FEBS Letters 579 (2005) 2364–2368 FEBS 29464

Caspase-3 can be pseudo-activated by a Ca^{2+}-dependent proteolysis at a non-canonical site

Maude Pelletier, Lisa Oliver, Khaled Meflah, François M. Vallette*

UMR 601 INSERM/Université de Nantes (Equipe 4), IFR 26, 9 quai Moncousu, 44035 Nantes Cedex 01, France

Received 11 February 2005; revised 14 February 2005; accepted 25 February 2005

Available online 29 March 2005

Edited by Vladimir Skulachev

Abstract We have shown previously that calcium could trigger nuclear fragmentation, which was associated with a caspase 3 (C3)-like activity [Juin, P., Pelletier, M., Oliver, L., Tremblais, K., Gregoire, M., Meflah, K. and Vallette, F.M. (1998) Induction of a caspase-3-like activity by calcium in normal cytosolic extracts triggers nuclear apoptosis in a cell-free system. J. Biol. Chem. 273, 17559]. Here, we report that this activation is associated with a non-canonical truncation of C3, which induces a weak DEVDase activity. The cleavage of C3 via calcium-dependent proteolysis is independent of caspase 9; lysate exposure to calcium prevents further cleavage and activation by the cytochrome *c* and dATP pathway. Altogether, our data suggest that calcium could favour a necrotic mechanism by inducing the generation of a form of C3 insensitive to mitochondrial activation.

Keywords: Caspase; Calcium; Apoptosis; Necrosis

1. Introduction

Calcium (Ca^{2+}) has been shown to play an essential role in some major central nervous system (CNS) diseases as it is involved in the degeneration and the death of neurones [1,2]. However, the actual mechanisms implicated in Ca^{2+}-induced apoptosis appear to be complex and involve several pathways including the activation of a family of proteases specifically involved in cell death programmes [1,2]. The relationship between caspases and Ca^{2+}-activated proteases, such as calpain, during the execution phase of apoptosis is an ongoing conundrum, which needs to be solved as considerable efforts are currently being made to develop highly specific inhibitors of calpain and caspase 3 (C3) for therapeutic applications. In many cases, an increase in the level of free intracellular [Ca^{2+}] in neurones results in the rapid activation of calpains, which leads to the degradation of several cytoskeletal and membrane proteins [1]. This Ca^{2+}-induced cell death, in combination with apoptosis, has been suggested to be involved in many physio-pathological situations in the CNS [1]. For example, many studies have shown that calpain, in conjunction with C3, was responsible for the cell death observed during trauma and/or ischemia [1]. However, the determination of the precise molecular pathways of the Ca^{2+}-induced cell death has been difficult to establish because of the contradictory results published on the consequence of calpain-induced caspase cleavage. For example, the cleavage by calpains has been shown to activate C3 in a hypoxia/ischemia model and to inhibit its activation in a Ca^{2+}-channel opener-treated cells [3,4]. Likewise, it has been reported that calpains could simultaneously inactivate C7 and C9 [5], while other studies have shown that calpains could induce the activation of C7 and C12 [6,7]. In addition to the cleavage of caspases, calpains have been shown to cleave several key apoptogenic proteins such as apoptotic protease activating factor-1 (Apaf-1) and Bax [8], suggesting that these proteases could also intervene in steps immediately preceding or following mitochondrial permeabilisation. All these results suggest that the nature of the induction in the rise of intracellular [Ca^{2+}] may favour either the activation or the inactivation of caspases. We have previously reported that the addition of high concentrations of Ca^{2+} to a cell extract was sufficient, via the induction of caspase activity, to trigger nuclear apoptosis in a cell-free assay and this independently of calpains [9]. We took profit of this simple experimental set up to study the effect of Ca^{2+} on the activation of caspases.

2. Materials and methods

2.1. Materials

Unless otherwise stated all reagents used in this study were from Sigma (St. Quentin, Fallavier, France). Commercial antibodies raised against C3 and C9 were from BDPharmingen (559565 and 556585, respectively). The fluorogenic peptides Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), a substrate for C3 and Ac-LEHD-AMC a substrate for C9 were obtained from Bachem (Budendorf, Switzerland). All cell culture material was obtained from Invitrogen (Cergy Pontoise, France).

