FEBS Letters 515 (2002) 8–12 FEBS 25720

Proteasome inhibitors prevent cytochrome *c* release during apoptosis but not in excitotoxic death of cerebellar granule neurons

Antonella Bobba[a,*], Nadia Canu[b], Anna Atlante[a], Vito Petragallo[a], Pietro Calissano[b], Ersilia Marra[a]

[a] *Centro di Studio sui Mitocondri e Metabolismo Energetico, CNR, Via Amendola 165/A, 70126 Bari, Italy*
[b] *Isituto di Neurobiologia e Medicina Molecolare, CNR, Viale Marx 15, 00137 Rome, Italy*

Received 4 December 2001; accepted 28 December 2001

First published online 11 January 2002

Edited by Veli-Pekka Lehto

Abstract In order to find out whether and how proteasomes participate in the processes leading cerebellar granule cells to death either in necrosis, due to glutamate neurotoxicity, or in apoptosis, due to K^+ shift, we measured the three proteasome activities by using specific fluorescent probes and investigated the effect of several proteasome inhibitors, including MG132, on the cytochrome *c* release taking place in the early phase of both apoptosis and necrosis. We show that differently from apoptosis, the early phase of necrosis does not require proteasome activation. Inhibition of proteasome activity can prevent cytochrome *c* release in cerebellar granule cells undergoing apoptosis, thus improving cell survival, but not necrosis. These findings show that proteasomes play an important role in the early phase of apoptosis but not that of necrosis, and that these two types of cell death differ from each other in their mechanism of cytochrome *c* release. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Cytochrome *c*; Proteasome; Apoptosis; Necrosis; Cerebellar granule cell

1. Introduction

One of the most outstanding problems in cell biology concerns the mechanism by which cell death occurs. Apoptosis and necrosis are two distinct modes of cell death that differ from each other, the former being physiological and the latter pathological. Since both inappropriate apoptosis and necrosis are linked to neurodegenerative diseases including Alzheimer's and Parkinson's diseases [1], the understanding of the molecular events that make apoptosis and necrosis different is worthy of special attention. In this regard, cultured rat cerebellar granule cells (CGCs) represent an excellent in vitro model that can be used to investigate the events underlying the two types of cell death. Indeed, CGCs undergo necrotic death when exposed to glutamate (GLU) and apoptotic death when the extra-cellular concentration of KCl utilized for their culture (25 mM) is reduced to 5 mM [2,3]. Both in apoptosis and in necrosis, cytochrome *c* (cyt *c*) is early released into the cytosol as an intact and functionally active protein from mitochondria that are essentially coupled and intact [4–7]. Moreover, in apoptosis, cyt *c* release has been found to be independent on the activity of caspases that can degrade the released cyt *c* accumulated in the cytosol [4].

Proteasomes play an important role in protein degradation in all eukaryotic cells as it is a conserved multicatalytic proteolytic complex [8,9]. Proteasome has been found to play a crucial role during the early commitment phase of apoptosis [10–12] and particularly in neuronal apoptosis [13,14], whereas the role of proteasome in cell neurotoxicity is not known. This prompted us to investigate whether and how proteasome participates in some events leading CGCs to either necrosis or apoptosis. In particular we investigated both the time-dependent changes of proteasome activity in necrosis and apoptosis, and the role of proteasome in cyt *c* release, showing that cyt *c* release is strictly dependent on the proteasome activity in apoptosis, but occurs in necrosis without proteasome involvement.

2. Materials and methods

2.1. Reagents

MG132 (N-CBZ-Leu-Leu-Leu-Al), MG115 (N-CBZ-Leu-Leu-norvalinal), ALLM (N-Ac-Leu-Leu-methioninal), Suc-LLVY-MCA (succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin), Boc-LSTR-MCA (N-*t*-boc-Leu-Ser-threonin-arginin-7-amido-4-methylcoumarin) and Z-LLE-βNap (N-CBZ-Leu-Leu-Glu-β-naphthylamide) were from Sigma (St. Louis, MO, USA). LC (lactacystin) was purchased from Calbiochem (La Jolla, CA, USA). The addition of inhibitors or other compounds was carried out concomitantly with either necrotic or apoptotic treatment. The final concentration of dimethyl sulfoxide (DMSO) was kept below 0.1%. Corresponding controls were treated either with the same inhibitor or with the same concentration of DMSO. 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 für Medizinische Neurobiologie, University of Magdeburg, Magdeburg, Germany).

