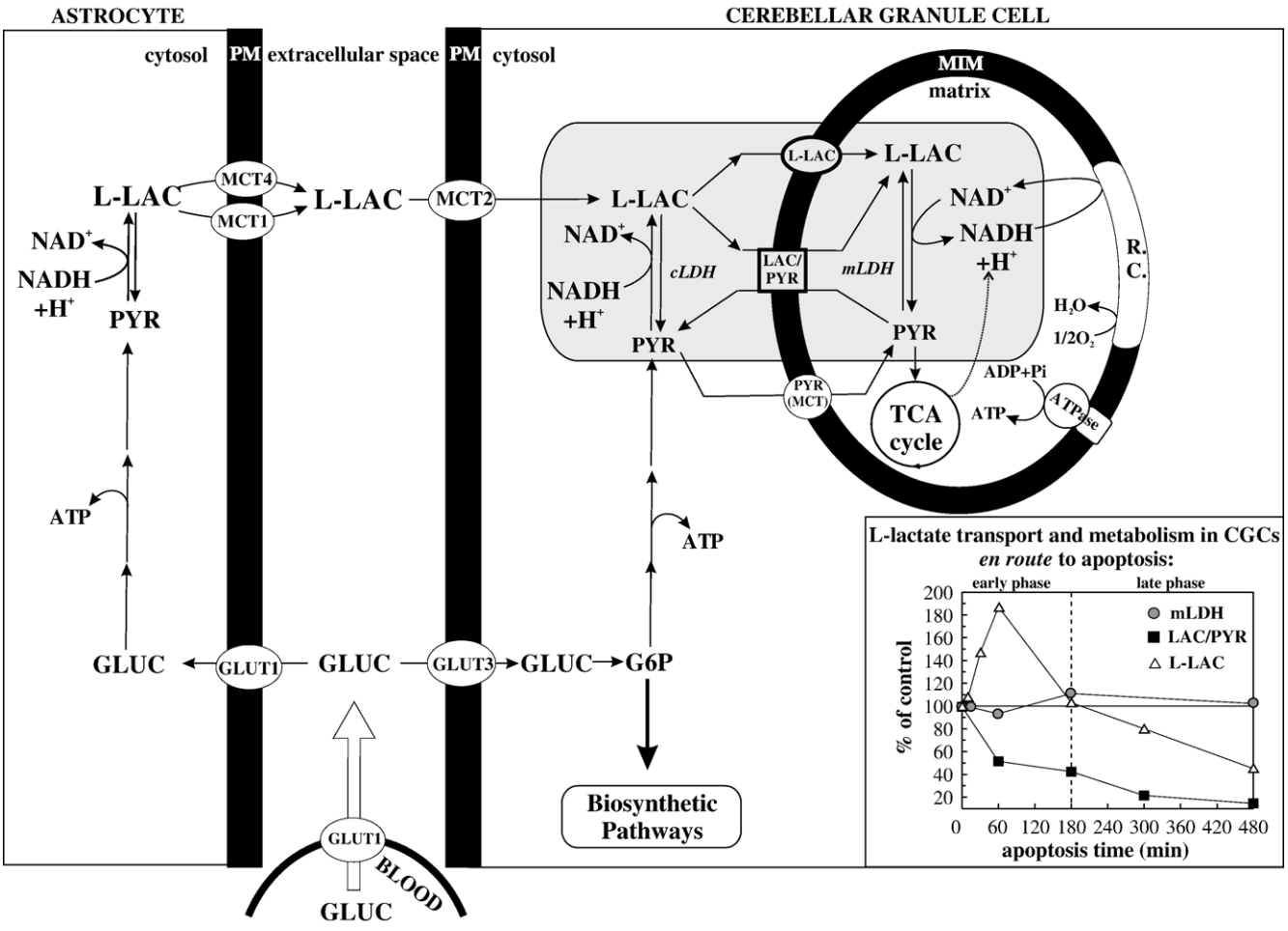


Fig. 1. Evidence for L-lactate uptake and metabolism in CGCs. (A) Oxygen uptake by CGCs added with L-lactate. CGCs maintained for 7 DIV (0.9 mg protein) were collected, suspended in 1 ml PBS and incubated in a polarographic vessel at 25 °C and oxygen uptake was measured. At the arrows the following additions were made: L-lactate (L-LAC, 3 mM), oxamate (OXA, 10 mM), rotenone (ROT, 0.2 μg/10μl), α-CCN^- (1 mM). Numbers along the curves are rates of oxygen uptake expressed as natom O/min × mg cell protein. Fluorescence emission spectra of CGCs. Cell suspension CGCs were incubated at 25 °C in 2 ml PBS, with protein concentration adjusted to 0.25 mg/ml. The spectra of fluorescence emission (λ_{exc} = 334 nm) were obtained as reported in Materials and methods. Cells, previously treated with FCCP (0.12 μM) (trace a) and then with rotenone (0.2 μg/10μl) for 2 min (trace b), were added with L-LAC (3 mM) in the absence (trace c) or presence of α-CCN^- (trace d) and spectra were carried out 2 min after each addition. α-CCN^- (1 mM) was preincubated with cells 2 min before L-LAC addition. The experiment was repeated four times with different cell preparations, giving values with 5–10% variation. Fluorescence intensity is reported in arbitrary units (a.u.). (B) Oxygen uptake by CGCs added with glucose. The experiment was carried out as in panel A. At the arrows the following additions were made: glucose (5 mM), oxamate (OXA, 10 mM), α-CCN^- (1 mM), ouabain (OUA, 1 μM), sodium–arsenite (ARS, 2 mM). Numbers along the curves are rates of oxygen uptake expressed as natom O/min × mg cell protein. Fluorescence emission spectra of CGCs. The experiment was carried out as in panel A. Cells, previously treated with FCCP (0.12 μM) (trace a) and then with rotenone (0.2 μg/10μl) for 2 min (trace b), were added with glucose (3 mM) (trace c). The experiment was repeated four times with different cell preparations, giving values with 5–10% variation. Fluorescence intensity is reported in arbitrary units (a.u.).

