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An increase in the ATP levels occurs in cerebellar granule cells en route to apoptosis in which ATP derives from both oxidative phosphorylation and anaerobic glycolysis

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Abstract

Although it is recognized that ATP plays a part in apoptosis, whether and how its level changes en route to apoptosis as well as how ATP is synthesized has not been fully investigated. We have addressed these questions using cultured cerebellar granule cells. In particular, we measured the content of ATP, ADP, AMP, IMP, inosine, adenosine and L-lactate in cells undergoing apoptosis during the commitment phase (0–8 h) in the absence or presence of oligomycin or/and of citrate, which can inhibit totally the mitochondrial oxidative phosphorylation and largely the substrate-level phosphorylation in glycolysis, respectively. In the absence of inhibitors, apoptosis was accompanied by an increase in ATP and a decrease in ADP with 1:1 stoichiometry, with maximum ATP level found at 3 h apoptosis, but with no change in levels of AMP and its breakdown products and with a relatively low level of L-lactate production. Consistently, there was an increase in the cell energy charge and in the ratio $([ATP][AMP])/[ADP]^2$. When the oxidative phosphorylation was completely blocked by oligomycin, a decrease of the ATP content was found both in control cells and in cells undergoing apoptosis, but nonetheless cells still died by apoptosis, as shown by checking DNA laddering and by death prevention due to actinomycin D. In this case, ATP was provided by anaerobic glycolysis, as suggested by the large increase of L-lactate production. On the other hand, citrate itself caused a small decrease in ATP level together with a huge decrease in L-lactate production, but it had no effect on cell survival. When ATP level was further decreased due to the presence of both oligomycin and citrate, death occurred via necrosis at 8 h, as shown by the lack of DNA laddering and by death prevention found due to the NMDA receptor antagonist MK801. However, at a longer time, when ATP level was further decreased, cells died neither via apoptosis nor via glutamate-dependent necrosis, in a manner similar to something like to energy catastrophe. Our results shows that cellular ATP content increases in cerebellar granule cell apoptosis, that the role of oxidative phosphorylation is facultative, i.e. ATP can also derive from anaerobic glycolysis, and that the type of cell death depends on the ATP availability.
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1. Introductory statement

In vitro cultured rat cerebellar granule cells (CGCs) die via necrosis when subjected to excessive and prolonged glutamate exposure [1,2] and via apoptosis when deprived of serum and depolarizing levels (25 mM) of extracellular potassium [3]. Therefore, these neurons constitute a versatile system to dissect the mechanisms which are common to apoptosis and necrosis and those which are unique. Furthermore, both death conditions are not induced by toxic or harmful manipulations as in other experimental paradigms [4–7], but, rather, by events which may occur at any time during the course of the life span of an organism. Thus, glutamate-evoked massive release is the most rapid event following neuronal oxygen shortage, while potassium deprivation causing apoptosis is the in vitro counterpart of in vivo deafferentation [8,9].

The processes leading to apoptosis have been investigated in detail and a role identified for an antioxidant system, a proteolytic system including proteasome and caspases, and for released cytochrome c (cyt c) [10–15]. In particular, we showed that released cyt c, working as a ROS scavenger and electron donor to cytochrome c oxidase, can generate an electrochemical proton gradient and drive the synthesis of ATP that is required for apoptosis, but not necrosis, to occur (see Refs. [15,16]).

It is clear that ATP-dependent steps take place in apoptotic signal transduction [17] including: (i) apoptosome complex formation [18,19] and processing of pro-caspase-9 [20]; (ii) chromatin condensation and apoptotic body formation [21]; (iii) phosphorylation of kinases and other pro-apoptotic proteins [22–24]; (iv) externalization of phosphatidylserine [25]. Moreover ATP concentration has been reported as a switch in the decision between apoptosis and necrosis [17,26,27].

It is not, however, known whether and how the ATP level can change and how ATP is synthesized en route to apoptosis. Thus, in this work we assayed the content of both the adenine nucleotides and AMP breakdown products as a function of time after induction of apoptosis. We found that ATP content increases en route to apoptosis with an increase in cell energy charge and in the activity quotient of the adenylate kinase reaction. Moreover we found that ATP itself is needed for apoptosis to occur in CGCs, and that it can be supplied by anaerobic glycolysis when oxidative phosphorylation is totally impaired.

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 chemicals were from Sigma Chemicals Co. (St Louis, MO, USA).

2.2. Cell cultures

Primary cultures of CGCs were obtained from dissociated cerebellar of 7-day-old Wistar rats as in [28]. Cells were plated in basal medium Eagle (BME) supplemented with 10% fetal calf serum (FCS), 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 [3]. 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. Apoptotic cells are referred to as S-K5 cells. 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.

2.4. Glutamate neurotoxicity induction

Glutamate exposure was performed 7 days after plating. Primary cultures were exposed for 30 min to glutamate (100 μM) at 25 °C in Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO$_3$, 2.3 mM CaCl$_2$, 5.6 mM glucose, 10 mM Hepes pH 7.4) in the presence of 1 μM glycine added in order to fully activate NMDA-sensitive glutamate recognition sites [29]. Cells were then replenished with BME containing 25 mM KCl, 2 mM glutamine and gentamicin (100 μg/ml) and put in the incubator. For the quantitative assessment of glutamate neurotoxicity (GNT), cell integrity and count were measured, as described below, after 12–24 h. Glutamate-treated cells and control cells are referred to as GNT- and C-GNT cells respectively.

2.5. Cell suspension and homogenate preparations

Before each experiment, the culture medium was removed and the plated CGCs were washed with phosphate-buffered saline (PBS) containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 15 mM KH$_2$PO$_4$ pH 7.4, and then collected by gentle scraping into a volume of PBS depending on the particular experiment. Although suspended granule cells lacked the morphological organization observed in culture dishes such as cell–cell and cell–substrate contacts, and had no neurites, they showed full viability. 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 [30], by confirming the ability of ouabain to block glucose transport into the cells [31], and by counting dead cells, identified as large phase-bright cell bodies, as in [32]. 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 at room temperature. With this procedure L-lactate dehydrogenase is released and subsequent treatment with Triton-X-100 does not cause further release.

2.6. Measurement of levels of both adenine nucleotides and AMP breakdown products

Concentrations of intracellular phosphorylated adenine nucleosides (ATP, ADP and AMP) and of AMP breakdown products (IMP, inosine and adenosine) in neuronal extracts were determined by HPLC as previously described [33]. Results were expressed as nmol/mg cell protein. Oligomycin (1.5 ng/ml) and/or citrate (10 mM), when present, were added to the serum-free BME (see above) simultaneously with induction of apoptosis.

2.7. Energy charge and ADK quotient activity calculations

The cellular energy charge was calculated from the formula: $(ATP+0.5\ ADP)/(ATP+ADP+AMP)$ (see Ref. [34]). The ADK quotient activity (AQ_{ADK}) was calculated from the formula: $([ATP][AMP])/[ADP]^2$.