2.2. Neuronal cultures

Primary cultures of CGCs were obtained from dissociated cerebellar of 7-day-old Wistar rats as in [15]. Cells were plated in basal medium with Eagle's salts (BME; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 25 mM KCl

*Corresponding author. Fax: (39)-80-5443317.
E-mail address: csmmab03@area.ba.cnr.it (A. Bobba).

Abbreviations: BME, basal medium with Eagle's salts; CGC, cerebellar granule cell; S-K25, control CGCs for the apoptotic paradigm; S-K5, apoptotic CGCs; C-CGC, control CGCs for the necrotic paradigm; GLU-CGC, necrotic CGCs; cyt *c*, cytochrome *c*; MK801, (+)-5-methyl-10,1-dihydro-5H-dibenzo(*a,d*)cyclohepten-5,10-imine hydrogen maleate

0014-5793/02/$22.00 © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(02)02231-7

and 2 mM glutamine (Life Technologies) on dishes (Nunc, Roskilde, Denmark) coated with poly-L-Lysine. Cells were plated at 2×10^6 per 35 mm dish or 6×10^6 per 60 mm dish. 1β-Arabinofuranosylcytosine (10 mM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells.

2.3. Induction of necrosis

Necrosis was induced as previously reported [2]. 6–7-day-in vitro (DIV) cultures were exposed to 100 μM GLU for 30 min 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 10 μM glycine. After this time, necrotic cells, referred to as GLU-CGC, were replenished with the original conditioned medium and put in the incubator. Control cells were treated as above but in the absence of GLU and are referred to as C-CGC.

2.4. Induction of apoptosis

Apoptosis was induced as in [3]. At 6–7 DIV, cells were washed twice and switched to a serum-free BME, containing 5 mM KCl (K5) and supplemented with 2 mM glutamine and 100 μg/ml gentamycin for the time 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 (K25) and are referred to as S-K25 cells.

2.5. Proteasome activity assay

Proteasomes can cleave peptides on the carboxyl side of hydrophobic, basic and acid residues. These proteolytic functions commonly referred to as the chymotrypsin-, trypsin- and caspase-like activity, can be measured by evaluating the hydrolysis of specific fluorogenic substrates [12,16,20]. Briefly, CGCs were detached and lysed in a buffer containing 20 mM Tris/HCl (pH 7.2), 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM ATP, 20% glycerol, 0.04% Nonidet P-40 as in [12,14,16]. After centrifugation, 10 μg of the supernatants was used for each assay. In particular, chymotrypsin-like activity was assayed with 50 μM Suc-LLVY-MCA, trypsin-like activity was assayed with 100 μM Boc-LSTR-MCA and caspase-like activity was assayed with 400 μM Z-LLE-βNap for 20, 30 and 60 min respectively. In every case the proteolytic activities were measured in a LS50 Perkin Elmer spectrofluorimeter using the following wavelength pairs: 380 nm excitation and 460 nm emission for MCA derivatives and 355 nm excitation and 410 nm emission for βNap compounds.