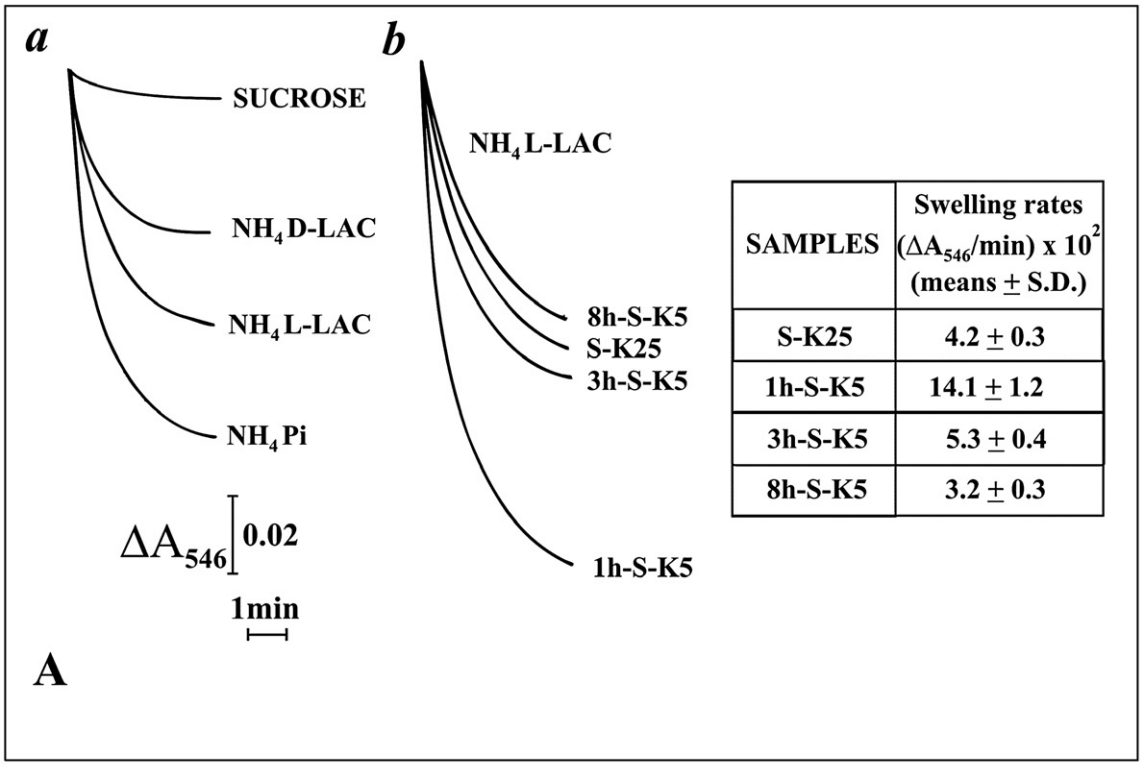
ods). Both fluorescence spectra exhibit a peak close to 456 nm due to the intracellular NAD(P)H. Following cell incubation with 3 mM L-LAC for 1–2 min, an increase of the fluorescence emission spectra was found (trace c). Such an increase did not occur when L-LAC was added in the presence of OXA (10 mM) (not shown), and was partially prevented by α-CCN$^-$(1 mM) (trace d), which themselves had no effect on the spectra (not shown). Fig. 1Aa, b, c shows that L-LAC can enter CGCs and that this occurs in a α-CCN$^-$ sensitive manner [12]; once inside the cell L-LAC causes NAD(P)H formation. This certainly occurs because L-LAC is oxidized to pyruvate in the cytoplasm via cLDH, but the possibility also arises that L-LAC is taken up by mitochondria and oxidized via the mLDH (see below) (see Scheme 1), both the cytosolic and the putative mitochondrial L-LAC oxidation being inhibited by OXA.

As a control, the same experiments were carried out by using glucose (3 mM) as an energy substrate: oxygen uptake was found at a rate of 16 natom O/min × mg cell protein (Fig. 1Ba). Glucose oxidation was partially prevented by α-CCN$^-$(1 mM) (4 natom O/min × mg cell protein), which can both enter the cells [44] and inhibit the mitochondrial PYR carrier [45], but completely blocked either by ouabain (OUA, 1 μM) (Fig. 1Ba), which prevents glucose transport in the cell [33], or by arsenite (ARS, 2 mM), an inhibitor of the pyruvate dehydrogenase which can enter cells [46], (Fig. 1Bb). Fluorescence spectra of CGCs incubated with glucose, carried out as described above for L-LAC (Fig. 1Bc), showed that glucose addition resulted in an increase of the fluorescence emission of CGCs (trace c) previously incubated with FCCP (trace a) and with rotenone (trace b). No change of fluorescence spectra was found in the presence of OUA (1 mM) (not shown).

Having confirmed that L-LAC can enter CGCs in our experimental conditions (see also [12]), the next step was to determine whether externally added L-LAC can enter CGC mitochondria. This was first done by monitoring mitochondrial swelling in 100 mM ammonium L-LAC solution, measured as a



Scheme 1. Transport and metabolism of L-lactate in cerebellar granule cell mitochondria and the occurrence of the L-lactate/pyruvate shuttle. For details see the text. Abbreviations: GLUC, glucose; G6P, glucose 6-phosphate; L-LAC, L-lactate; MIM, mitochondrial inner membrane; PM, plasma membrane; PYR, pyruvate; R.C., Respiratory Chain. Plasma membrane transporters: GLUT3, neuronal glucose transporter; GLUT1, astrocyte and endothelial cell glucose transporter; MCT2, neuronal monocarboxylate transporter; MCT1, MCT4, astrocyte monocarboxylate transporters. Mitochondrial translocators: L-LAC, L-lactate/H$^+$ symporter; LAC/PYR, L-lactate/pyruvate antiporter; PYR (MCT), pyruvate translocator, i.e. monocarboxylate translocator. Enzymes: cLDH, cytosolic L-lactate dehydrogenase; mLDH, mitochondrial L-lactate dehydrogenase. In the inset: Changes in the rate of L-lactate mitochondrial transport and metabolism *en route* to apoptosis. Either mLDH activity (●), L-lactate uptake (Δ) or L-lactate/pyruvate exchange (■) rate, measured after the addition of 1 mM L-lactate as described for Figs. 3C, 4 and 5, respectively, are reported as a function of the apoptosis time. The rates are expressed as percentage of the control (equal to 100 at each time).



SAMPLES	Swelling rates (ΔA_{546}/min) x 10^2 (means ± S.D.)
S-K25	4.2 ± 0.3
1h-S-K5	14.1 ± 1.2
3h-S-K5	5.3 ± 0.4
8h-S-K5	3.2 ± 0.3