2.1.1. Cell culture. The human neuroblastoma cell line SH-SY5Y was grown in DMEM supplemented with 10% heat-inactivated foetal calf serum and antibiotics, as described in [10]. The human promyelocytic leukaemia HL60 and HL60/ADR cell lines were grown as described previously [11]. Control cellular extracts (CCE) were obtained from SH-SY5Y, HL60 and HL60/ADR using a protocol similar to that described earlier for a rat glioma cell line [9].

2.2. Quantification of C3-like activity

CCE (250 μg) was incubated in the presence of the indicated concentrations of $CaCl_2$ or cytochrome *c* (cyt *c*). In the latter case, dATP (200 μM) was also added to CCE. The C3 or C9 activity was monitored by measuring the degradation of the fluorometric substrate Ac-DEVD-AMC or Ac-LEHD-AMC as previously described [9].

*Corresponding author. Fax: +33 240084082.
E-mail address: francois.vallette@univ-nantes.fr (F.M. Vallette).

Abbreviations: C3, caspase 3; pC3, procaspase 3; cyt *c*, holocytochrome *c*; tC3, truncated C3; CNS, central nervous system; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-AMC

0014-5793/$30.00
doi:10.1016/j.febslet.2005.02.079

2.3. Immunoblots and immunodepletion

Immunoblots were performed as described earlier [9]. For the immunodepletion of C3 or C9, 20 μg antibody was incubated at 4 °C for 24 h with 300 μg CCE. Then 30 μl Protein G–agarose (Sigma) was added and incubated for a further 24 h at 4 °C. The mixture was then centrifuged 10 min at 10000×*g* at 4 °C and the resulting cytosols adjusted to 250 μg prior to incubation with Ca^{2+} and/or cyt *c*/dATP. The presence and the processing of C3 and C9 were analysed by immunoblots. The depletion of C3 and C9 was verified in parallel by reprobing the membrane with the appropriate antibody. Note that these antibodies reacted with both the proenzyme and the active subunit.

3. Results

3.1. Comparative induction of DEVDase activity in non-apoptotic cell extracts by increasing the concentration of Ca^{2+} and cyt c

We have previously reported that a C3-like activity measured by the hydrolysis of the fluorometric peptide Ac-DEVD-AMC could be generated by the addition of Ca^{2+} to non-apoptotic cellular extracts (i.e., CCE) while the addition of low concentrations of cyt *c* (i.e., 2 μM) were ineffective [9]. We examined the dose-dependent induction of DEVDase activity using 250 μg CCE prepared from the human neuroblastoma cell line SH-SY5Y. As shown in Fig. 1A, a significant increase in DEVDase activity was observed only at Ca^{2+} concentrations superior to 100 μM ($P \leqslant 0.05$), peaked at 300 μM (3-fold increase; $P \leqslant 0.00$) and decreased at higher concentrations. Note that as described previously [9], the addition of Ca^{2+} had no effects on the DEVDase activity measured in cellular extracts obtained from apoptotic cells (data not shown).