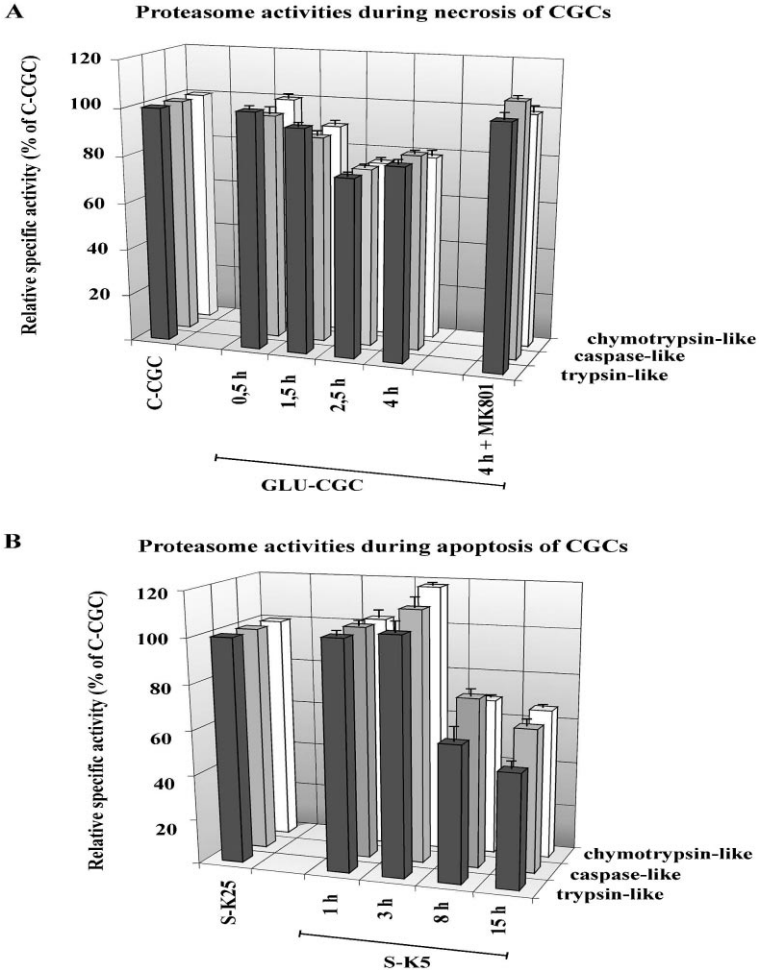

Fig. 1. Proteasome activities in CGCs during apoptosis or necrosis. A: In order to induce necrosis, rat CGCs, cultured for 7 DIV, were incubated in Locke's solution in the presence of 100 μM GLU-CGC or with the same volume of Locke's solution alone (C-CGC) for 30 min. After this time, both necrotic (GLU-CGC) and control cells (C-CGC) were replenished with the original conditioned medium and put in the incubator for the indicated time. Where indicated, 1 μM MK801 was added simultaneously with GLU. B: In order to induce apoptosis, rat CGCs, cultured for 7 DIV, were washed and incubated in serum-free medium with 25 mM KCl (S-K25, i.e. control cells) or switched to 5 mM KCl serum-free medium (S-K5, i.e. apoptotic cells) for the indicated times. Both in the case of necrosis and apoptosis, at the time indicated after the administration of the stimulus, supernatants were prepared from cell suspensions (see Section 2). 10 μg supernatants was incubated with the fluorogenic substrates specific for chymotrypsin-like (Suc-LLVY-MCA), trypsin-like (Boc-LSTR-MCA) and caspase-like (Z-LLE-βNap) activities at 25°C for 20, 30 and 60 min respectively. Specific activities are expressed as the percentage of activities of control cells which have been given a value of 100. Results are means ± S.D. of at least three separate experiments carried out with different cell preparations obtained from different groups of animals.

2.6. Western blot analysis

Immunoblot analysis was carried out on cytosolic extracts from control (S-K25) and apoptotic cultures (S-K5), as in [4].

2.7. Oxygen uptake studies

O_2 consumption was measured polarographically at 25°C as in [17,18] 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 O_2 uptake as low as 0.5 natoms/min/mg cell protein.

2.8. Assessment of neuronal viability

Viable CGCs were quantified by counting the number of intact nuclei after lysing the cells in detergent-containing solution as described in [19].