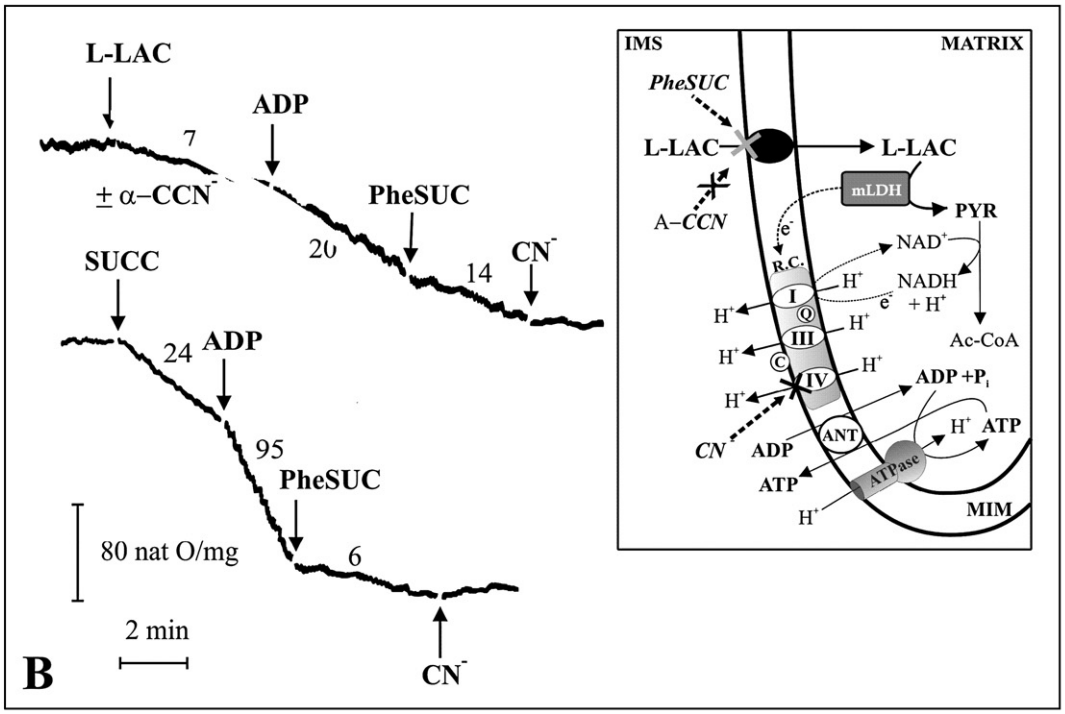


Fig. 2. Metabolism of externally added L-lactate by CGC mitochondria. (A) Swelling in ammonium L-lactate solution of mitochondria from cells undergoing apoptosis. In (a) CGC homogenate (0.5 mg protein) from S-K25 cells was added to 0.1 M ammonium phosphate (NH_4Pi), 0.1 M ammonium L-lactate (NH_4L-LAC), 0.1 M ammonium D-lactate (NH_4D-LAC) or 0.25 M sucrose. In (b) CGC homogenate (0.5 mg protein) from either S-K25 or S-K5 cells taken at the indicated times was rapidly added to 2 ml of 0.1 M ammonium L-lactate (NH_4L-LACT). In the table the means ± S.D. of the initial swelling rates are reported. Mitochondrial swelling was monitored as described in Materials and methods. (B) Oxygen uptake by homogenates from CGCs incubated with L-lactate and succinate. Homogenate (1 mg cell protein) from rat CGCs cultured for 7 DIV was incubated in a polarographic vessel at 25 °C and oxygen uptake was measured as a function of time. At the arrows the following additions were made: L-lactate (L-LAC, 5 mM), succinate (SUCC, 5 mM), ADP (1 mM), phenylsuccinate (PheSUC, 10 mM), potassium cyanide (CN^-, 1 mM). α-CCN^- (1 mM) was added 1 min before L-LAC. Numbers along the curves are rates of oxygen uptake expressed as natom O/min × mg cell protein. The inset shows a scheme representing mitochondrial L-LAC uptake and oxidation. For details see the text. Main abbreviations: Ac-CoA, acetyl coenzyme A; ANT, adenine nucleotide translocator; α-CCN^-, α-cyanocinnamate, IMS, intermembrane space; L-LAC, l-lactate; mLDH, mitochondrial L-lactate dehydrogenase; MIM, mitochondrial inner membrane; PheSUC, phenylsuccinate; PYR, pyruvate.

decrease in absorbance at 546 nm, which occurs when mitochondria are incubated in an isotonic solution of the ammonium salt of a penetrant anion (see [40]). Mitochondrial swelling was monitored in homogenates from either control cells (S-K25) or from cells undergoing apoptosis at different times *x*, expressed in hours (h) or minutes (min), after apoptosis induction (which will be referred to as *x*time-S-K5). In both cases the assumption is made that the non-mitochondrial fraction of the homogenate has no effect on the absorbance of the suspension over the investigated times.

Rapid and extensive swelling of S-K25 homogenate mitochondria occurred in 100 mM ammonium L-LAC solution as shown in Fig. 2Aa. Swelling in 100 mM ammonium D-LAC occurred at a slower rate and to a lesser extent as in [47]. This demonstrates that these mitochondria take up L-LAC, likely in a proton compensated carrier–mediated manner [47]. Both the rate and the extent of swelling were lower than that which occurred with 100 mM ammonium phosphate, a highly penetrant anion (see [41]). No significant swelling was found in 250 mM sucrose, showing the intactness of the mitochondrial membranes. Surprisingly enough, swelling of mitochondria from 1 h-S-K5 cells in ammonium L-LAC occurred with rate and extent higher (about 280 and 70% respectively) than in the control (Fig. 2Ab). At longer times of apoptosis both the rate and extent of swelling decreased to values similar to those of the control cells, but were lower than those of the control at 8 h.

In a series of experiments, the rates of swelling in ammonium L-LAC solutions were normalized to that of ammonium phosphate in the same experiments. We confirmed statistically that the swelling rate up to 1 h after apoptosis induction was significantly higher (about 160%) compared with S-K25 cells (see the table of Fig. 2A where the means ± S.D. of the initial rates of swelling are reported).

Having established that L-LAC can enter mitochondria, the ability of externally added L-LAC to cause uptake of oxygen was checked using cell homogenates (1 mg cell protein), as in [48] (Fig. 2B). As a result of the addition of 5 mM L-LAC, either in the absence or presence of 1 mM α-CCN^-, oxygen uptake occurred at a rate of about 7 natom O/min × mg cell protein (State 4); following addition of 1 mM ADP (State 3), an increase in rate of about 185% was found. The respiratory index, i.e. oxygen uptake rate after ADP addition/oxygen uptake rate before ADP addition, with L-LAC was 2.9, showing that mitochondria were coupled, and more importantly that they can metabolize L-LAC in an energy competent manner. The intactness of mitochondria in homogenates was also established by confirming the absence of activities of adenylate kinase and glutamate dehydrogenase