2.8. Phosphofructokinase activity assay

Total phosphofructokinase activity was assayed in a standard coupled spectrophotometric assay at 25 °C and pH 7.2 to mimic intracellular pH, essentially as in [35]. The reaction mixture contained 50 mM Tris–HCl, 5 mM KCl, 5 mM $MgCl_2$, 0.2 mM NADH, 2 mM ATP, 0.4 unit/ml aldolase, 2.5 units/ml triose-phosphate isomerase, 0.4 unit/ml 3-glycerophosphate dehydrogenase and 0.5 mg cell homogenate. The reaction was initiated by adding 5 mM fructose 6-phosphate and the change in absorbance at 340 nm was followed using a Perkin-Elmer Lambda 5 spectrophotometer. All assays were run in duplicate and mean values reported.

2.9. Measurement of L-lactate production

Cells were plated in 9-cm diameter dishes (15×10^6 cells/dish), and after 7 DIV apoptosis was induced as described above. At different times (see figure legends) aliquots of the medium were collected and the extracellular L-lactate concentrations were measured by the method of Brandt [36]; these give a reliable estimate of L-lactate production inside the cells [37].

2.10. Polarographic measurements

O_2 consumption was measured polarographically using a Gilson 5/6 oxygraph with a Clark electrode, as in [38–40]. Cells were suspended in PBS (about 0.2 mg protein) and 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 which did not damage the cells in suspension. Oxygen uptake was initiated by adding glucose to the cell suspension. The rate of oxygen uptake was measured from the tangent to the initial part of the progress curve and expressed as natom O $min^{-1}mg^{-1}$ protein. The sensitivity of the instrument was set so as allow rates of O_2 uptake as low as 0.5 natom $min^{-1}mg^{-1}$ protein to be measured.

2.11. DNA fragmentation analysis

Fragmentation of DNA was performed as in [41]. Briefly CGCs (6×10^6) were plated in poly-L-lysine-coated 60 mm tissue culture dishes, collected with cold phosphate-buffered saline (PBS pH 7.2) and, after removal of the medium and washing once with cold PBS, CGCs were centrifuged at $3500 \times g$ for 5 min. The pellet was lysed in 10 mM Tris, 10 mM EDTA, 0.2% Triton X-100 (pH 7.5). After 30 min on ice, the lysates was centrifuged at $17,000 \times g$ for 10 min at 4 °C. The supernatant was digested with proteins K and then extracted twice with phenol-chloroform/isoamyl alcohol (24:1). The aqueous phase, containing soluble DNA, was recovered and nucleic acids were precipitated with sodium acetate and ethanol overnight. The pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). After digestion with RNAse A (50 ng/ml at 37 °C for 30 min), the sample was subjected to electrophoresis in a 1.8% agarose gel and visualized by ethidium bromide staining. Soluble DNA from equal numbers of cells was loaded in each lane.

2.12. Lactate dehydrogenase activity

The activity of lactate dehydrogenase (LDH) released into the culture medium was determined spectrophotometrically according to [42] and expressed as percent of maximum LDH released at 24 h after the induction of glutamate neurotoxicity. An aliquot (100 μl) of culture medium (2×10^6 cells/2 ml culture medium) was added to 2 ml of 50 mM Tris–HCl buffer pH 7.4 in the presence of 0.2 mM NADH. The assay reaction was started by adding 0.6 mM pyruvate.

2.13. Assessment of neuronal viability

Viable CGCs were quantified by counting the number of intact nuclei after dissolving the cells in detergent-containing solution as described by [32]. Dead cells were identified as large phase-bright cell bodies by using phase-contrast

microscopy. Cell survival was expressed as the percentage of intact cells with respect to control cells, i.e. S-K25 cells, kept under the same experimental conditions. In control experiments, 95–97% integrity was found after 24 h.

2.14. Statistical analysis and computing

All statistical analyses in this study were performed by SPSS software. The data were representative of at least three independent neuronal preparations (with comparable results) each one in triplicate. They 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. Content of adenine nucleotides and of AMP breakdown products in CGCs undergoing apoptosis

The experimental protocol used in the experiments reported here involved shifting the original culture of neurons from a medium containing 10% FCS to a serum-free medium. This could give rise to immediate drastic changes in cellular viability and energy status. Hence, in a preliminary experiment, the oxidation of glucose by intact cells and oxidation of succinate by cell homogenates were monitored by polarography, as in [39], in control cells, in the presence (S+K25) or in the absence of serum (S-K25), and in cells en route to apoptosis (S-K5). In three separate experiments oxidation of glucose (10 mM) proceeded at rates of 68 ± 4, 55 ± 3 and 46 ± 4 natom O/min$\times10^6$ cells for S+K25, S-K25 and S-K5 respectively. Oxygen uptake by cell homogenates to which succinate (5 mM) had been added occurred with rates of 30 ± 4, 24 ± 4 and 20 ± 2 natom O/min$\times10^6$ cells. These rates were increased by the addition of ADP (1 mM) to give respiratory control ratios of 5.5, 4.6 and 3.5 for S+K25, S-K25 and S-K5 respectively.

Measurements of the content of ATP, ADP, AMP, IMP, adenosine and inosine in cerebellar granule cells in which apoptosis was induced via potassium shift (S-K5 cells) and in control cells (S-K25 cells) were made by using HPLC as described by [33]. Initial concentrations of ATP and ADP in control cells were 0.95 ± 0.07 and 0.97 ± 0.07 nmol/mg protein respectively and remained constant over a 0–8 h time range (Fig. 1). In the early phase of apoptosis (up to 1 h), no significant changes in the nucleotide levels were found compared with the relevant controls.

However, in CGCs analyzed 1.5 h after potassium shift, an increase of about 10% ($P<0.01$) in ATP level was found with the maximum increase (about 30%, $P<0.001$) reached at 3 h. The increase was statistically significant as
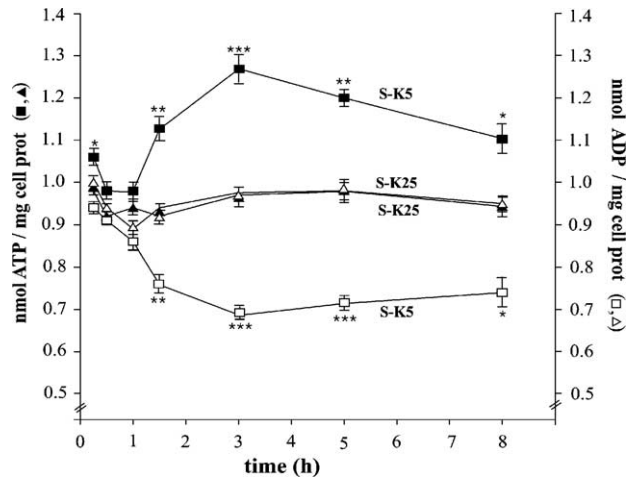

