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Early release and subsequent caspase-mediated degradation of cytochrome *c* in apoptotic cerebellar granule cells

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Abstract Cytochrome *c* (cyt *c*) release was investigated in cerebellar granule cells used as an in vitro neuronal model of apoptosis. We have found that cyt *c* is released into the cytoplasm as an intact, functionally active protein, that this event occurs early, in the commitment phase of the apoptotic process, and that after accumulation, this protein is progressively degraded. Degradation, but not release, is fully blocked by benzyloxycarbonyl-Val-Ala-Asp-fluoromethylchetone (z-VAD-fmk). On the basis of previous findings obtained in the same neuronal population undergoing excitotoxic death, it is hypothesized that release of cyt *c* may be part of a cellular attempt to maintain production of ATP via cytochrome oxidase, which is reduced by cytosolic NADH in a cytochrome b_5-soluble cyt *c*-mediated fashion.

Key words: Apoptosis; Mitochondrion; Cerebellar granule cell; Cytochrome *c*

1. Introduction

In vitro cultured cerebellar granule cells (CGCs) undergo a massive process of apoptosis when the extracellular concentration of KCl employed for their culturing (25 mM) is shifted to 5 mM. KCl-triggered apoptosis of CGCs is characterized by DNA fragmentation [1], TUNEL positivity and formation of apoptotic bodies [2] and is attributed to an intracellular Ca^{2+} drop [3]. The apoptotic process is inhibited by transcriptional and translational inhibitors, by IGF-I, forskolin, cAMP [1], as well as by neurotransmitters and neuromodulators such as ADP and adenosine [2]. This peculiar neuronal response, characterized by activation of apoptosis following membrane repolarization, has been hypothesized as an in vitro response that could mimic an in vivo prolonged state of neuronal depolarization [1,3].

Among the intracellular organelles and structures involved in the apoptotic process of CGCs, mitochondria appear to be particularly involved since their ability to produce ATP via oxidative phosphorylation is already 50% reduced 2 h after the apoptotic trigger [4]. A further involvement of mitochondria in apoptosis has recently been pointed out by the finding that one of the constituents of the respiratory chain, cytochrome *c* (cyt *c*), is released from mitochondria into the cytosol of several cell types [5,6]. A similar cytosolic accumulation has also been reported in CGCs treated with the neurotoxin 1-methyl-4-phenylpyridinium [7] or with the anti-microtubular agent colchicine [8].

Although the presence of cyt *c* in the cytoplasmic compartment of several cell types undergoing apoptosis constitutes a frequent finding, nothing is known about the mechanism(s) that causes such an unpredictable event, the fate of this protein once released into the cytoplasm and its possible biological significance, whether as a side effect of the mitochondrial functional impairment, a cellular attempt to cope with the destructive apoptotic message or part of the pathway eventually leading to cell death.

In order to investigate some of these questions, we selected the in vitro model of CGCs because, to our knowledge, they constitute the most suitable in vitro model of primary neuronal cultures whereby such a programmed death can be analyzed. Moreover, the possibility of inducing apoptosis by a relatively physiological manipulation, consisting of repolarizing the membrane potential via the KCl shift from 25 to 5 mM, provides a mechanism by far less drastic than that of using toxic [7] or other harmful manipulations [8] that do not mimic a possible in vivo situation. It will be shown that cyt *c* is released by mitochondria as a very early event and that after a progressive accumulation, it is degraded by the specific apoptotic pathway of cytosolic caspases.

2. Materials and methods

2.1. Reagents

Anti-cyt *c* antibodies (7H8-2C12) were purchased from Pharmingen (San Diego, CA, USA). Anti-glutamate dehydrogenase (GDH) antibodies were kindly supplied by Dr F. Rothe (Institut fur Medizinische Neurobiologie, University of Magdeburg, Magdeburg, Germany). Anti-cytochrome oxidase (subunit IV) antibodies (20E8-C12) were purchased from Molecular Probes (Eugene, OR, USA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham. Cycloheximide and actinomycin D were from Sigma Chemical (St. Louis, MO, USA). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylchetone (z-VAD-fmk) was obtained from Calbiochem (La Jolla, CA, USA).