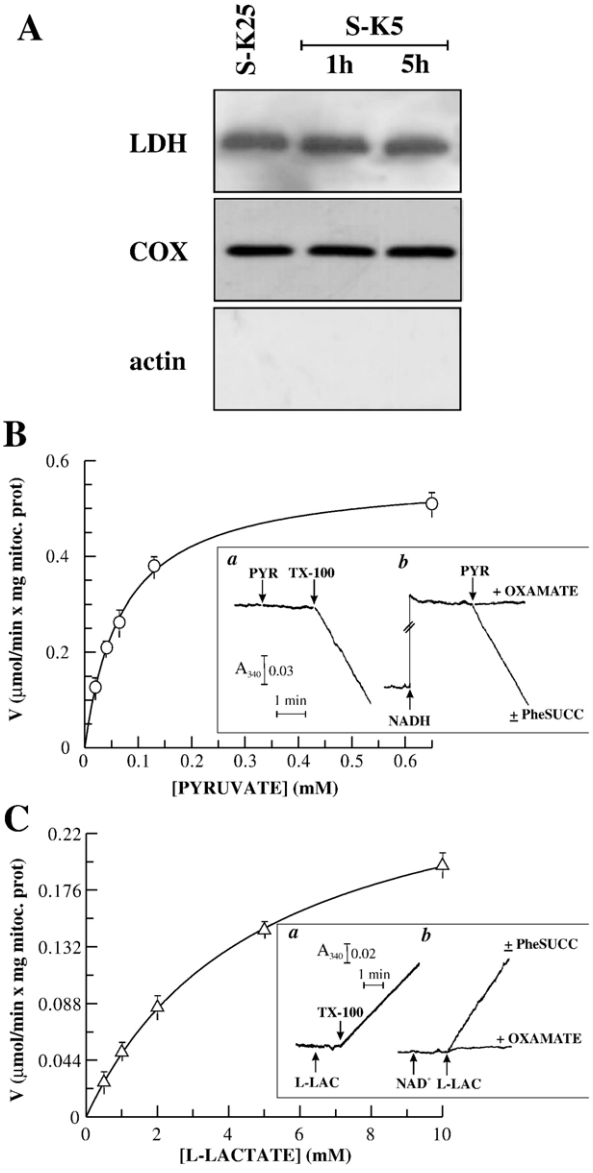


Fig. 3. Western blot of mitochondrial extracts using LDH specific antibody and LDH assay in mitochondria from cells undergoing apoptosis. (A) Solubilized mitochondrial proteins (4 µg of total mitochondrial protein loaded in each lane) from either control (S-K25) or apoptotic (S-K5) cells were obtained at 1 and 5 h and analyzed by Western blotting analysis using LDH specific antibody, as described in Materials and methods. Mitochondrial purity was confirmed by detection of the presence of the mitochondrial marker protein COX and by the absence of the cytosolic marker protein actin. (B, C) Mitochondrial fractions (0.02 mg protein), dissolved with TX-100 (0.2%), from S-K25 were incubated in phosphate buffer 0.1 M pH 7.6 to a final volume of 1 ml and previously added with rotenone (0.2 µg/10µl) to prevent the NADH oxidation. Both the dependence of the rate of NADH oxidation on increasing PYR concentrations (B) and also that of NAD^+ reduction on increasing L-LAC concentrations (C) were measured. When PYR was used as mLDH substrate, mitochondria were incubated in the presence of TX-100, after which NADH (0.2 mM) followed by PYR (B) were added to start the reaction. When L-LAC was used as mLDH substrate, mitochondria were incubated in the presence of TX-100, after which NAD^+ (0.2 mM) followed by L-LAC (C) were added to start the reaction. In the insets either healthy (a) or solubilized mitochondria (b) (0.02 mg protein) were used. When PYR was used as mLDH substrate, mitochondria were incubated in the presence of NADH (0.2 mM) (Ba) or the detergent (Bb) respectively, after which PYR (0.65 mM) followed by TX-100 (0.2%) (Ba) or NADH (0.2 mM) followed by PYR (0.65 mM) (Bb) were added to start the reaction. When L-LAC was used as mLDH substrate, mitochondria were incubated in the presence of NAD^+ (1 mM) (Ca) or TX-100 (0.2%) (Cb) respectively, after which L-LAC (10 mM) followed by TX-100 (0.2%) (Ca) or NAD^+ (0.2 mM) followed by L-LAC (10 mM) (Cb) were added to start the reaction. Oxamate (OXA, 10 mM) and phenylsuccinate (PheSUC, 10 mM) were previously incubated with healthy (in a) or solubilized mitochondria (in b). The rate of absorbance change at 334 nm, measured as the tangent to the initial part of the progress curve, is expressed as µmol/min × mg mitochondrial protein.

Table 1
Lactate dehydrogenase activity assay in cells undergoing apoptosis

Mitochondrial L-LDH				
Time after apoptosis induction	$K_m^{L\text{-}LAC}$ (mM)	V_{max} (μmol/min × mg prot)	K_m^{PYR}(mM)	V_{max} (μmol/min × mg prot)
S-K25 15′	4.9±0.45	0.29±0.005	0.070±0.006	0.57±0.01
S-K5 15′	4.4±0.49	0.27±0.01	0.065±0.004	0.58±0.01
S-K25 1 h	5.0±0.50	0.30±0.010	0.070±0.005	0.55±0.02
S-K5 1 h	4.7±0.75	0.27±0.02	0.073±0.005	0.52±0.01
S-K25 3 h	4.9±0.35	0.28±0.05	0.070±0.005	0.60±0.03
S-K5 3 h	3.9±0.72	0.28±0.02	0.071±0.002	0.59±0.01
S-K25 8 h	5.2±0.20	0.27±0.02	0.075±0.005	0.57±0.02
S-K5 8 h	3.9±0.45	0.28±0.01	0.076±0.009	0.56±0.02

Mitochondria (0.02 mg protein), from either control (S-K25) or apoptotic (S-K5) CGCs, were solubilized with TX-100 (0.2%) and then incubated at 25 °C in phosphate buffer 0.1 M pH 7.6 to a final volume of 1 ml. The assay was performed as in Fig. 3B and C. The rate of absorbance increase (decrease), measured as the tangent to the initial part of progress curve, is expressed as μmol of $NADP^+$ reduced (NADPH oxidized)/min × mg of mitochondrial protein. Results are means with the standard deviation (±S.D.) of triplicate measurements and representative of at least 5 different experiments carried out with different cell preparations prepared from different groups of animals. ANOVA and Bonferroni test: no statistically significant differences were found for any of the samples at any of the time considered.

[36], which are marker enzymes of the mitochondrial intermembrane space and matrix respectively, in post-mitochondrial supernatants (for details see Materials and methods). In the same experiment, the control respiratory index with succinate used a respiratory substrate (5 mM) was 3.9. In three separate experiments oxidation of L-LAC (5 mM) proceeded at rates of 7±4 and 20±5 natom O/min × mg cell protein in *states 4* and *3* respectively; in the same experiments, oxygen uptake by cell homogenates to which succinate (SUCC, 5 mM) had been added occurred with rates of 24±4 and 95±10 natom O/min × mg cell protein in *states 4* and *3* respectively.

In both cases, phenylsuccinate (PheSUC, 10 mM), a non-penetrant compound which can inhibit a variety of carriers, including the L-LAC carriers in heart and liver mitochondria [28,29], but excluding both the PYR carrier and the ANT (for refs see [41]), was found to reduce the rate of oxygen consumption due to L-LAC and SUCC (35 and 95%, respectively); both processes were completely blocked by potassium cyanide (CN^-, 1 mM) which inhibits the cytochrome *c* oxidase (COX). The inhibition by PheSUC rules out the possibility that oxygen uptake was dependent on the oxidation of PYR generated outside mitochondria, by the action of contaminating cLDH and NADH, and taken up by mitochondria via its own carrier.

Similar experiments, not reported in detail, were carried out with homogenates from cells *en route* to apoptosis. As already reported in [36] a decrease in the oxygen uptake was found as a function of time of apoptosis.

A possible explanation of the results in Fig. 2B is the following: externally added L-LAC enters mitochondria via a PheSUC-sensitive, but α-CCN^- insensitive, carrier. Inside mitochondria, L-LAC is oxidized to PYR via a putative mLDH, and the NADH formed drives formation of the electrochemical proton gradient used for ATP synthesis and export (see inset to Fig. 2B).

3.2. The existence of LDH in CGC mitochondria

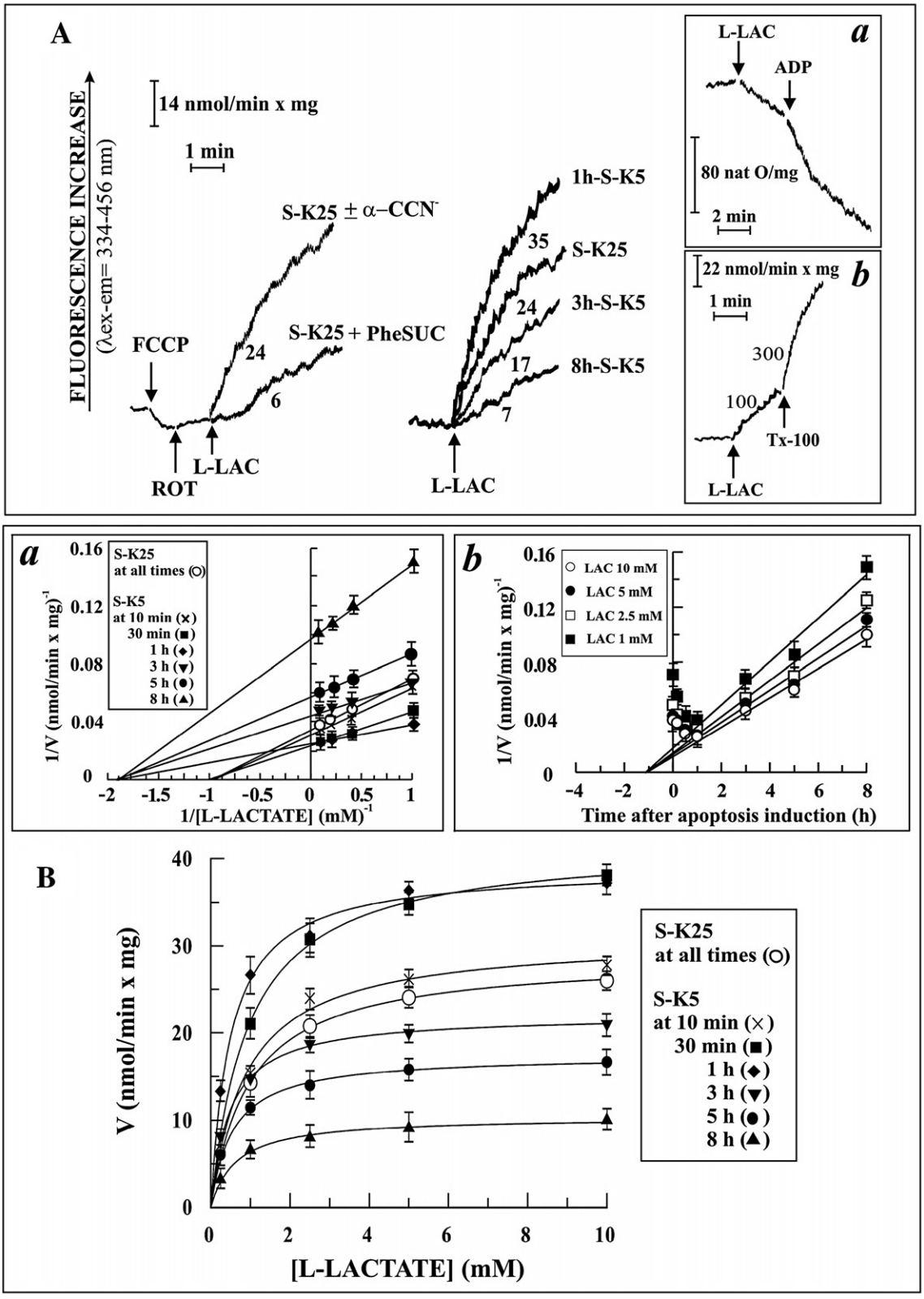
To confirm that mitochondria from CGCs contain their own mLDH and to ascertain whether the mLDH protein level changes *en route* to apoptosis, an immunological analysis was carried out by using anti-LDH antibody (Fig. 3A). This showed the occurrence of the novel mitochondrial LDH at a protein level essentially similar in mitochondria from either 1 h/5 h-S-K5 cells or S-K25 at the same time (not shown in detail). Each mitochondrial sample was also subjected to Western blotting for beta-actin, as a cytosolic marker, using a monoclonal anti-beta-actin antibody. No cross reacting material was found even as monitored for times up to 2 h showing that all mitochondrial preparations were completely free of cytosolic contamination. COX was used as a mitochondrial marker. Challenge with a monoclonal anti-COX subunit IV antibody showed constant levels of protein at the investigated times.