Fig. 1. Content of ATP and ADP in CGCs undergoing apoptosis. Rat CGCs (6×10^6/well) at 7 DIV were incubated either in high potassium (S–K25) ($\blacktriangle$,$\triangle$) or in low potassium (S–K5) ($\blacksquare$,$\square$) serum-free culture medium for the reported times. The amounts of ATP ($\blacktriangle$,$\blacksquare$) and ADP ($\triangle$,$\square$) were determined in neutralized extracts and expressed as nmol/mg cell protein. Reported values are the mean of five independent neuronal preparations (with comparable results) each one in triplicate with the standard deviation ($\pm$S.D.). ANOVA and Bonferroni test: statistically significantly different with *$P<0.05$, **$P<0.01$ and ***$P<0.001$ when comparing S-K5 with S-K25. ADP in S-K5: No statistically significant differences when comparing each time with the preceding time, except for 60 vs. 90 min ($P<0.05$). $P<0.05$ when comparing 30/60 vs. 90 min; $P<0.01$ when comparing 15 vs. 300 min; $P<0.001$ when comparing 15 vs. 90/180 min. ATP in S-K5: No statistically significant differences when comparing each time with the preceding time. $P<0.05$ when comparing 15 vs. 180 min and 180 vs. 480 min; $P<0.01$ when comparing 60 vs. 300; $P<0.001$ when comparing 30 vs. 180 and 60 vs. 180 min. No statistically significant differences were found when comparing S-K25 at all times for both ADP and ATP.

judged by ANOVA with Bonferroni post-hoc test. Subsequently the level of ATP declined, but was still significantly higher than in controls (about 15% increase at 8 h, $P<0.05$). Consistently, the content of ADP decreased in a fairly good agreement with 1:1 stoichiometry between ATP and ADP levels. No changes in the concentration of AMP or of AMP breakdown products (IMP, adenosine and inosine) were observed in 0–8 h time range of both S-K25 and S-K5 cells. The average values of the AMP, IMP, adenosine and inosine concentrations were 0.38 ± 0.06, 0.19 ± 0.04, 0.15 ± 0.03 and 0.40 ± 0.06 nmol/mg protein respectively.

As can be seen in Table 1 the cell energy charge, used as a sensitive indicator of cell energy status [43,44] and expressed as $\varphi=([ATP]+\frac{1}{2}[ADP])/([ATP]+[ADP]+[AMP])$, does not significantly change up to 1 h after the induction of apoptosis. However over 1.5 h to 3 h a statistically significant ($P<0.05$) increase in energy charge was found with a maximum at 3 h. No significant change in the control φ was found.

Since ATP, ADP and AMP levels are regulated by the adenylate kinase (2ADP<=>AMP+ATP), the activity quotient for this reaction was also calculated from the same data (Table 1). The value was about 0.4 in control cells for all times and S-K5 cells up to 1 h, but increased to about 1

Table 1
Energy charge (φ) and adenylate kinase activity quotient (AQ_{ADK}) in both S-K25 and S-K5 cells

Time (min)	φ		AQ_{ADK}	
	S-K25	S-K5	S−K25	S-K5
15	0.62±0.01	0.63±0.015	0.39±0.064	0.51±0.10
30	0.64±0.00	0.64±0.03	0.38±0.012	0.41±0.001
60	0.64±0.038	0.63±0.025	0.40±0.059	0.49±0.010
90	0.63±0.027	0.68±0.016*	0.38±0.051	1.04±0.12**
180	0.63±0.018	0.69±0.015*	0.41±0.055	0.98±0.12**
300	0.64±0.025	0.67±0.015	0.37±0.053	1.00±0.16**
480	0.62±0.017	0.67±0.019	0.39±0.046	0.76±0.15**

$\varphi=([ATP]+1/2[ADP])/([ATP]+[ADP]+[AMP])$. $AQ_{ADK}=([ATP][AMP])/[ADP]^2$. Data represent the means (±S.D.) of energy charge and adenylate kinase activity quotient values as obtained from Fig. 1 and from the AMP assay. AMP level is 0.38±0.06 nmol/mg protein.

* ANOVA and Bonferroni test: statistically significantly different with $P<0.05$ when comparing S-K5 with S-K25 at the same time.

** ANOVA and Bonferroni test: statistically significantly different with $P<0.01$ when comparing S-K5 with S-K25 at the same time.

in 1.5–8 h S-K5 cells ($P<0.01$); this is in fairly good agreement with the equilibrium constant for the reaction [45]. Notice that the activity of adenylate kinase, assayed as in [46], did not change en route to apoptosis, its value being about 18 nmol $NADP^+$ reduced×min^{-1} ×mg cell protein.

3.2. Content of adenine nucleotides and L-lactate production in S-K5 cells treated with oligomycin and/or citrate

Since ATP can be synthesized both via oxidative phosphorylation in mitochondria and via substrate-level phosphorylation, mostly in cytosol where glycolysis occurs, we investigated the contribution of the two phosphorylation types to ATP level in apoptosis. First, control was made of the intactness of cell membranes: this was established in the experiment of Fig. 2Aa. Externally added glucose (10 mM) causes oxygen consumption as a result of a multistep process consisting of glucose transport across the cell membrane, glycolysis, pyruvate uptake by mitochondria where oxidative decarboxylation via the pyruvate dehydrogenase complex occurs, citric cycle and oxidative phosphorylation. The lack (when added before glucose) and the inhibition (when added during the glucose oxidation) of oxygen consumption due to ouabain (1 μM), which prevents glucose transport into cells [31], show that cell membrane is not freely permeable to glucose, i.e. that the cells are intact. In order to investigate how oxidative phosphorylation participates in CGC apoptosis (see Ref. [47]) we used oligomycin (1.5 ng/ml), a potent inhibitor of mitochondrial ATP synthase. Notice that in the oxidative phosphorylation ATP is synthesized by using the electro-
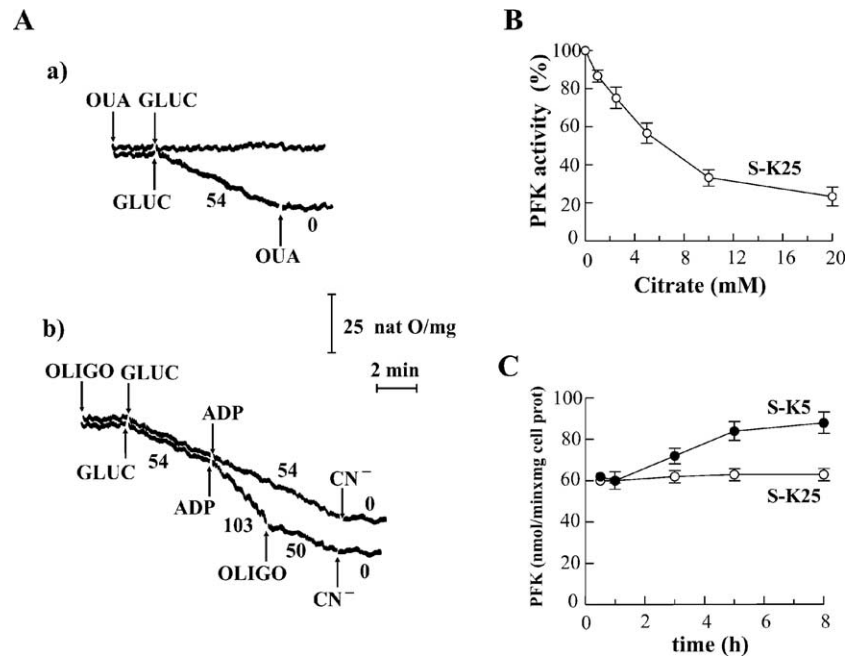