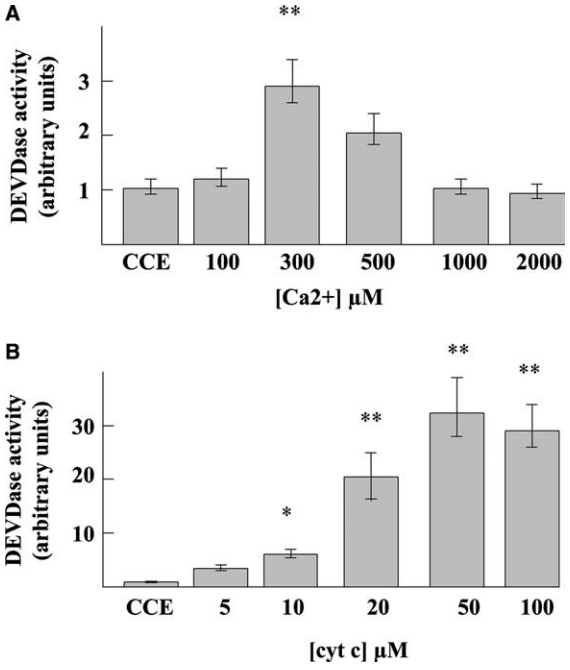

Fig. 1. Activation of DEVDase activity in CCE. The human neuroblastoma cell line, SHSY5Y, was cultivated in 10% FCS in RPMI at 37 °C, 5% CO_2 for 7 days before the preparation of the CCE (immunodepleted or not in C3) as described in Section 2. CCE (250 μg) were incubated for 1 h at 37 °C in the presence of increasing concentration of $CaCl_2$ (from 100 μM to 2 mM) (A) or for 20 min at 37 °C in the presence of 200 μM dATP and increasing concentrations of cyt *c* (from 5 to 100 μM) (B). At the end of the incubation the DEVDase activity was measured using the fluorogenic substrate Ac-DEVD-AMC as described in Section 2. The activity was normalised to 1 for the CCE incubated in the absence of Ca^{2+} and/or cyt *c*/dATP. Data shown are the mean of at least five independent experiments (means ± S.D.). $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$.

On the other hand, the addition of cyt *c* plus dATP (see Section 2), provoked a rapid and important increase in DEVDase activity at concentrations superior to 10 μM ($P \leqslant 0.05$) and attained a maximum at 50 μM showing a 30-fold increase (Fig. 1B). However, contrary to what we observed with Ca^{2+}, high concentrations of cyt *c* (i.e., $\geqslant$ 50 μM) were capable of inducing similar levels of DEVDase activity in these extracts (Fig. 1B). The dose/response induction of DEVDase activity suggested that the pathways involved in Ca^{2+}-induced DEVDase activity were different from those involved in cyt *c*-induced DEVDase activity.

3.2. Calcium-induced DEVDase activity cannot be further enhanced by cyt c

We examined the relationships between these two induction pathways of DEVDase activity by sequential activation of these activities in CCE derived from the human neuroblastoma cell line SH-SY5Y. We used concentrations of Ca^{2+} and cyt *c*, which generated the maximal DEVDase activities (i.e., 300 μM $CaCl_2$ and 50 μM cyt *c*). Quite spectacularly, pre-treatment of the CCE with Ca^{2+} inhibited the cyt *c*/dATP-induced activation of Ac-DEVD-AMC as represented in Fig. 2. As illustrated in Fig. 2, the DEVDase activity was maintained at a level similar to that observed with Ca^{2+} alone. Concurrently, the addition of 300 μM Ca^{2+} to a C3-depleted CCE (see Section 2) had no effect on the induction of DEVDase activity (Fig. 2), showing that this activity was due to C3 (Fig. 2).

Immunoblot analyses of these extracts showed that Ca^{2+} generated the cleavage of the 32 kDa procaspase 3 (pC3) into a p25 kDa band (Fig. 2A) which we called truncated C3 (tC3). At concentrations of Ca^{2+} superior to 300 μM the tC3 progressively disappeared to be completely degraded at high concentration (i.e., 600 μM) (Fig. 2B). Of note, an important amount of undegraded p32 C3 was still observed even at high calcium concentrations (Fig. 2B), which was not activated by cyt *c*/dATP (data not shown). When CCE were treated with cyt *c* and dATP, two bands of, respectively, 20 and 17 kDa were observed (Fig. 3A). This data is consistent with previous results showing the activated forms of C3 detected during apoptosis [12,13]. In agreement with the DEVDase activities reported in Fig. 2, the pre-treatment of CCE with Ca^{2+} prior to the addition of cyt *c*/dATP did not result in the appearance of the p20 and p17 forms of C3 but to that of tC3 (Fig. 3A).