In parallel, we tried to assay the activity of mLDH in isolated mitochondria. To do this mitochondria were added to 1-ml medium containing either NADH (0.2 mM) and PYR (1 mM) (Fig. 3B) or NAD^+(1 mM) and L-LAC (1 mM) (Fig. 3C) to follow the LDH reaction in the sense of NADH oxidation or NAD^+ reduction respectively, and the absorbance at 334 nm measured as a function of time (for details see Materials and methods). No NADH oxidation/NAD^+ reduction was found following the addition of intact mitochondria, previously treated with rotenone (0.2 μg/10μl) to prevent NADH oxidation, thus showing the absence of NAD^+-linked LDH in the external mitochondrial compartments. As a result of addition of TX-100 (0.2%) to the mitochondria, LDH activity was found as revealed by the decrease/increase of absorbance respectively (traces a of insets to Fig. 3B and C). The same changes were observed when solubilized mitochondria were used (traces b of insets to Fig. 3B and C). This shows that mLDH is located in the inner mito-

Fig. 4. Fluorimetric investigation of the redox state of the intramitochondrial pyridine nucleotides caused by externally added L-lactate. (A) CGC homogenate (1 mg protein), in 2 ml medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.2), from either S-K25 or S-K5 cells taken at the indicated times of apoptosis, was incubated at 25 °C and treated with FCCP and then with rotenone (ROT, 0.2 μg/10μl). Reduction of pyridine nucleotides was followed fluorimetrically (λ_{exc}=334 nm; λ_{em}=456 nm) as a function of time. At the arrows L-lactate (L-LAC, 5 mM) was added to the suspension. Where indicated, PheSUC (10 mM) or α-CCN^- (1 mM) were previously incubated with the homogenate. The rate of fluorescence increase, measured as the tangent to the initial part of the progress curve, is expressed as nmol of intramitochondrial $NAD(P)^+$ reduced/min × mg of cell protein. The inset (a) reports the P/O ratio calculated by adding 0.1 mM ADP to homogenate added with 5 mM L-LAC. The inset (b) reports an experiment with added TX-100: TX-100 addition (0.2%) follows 5 mM L-LAC addition to homogenate previously incubated with 1 mM $NAD(P)^+$. The rates of $NAD(P)^+$ reduction are expressed as a percentage of control (100%), i.e. in the absence of TX-100. (B) Dependence of the rate of L-lactate uptake as a function of the L-LAC concentration was investigated in S-K25 and S-K5 homogenate taken at the indicated times of apoptosis. In the insets are reported the double reciprocal (a) and the Dixon-like (b) plots, obtained by replotting the rate data of panel B as a function of apoptosis times. In both cases, the rates (*V*), measured as the tangent to the initial part of the progress curve, are expressed in nmol of $NAD(P)^+$reduced/min × mg cell protein.

chondrial compartments. Externally added OXA (10 mM) was found to completely block the mLDH reaction in whichever direction it was investigated. In the same experiment, we found that PheSUC (10 mM) was ineffective in inhibiting the NADH/NAD^+ oxidation/reduction (traces b of insets to Fig. 3B and C).

In the light of these results we used the mitochondrial fractions dissolved with TX-100 both from S-K25 (Fig. 3B and C, Table 1) and from S-K5 cells at different times after induction of apoptosis (Table 1) to investigate the kinetic properties of the enzyme. We measured the dependence of the rate of NADH



oxidation on increasing PYR concentrations and also that of NAD^+ reduction on increasing L-LAC concentrations with the results shown in Fig. 3B and C respectively. Saturation kinetics were found in both cases. Consistent with the results in Fig. 3A, no significant differences in V_{max} values were found when the mLDH reaction was monitored with either substrate pair in S-K5 cells undergoing apoptosis compared with S-K25 control cells (Table 1). Similarly, no changes in the K_m values were found.

3.3. L-lactate transport into mitochondria

The stereospecific spontaneous swelling shown in Fig. 2A may suggest that L-LAC produced in the cytosol can cross the mitochondrial membrane in a carrier mediated manner which results in proton compensated L-LAC symport. In order to gain some insight into this point, we investigated, using a fluorimetric method, the ability of externally added L-LAC, to reduce the intramitochondrial pyridine dehydrogenase cofactors (see [41]). We first checked that in spite of the presence outside mitochondria of the cytosolic LDH and of the pyridine nucleotide pool, no change of fluorescence occurred when L-LAC was added to the post-mitochondrial supernatant. This allowed us to measure the reduction of the intramitochondrial $NAD(P)^+$in S-K25 cell homogenates previously incubated with FCCP and then with rotenone (see above to Fig. 1). When the fluorescence had stabilized, addition of L-LAC (5 mM) resulted in a fast increase in fluorescence due to reduction of $NAD(P)^+$(Fig. 4A). The fluorescence increase was unaffected by α-CCN^- (1 mM), whereas PheSUC (10 mM) largely reduced the rate of L-LAC-dependent NAD(P)H formation (75% inhibition). In a control experiment, it was confirmed that PheSUC (10 mM) does not affect the oxidation of β-hydroxy-butyrate which is known to enter mitochondria by diffusion [49], thus excluding the possibility that PheSUC can cause any non-specific effect on the mitochondrial membrane. To confirm that mitochondrial LDH is a pyridine nucleotide-dependent enzyme, in a set of experiment carried out as in Fig. 2B, the P/O ratio was determined using 0.1 mM ADP and found to be 2.85±0.08 (inset a to Fig. 4A).