Fig. 2. (A) Effect of either ouabain or oligomycin on the glucose-dependent oxygen consumption. CGC homogenate (0.2 mg cell protein) was incubated at 25 °C in a water-jacketed glass vessel and consumption of O_2 was monitored polarografically. Glucose (GLUC, 10 mM), ouabain (OUA, 1 μM), ADP (1 mM), oligomycin (OLIGO, 1.5 ng/ml) and potassium cyanide (CN$^-$, 1 mM) were added at the times indicated. Rates of oxygen uptake are expressed as natom O/min×mg cell protein. (B) Citrate inhibition on phosphofructokinase activity. Phosphofructokinase activity in S-K25 cells homogenate was assayed (see below) in the absence or presence of citrate at the indicated concentrations. The rate values were expressed as a percentage of the rate value calculated in the absence of citrate (considered as the 100%). (C) Phosphofructokinase activity assay in cells undergoing apoptosis. CGC homogenate (0.5 mg cell protein) was incubated at 25 °C. The reaction mixture contained 50 mM Tris–HCl, 5 mM KCl, 5 mM MgCl$_2$, 0.2 mM NADH, 2 mM ATP, 0.4 unit/ml aldolase, 2.5 units/ml triose–phosphate–isomerase, and 0.4 unit/ml 3-glycerophosphate dehydrogenase. The reaction was initiated by adding 5 mM fructose-6-phosphate and the change in absorbance at 340 nm was followed. The rate of absorbance decrease, measured as the tangent to the initial part of the progress curve, is expressed as nmol/min×mg cell protein. All assays were run in duplicate and mean values reported ±S.D.

chemical proton gradient generated by the electron flow along the respiratory chain, then the absence of the oxidative phosphorylation can be assessed by verifying that the addition of ADP does not result in the increase of oxygen uptake by the cells. Thus, to ascertain that CGC incubation with oligomycin results in a complete inhibition of the oxidative phosphorylation, we checked the ability of externally added oligomycin to inhibit the stimulation of glucose oxidation due to ADP (Fig. 2Ab), as in [39]. The

addition of glucose (10 mM) resulted in a linear decrease in oxygen content (v=54 natom O/mg cell protein), with externally added ADP increasing oxygen uptake rate (v=103 natom O/mg cell protein). The subsequent addition of oligomycin (1.5 ng/ml) reduced the rate of oxygen uptake up to 50 natom O/mg cell protein, i.e. to the same rate of O_2 uptake exclusively due to glucose oxidation in the absence of ATP synthesis, therefore showing an essentially complete inhibition of the oxidative phosphor-
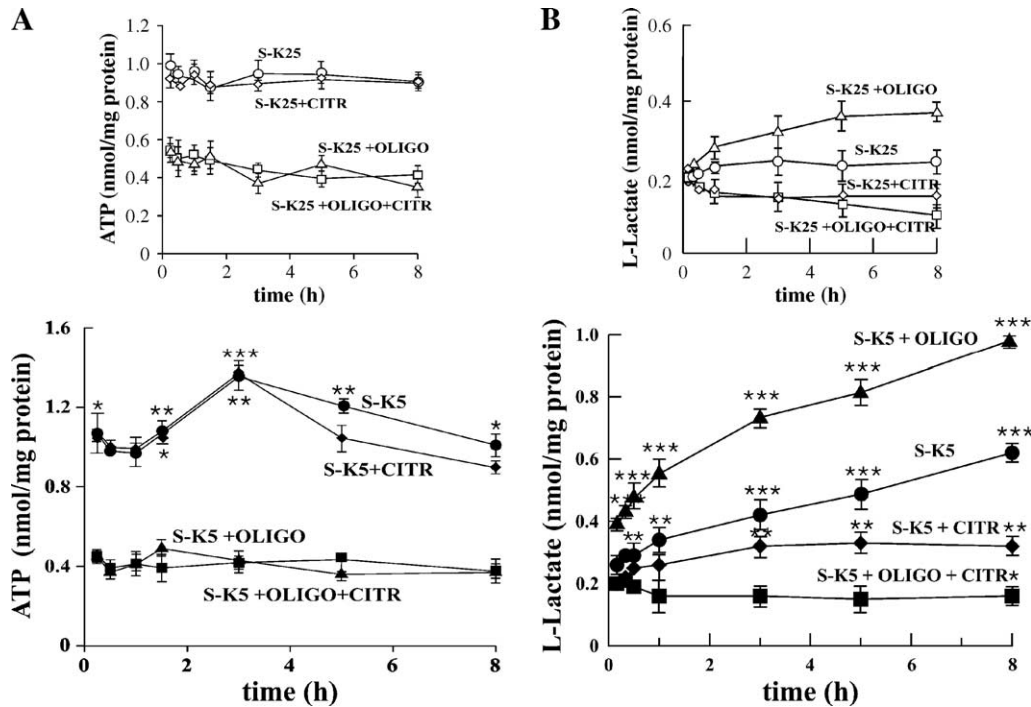

Fig. 3. Effect of oligomycin and/or citrate both on the level of ATP and on the L-lactate production in CGCs undergoing apoptosis. (A) ATP level. The dependence of level of ATP on time after potassium shift was determined for both S-K25 (in the inset) and S-K5 cells incubated in the presence of oligomycin (1.5 ng/ml) (△,▲) or citrate (10 mM) (◇,◆) or both of them (□,■), or in the absence of the inhibitors (○,●). At the times indicated, the amount of ATP was determined (see also Fig. 1). Reported values are the mean of five independent neuronal preparations (with comparable results) each one in triplicate with the standard deviation (±S.D.). ANOVA and Bonferroni test: statistically significantly different with *P<0.05, **P<0.01 and ***P<0.001 when comparing S-K5 with the respective S-K25 samples at the same time. The lack of asterisk/s indicate no statistically significant difference from the control. ANOVA and Bonferroni test for S-K5: P<0.001 when comparing S-K5 vs. (S-K5+OLIGO), S-K5 vs. (S−K5+OLIGO+Citrate); (S-K5+Citrate) vs. (S-K5+OLIGO+Citrate); (S-K5+Citrate) vs. (S−K5+OLIGO); no statistically significant differences when comparing S-K5 vs. (S−K5+Citrate) (except at 300 min with P<0.001) or (S-K5+OLIGO) vs. (S−K5+OLIGO+Citrate). No statistically significant differences when comparing each time with the preceding for S-K5 (P<0.05 when comparing 15 vs. 180 min and 180 vs. 480 min; P<0.01 when comparing 60 vs. 300; P<0.001 when comparing 30 vs. 180 and 60 vs. 180 min); for (S-K5+OLIGO); for S-K5+Citrate (P<0.05 at 30 vs. 180 min and 60 vs. 180 min; P<0.001 at 180 vs. 480 min); for (S-K5+OLIGO+Citrate). ANOVA and Bonferroni test for S-K25: P<0.001 at all times when comparing S-K25/(S-K25+Citrate) vs. (S−K25+OLIGO)/(S-K25+OLIGO+Citrate). No statistically significant differences when comparing S-K25 vs. (S-K25+Citrate) or (S-K25+OLIGO) vs. (S-K25+OLIGO+Citrate). No statistically significant differences when comparing each S-K25 sample at each time with the preceding time. (B) L−Lactate production. The amount of L-lactate produced was determined as a function of potassium shift time for both S-K25 (in the inset) and S−K5 cells incubated in the presence of oligomycin (1.5 ng/ml) (△,▲) or citrate (10 mM) (◇,◆) or both of them (□,■), or in the absence of the inhibitors (○,●). Reported values are the mean of four independent neuronal preparations (with comparable results) each one in triplicate with the standard deviation (±S.D.). ANOVA and Bonferroni test: statistically significantly different with *P<0.05, **P<0.01, ***P<0.001 when comparing S−K5 with the respective S-K25 samples at the same time. ANOVA and Bonferroni test for S-K5: P<0.001 when comparing all the samples with each other, except P<0.05 and P<0.01 when comparing S-K5 vs. (S-K5+Citrate) or S-K5 vs. (S−K5+OLIGO) at 10–20 min and 30 min respectively. No statistically significant differences when comparing each time with the preceding for S−K5, except P<0.05 when comparing 300 vs. 480 min; no statistically significant differences when comparing each time with the preceding for S−K5+OLIGO, except P<0.001 when comparing 60 vs. 180 min and P<0.05 when comparing 300 vs. 480 min; no statistically significant differences when comparing each time with the preceding for (S−K5+Citrate) and (S−K5+OLIGO+Citrate). ANOVA and Bonferroni test for S−K25: No statistically significant differences when comparing all the samples with each other, except P<0.05 when comparing S-K25 vs. (S-K25+OLIGO+Citrate) at 480 min; P<0.01 when comparing S−K25 vs. (S−K25+OLIGO) at 300 min; P<0.001 when comparing S−K25 vs. (S−K25+OLIGO) at 480 min and (S−K25+OLIGO) vs. (S−K25+Citrate)/(S-K25+OLIGO+Citrate) at the 180–480 min time range. No statistically significant differences when comparing each S−K25 sample at each time with the preceding time, but for (S-K25+OLIGO): P<0.05 when comparing 30 vs. 300 min and 60 vs. 480 min; P<0.01 when comparing 10 vs. 300 min and 10/20 vs. 480 min.