It has been reported that calpain mediated a N-terminal truncation of C9, which could activate C3 [5,14]. Analysis of C9 processing in CCE incubated with Ca^{2+} and/or cyt *c*/dATP revealed that both treatments generated the proteolysis of C9 into the mature p37 kDa form (Fig. 3B). In order to determine the importance of this cleavage in both Ca^{2+} and cyt *c*/dATP activation of C3, CCE were depleted of C9 by immunoprecipitation using an anti-C9 antibody (see Section 2). Immunodepletion was verified by immunoblotting of the immunodepleted CCE with anti-C9 antibodies (data not shown). C9-immunodepleted CCE was then subjected to Ca^{2+} and/or cyt *c*/dATP treatment before immunoblot analysis of C3 cleavage. As shown in Fig. 3C, the immunodepletion of

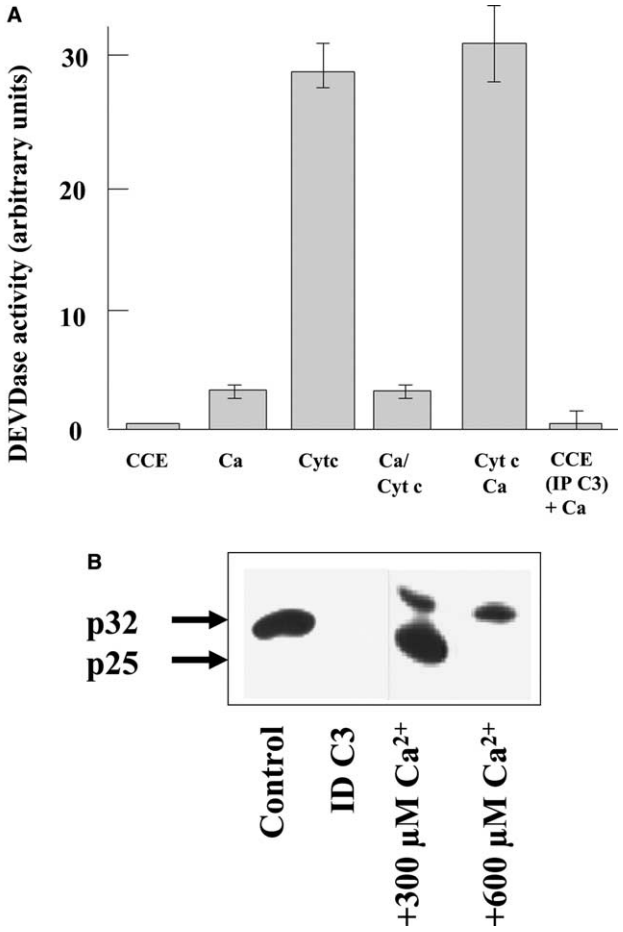

Fig. 2. Effect of Ca^{2+} pre-treatment on cyt *c*/dATP induction of DEVDase activity in CCE. CCE (250 µg) were incubated for 1 h at 37 °C in the presence of 300 µM $CaCl_2$ (Ca) or 20 min at 37 °C in the presence of 50 µM cyt *c* plus 200 µM dATP (cyt *c*) before determining the DEVDase activity (A) and immunoblot analysis of the presence of C3 (B). In some case, CCE were immunodepleted using 5 µl of the polyclonal antibody directed against human C3 (see Section 2). CCE were also incubated with $CaCl_2$ prior to the incubation with cyt *c*/dATP (Ca/cyt *c*) or with cyt *c*/dATP prior to incubation with $CaCl_2$ (cyt *c*/Ca). The DEVDase activity was quantified as in Fig. 1.

C9 did not affect Ca^{2+}-induced C3 cleavage while, as expected, it prevented that of cyt *c*/dATP. This result showed that C9 was not involved in the Ca^{2+}-induced DEVDase activity.

These results suggest that Ca^{2+}-induced proteolysis triggered a direct aberrant processing of tC3, which could not be further processed into the active C3 subunits (i.e., p20 and p17).

3.3. Mutations in the N-terminus of the p20 subunit of C3 prevent its activation by Ca^{2+} and by cyt c

We have extended our observation to CCE obtained from the HL60 cell line. As shown in Fig. 4, upon incubation with 300 µM $CaCl_2$, pC3 was degraded in a tC3 form which corresponds to a p29 kDa cleavage product of C3. This cleavage was accompanied by a 2-fold increase in DEVDase activity ($P \leqslant 0.05$) (Fig. 4). The addition cyt *c*/dATP induced a 20-fold increase in the DEVDase activity and the appearance of 2 major polypeptides of, respectively, p20 and p17 kDa in the CCE (Fig. 4), as observed in the CCE of SH-SY5Y.