3. Results

As already reported, proteasomes play a primary role in CGCs undergoing apoptosis [14] with a slight but consistent increase in the three peptidase activities of proteasome in the early phase of cell death followed by a progressive decline. On the other hand, it is still unknown whether and how proteasome activity changes in GLU neurotoxicity. Thus, cells undergoing necrosis (GLU-CGC), apoptosis (S-K5) and their respective controls (C-CGC and S-K25 cells) were homogenized and centrifuged with the chymotrypsin-, trypsin- and caspase-like activity of the proteasome assayed in the supernatant fractions as reported in Section 2 (Fig. 1). No change in proteasome activities was found with respect to the control in the early phase of GLU neurotoxicity (up to 30 min) or when GLU neurotoxicity induction was prevented by MK801 ((+)-5-methyl-10,1-dihydro-5H-dibenzo(*a,d*)cyclohepten-5,10-imine hydrogen maleate). After 1.5 h a progressive decline was observed and a 20% decrease was detected at 2.5 h which remained constant up to 4 h (Fig. 1A).

In close agreement with [14], proteasome activity time

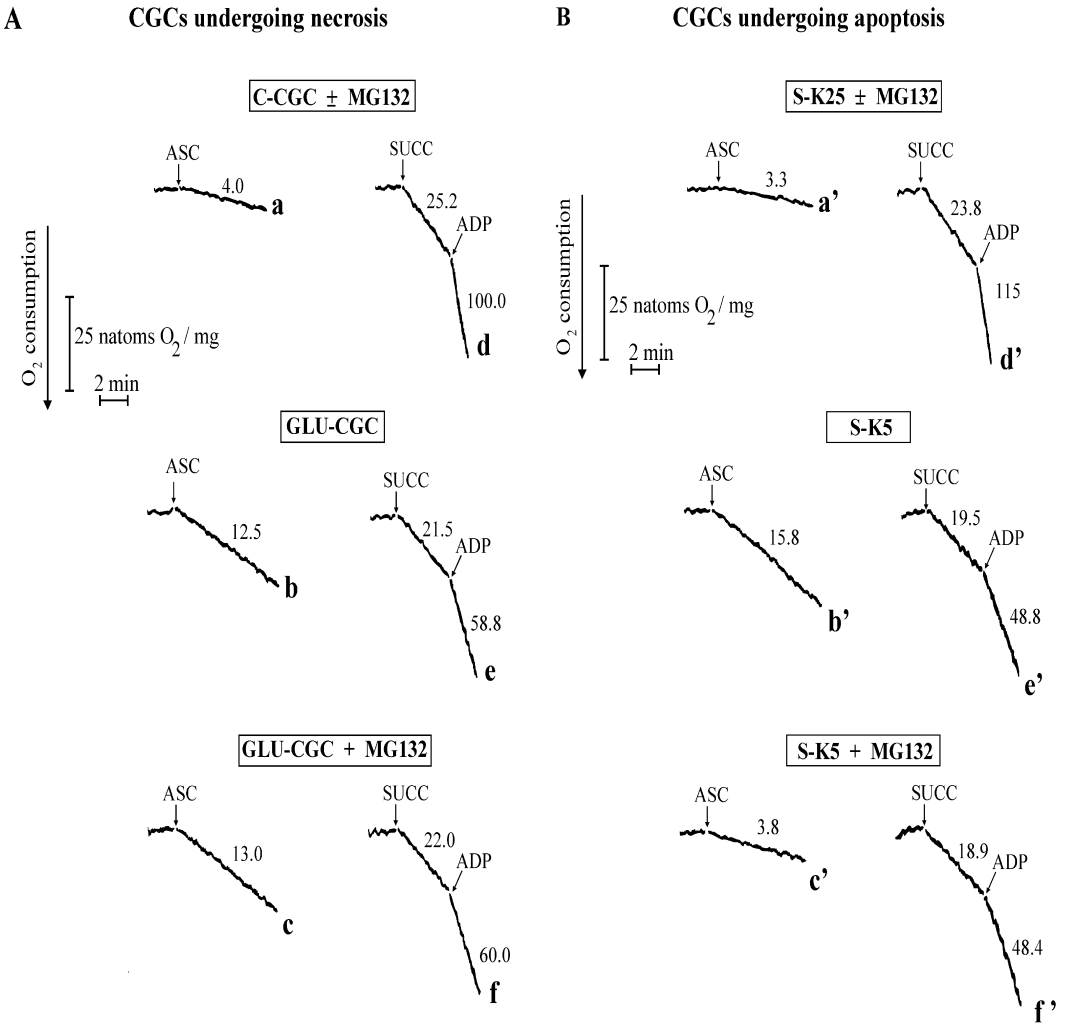

Fig. 2. Cyt *c* release in CGCs undergoing either necrosis or apoptosis: effect of MG132. A: In order to induce GLU neurotoxicity, rat CGCs, cultured for 7 DIV, were incubated in Locke's solution in the presence of 100 μM GLU-CGC or with the same volume of Locke's solution alone (C-CGC) for 30 min, either in the absence or presence of 5 μM MG132. B: In order to induce apoptosis, rat CGCs, cultured for 7 DIV, were washed and incubated in serum-free medium with 25 mM KCl (S-K25, i.e. control cells) or switched to 5 mM KCl serum-free medium (S-K5, i.e. apoptotic cells) for 3 h, either in the absence or presence of 5 μM MG132. In both apoptotic and necrotic conditions, cells were collected, suspended in phosphate-buffered saline and homogenized (see text for details). In A and B, a–c and a′–c′, aliquots (about 0.2 mg cell protein) were incubated at 25°C in the presence of rotenone (3 μM), antimycin (0.8 μM) and myxothiazole (6 μM) in a water-jacketed glass vessel and oxygen consumption was started by adding 5 mM ascorbate (ASC). In A and B, d–f and d′–f′, homogenates were incubated at 25°C in the presence of rotenone and oxygen consumption was started by adding 5 mM succinate. Then, ADP (0.5 mM) was added to verify mitochondrial coupling. Numbers along the traces are rates of oxygen uptake expressed in natoms O_2/min/mg cell protein. In five experiments, carried out using different cell preparations, variations of up to 5% were found.