2.2. Cell cultures

Primary cultures of cerebellar granule neurons were obtained as described in [9]. Before each experiment, both the cell viability and integrity were analyzed as already reported in [10]. Cell homogenates from a cell suspension were obtained with 20 strokes performed in a Dounce potter at room temperature. The integrity of mitochondria in the homogenate was assessed by measuring the activities of both adenylate kinase (ADK, E.C. 2.7.4.3) and GDH (E.C. 1.4.1.3), which are marker enzymes of the mitochondrial intermembrane space and matrix, respectively. The percentage of damaged mitochondria was lower than 1.5%. Mitochondrial coupling, which reflects the ability

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Abbreviations: ADK, adenylate kinase; BME, basal medium with Eagle's salts; S-K25, serum-free BME supplemented with 25 mM KCl; S-K5, serum-free BME containing 5 mM KCl; CGC, cerebellar granule cell; Cyt *c*, cytochrome *c*; GDH, glutamate dehydrogenase; PBS, phosphate-buffered saline; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylchetone

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of mitochondria to produce ATP, was evaluated as in [4,11]. The cell protein content was determined according to Waddel and Hill [12], with bovine serum albumin as a standard.

2.3. Induction of apoptosis

Apoptosis was induced as previously reported [1]. At 6–7 DIV, cells were washed twice and switched to a serum-free basal medium with Eagle's salts (BME), containing 5 mM KCl (S-K5), and supplemented with 2 mM glutamine and 100 μg/ml gentamicin, for the time reported in the figure legends. Controls were treated identically but maintained in serum-free BME medium supplemented with 25 mM KCl (S-K25). Cell death due to apoptosis was evaluated 24 h after the shift of $[K^+]$ by counting viable cells, according to Volontè et al. [13]. Inhibitors were added to the cells simultaneously with the $[K^+]$ shift, unless otherwise stated, with the final concentration of DMSO kept below 0.1%. Corresponding controls were treated with the same concentration of DMSO.

2.4. Immunoblot analysis

Immunoblot analysis was performed on cytosolic and mitochondrial extracts from control and apoptotic cultures. In both cases, cells were washed once with phosphate-buffered saline (PBS) and collected by centrifugation at $2000\times g$ for 5 min at 4°C. The cell pellet was resuspended in 500 μl of extraction buffer containing 250 mM sucrose, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM 1,10-phenantroline, 0.1 mM PMSF pH 7.4. The cells were homogenized in a Teflon/glass homogenizer (10 strokes) and after 5 min on ice, the suspension was centrifuged at $15\,000\times g$ for 30 min. The supernatants (i.e. cytosolic fractions) were removed and stored at −80°C until analyzed by gel electrophoresis. The pellets were resuspended in 500 μl of 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 M DTT, 1 mM 1,10-phenantroline, 0.1 mM PMSF pH 7.4 and homogenized on ice (10 strokes). After centrifugation at $750\times g$ for 10 min at 4°C to pellet nuclei, 400 μl of the resulting supernatant was supplemented with 400 mM NaCl and 1% Triton X-100. After 5 min incubation on ice, the samples were centrifuged at $15\,000\times g$ for 5 min at 4°C to remove insoluble materials. Supernatants, corresponding to the solubilized mitochondrial fraction, were aliquoted and stored at −80°C. 10 μg of either cytosolic or mitochondrial proteins was loaded onto a 15% SDS-polyacrylamide gel, separated and transferred to a PVDF membrane which was probed with the different antibodies. Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using enhanced chemiluminescence Western blotting reagents (Amersham). Relative optical densities and areas of bands were quantified using the GS-700 Imaging Densitometer implemented with Molecular Analyst Software (Bio-Rad).