The incubation of HL60 CCE with Ca^{2+} prior to the addition of cyt c inhibited the induction of DEVDase activity

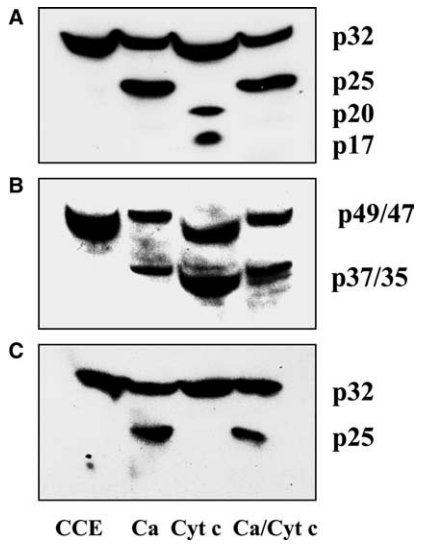

Fig. 3. Cleavage of C3 and C9 upon Ca^{2+} and/or cyt *c*/dATP treatments. Immunoblots were performed as described in Section 2. CCE (250 µg) were analysed using an antibody directed against C3 (A) or C9 (B) at a 1:1000 dilution. CCE were treated as in Fig. 2 prior to SDS–PAGE and immunoblot. Immunoblots illustrated are representative of five independent experiments. (C) CCE immunodepleted in C9 still respond to Ca^{2+} but not to cyt *c*/dATP. CCE were immunodepleted using 5 µl of the polyclonal antibody directed against human C9 (see Section 2). The processing of C3 was analysed by immunoblots as in A. Immunoblot illustrated is representative of five independent experiments. The depletion of C9 was verified in parallel by reprobing the membrane with the anti-C9 antibody (data not shown).

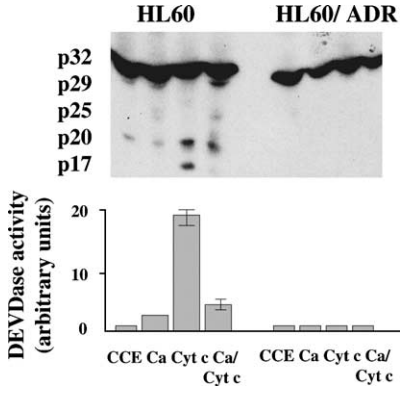

Fig. 4. The role of Ca^{2+} in the activation of C3 in HL60 and in HL60/ADR cells: CCE were prepared form the human leukaemia cell lines as described in Section 2 and were treated with Ca^{2+} and/or cyt *c* then DEVDase activities and the processing of C3 were performed as in Figs. 2 and 3. As with SH-SY5Y CCE, Ca^{2+} (Ca) induced a weak but significant increase in DEVDase activity in HL60 CCE. This was paralleled by the processing of C3 into tC3 with a molecular weight of 29 kDa, which could not be further activated by the addition of cyt *c*/dATP (cyt *c*). Similar experiments performed with CCE derived from HL60/ADR showed no activation of C3 by Ca^{2+} or by cyt *c*. Immunoblots showed are representative of at least three independent experiments. DEVDase activity (means ± S.D.) was calculated from three different experiments.

and that of the p17 kDa C3 subunit but only partially that of the p20 kDa form of C3. These results suggest that although Ca^{2+} produced a similar activation/inhibition of the DEVDase activity, the proteolytic processing of the pC3 involved differed from that observed in SH-SY5Y.

We have recently described a mutant of C3, present in a subcell line HL60 resistant to adrianomycin (HL60/ADR) [11]. This mutant, which we called C3-NTM, is modified in the region encompassing amino acids 30–36 with the sequence GISLDNS in wild type C3 (i.e., in HL60) and MSWDTG in C3NTM (i.e., in HL60/ADR). We have shown that these mutations impair the activation of C3 in HL60/ADR upon induction of apoptosis [11]. As illustrated in Fig. 4, neither the addition of cyt *c* nor that of Ca^{2+} induced the proteolytic activation of C3NTM, which suggests that the mutated amino acids are involved in both activating processes.