To determine whether the rate of oxidation of L-LAC via the putative LDH was limited by the rate of its uptake, TX-100 (0.2%) was added to the cell homogenate as in [39] (inset b to Fig. 4A). In this case, 1 mM $NADP^+$ was used as the coenzyme rather than NAD^+, both because of the release of intramitochondrial NAD^+ which is a result of TX-100 addition and because the newly synthesized NADPH cannot be oxidized by the solubilized mitochondria. A large increase (about 200%) in the rate of pyridine nucleotide reduction was found showing that the rate of oxidation of L-LAC reflects the rate of L-LAC uptake across the mitochondrial inner membrane.

To ascertain whether and how L-LAC uptake by mitochondria could change in apoptosis, the rates of the process were measured in homogenates from either S-K25 or S-K5 cells taken at different times after induction of apoptosis. In this case 5 mM L-LAC was used. Whereas the rate of NAD(P)H formation in control homogenates remained essentially constant up to 8 h (not shown), this rate was enhanced in 1 h-S-K5 cells up to about 145% of the control, with a subsequent decrease at longer times: a decrease up to about 65% was found with homogenate from 8 h-S-K5 (Fig. 4A). It should be noted that, because of the impairment of the ANT known to occur *en route* to apoptosis (see [48]), the P/O ratio could not be calculated. Notice that TX-100 experiment, carried out as reported above, showed that the rate of fluorescence increase mirrored the rate of L-LAC transport (see inset b to Fig. 4A).

In the light of the transport paradigm according to which mitochondria first provide net carbon uptake and then allow for efflux of newly synthesized compounds (see [41]) and since the L-LAC/PYR shuttle takes place in heart and liver as already shown in [28,29], we tried to reconstruct the L-LAC/PYR shuttle *in vitro*, as in [28], by checking whether addition of L-LAC (5 mM) can cause efflux of PYR from the mitochondria (Fig. 5A); the concentration of PYR outside mitochondria was negligible as shown by the fact that no decrease in the absorbance measured at 334 nm was found when NADH+LDH, i.e. the PYR detecting system, were added to mitochondrial suspensions. As a result of addition of 5 mM L-LAC, a decrease in absorbance of NADH occurred with a rate equal to 19 nmol NADH oxidized/min×mg cell protein, which indicates the appearance of PYR in the extramitochondrial phase. α-CCN^-(1 mM) did not affect this rate, thus showing that the putative L-LAC/PYR antiporter is distinct from the PYR carrier. PheSUC (10 mM) caused about 60% inhibition of the rate of NADH absorbance decrease.

In a parallel experiment, homogenates were incubated with LDH plus NADPH (0.2 mM), which cannot be oxidized by complex I of mitochondrial respiratory chain, then (5 mM) L-LAC was added; as a result of the addition of the detergent TX-100 (0.2%) (inset to Fig. 5A), an increase (about 300%) in the rate of NADPH oxidation was found in homogenates from both S-K25 and in S-K5 CGCs (not shown), thus further showing that PYR efflux across the mitochondrial membrane is the rate limiting step of the measured rate of absorbance change.

The rates of NADH oxidation caused by addition of 5 mM L-LAC to S-K5 cell homogenates decreased from 15 to 12 and finally to 5 nmol NADH oxidized/min/mg cell protein with 1 h, 3 h and 8 h-S-K5 cells respectively.

A possible explanation of these findings is that L-LAC enters mitochondria via a L-LAC transporter as well as in exchange for endogenous PYR which originates in the matrix from the action of the mLDH. The newly synthesized PYR can, in turn, move to the extramitochondrial phase in exchange for further L-LAC in a carrier-mediated manner (see Scheme 1).

To gain some insight into the mechanism by which both control and apoptotic CGCs take up external L-LAC, the dependence of the rate of NAD(P)H formation inside mitochondria and of the appearance of PYR in the extramitochondrial phase on increasing L-LAC concentration was investigated with homogenates from S-K25 and S-K5 CGCs at different times after induction of apoptosis (Figs. 4B and 5B). As expected in the case of a carrier mediated transport, a hyperbolic dependence on L-LAC concentration was found. Interestingly, for CGCs up to 30 min after induction of apoptosis, a significant increase in the V_{max} values of L-LAC uptake (Fig. 4B and inset a) (see also Fig. 2) was found with respect to the control (41±4 and 28±