2.5. Oxygen uptake studies

O_2 consumption was measured polarographically at 37°C, as in [4,10,11], by means of a Gilson 5/6 oxygraph, using a Clark electrode. The instrument sensitivity was set to a value which allowed us to follow rates of O2 uptake as low as 0.5 natoms/min/mg protein.

2.6. ADK and GDH assay

ADK activity was measured photometrically at 37°C essentially as reported in [14] in the presence of 0.2 μg of oligomycin plus 1 μM atractyloside. GDH activity was assayed photometrically at 37°C essentially as described in [15]. Given that no significant activity was usually measured owing to the integrity of the mitochondria, a further check was made in Triton X-100-treated cell homogenate.

3. Results

Cyt *c* release from mitochondria was investigated by checking its presence in the cytosolic fractions of apoptotic CGCs (S-K5) by protein immunoblot analysis. In order to exclude the presence of intact mitochondria in the cytosolic fractions,

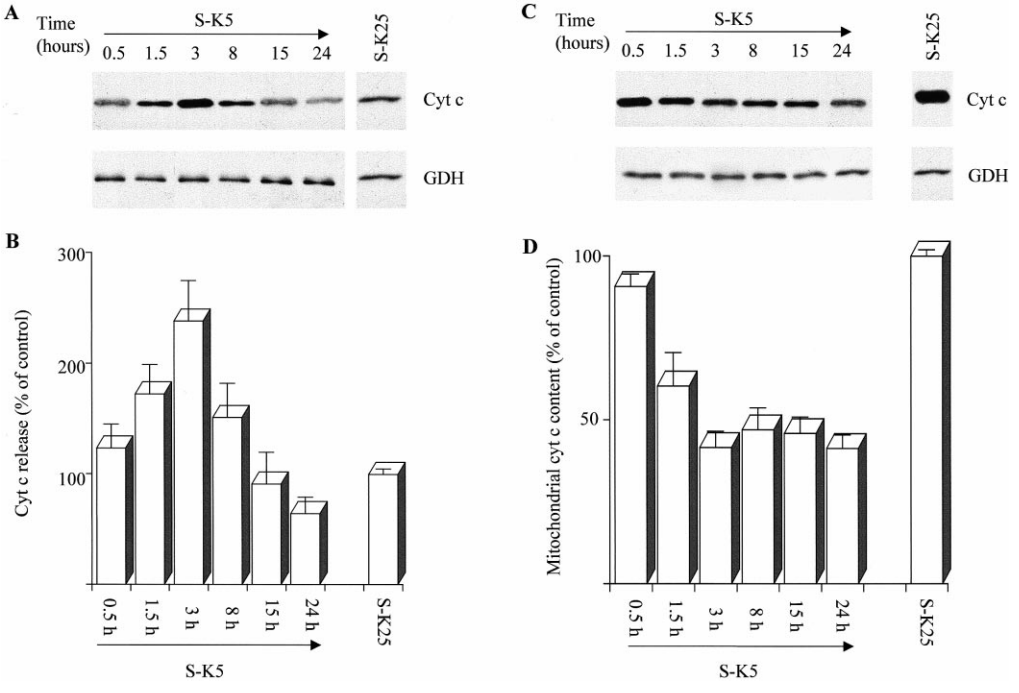

Fig. 1. Western blot analysis of cyt *c*. (A) Cytosolic fractions from either control (S-K25) or apoptotic (S-K5) CGCs were obtained at 0.5, 1.5, 3, 8, 15 and 24 h as indicated, after serum and potassium removal and analyzed by Western blotting analysis as described in Section 2. (B) The cytosolic content of cyt *c* was quantified by densitometric scanning of the film and expressed, after normalization, as a percentage of control cells (S-K25), where control cells were analyzed at the different times and to which a value of 100 has been given. (C) Mitochondrial fractions from either control (S-K25) or apoptotic (S-K5) CGCs were obtained at 0.5, 1.5, 3, 8, 15 and 24 h as indicated, after serum and potassium removal and analyzed by Western blotting analysis as described in Section 2. Antibodies against GDH were used to normalize the protein amount loaded onto the gel. (D) The amount of cyt *c* was quantified by densitometric scanning of the film and expressed as a percentage of control cells (S-K25), where control cells were analyzed at the different times and to which a value of 100 has been given. Results are means ± S.D. of triplicate measurements and representatives of at least 10 different experiments carried out with different cell preparations prepared from different groups of animals.