courses in S-K5 cells show no significant change 1 h after induction of apoptosis. However, 3 h after potassium shift, a small but statistically significant activation of proteasome activities was detected (Fig. 1B), followed by a progressive reduction during incubation occurring up to 15 h, when the values of the chymotrypsin-, trypsin- and caspase-like activity were found to be 67 ± 2%, 51 ± 5%, and 64 ± 5% respectively.

In order to check whether proteasome can affect cyt *c* release that occurs in the early phase of both necrosis and apoptosis, cyt *c* release was measured in the absence or presence of some proteasome inhibitors at definite times after death induction. In the case of necrosis, analysis was performed 30 min after death induction when the proteasome activities had not decreased yet. In apoptosis, analysis was performed 3 h after K^+ deprivation, when maximum proteasome activation (this paper and [14]) and cyt *c* release [4] were reached. In both cases, cyt *c* release was monitored by means of polarographic measurements of cyt *c*-dependent ascorbate oxidation in cell homogenate [4–6] (Fig. 2).

In this case, the capability of CGC homogenate to oxidize ascorbate at a higher rate than that of the control reflects cyt *c* release from mitochondria. In fact, ascorbate per se cannot be oxidized by mitochondria in the absence of cyt *c*. Extra-mitochondrial cyt *c*, once reduced by ascorbate, can induce oxygen consumption via cyt *c* oxidase [6]. In agreement with [4–7], both necrosis and apoptosis induction cause release of fully functioning cyt *c*, as revealed by the increase in the rate of ascorbate (5 mM) oxidation by necrotic and apoptotic cell homogenates (Fig. 2A,B, b,b′) compared to control cells (Fig. 2A,B, a,a′ respectively; 12.5 and 4 natoms O_2/min/mg cell protein measured in GLU-CGC and in C-CGC respectively; 15.8 and 3.3 natoms O_2/min/mg cell protein measured in S-K5 and in S-K25 respectively). Such an increase was found to be statistically significant with a $P < 0.05$ value detected in five and in four experiments carried out with GLU-CGC and S-K5 cells respectively. Interestingly, externally added MG132, a reversible proteasome inhibitor [21], was found to prevent the increase in oxygen uptake due to ascorbate, i.e. cyt *c* release, in cells undergoing apoptosis (Fig. 2B, c′), but not in those undergoing necrosis (Fig. 2A, c).