4. Discussion

The cross-talk between the Ca^{2+} and the caspase proteolytic pathways during apoptosis has been extensively studied especially in the CNS. This cross talk appears to involve many pathways but it is not known if calcium participates in a continuum or in a switch between the apoptotic and the necrotic pathways. Conflictual reports have been published on the relationship between calpain and caspases during cell death probably due to the multiplicity of cellular targets of these proteases and the different experimental conditions used in these studies. We have shown that the addition of Ca^{2+} to cellular extracts at concentration compatible with that observed during ischemia/reperfusion [15] induced a weak but significant activation of C3. This activation was sufficient to trigger caspase-dependent nuclear apoptosis in a cell-free assay [9]. We have re-evaluated this process in the present study and have found that this apparent activation leads to the generation of a form of C3 with a molecular weight of 25 kDa in SH-SY5Y and 29 kDa in HL60, which we called tC3 for truncated C3. This difference in the size of the fragment generated by Ca^{2+} in the different cell lines suggests the implication of distinct proteases which are still to be determined. Studies with the C3NTM allowed us to identify a region, which is involved in both Ca^{2+}- and cyt *c* activation of pC3 in HL60. The mechanisms by which this sequence regulates both Ca^{2+} and cyt *c* activation of pC3 are still unclear and probably differ from one cell line to another one. It should be noted, that a large proportion of pC3 appears to be resistant to cyt *c* and Ca^{2+}-induced activation (see the proportion of the p32 kDa band remaining after cyt *c* or Ca^{2+} treatments in Figs. 2–4). This raises the question of a possible action of calcium on other components of the apoptosome such as apaf-1 as previously described [16]. Indeed, we observed a degradation of apaf-1 in cell lysates treated with calcium (data not shown) which thus could make impossible the activation of pC3 not cleaved by the calcium related process. We also show that the Ca^{2+}-induced C3 activity is independent of the apoptosome as it does not require the apical C9 as shown by the immunodepletion experiments (Fig. 3). McGinnis et al. [16] have recently reported that maitotoxin, a potent Ca^{2+}-channel opener, induces cyt *c* release from apoptosis in SH-SY5Y which was not followed by caspases activation. Consistent with our results, pC3 appeared under theses conditions to undergo a non-conventional cleavage [16].

Taken together, our results suggest that calpain or another Ca^{2+}-induced protease can partially activate pC3 in a cellular extract but this processing precludes the apoptotic activation generated by the mitochondria. The amount of caspase required for the completion of apoptosis diverges from one cell to another and/or from one apoptotic inducer to another, presumably because of the presence in the cytosol of XIAPs [17]. Thus, one can postulate that Ca^{2+} can induce cell death under conditions, which requires low caspase activity but that it becomes an inhibitor when higher C3 activities are necessary. This feature could be related to a paradoxical role of caspases in neuroprotection and CNS remodelling [18]. However, the concentration of Ca^{2+} necessary to "activate" C3 is more compatible with necrosis than apoptosis. One can thus postulate that under low Ca^{2+} concentrations, cyt *c* can activate C3 and that, once a high level of Ca^{2+} is reached, its activation by the mitochondrial pathway is inhibited. Interestingly, the degradation of Apaf-1, another member of the apoptosome has been shown to be induced by Ca^{2+}-activated proteases [19]. Taken together, these results suggest that degradation of Apaf-1 and C3 by Ca^{2+} prevent cyt *c* activation of C3 during certain types of Ca^{2+}-mediated cell death and also could explain the mixed apoptotic/necrotic features observed in certain types of pathological cell death.

Acknowledgements: We thank Dr. Babajko (INSERM U515) for the gift of the SH-SY5Y cell line. This work was supported by grants by the INSERM and ARC. M.P. was a recipient of an ARC fellowship.