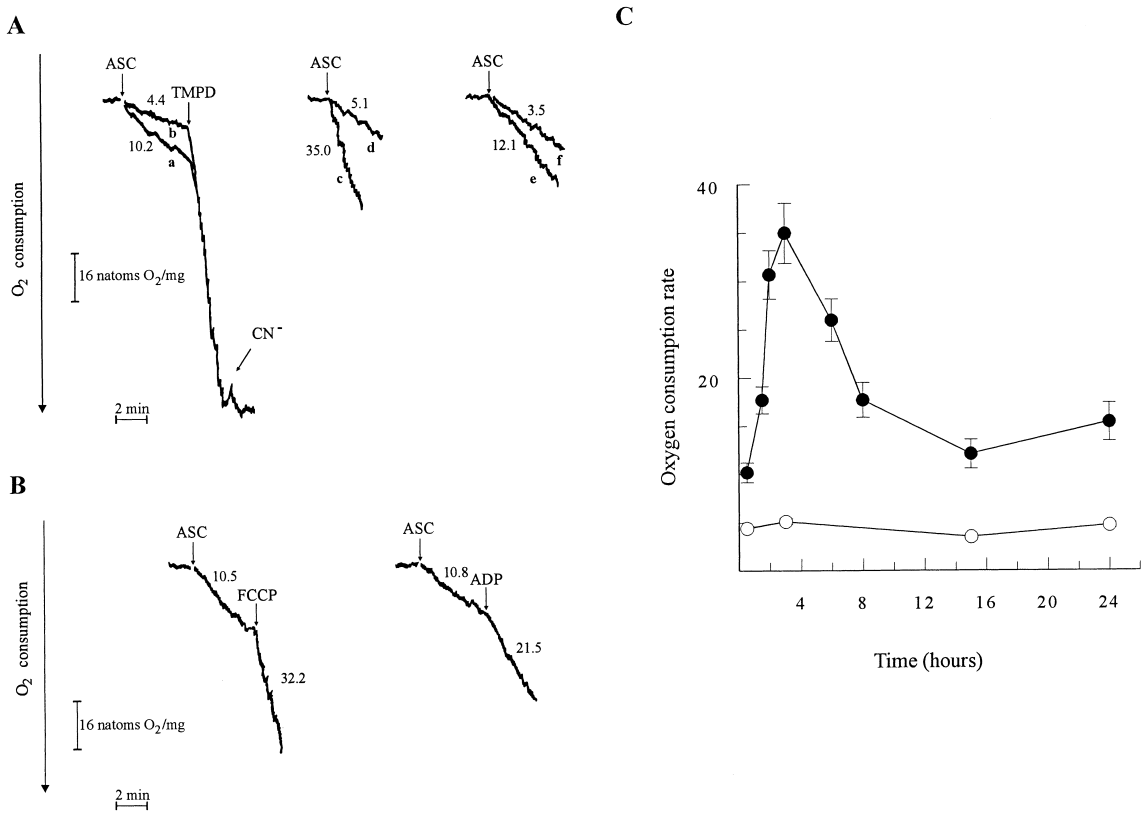

Fig. 2. Released cyt *c* is functionally active. CGCs were switched from high K^+ (S-K25) to low K^+ (S-K5) serum-free culture medium for the indicated times, subsequently collected, suspended in PBS and homogenized (see text for details). Aliquots (about 0.2 mg protein) were incubated in the presence of rotenone (3 μM), antimycin A (0.8 μM) and myxothiazole (6 μM) at 37°C in a water-jacketed glass vessel to monitor O_2 consumption polarographically. (A) S-K5 neurons at 30 min (a), 3 h (c) and 15 h (e) after apoptosis induction or the corresponding S-K25 neurons (b, d and f) were used. The following additions were made: ASC (5 mM), TMPD (0.2 mM), cyanide (CN^-, 1 mM). (B) S-K5 neurons at 30 min were used. The following additions were made: ASC (5 mM), FCCP (0.01 μM), ADP (1 mM). Numbers along the traces are rates of oxygen uptake expressed as natoms O_2/min/mg cell protein. In five experiments, carried out using different cell preparations, variations up to 5% were found. (C) The dependence of the ascorbate-dependent oxygen uptake rate versus the duration of low K^+ treatment was studied for control (○) and low K^+ CGCs (●), as reported above. The initial rate of the reaction was expressed as natoms O_2/min/mg cell protein. Results are means ± S.D. of triplicate measurements and representatives of at least five different experiments carried out with different cell preparations prepared from different groups of animals.

filters were probed with monoclonal antibodies against cytochrome oxidase (subunit IV) (not shown). Moreover, in order to account for possible cyt *c* release due to mitochondrial damage occurring during the cell fractionation procedure, the same filter was probed with a polyclonal antibody against GDH, a marker enzyme of the