ylation. Consistently, oligomycin, when added before glucose, proved to prevent any stimulation of the rate of oxygen uptake by externally added ADP. In a parallel experiment, the capability of ADP (1 mM) to enter CGCs was confirmed as shown by ADP accumulation in the cell soluble fraction measured via HPLC. Levels of ADP were about 1.4 and 0.9 nmol/mg cell protein in CGCs added or not with ADP, respectively.

Having established that no oxidative phosphorylation could occur under the conditions used, we determined the effect of oligomycin on the level of ATP in control and apoptotic cells (Fig. 3A). When S-K25 cells were incubated in the presence of oligomycin, the level of ATP remained approximately constant at a value about 50% lower than that in the absence of the inhibitor. In S-K5 cells the ATP level decreased about 60% in 0–90 min ($P<0.001$) and 5–8 h range ($P<0.001$), whereas the decrease was higher in 90–180 min range ($P<0.001$) when no maximum ATP level was found.

The levels of ADP, AMP, IMP, adenosine and inosine were also measured as a function of time in S-K5 cells treated as above and are reported as the percentage increase or decrease (Table 2) compared with the level values in S-K5, along with the calculated values of the energy charge. At all times a decrease in level of ADP and an increase in level of AMP were found with respect to the control, i.e. in the absence of oligomycin. These changes account for the decrease in the energy charge. The increase in content of IMP, adenosine and inosine as a result of the addition of oligomycin was found for S-K5 as well as S-K25 cells.

In order to ascertain at what extent ATP synthesis during apoptosis could also derive from substrate-level phosphorylation, which occurs in glycolysis, we used citrate, which can enter neurons via a Na^{+}-coupled citrate transporter [48,49] and operates as an allosteric inhibitor of phosphofructokinase (PFK) [50,51], thus inhibiting glycolysis. Preliminarily, as a control, we incubated CGCs

with increasing concentrations of citrate and assayed PFK activity in the cell homogenates (Fig. 2B) as in [35]. 50% of PFK activity was found in the presence of about 6 mM citrate. About 20% residual activity was still observed even at high citrate concentration (above 20 mM) as in [51–53]. That citrate cannot completely inhibit PFK was confirmed by plotting the fractional inhibition i ($i=1-Vi/Vo$) (where Vi and Vo are the rates of the reaction in the presence or in the absence of the inhibitor respectively), as a function of inhibitor concentration in a graph of the form $1/i$ vs. $1/[I]$. Y axis intercept was higher than 1, showing that a residual rate of reaction occurs even in the presence of infinite inhibitor concentration (data not shown). Interestingly, when assaying the PFK activity in cell homogenates at different times after apoptosis induction, we have first observed an increase in PFK activity starting 3 h after apoptosis induction (Fig. 2C).

The effect of citrate on ATP level in both S-K25 and S-K5 cells was analyzed (Fig. 3A). Citrate treatment had no effect on the ATP content of either S-K25 or S-K25 cells plus oligomycin; in the case of S-K5 CGCs, citrate did not reduce the ATP level of S-K5 cells up to 5 h, whereas a small reduction of ATP level was found for 5–8 h range ($P<0.05$). No reduction was found in S-K5 cells added with oligomycin.

In order to confirm that in apoptosis ATP can derive from glycolysis, the production of L-lactate, which itself is both a valuable intercellular exchange molecule in the neuron-glia metabolic interactions [54] and a preferential fuel for neurons [55], was investigated in S-K5 and S-K25 cells both in the absence and in the presence of oligomycin and/or citrate (Fig. 3B). In the absence of oligomycin, S-K5 cells produced higher levels of L-lactate than did S-K25 cells at all times studied ($P<0.001$). The effect was already apparent at 1 h (a 25% higher level) and became progressively greater up to 8 h (160%). The addition of

Table 2
The effect of oligomycin on the content of adeninucleotides and derivatives and on the energy charge (φ) in S−K5 cells

Time (min)	Compounds					φ (% decrease)
	ADP (% decrease)	AMP (% increase)	IMP (% increase)	Adenosine (% increase)	Inosine (% increase)	
15	19**	22*	250***	0	12*	24***
30	21**	27*	240***	25*	15*	25***
60	23**	26*	200***	16*	13	26***
90	16*	25*	254***	16*	11	24***
180	23*	23**	116***	7	16*	28**
300	18*	25**	73***	7	12	33**
480	23*	23*	70**	9	15*	29***

Rat CGCs (6×10^{6}/well) at 7 DIV were incubated in low potassium (S-K5) serum-free culture medium in the presence of oligomycin (1.5 ng/ml) for the reported times. The amounts of ADP, AMP, IMP, adenosine and inosine were determined in neutralized extracts and expressed as nmol/mg cell protein. $\varphi=([ATP]+1/2[ADP])/([ATP]+[ADP]+[AMP])$. In each case, the percentage increase or decrease comparing with the values of ADP, AMP,IMP, adenosine and inosine content and of the energy charge in S-K5 is given. ADP content and energy charge values are reported in Fig. 1 and Table 1 respectively. The AMP, IMP, adenosine and inosine values in oligomycin-untreated S−K5 cells are 0.38±0.06, 0.19±0.04, 0.15±0.03 and 0.40±0.06 nmol/mg protein respectively.

  * ANOVA and Bonferroni test: statistically significantly different with $P<0.05$ when comparing S−K5+OLIGO with S-K5 at the same time.