References

[1] Mattson, M.P. and Chan, S.L. (2003) Calcium orchestrates apoptosis. Nat. Cell Biol. 5, 1041–1043.
[2] Mattson, M.P., Duan, W., Pedersen, W.A. and Culmsee, C. (2001) Neurodegenerative disorders and ischemic brain diseases. Apoptosis 6, 69–81.
[3] Blomgren, K., Zhu, C., Wang, X., Karlsson, J.-O., Leverin, A.-L., Bahr, B.A., Mallard, C. and Hagberg, H. (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia. A mechanism of "pathological apoptosis"?. J. Biol. Chem. 276, 10191–10198.
[4] McGinnis, K.M., Gnegy, M.E., Park, Y.H., Mukerjee, N. and Wang, K.K.W. (1999) Procaspase-3 and poly (ADP)ribose polymerase (PARP) are calpain substrates. Biochem. Biophys. Res. Commun. 263, 94–99.
[5] Chua, B.T., Guo, K. and Li, P. (2000) Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. J. Biol. Chem. 275, 5131–5135.
[6] Ruiz-Vela, A., Gonzalez de Buitrago, G. and Martinez-A, C. (1999) Implication of calpain in caspase activation during B cell clonal deletion. EMBO J. 18, 4988–4998.
[7] Nakagawa, T. and Yuan, J. (2000) Cross-talk between two cysteine protease families: activation of caspase-12 by calpain in apoptosis. J. Cell Biol. 150, 887–894.
[8] Fischer, U., Janicke, R.U. and Schulze-Osthoff, K. (2003) Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. 10, 76–100.
[9] Juin, P., Pelletier, M., Oliver, L., Tremblais, K., Gregoire, M., Meflah, K. and Vallette, F.M. (1998) Induction of a caspase-3-like activity by calcium in normal cytosolic extracts triggers nuclear apoptosis in a cell-free system. J. Biol. Chem. 273, 17559–17564.
[10] Chambery, D., de Galle, B. and Babajko, S. (1998) Retinoic acid stimulates IGF binding protein (IGFBP)-6 and depresses IGFBP-2 and IGFBP-4 in SK-N-SH human neuroblastoma cells. J. Endocrinol. 159, 227–232.
[11] Pelletier, M., Cartron, P.F., Delaval, F., Meflah, K., Vallette, F.M. and Oliver, L. (2004) Caspase 3 activation is controlled by a sequence located in the N-terminus of its large subunit. Biochem. Biophys. Res. Commun. 316, 93–99.
[12] Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M. and Lazebnik, Y.A., et al. (1995) Identification and inhibition of

the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.
[13] Han, Z., Hendrickson, E.A., Bremner, T.A. and Wyche, J.H. (1997) A sequential two step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. J. Biol. Chem. 272, 13432–13436.
[14] Lankiewicz, S., Marc Luetjens, C., Truc Bui, N., Krohn, A.J., Poppe, M., Cole, G.M., Saido, T.C. and Prehn, J.H. (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J. Biol. Chem. 275, 17064–17071.
[15] Siesjo, B. and Bengtsson, F. (1989) Calcium fluxes, calcium antagonists, and calcium related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. J. Cereb. Blood Flow Metab. 9, 127–140.
[16] McGinnis, K.M., Gnegy, M.E., Falk, N., Nath, R. and Wang, K.K.W. (2003) Cytochrome *c* translocation does not lead to caspase activation in maitotoxin-treated SH-SY5Y neuroblastoma cells. Neurochem. Int. 42, 517–523.
[17] Huang, Y., Lu, M. and Wu, H. (2004) Antagonizing XIAP-mediated caspase-3 inhibition. Achilles' heel of cancers?. Cancer Cell. 5, 1–2.
[18] McLaughlin, B. (2004) The kinder side of killer proteases: caspase activation contributes to neuroprotection and CNS remodeling. Apoptosis 9, 111–121.
[19] Reimertz, C., Kogel, D., Lankiewicz, S., Poppe, M. and Prehn, J.H. (2001) Ca(2+)-induced inhibition of apoptosis in human SH-SY5Y neuroblastoma cells: degradation of apoptotic protease activating factor-1 (APAF-1). J. Neurochem. 78, 1256–1266.