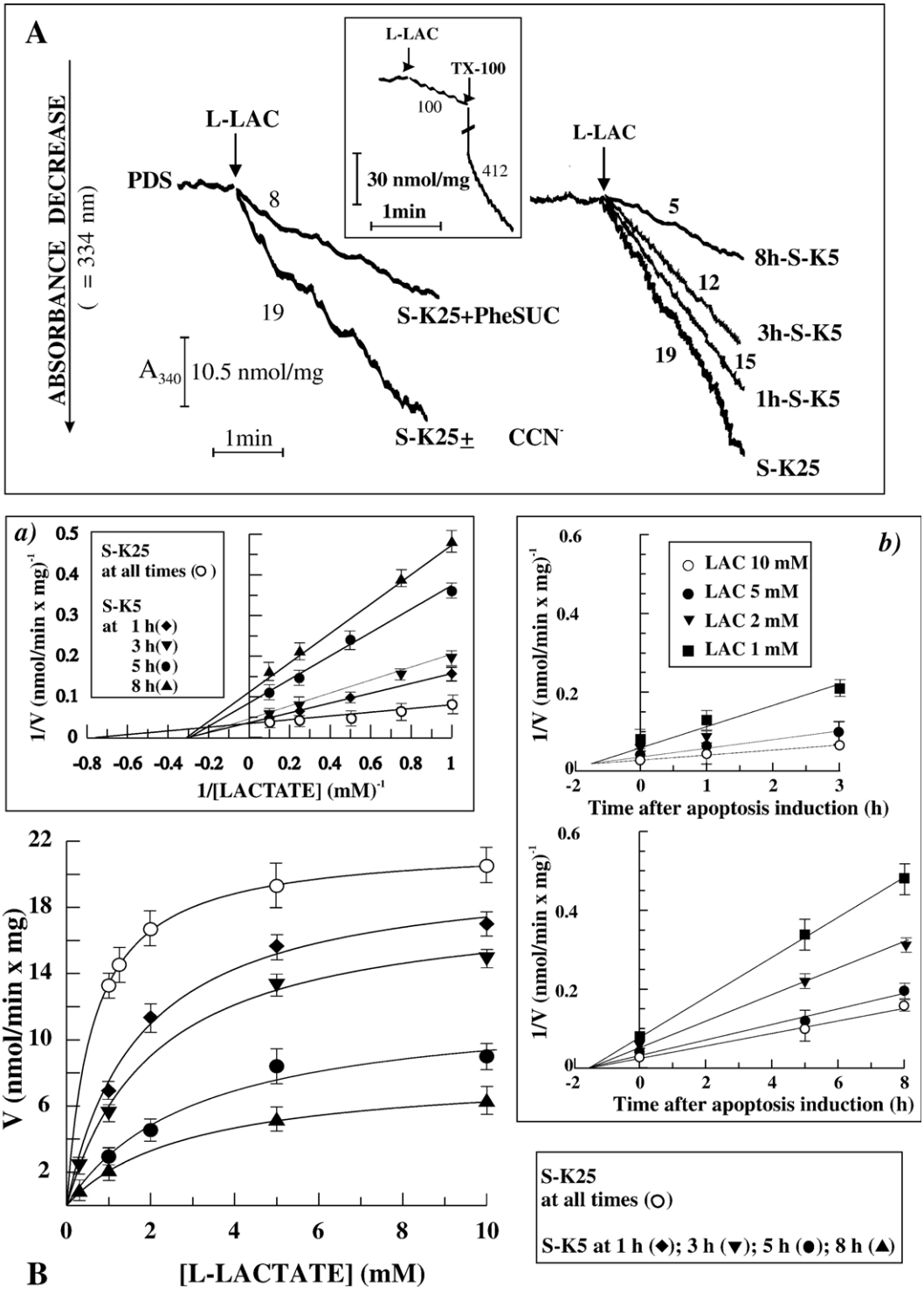


Fig. 5. Photometric investigation of the redox state of the extramitochondrial pyridine nucleotides caused by externally added L-lactate. (A) Appearance of PYR due to addition of L-LAC (5 mM) to CGC homogenate (1 mg protein) from S-K25 or S-K5 cells at the indicated times after apoptosis induction, incubated in the presence of the pyruvate detecting system (PDS), was monitored as described in Materials and methods. Where indicated, PheSUC (10 mM) and α-CCN^{-} (1 mM) were previously incubated with the PDS. The inset reports an experiment with added TX-100: TX-100 (0.2%) addition follows 5 mM L-LAC addition. Experimental conditions are the same, except that mitochondria were incubated with 0.2 mM NADPH (see Materials and methods). Numbers along the curves are rates of pyridine nucleotide reduction expressed as a percentage of control (considered as the 100%), i.e. in the absence of TX-100. (B) Dependence of the rate of L-LAC/PYR exchange as a function of the L-LAC concentration was investigated in S-K25 and S-K5 homogenates taken at the indicated times of apoptosis. In the insets are reported the double reciprocal (a) and the Dixon-like (b) plots, obtained by replotting the rate data of panel B as a function of apoptosis times, respectively. In both cases, the rates (V), measured as the tangent to the initial part of the progress curve, are expressed in nmol of NADH oxidized/min × mg cell protein.

3 nmol NAD(P)$^+$ reduced/min × mg cell protein respectively) with no change in the K_m values, measured as the L-LAC concentration which gave half maximum rate of transport, which were about 1 mM. In 1 h-S-K5 cells the increased V_{max} remained constant, but K_m decreased to 0.5 mM. Later *en route* to apoptosis a large decrease of V_{max} of the putative L-LAC translocator was found (10 ± 2 nmol NAD(P)$^+$reduced/min × mg cell protein at 8 h), without any substantial change in the K_m value (about 0.5 mM) (see also ***inset*** to Scheme 1 where the rate of both the transport and the mLDH reaction are reported as a function of the apoptosis times, as calculated at 1 mM L-LAC).

Conversely, in the case of L-LAC/PYR exchange, early inhibition was found (Fig. 5B and inset a): up to 3 h apoptosis, V_{max} remained constant, but there was an early decrease in the affinity for L-LAC with K_m value equal to about 3 mM at 3 h apoptosis, according to competitive-like inhibition. The type of inhibition changes later: although in 1 h–8 h apoptosis time K_m remained unchanged, decrease of the V_{max} with respect to the control was found 5 h after induction of apoptosis (12 ± 0.8 and 22 ± 1.2 nmol NADH oxidized/min × mg cell protein respectively) with a further decrease to 8 nmol NADH oxidized/min × mg cell protein at 8 h after apoptosis induction (Fig. 5B) (see also inset b to Fig. 5B). This clearly shows that a non-competitive-like inhibition occurs in cells undergoing late apoptosis, characterized by a marked decrease in the L-LAC/PYR exchange capacity which takes place later after the initial change in L-LAC affinity.

When the data in Fig. 4B were plotted as 1/v vs. the apoptosis induction time in a Dixon-like plot (see inset b to Fig. 4B), as expected, an increase in the rate of L-LAC uptake was found up to 1 h at all the L-LAC concentrations used. Interestingly, when the experimental points obtained in 1–8 h apoptosis times were plotted, a non-competitive like inhibition was found at 1–10 mM L-LAC concentrations, thus suggesting that the already activated uptake decreased as a result of increasing impairment of the carrier efficiency.

Straight lines were obtained in the case of the L-LAC/PYR exchange as investigated at all times apoptosis when the data in Fig. 5B were plotted as 1/v vs. the apoptosis induction time in a Dixon-like plot (see inset b to Fig. 5B). A statistical analysis showed that the intercepts to the ordinate axis (both in the competitive-like and in the non-competitive like inhibition) obtained by treating the experimental points by linear regression, coincided with the corresponding experimental controls. This clearly shows that the measured inhibition is related to the same parameter measured in the control (see above), i.e. the rate of L-LAC/PYR exchange.

4. Discussion

4.1. Mitochondrial metabolism of L-lactate in CGCs

Having confirmed that, as needed for the astrocytes-neuron shuttle to occur [4–9], CGCs can take up externally added L-LAC [10,12] and metabolize it inside the cell (Fig. 1 and Scheme 1), we also show for the first time that mitochondria from neurons, in particular from cerebellar granule cells, can metabolize externally added L-LAC. Mitochondria take up L-LAC via a putative L-LAC/H$^+$ symporter and oxidize it via the NAD(P)-linked mLDH. Moreover, we show that the newly synthesized PYR can be exported outside mitochondria by virtue of the existence of a putative L-LAC/PYR antiporter which allows for operation of an L-LAC/PYR shuttle which could play a major role in the oxidation of cytosolic NADH (see Scheme 1).

The existence of mLDH in CGCs was shown by both immunological (Fig. 3A) and functional studies (Fig. 3B, C). In this case bioinformatics were of no help since none of the LDH subunits revealed by a current gene search is predicted to be targeted to mitochondria. In the light of Fig. 3 we show that, differently from [50] where the presence of a mitochondrial L-LAC oxidation complex on the outer surface of the inner mitochondrial membrane was proposed, mLDH is located in the mitochondrial inner compartment.

In distinction to what occurs in potato tuber mitochondria, in which L-LAC oxidation due to the mLDH does not result in the generation of the electrochemical proton gradient [51], mitochondrial oxidation of L-LAC in CGCs occurs in an energy-competent manner, as shown by the ADP stimulation of oxygen consumption rate. This means that intramitochondrial PYR and NADH metabolism and the PYR efflux can drive the reaction towards products in spite of the thermodynamic imbalance.

It should be emphasised that we have provided the first functional evidence for the existence of two putative translocators which allow for L-LAC traffic across the mitochondrial membrane: a putative L-LAC/H$^+$ symporter and a putative L-LAC/PYR antiporter (see Scheme 1). In distinction with what occurs for L-LAC transport into the cells (see [10–12]), current database searching yields no information on L-LAC transport across the mitochondrial inner membrane because even though many genes are reported to be mitochondrial carriers, most of them have no function assigned to them. The clear lesson from this is that functional studies remain essential to improve knowledge of mitochondrial transport and metabolism. That being said, not all experimental techniques frequently used to study transport processes were available to us. In particular, for technical reasons including the low amount of cell homogenate and the inadequacy of isotopic techniques for the study of transport processes in which metabolites newly synthesized in the mitochondrial matrix are exported outside mitochondria still labelled (see [41]), a detailed investigation of L-LAC transport by using labelled compounds was impossible. In the present work we resorted to the use of spectroscopic techniques using mitochondria in which metabolic processes were essentially unaffected. The saturation kinetics (with V_{max} values different for the investigated transports) and the inhibition by non-penetrant compounds strongly suggest the occurrence of transport processes mediated by two separate translocators which are also different from the PYR (monocarboxylate) carrier (see Figs. 4 and 5).

In any cases the rate of transport across the mitochondrial membranes regulates the L-LAC metabolism as shown by the TX-100 experiments.