  ** ANOVA and Bonferroni test: statistically significantly different with $P<0.01$ when comparing S−K5+OLIGO with S-K5 at the same time.

  *** ANOVA and Bonferroni test: statistically significantly different with $P<0.001$ when comparing S−K5+OLIGO with S-K5 at the same time.

oligomycin had a profound effect on the production of L-lactate in S-K5 cells, with a rapid increase over the first hour and then a somewhat slower increase ($P<0.001$) up to a value 260% higher than that in S-K25 cells. In S-K25 cells, L-lactate production was somewhat increased by the presence of oligomycin, but the changes were only about 20 and 50% at 3 and 8 h ($P<0.05$), respectively. The addition of citrate (10 mM) did not statistically significantly reduce L-lactate production in S-K25 cells, whereas a progressive statistically significant inhibition was found in
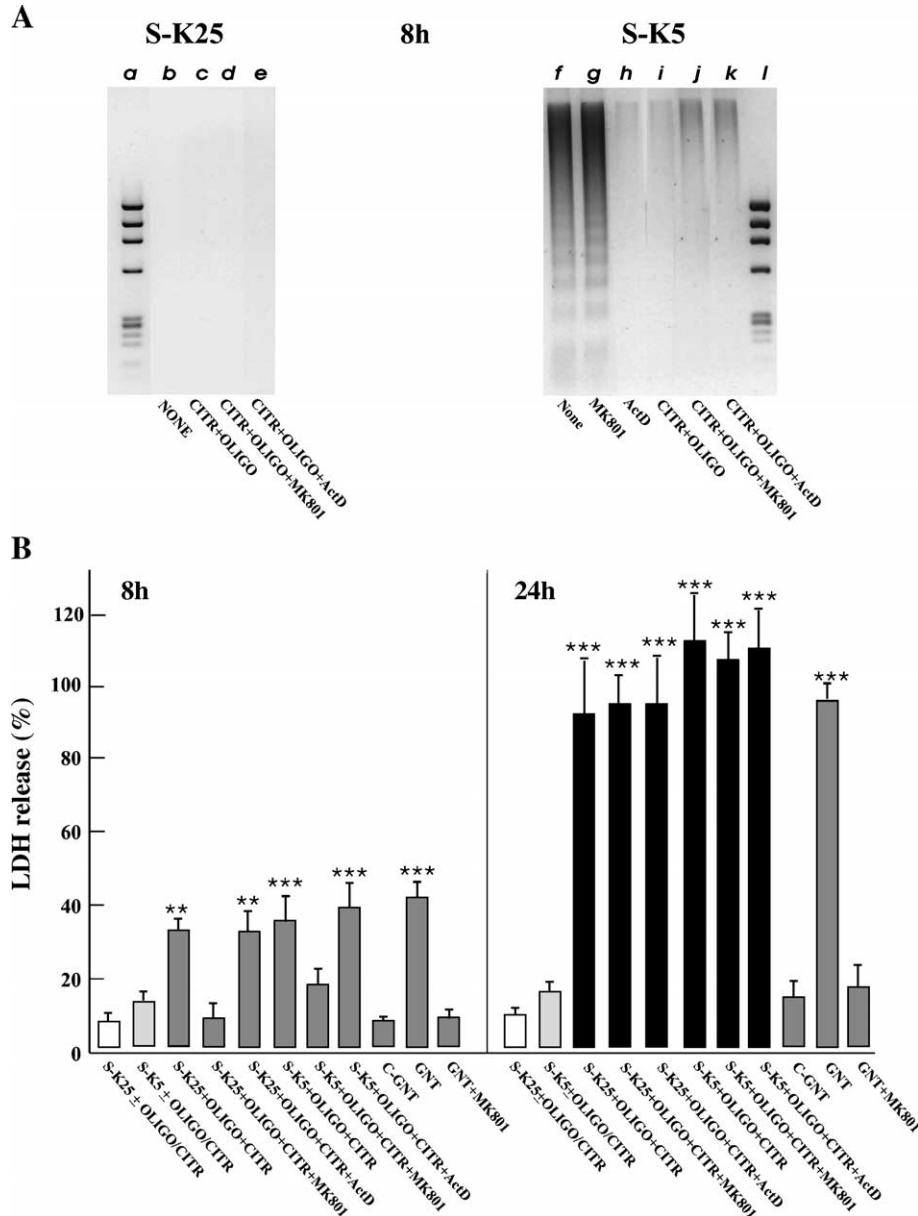

Fig. 4. DNA fragmentation and LDH release sensitivity to oligomycin, citrate, actinomycin D and MK801. (A) DNA fragmentation. Soluble DNA was extracted from either neurons switched to serum-free culture medium containing low K$^+$ (5 mM) in the absence (lane f) or presence of MK801 (lane g) or actinomycin D (Act D, 1 μg/ml) (lane h) and of oligomycin plus citrate in the absence (lane i) or presence of MK801 (lane j) or actinomycin D (Act D, 1 μg/ml) (lane k). Lanes b–e contain DNA from control cells maintained in high K$^+$ (25 mM) in the absence (lane b) or in the presence of oligomycin plus citrate in the absence (lane c) or in the presence of MK801 (lane d) or Act D (lane e). DNA from equal numbers of plated cells (6×10^6) was loaded in each lane. Size marker was *Hae*III-digested ϕX174 phage DNA (lanes a and l). (B) Lactate dehydrogenase activity in the extracellular culture medium. Cells were switched to serum-free medium containing low K$^+$ (S-K5) for 8 and 24 h or treated with glutamate (see Materials and methods) for the same times (GNT). Where indicated oligomycin (OLIGO, 1.5 ng/ml), citrate (CITR, 10 mM), MK-801 (1 μM) or actinomycin D (Act D, 1 μg/ml) were present. LDH activity was assayed, as reported in the Materials and methods, in S-K5, in the presence or absence of inhibitors, and GNT culture medium as well as in the respective controls (S-K25 or C-GNT). Results represent mean±S.D. of triplicate cultures and are expressed as percent maximum LDH activity released in GNT at 24 h (100%). In S-K25, in the presence or absence of citrate or oligomycin or both of them, and C-GNT cultures, LDH activity in the extracellular medium was negligible up to 96 h (not shown). **$P<0.01$ ***$P<0.001$ as compared to the respective untreated cells.

S-K5 cells ($P<0.001$). In the presence of both oligomycin and citrate, L-lactate production was markedly reduced in apoptotic cells ($P<0.001$), whereas in S-K25 cells L-lactate production remained similar to that of cells treated with citrate alone.

3.3. ATP level impairment and death type in CGCs

In the light of the above findings, we investigated whether and how the incubation of the cells with oligomycin and/or citrate modified both their survival and the death type. This was made at 8 and 24 h after apoptosis induction. To confirm that CGCs die via apoptosis, DNA laddering, which is a specific hallmark of apoptosis, was checked at 8 h (Fig. 4A) and 24 h, in both S-K25 and S-K5 cells. DNA laddering was found only in S-K5 cells, and it was completely prevented in the presence of the transcriptional inhibitor actinomycin D (Act D, 1 μg/ml), but not of MK801 (1 μM) a selective NMDA receptor antagonist which prevents glutamate-necrosis [9]. In the presence of oligomycin plus citrate,

at 8 h, cell death did not occur via apoptosis, since no laddering occurred either in the absence or presence of Act D or MK801, but via necrosis as demonstrated by the occurrence of the lactate dehydrogenase release prevented by MK801, but not by Act D as in [3,14] (Fig. 4B). The situation at 24 h is different: In the presence of oligomycin plus citrate, LDH release was found, but it was completely insensitive to MK801 both in S-K25 and in S-K5. No significant LDH release was found in control cells in both the investigated times. As expected, in the light of [9], LDH release was found in the culture medium of cells in which glutamate neurotoxicity (GNT) had previously been induced as in [16].