mitochondrial matrix. A typical experiment is reported in Fig. 1A with the densitometric analysis of several experiments of both the cyt *c* and the GDH blots shown in Fig. 1B. The GDH amount in each lane was used to normalize the corresponding amount of cyt *c* revealed on the same filter. Cyt *c* is already detectable in the cytosolic fraction 30 min after apoptosis induction, its amount reaches a maximum after 3 h with a 238 ± 36% increment over controls and declines thereafter, being roughly similar to that measured in controls after 24 h.

The amount of cyt *c* in the corresponding mitochondrial fractions, evaluated in the same cellular preparation, is reported in Fig. 1C and D. An early decrease in the amount of mitochondrial cyt *c* occurs in the first 30 min, reaches a minimum after 3 h and remains constantly low in the following 24 h.

In order to ascertain whether the cyt *c* released into the

Table 1
Detection of both ADK and GDH in the cytosolic fraction and cell homogenate of CGCs undergoing apoptosis

Enzymatic activity	S-K25		S-K5	
	Cytosolic fraction	Homogenate+TX-100	Cytosolic fraction	Homogenate+TX-100
ADK	0.14 ± 0.08	40 ± 2.2	0.5 ± 0.06	41 ± 1.9
GDH	1.8 ± 0.33	421 ± 20	2.1 ± 0.41	430 ± 22

Either the cytosolic fraction or the homogenate in the presence of Triton X-100 (TX-100, 0.5%) from control (S-K25) or CGCs undergoing apoptosis (S-K5) (about 0.2 mg protein) were incubated at 37°C in 1.5 ml PBS and assayed, at 3 h after apoptotic stimulus, for both ADK and GDH activity, as reported in Section 2. Enzymatic activity is expressed as nmol $NADP^+$ reduced (NADH oxidized)/min/mg cell protein for ADK and GDH, respectively. The values of 10 enzymatic activities (± S.E.M.) obtained from six experiments, carried out by using different cell preparations, are reported.