This paper provides a novel mechanism for L-LAC metabolism in the brain in which mitochondria play a major role (see

Scheme 1). L-LAC, generated in the astrocytes from taken up glucose (see [1,10–12]), is transported to the extracellular space and to the neurons via MCT1 and MCT4, and MCT2 respectively, in the intercellular L-lactate shuttle [4–6,10,11,25]. Inside neurons, in addition to cytosolic oxidation of L-LAC via the cLDH, mitochondria themselves can contribute to the removal of cytosolic L-LAC by importing it via the putative L-LAC translocators and by oxidizing it to PYR via mLDH. The newly synthesized PYR can efflux from mitochondria via the putative L-LAC/PYR antiporter, thus constituting an intracellular L-LAC/PYR shuttle (see below). It was argued that since the NAD^+/NADH ratio is about 1000 in liver cytosol and less than 10 in the matrix (see [18]), this should prevent any mitochondrial L-LAC metabolism since the lactate/pyruvate ratio is about 100 times higher in the mitochondria than in the cytoplasm; accordingly, L-LAC oxidation is basically much more likely in the cytoplasm than in the mitochondria. However given that in brain mitochondria the NAD^+ concentration is 8–20 fold higher than that of NADH [52] and that PYR is actively oxidized via PYR dehydrogenase [53], we suggest that mLDH *in vivo* catalyses essentially L-LAC oxidation. Ultimately, the removal of the oxidation product by carrier mediated transport and mitochondrial metabolism overcomes any thermodynamic difficulty.

Although a significant portion was incorporated into lipids, mostly sterols, in early neonatal rat brain L-LAC was mainly oxidized to CO_2 (see [1,21] and references therein). Our finding, obtained with cells in growth, is consistent with this (see [1] and references therein) and we might suggest that in this case L-LAC is taken up by its own carrier; because of mLDH activity, the net carbon uptake results in PYR formation (see Scheme 1). Interestingly, the K_m for L-LAC of the L-LAC/H^+ symporter is quite close to the cytosolic L-LAC concentration (0.5 and 1 mM, respectively), thus suggesting that its activity would be responsive to L-LAC concentration changes which could occur within the cytosol.

On the other hand, our results are consistent with the postulate/proposal of Schurr (see [2,17]) that L-LAC is the only major product of cerebral glycolysis and that it can be metabolised inside mitochondria. The mLDH whose existence we have shown may be the hypothesized LDH1 suggested to be the mitochondrial isoform which converts L-LAC to PYR, as the first enzyme of the mitochondrial oxidative pathway, supplying PYR to the tricarboxylic acid cycle. However, in distinction to Schurr [2], we show that L-LAC traffic across the mitochondrial membranes occurs via two separate carriers distinct from that which transports PYR (MCT in Schurr's terms) and that the mLDH is located in the inner mitochondrial compartment.

The role of the L-LAC carriers in neuronal metabolism requires comment. In agreement with the metabolite transport paradigm (see [41]), the putative L-LAC/H^+ symporter allows for net uptake of L-LAC, while the putative L-LAC/PYR antiporter participates in the L-LAC/PYR shuttle, reconstructed here, which can contribute to the oxidation of cytosolic NADH. Indeed, such a transfer of reducing equivalents is essential for maintaining the favourable NAD^+/NADH ratio required for the oxidative metabolism of glucose and synthesis of neurotransmitters in brain. Notice that in addition to the malate–aspartate shuttle and the glycerol–phosphate shuttle (see [5,43]), we have already shown the occurrence of the malate/oxaloacetate shuttle in brain [54] and in particular in CGCs [43]. The results that we report here add the L-LAC/PYR shuttle as a contributor to oxidation of external NADH. Comparison made between this shuttle and the glycerol–phosphate and the malate/oxaloacetate shuttles suggests that the L-LAC/PYR shuttle plays a major role: we calculate that at physiological cytosolic substrate concentration (about 1 mM) [55,56] the rates of NADH oxidation would be about 5, 8 and 13 nmol/min × mg cell homogenate for the glycerol-3-phosphate, malate/oxaloacetate and L-LAC/PYR systems respectively. At present we have no conclusive data on the contribution of the malate/aspartate shuttle, however initial experiments dealing with the reconstruction of the malate/aspartate shuttle, as in [43], suggests a minor role for this shuttle.

4.2. L-Lactate mitochondrial metabolism in CGCs undergoing apoptosis

We have previously shown that L-LAC production increases in CGCs *en route* to apoptosis [20], and hence a mechanism is required to remove extra cytosolic L-LAC. Thus we propose that mitochondria, at least in the early phase of apoptosis as well as at high L-LAC cytosolic concentration, work as a safety valve to remove excess L-LAC. One could argue that cytosolic L-LAC can be exported by neurons via the plasma membrane MCT2 (see [12,57]), but this seems to be improbable on the basis of both the L-LAC concentration gradient between the extracellular and intracellular phases within the brain and the kinetic properties of the specific neuronal monocarboxylate transporter which has high affinity for extracellular L-LAC [11,55,58].

Thus, although the basic mitochondrial L-LAC metabolism in S-K5 cells is similar to that of the S-K25 cells, we show that L-LAC metabolism changes in CGCs *en route* to apoptosis as a response to the apoptosis dependent cell modifications (see the inset to Scheme 1). In the light of (Fig. 3, Table 1), we conclude that any change in L-LAC metabolism in CGCs *en route* to apoptosis does not depend on mLDH activity, but on the carrier mediated transport which regulates, at least *in vitro*, L-LAC mitochondrial metabolism in both normal and apoptotic CGCs.

In early apoptosis, in spite of initial impairment of the oxidative phosphorylation, ATP is needed to activate the caspase cascade together with the released cytochrome *c* [59]. Thus, since in early apoptosis, increase in the V_{max} without substantial changes in K_m is found, we propose that L-LAC mitochondrial metabolism plays a double role: it removes L-LAC from the cytosol, thus preventing cell acidification (see [60]), and favours ATP production by supplying carbon skeleton to mitochondria, these processes decreasing with the apoptosis progression. At longer times of apoptosis a mixed non-competitive-like inhibition of the L-LAC uptake was found, perhaps as a consequence of the mitochondrial damage. How early activation and late impairment of the carrier occur remains a matter of investigation, however since increase of ROS production occurs in the early phase of apoptosis with caspase activation occurring essentially later [61], we might speculate that the increase of the L-LAC symporter activity over the first

hour of apoptosis is a ROS-dependent activation, whereas the caspase-dependent inhibition might occur later, following caspase activation. Notice that since in early apoptosis a gradual impairment of the generation of the electrochemical proton gradient takes place, an energy dependent increase of the L-LAC transport can be ruled out.

In contrast, the putative L-LAC/PYR antiporter, as investigated in S-K5 cells during apoptosis, is inhibited already in the early apoptosis, perhaps due to increasing impairment of the mitochondrial metabolism in which no shuttle is required. The impairment of the putative L-LAC/PYR antiporter occurs in a double manner as already shown for the adenine nucleotide translocator [48]. In early apoptosis, the inhibition takes place in a competitive-like manner (no change in the V_{max} and increase in K_m), whereas at longer time there is a decrease in the transport efficiency (V_{max}), without further K_m change as in a non-competitive like inhibition. As stressed above, we maintain the hypothesis that the initial inhibition is ROS-dependent, whereas the late inhibition might be caspase-dependent. Whether and how ROS production and caspase activation can affect L-LAC transport via the two carriers is at present under investigation using CGCs undergoing apoptosis under conditions in which antioxidant and/or proteolytic systems are selectively blocked, as in [61].

Thus we conclude that the fate of L-LAC produced in the neurons during apoptosis is also linked to the novel mitochondrial metabolism described in this paper.

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