In another set of experiments, we checked cell survival at 8 and 24 h, in the presence of a variety of compounds including actinomycin D (1 μg/ml,) MK801 (1 μM), citrate (10 mM) and oligomycin (1.5 ng/ml) added separately or in various cocktails.

The results are given in Fig. 5. In S-K25 cells, 100% survival was found in the presence of oligomycin and citrate, added separately in the absence or presence of
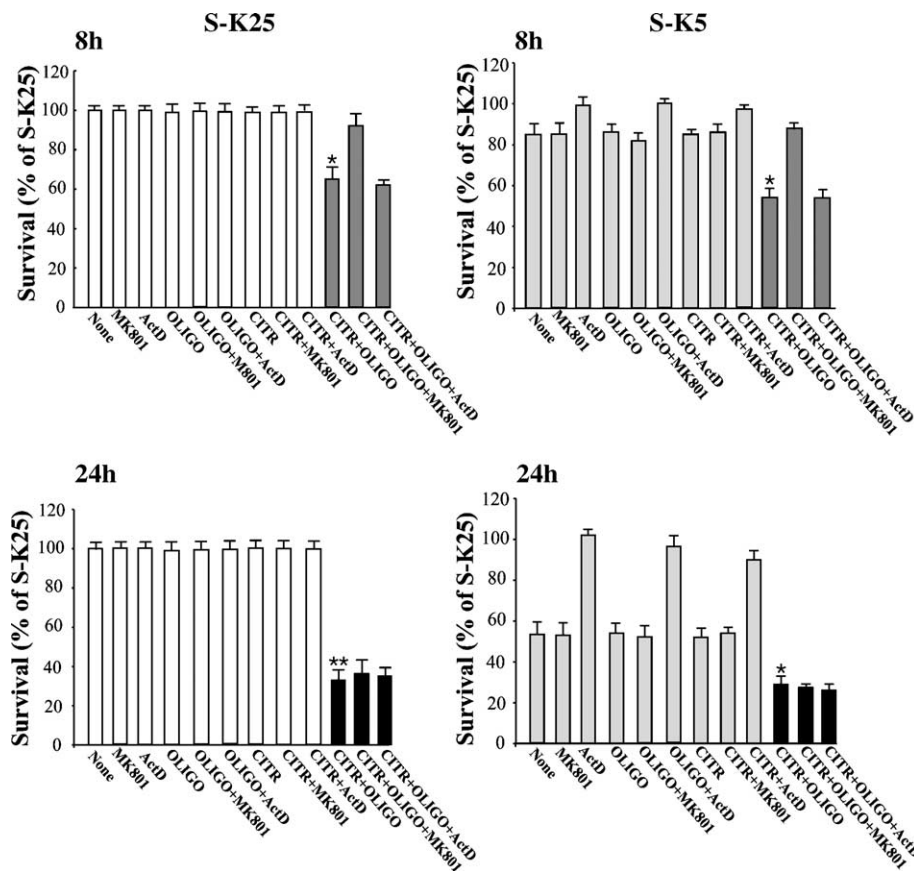

Fig. 5. Type of death: the effects of oligomycin, citrate, actinomycin D and MK801. Rat CGCs (2×10^6/well) at 7 DIV were incubated either in high potassium (S-K25) or in low potassium (S-K5) serum-free culture medium. Where indicated oligomycin (OLIGO, 1.5 ng/ml), citrate (CITR, 10 mM), MK-801 (1 μM) or actinomycin D (Act D, 1 μg/ml) were present in the medium. At 8 and 24 h, the viability of S-K25 and S-K5 cells was determined by counting the number of intact nuclei. Cell viability is expressed as the percentage of the S−K25 cells taken as 100%. Control values were 100 ± 10. Results are means±S.D. of triplicate measurements and representative of six different experiments carried out with different cell preparations from different groups of animals. S−K25 cells: **$P<0.01$ and ***$P<0.001$ as compared to untreated cells at 8 h and 24 h respectively; S-K5 cells: **$P<0.01$ as compared to untreated cells.

MK801 or Act D, which per se were without effect on the survival; only citrate plus oligomycin resulted in survival decrease up to 60 and 40% at 8 and 24 h respectively. Such an impairment was sensitive to Act D and MK801 in different time-dependent manner: at 8 h, MK801, but not Act D, proved to prevent cell death, whereas at 24 h survival decrease remained up to 40% (P<0.001) in a manner independent on the presence of Act D or MK801.

In S-K5 CGCs, survival decreased up to 80 and 60% at 8 and 24 h respectively; in both cases Act D proved to prevent cell death completely, whereas MK801 failed to do this. In the presence of either oligomycin, i.e. when oxidative phosphorylation does not occur, or citrate, which largely prevent ATP synthesis in glycolysis, CGCs died essentially via apoptosis in a manner indistinguishable from the CGCs undergoing apoptosis; consistently they were rescued by the addition of Act D, but they were insensitive to MK801. When S-K5 CGCs were treated with both oligomycin plus citrate, survival was further reduced by about 30–40% in a manner statistically significant (P<0.01). Act D was without effect both at 8 h and at 24 h, whereas, MK801 prevented death at 8 h, but was ineffective at 24 h in a manner similar to that shown for S-K25 cells.

Under this latter condition, prevention is detectable at 8 h in the presence of MK801 indicating that up to this time, death due to complete ATP inhibition occurs via release of the endogenous glutamate pool (see Ref. [56,57]).

4. Discussion

The results reported in this paper shed new light on some aspects of energetic metabolism in neuronal apoptosis. In particular, we show an increase of the ATP level in cerebellar granule cells undergoing apoptosis with maximum in 180 min after apoptosis induction; such an increase results in the increase of the cell energy charge and in the alteration of the steady state levels of the adenine nucleotides towards equilibrium. Moreover investigating as to how ATP is synthesized, we show that apoptosis can occur even when the oxidative phosphorylation is completely blocked, being the glycolitic substrate-level phosphorylation the main ATP source. Finally, we show that in dependence of the ATP availability, the manner by which cells die in vitro can change, with apoptosis, necrosis due to glutamate excitotoxicity or to "energy catastrophe" occurring in time as a function of decreasing ATP level.

These points will be discussed separately.