cytosol is fully functional, the rate of ascorbate-dependent oxygen consumption in the absence of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (Fig. 2) was measured polarographically in CGC homogenate, essentially as reported in [10]. S-K25 and S-K5 cells were compared with respect to their capability to oxidize ascorbate (5 mM). The rate of oxygen uptake increases from about four to 10 natoms O_2/min/mg cell protein 30 min after the start of apoptotic treatment (Fig. 2A, a and b). In both cases, the externally added TMPD (0.2 mM) was found to strongly stimulate the rate of oxygen uptake. Cyanide, a classic inhibitor of mitochondrial cytochrome oxidase activity, completely prevents oxygen consumption. To verify that the release of cyt *c* takes place from intact mitochondria, in the same experiment, the activity of the mitochondrial ADK and GDH was assayed in either control or cells undergoing apoptosis (Table 1). Neither difference in the ADK activity, measured in S-K25 and S-K5, was found as judged by a statistical analysis ($P > 0.1$) nor significant activity was present in the respective supernatants, i.e. cytosolic fraction, without Triton X-100 addition (not shown). The intactness of the mitochondrial inner membrane was also checked by assaying GDH, which is a marker of the mitochondrial matrix. A negligible GDH activity was found both in cell homogenate and in the cytosolic fraction, whereas its activity has been proved ($P > 0.3$) following treatment of cells with Triton X-100. Moreover, in order to verify mitochondrial coupling, FCCP and ADP were added to S-K25 and S-K5 cells (Fig. 2B) as in [11]. Both of them proved to stimulate the rate of oxygen consumption with ICR values equal to about three and two, respectively.

The percentage increase in the rate of oxygen uptake caused by ascorbate addition to either apoptotic or control CGCs as a function of the apoptotic treatment time is reported in Fig. 2C. Interestingly, a few min (30) of low [K^+] exposure causes a remarkable increase in the ascorbate-dependent oxygen uptake rate with respect to control cultures, indicating the presence of a pool of soluble cyt *c*. Further stimulation was found up to 3 h after the switch to low [K+] (35 natoms O_2/min/mg cell protein) (Fig. 2A, c and d). Similarly to the finding reported in Fig. 1, a significant decline in the rate of oxygen uptake, i.e. in the amount of fully functional cyt *c*, was found in the following 3–24 h range (Fig. 2A, e and f) after the apoptosis trigger.

When both S-K5 and S-K25 cells were incubated in the presence of inhibitors of transcription and translation such as actinomycin D (1 μg/ml) and cycloheximide (10 μg/ml), respectively, as well as growth factors such as IGF-I (25 ng/ml), which have previously been reported to inhibit apoptosis in CGCs to a varying extent [1], neither cyt *c* protein nor cyt *c* activity was detected in the extramitochondrial phase, clearly showing that these compounds can prevent cyt *c* release and that this release is specifically associated with the apoptotic process (not shown).

Since a link between cyt *c* release and the activity of caspases has been reported [16], experiments were carried out to investigate the possible involvement of this class of proteases in early cyt *c* release from mitochondria and in the subsequent cellular decrease observed in this study. Thus, z-VAD-fmk (100 μM), a broad-range specific caspase inhibitor (for review, see Cohen et al. [17]), was added to CGCs during apoptotic treatment (Table 2) under two experimental conditions: contemporarily with the shift to low [K^+] (line 4) corresponding to the commitment phase of apoptosis [3] or after 3 h (lines 6–8), i.e. when cyt *c* is apparently on its route of degradation. Cyt *c* activity was followed as above. In the first type of experiments, cyt *c* activity was detected in the cytosol (line 4) with no significant change compared to that found in apoptotic CGCs in the absence of the caspase inhibitor (line 2). Interestingly, no decrease in cyt *c* content was found in the 3–24 h range of apoptosis if the inhibitor was added to the plate 3 h after the induction of apoptosis (lines 6–8), thus showing that z-VAD-fmk has no effect on the release of cyt *c*, but it can prevent long term cyt *c* degradation.