In the same experiment, as a control, the effect of MG132 on succinate oxidation was also checked. In good agreement with [17,18], GLU-CGC and S-K5 cell homogenates were found to be less effective than their respective controls when both state 3 and state 4 respiration were measured (Fig. 2A,B, e,e′ as compared to d,d′). Interestingly, MG132 does not affect electron flow in the respiratory chain due to succinate oxidation in any cell samples, either in state 4 or 3 (Fig. 2A,B, d-d′, and f,f′ as compared to e,e′).

The release of cyt *c* was also investigated in S-K25 cells treated with other proteasome inhibitors namely LC, a microbial metabolite which irreversibly inhibits all three proteasome activities [22], and MG115, the closely related MG132 inhibitor, which reversibly inhibits the proteolytic activities of proteasome [21]. To account for the possible contribution to cyt *c* release due to calpain and cathepsin, both partially inhibited by MG132 and MG115, the effect of ALLM on cyt *c* release was also checked. ALLM is a permeable inhibitor of both calpain and cathepsin.

All the proteasome inhibitors proved to be statistically significant ($P < 0.001$) in preventing cyt *c* release in apoptosis, as polarographically measured (Fig. 3A). Similar results were obtained with Western blot analysis carried out in parallel on the cytosolic fractions (Fig. 3B). Polyclonal antibodies against GDH were used to normalize the corresponding amount of cyt *c* revealed on the same filter as reported in [4]. Cyt *c* release which occurs under apoptosis (S-K5, Fig. 3, lane b) was fully prevented by MG132, MG115 and LC (Fig. 3, lanes d, e, f) but only to a low degree by ALLM (Fig. 3, lane c).

In order to find out whether proteasome inhibition can somehow affect the apoptosis time course, cell survival was monitored in both S-K25 and S-K5 cells in the absence or presence of the above reported inhibitors (Fig. 4). None of them proved to affect control cell survival for up to 15 h (not shown). However, LC and MG132, but not ALLM, were found to rescue cells from apoptosis.