4.1. ATP level in CGCs apoptosis

Our data confirm that ATP is strictly required for apoptosis to occur (see [18–25]). However our data have the added dimension of showing that the level of ATP increases with maximum in 180 min in CGCs en route to apoptosis. Such a conclusion is unique. Indeed, our experimental system allows for following the apoptosis time-course already 10 min after apoptosis induction, thus making possible the investigation of the death type and progression, of the mitochondrial function and of a variety of processes/compounds involved in apoptosis, including the antioxidant and the proteolytic systems, the proteins released from mitochondria and the adenine nucleotides and derivatives. In other systems, a general ATP depletion has been shown in apoptosis: for instance ATP depletion was associated with apoptotic cell death in glia cells and neuronal subpopulations as well as in rat embryonal or adult cortical neurons [58–60]. That this depends on features of the experimental system used and/or on the type of neuronal cell death insult remains to be established.

We found that the gradual ATP increase results in the increase of the cell energy charge and in the alteration of the steady state levels of the adenine nucleotides towards equilibrium as shown by the increase in the ratio [ATP][AMP]/[ADP]^2 up to a value of 1 (Table 1), which is similar to the reported equilibrium constant for the adenylate kinase reaction [45]. Notice that the activity of adenylate kinase does not change en route to apoptosis.

The increase in the content of ATP in S-K5 cells is accompanied by a stoichiometric decrease in the level of ADP (Fig. 1), but by unchanged levels of AMP and its breakdown products including IMP, inosine and adenosine. This strongly suggests that phosphorylation rather than new synthesis of adenine nucleotides is responsible for the extra content of ATP. It is noteworthy that the maximum level of ATP is reached at 3 h after apoptosis induction that is the time at which maximum cyt c release was found from cerebellar granule cells undergoing apoptosis [40]; since the released cyt c can generate the electrochemical proton gradient and drive ATP synthesis in isolated mitochondria in vitro [15], it might be that cyt c contributes to ATP extra-synthesis during CGC apoptosis in addition to that produced in glycolysis.

4.2. How ATP is synthesized in CGCs en route to apoptosis and the L-lactate production

Particular interest attaches to how CGCs provide ATP in apoptosis. In S-K5 cells we have found increasing production of L-lactate en route to apoptosis: this suggests that glycolysis contributes to ATP synthesis via substrate-level phosphorylation, even if this occurs to a minor extent compared with ATP synthesis by oxidative phosphorylation. Consistently, we showed that complete inhibition of the oxidative phosphorylation due to oligomycin resulted in a significant decrease in cellular energy charge; the latter arose from a reduction in ATP and a minor decrease in ADP, but with a significant increase in AMP level (Table 2). On the other hand, we found that in those circumstances, in

which a very substantial increase in the production of L-lactate occurred (Fig. 3B), the cells died by apoptosis even in the presence of oligomycin (Fig. 5). This strongly suggests that ATP itself is used for apoptosis to occur independently from how it is synthesized. Citrate proved to inhibit the production of L-lactate, but with a different effectiveness in control and apoptotic cells; the reason for this difference is at present unknown. As oligomycin, citrate alone had no effect on the survival of S-K5 cells as well as on the death type which is still apoptosis. Indeed, the ATP levels measured under these conditions are different, however we can speculate that in the presence of oligomycin ATP is synthesized, as shown by L-lactate production, but it is rapidly used in processes necessary for apoptosis to occur. Thus, we are forced to conclude that glycolysis can provide the ATP required for apoptosis when oxidative phosphorylation is impaired. The contribution of glycolysis to the synthesis of ATP required for apoptosis could be of particular importance in situations where mitochondrial function is impaired, such as in oxygen deficit in ischemic brain. This is consistent with the increase in the activity of phosphofructokinase as a function of time observed in cell homogenates (Fig. 2C) in which mitochondria coupling begin to decrease when survival is essentially 100% [39]. The facultative role of mitochondrial oxidative phosphorylation in apoptosis is also consistent with reports which show that apoptosis can occur in cells lacking functional mitochondria [61–63], and that anaerobic glycolysis with L-lactate production can perpetuate cell survival when mitochondrial ATP synthesis is suppressed in striated muscle cells [64]. One could argue that no ATP synthesis could occur under our conditions in which both glycolysis and oxidative phosphorylation are inhibited, however, the failure of citrate in inhibiting completely the phosphofructokinase as well as the increase of activity quotient of the adenylate kinase reaction could explain the found ATP level. Interestingly, it was reported that ATP synthesized in the glycolysis plays a critical role in preventing the collapse of the mitochondrial membrane potential and thus apoptotic cell death [65]. Interestingly, we have found a decrease in the capability of the cell homogenate to generate mitochondrial membrane potential as a result of externally added β-OH-butyrate occurring en route of apoptosis. Nonetheless, since cells can still survive both under conditions in which membrane potential is generated by hydrolysis of the glycolitic ATP [65] and when membrane potential decreases, we are forced to conclude that ATP itself independently on how it is synthesized can support cell viability.

4.3. Type of death in CGCs

We finally show that the ATP availability plays a major role in determining how CGCs die in vitro. That cells die via apoptosis at least up to 8 h, when either oxidative or substrate level phosphorylation are impaired, is shown by the occurrence of DNA laddering, which is a specific apoptosis marker, as well as by the prevention of the death caused by actinomycin D. MK801 caused no death prevention; such a finding could be used to further confirm that death occurs via apoptosis.

Of particular note is the observation that, in the presence of oligomycin plus citrate, the mechanism of cell death changes. In 8 h S-K5 cells, oligomycin and citrate, when added together, resulted in cell death by necrosis as shown both by the lack of DNA laddering, by the LDH release and by the evidence that the CGCs could be rescued by the addition of MK801, but not of actinomycin D. Although the explanation for this deserves further investigation, it does not affect our essential conclusion that an elevated level of ATP provides an additional determinant of whether neuronal cell death occurs via apoptosis or necrosis. It should be noted that another similar determinant is the level of ROS [14,66].

Our results provide new information about the mechanism by which the death progression takes place: at 24 h, no change in either death type, which remains prevented by Act D, or extent takes place due to the presence of either oligomycin or citrate. When the inhibitors were used together a higher extent of death was found, which showed peculiar inhibitor sensitivity: both Act D and MK801 did not prevent both LDH release and cell death; then we propose that death occurs as a necrosis non-dependent from glutamate release (see Ref. [56,57]), as suggested by the almost complete ATP depletion (not shown). This point could be considered when discussing the experimental conditions of ischemia, in which the rapid administration of an NMDA receptor antagonist, such as MK801, could avoid the rapid cell necrosis.

Thus, we suggest that the ATP drop rapidly following oxygen shortage during an ischemic condition is accompanied by membrane depolarization due to functional failure of ATPase and consequent massive glutamate release which, in turn, causes a large calcium influx and necrotic death.

In fairly good agreement with our findings, Leist et al. [26] provided evidence that human T cells triggered to undergo apoptosis, by treatment with staurosporin or by CD95 stimulation, are instead forced to die by necrosis when energy levels are rapidly compromised; moreover Volbracht et al. [5] reported that in CGC exposed to colchicine or nocodazole, ATP-depleting agents prevented caspase activation, the translocation of the phagocytosis marker, phosphatidylserine, and apoptotic death, but the repletion of ATP by enhanced glycolysis restored all apoptotic features.

Thus, cells en route to apoptosis differ from those undergoing necrosis in that the level of ATP increases in apoptosis, whereas in necrosis it is strongly reduced [5,17,26,27,38,67–69]. Whether this decrease is a primary cause or a consequence of the processes leading to cell death remains to be established.

Acknowledgements

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