4. Discussion

In this paper, evidence is given that apoptosis of CGCs cultured in low potassium medium is accompanied by release of cyt *c*, which starts to accumulate in the cytosol at a very early stage with the maximum accumulation reached after 3 h. Cyt *c* release is shown in this paper both via immunoblotting and via polarographic experiments which have the added dimension to show that the released cyt *c* is fully functional. The time courses of the cyt *c* level both in the cytosolic and in the mitochondrial fractions deserve special discussion. Indeed, in the first 3 h of treatment, the increase in the extramitochondrial phase and the decrease in the mitochondria appear to be correlated. However, a clear difference is found after a long term treatment when the cyt *c* amount remains constant in the mitochondria, even though at a concentration lower than in

Table 2
Effect of z-VAD-fmk on cyt *c* release from CGCs undergoing apoptosis as measured polarographically

Line	Treatment	Oxygen consumption rate (natoms O_2/min/mg cell protein)		
	t = 0	*t*+3 h	*t*+8 h	*t*+18 h
1	S-K25S	4.5 ± 0.1		
2	S-K5	34.4 ± 4.4	17.7 ± 1.8	12.1 ± 1.5
3	S-K25+DMSO	4.4 ± 0.3		
4	S-K5+z-VAD-fmk	35.5 ± 4.8		
5	S-K25	+DMSO	4.8 ± 0.3	
6	S-K5	+z-VAD-fmk	32.7 ± 3.9	
7	S-K25	+DMSO		5.2 ± 0.1
8	S-K5	+z-VAD-fmk		36.2 ± 3.9

Homogenates from either control (S-K25) or CGCs undergoing apoptosis (S-K5) (about 0.4 mg protein) were incubated at 37°C in 1.5 ml PBS in the presence of rotenone (3 μM), antimycin A (0.8 μM) and myxothiazole (6 μM). Experimental conditions as reported in Fig. 2. Addition of z-VAD-fmk (100 μM) was done at the indicated times. Oxygen consumption after addition of ascorbate (5 mM) was recorded in each test and the initial rate of the reaction is expressed as natoms O_2/min/mg cell protein. In five experiments, carried out using different cell preparations, variations up to 5% were found.

controls, but it strongly decreases in the cytosol. A correspondent decrease in the activity of cyt *c* was also detected. These experimental findings show that cyt *c* is released during the phase of apoptotic commitment and is degraded during the execution phase. Such a conclusion is consistent with z-VAD-fmk experiments which give clear experimental evidence that cyt *c* release is not mediated by caspases, since it is not blocked by z-VAD-fmk as also detected in other cell systems [5,18], although this inhibitor largely blocks the apoptotic process [19]. On the other hand, z-VAD-fmk clearly inhibited the later decrease in both cyt *c* content and activity once administered at the time when the cytoplasmic cyt *c* level reached its maximum (i.e. 3 h). This finding indicates for the first time that cyt *c* is degraded in a caspase-dependent way. It should be noted that different results have recently been reported in studies carried out with a different neuronal cell type [18].

Previous studies performed on CGCs undergoing necrotic death upon exposure to glutamate [10] revealed that cyt *c* was released from mitochondria and participates in the oxidation of cytosolic NADH via NADH-b5 oxidoreductase. In the light of this report and concerning the possible functional significance of cyt *c* release during the apoptotic process, our findings are in favor of the proposal that the early release could contribute, via NADH-b5 oxidoreductase, to the maintenance of adequate cell ATP [20], which, in turn, could play a role in the cellular integrity or, alternatively, in the execution phase of the apoptotic program. Concomitantly, cyt *c* could also initiate the caspase cascade as proposed by [5,16] and confirmed by the finding that CPP32-like protease activity only starts to increase in apoptotic CGCs after 3 h from the administration of an apoptotic stimulus (i.e. after cytosolic cyt *c* has reached its maximum level).

The picture that emerges from the experimental findings reported in this paper is one in which the cyt *c* level in the cytosol is controlled by (i) the cytosolic cyt *c* increase due to its release from mitochondria and (ii) its decrease due to its degradation. A system including a controlled cyt *c* level will be much more sensitive to any regulatory effects than a system lacking the degradation step.

The leak of cyt *c* from mitochondria and its release into the cytosol may play a pivotal role in the regulation of the programmed cell death pathways in CGCs. At present, the mechanism by which cyt *c* release occurs remains to be established.

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