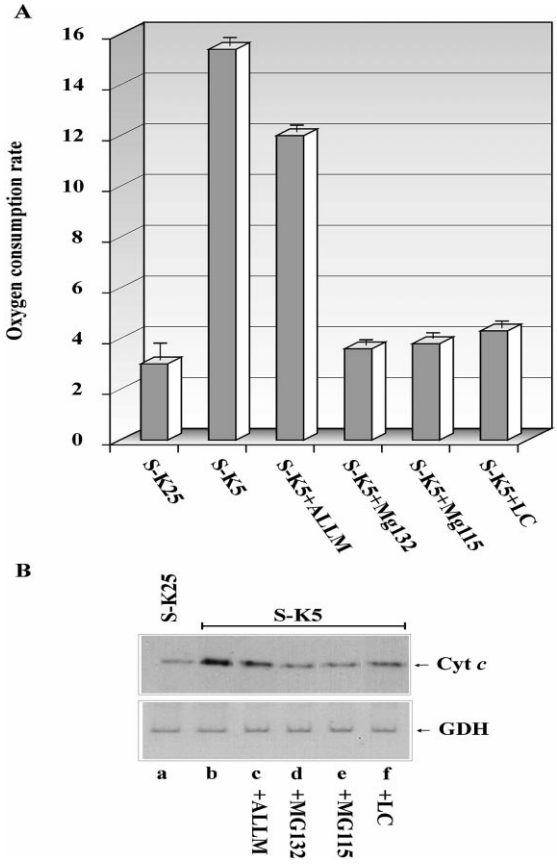

Fig. 3. Effect of proteasome inhibitors on cyt *c* release in CGCs undergoing apoptosis. Rat CGCs, cultured for 7 DIV, were incubated either in high potassium or in low potassium serum-free culture medium for 3 h in the absence or presence of different inhibitors: LC 100 μM, MG132 5 μM, MG115 5 μM, ALLM 40 μM. A: Oxygen consumption was caused by externally added ascorbate. Homogenates (about 0.2 mg protein) from either S-K25 or S-K5 cells were incubated at 25°C in 1.5 ml phosphate-buffered saline in the presence of rotenone (3 μM), antimycin (0.8 μM) and myxothiazole (6 μM). Oxygen consumption was started by adding 5 mM ascorbate and the initial rate of the reaction was expressed in natoms O_2/min/mg cell protein. Results are means ± S.D. of triplicate measurements and are representative of at least six separate experiments carried out with different cell preparations obtained from different groups of animals. B: Western blot analysis of released cyt *c*. Cytosolic fractions from either S-K25 or S-K5 cells were analyzed by Western blotting analysis. Antibodies against GDH were used to normalize the amount of protein loaded onto the gel.

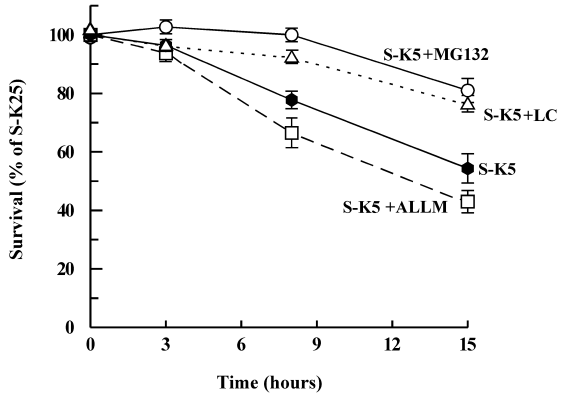

Fig. 4. Effect of proteasome inhibitors on cell survival in CGCs undergoing apoptosis. Rat CGCs at 7 DIV were incubated either in high potassium (S-K25) or in low potassium (S-K5) serum-free culture medium in the absence or presence of different inhibitors: LC 100 μM, MG132 5 μM, ALLM 40 μM. At different times cell viability was determined by counting the number of intact nuclei.

In a parallel experiment, these inhibitors were found not to affect the release of cyt *c* or cell survival in CGCs undergoing necrosis (not shown).

4. Discussion

In this paper we compare the proteasome activity time profiles in CGCs undergoing either necrosis or apoptosis, and, moreover, we investigate the physiological role played by proteasome in cyt *c* release that occurs in both types of cell death. This study, together with [14], shows that the early phase after apoptosis and necrosis induction in CGCs is accompanied by a contrasting change in the three proteasome activities which increase in apoptosis but show no significant change in necrosis. For incubation longer times, a general decrease was found in both types of cell death. We show that during apoptosis proteasome is required to trigger the death process, in fact when its activity is inhibited, both cyt *c* release and neuronal death are prevented. Such a conclusion derives from the inhibition experiments. In this regard, we rule out any side effect due to the inhibitors, such as electron flow impairment, in the light of the failure of all the inhibitors used to affect either oxygen consumption or mitochondrial coupling in the succinate experiments. At present the proteasome mechanism must remain a matter of speculation. Proteasomes could act on unknown factors involved in cyt *c* release, for instance Bcl2, which is a mitochondrial-localized anti-apoptotic protein candidate, to undergo degradation via proteasomes.

The picture that emerges from this paper and from [4] is that the cytosolic level of the released cyt *c* in apoptosis is controlled by at least two different proteolytic systems: (i) the proteasome complex which mediates cyt *c* release (this paper) and (ii) caspases which start to degrade cyt *c* accumulated in the cytosol [4].

Furthermore, this paper gives a clear indication that proteasomes are not involved in the early phase of necrosis progression and that they do not affect cyt *c* release in this type of cell death, as also confirmed by the finding that proteasome inhibitors fail to rescue CGCs undergoing excitotoxicity (Bobba, Atlante, Marra, personal communication).

In the light of the findings reported in this paper, we conclude that the release of cyt *c* in CGCs undergoing necrosis or apoptosis occurs in at least two distinct manners.

Acknowledgements: The authors thank Prof. S. Passarella for his advice and stimulating discussion during the preparation of the manuscript and Mr. Richard Lusardi for linguistic consultation. This work was partially financed by PRIN 'Bioenergetica e Trasporto di Membrana' (MURST) and by C.N.R. Target Project on Biotechnology to P.C.

References

[1] Honig, L.S. and Rosenberg, R.N. (2000) Am. J. Med. 108, 317–330.
[2] Johnson, J.W. and Ascher, P. (1987) Nature 325, 529–531.
[3] D'Mello, R., Galli, C., Ciotti, T. and Calissano, P. (1993) Proc. Natl. Acad. Sci. USA 90, 10989–10993.
[4] Bobba, A., Atlante, A., Giannattasio, S., Sgaramella, G., Calissano, P. and Marra, E. (1999) FEBS Lett. 457, 126–130.
[5] Atlante, A., Gagliardi, S., Marra, E., Calissano, P. and Passarella, S. (1999) J. Neurochem. 73, 237–246.
[6] Atlante, A., Calissano, P., Bobba, A., Azzariti, A., Marra, E. and Passarella, S. (2000) J. Biol. Chem. 275, 137159–137166.
[7] Atlante, A., Calissano, P., Bobba, A., Giannattasio, S., Marra, E. and Passarella, S. (2001) FEBS Lett. 497, 1–5.
[8] Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and Goldberg, A.L. (1994) Cell 78, 761–771.
[9] DeMartino, G.N. and Slaughter, C.A. (1999) J. Biol. Chem. 274, 22123–22126.
[10] Grimm, L.M., Goldberg, A.L., Poirier, G.G., Schwartz, L.M. and Osborne, B.A. (1996) EMBO J. 15, 3835–3844.
[11] Hirsch, T., Dallaporta, B., Zamzami, N., Susin, A.A., Ravagnan, L., Marzo, I., Brenner, C. and Kroemer, G. (1998) J. Immunol. 161, 35–40.
[12] Beyette, J., Mason, G.G., Murray, R.Z., Cohen, G.M. and Rivett, A.J. (1998) Biochem. J. 332, 315–320.
[13] Sadoul, R., Fernandez, P.-A., Quiquerez, A.-L., Martinou, I., Maki, M., Schroter, M., Becher, J.D., Irmler, M., Tschopp, J. and Martinou, J.-C. (1996) EMBO J. 15, 3845–3852.
[14] Canu, N., Barbato, C., Ciotti, M.T., Serafino, A., Dus, L. and Calissano, P. (2000) J. Neurosci. 15, 589–599.
[15] Levi, G., Aloisi, F., Ciotti, M.T. and Gallo, V. (1984) Brain Res. 290, 77–86.
[16] Rivett, A.J., Savory, P.J. and Djaballah, H. (1994) Methods Enzymol. 244, 331–350.
[17] Atlante, A., Gagliardi, S., Marra, E. and Calissano, P. (1998) Neurosci. Lett. 245, 127–130.
[18] Atlante, A., Gagliardi, S., Minervini, G.M., Marra, E., Passarella, S. and Calissano, P. (1996) NeuroReport 7, 2519–2523.
[19] Volontè, C., Ciotti, M.T. and Battistini, L. (1994) Cytometry 17, 274–276.
[20] Orlowsky, M. and Wilk, S. (2000) Arch. Biochem. Biophys. 383, 1–16.
[21] Figueiredo-Pereira, M.E., Berg, K.A. and Wilk, S. (1994) J. Neurochem. 63, 1578–1581.
[22] Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J. and Schreiber, S.L. (1995) Science 268, 726–731.