Differential degradation of calpastatin by μ- and m-calpain in Ca^{2+}-enriched human neuroblastoma LAN-5 cells

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Abstract In neuroblastoma LAN-5 cells during calpain activation, in addition to the two expressed 70 kDa and 30 kDa calpastatin forms, other inhibitory species are produced, having molecular masses of 50 kDa and 15 kDa. At longer times of incubation, both native and new calpastatin species disappear. The formation of these new calpastatins as well as the decrease in intracellular total calpastatin activity are mediated by calpain itself, as indicated by the effect of the synthetic calpain inhibitor I, which prevents both degradative processes. Analysis of the calcium concentrations required for the two processes indicates that the first conservative proteolytic event is mediated by μ-calpain, whereas the second one is preferentially carried out by m-calpain. The appearance of the 15 kDa form, containing only the calpastatin repetitive inhibitory domain and identified also in red cells of hypertensive rats as the major inhibitor form, can be considered a marker of intracellular calpain activation, and it can be used for the monitoring of the involvement of calpain in pathological situations.

Key words: Calpastatin degradation; μ-Calpain; m-Calpain; LAN-5 cell

1. Introduction

Calpain belongs to a family of thiol-proteinases having an absolute requirement for Ca^{2+} ions [1–3]. Although many calpain isoforms have been identified, those most relevant seem to be the ubiquitary isozymes called μ-calpain and m-calpain, requiring low and high calcium concentrations, respectively. These proteinases are present in all mammalian tissues and are involved in a variety of processes including cell proliferation [4,5], differentiation [6–8], vesicle secretion and others [9,10].

Both calpain isozymes reside in the cell cytosol in an inactive conformation that is converted into the active one by an intramolecular process triggered by the binding of calcium ions to specific sites localised in the calmodulin-like domain. The initial Ca^{2+}-induced conformational change promotes both the organisation of essential amino acidic residues at the active site and the removal of a segment from its N-terminal region, a process occurring in two autoproteolytic steps. The digested calpain form requires much less Ca^{2+}, as compared to the native form. This activation process is favoured by phospholipids [11,12], activator proteins [13,14] and metabolites [15]. The negative natural modulator is a protein called calpastatin [1–3]. This protein is characterised by a peculiar molecular structure [16] having four repetitive inhibitory domains and the N-terminal domain (L-domain) whose function has not yet been defined. In rat brain, multiple calpastatin forms are constitutively expressed; the more represented species are a 70 kDa form containing in addition to L-domain four repetitive domains, and a truncated form (~30 kDa) having just one inhibitory domain [17–20]. More recently, we have observed that in various cellular types calpastatin is aggregated in a specific cell region and is released from its association by an increase in intracellular $[Ca^{2+}]$ [21]. Hence, the same signal inducing calpain activation is also involved in a redistribution of calpastatin, a process required for the formation of the enzyme–inhibitor complex with the native as well as the activated calpain forms.

Furthermore, calpastatin can be digested by calpains [22–24]. Evidence in favour of this specific proteolysis has been collected both in vitro and in vivo experiments. In reconstructed systems, it has been demonstrated that μ-calpain slowly digests calpastatin, without loss of inhibitory efficiency. On the contrary, m-calpain produces a more dramatic digestion of calpastatin, yielding inactive fragments [24]. A loss of calpastatin activity has been reported in different pathological conditions, including essential hypertension both in humans [25] and in genetic hypertensive rats of Milan strain [26,27]. In this disease, it has been postulated an intracellular increase in Ca^{2+} leading to cellular changes, including cell shape, and volume [28]. However, in patients treated for long times with anti-hypertensive drugs, together with the normalisation of arterial blood pressure, an increase in calpastatin to control levels has been detected [29]. These data suggest that a proteolytic process is responsible for these changes.

We have now analysed, at the molecular level, calpastatin degradation processes occurring in neuronal cells following an increase in intracellular $[Ca^{2+}]$. Characterisation of those processes leading to calpastatin fragmentation and inactivation may be relevant for the identification of the involvement of calpain also in the triggering of pathological conditions.

2. Materials and methods

2.1. Materials

Human neuroblastoma LAN-5 cells were cultured as reported previously [30]. Ca^{2+}-ionophore A23187, leupeptin and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma Chem. Co. Calpain inhibitor I [31] was purchased from Boehringer Mannheim. Calpain inhibitor I was found to be equally effective in inhibiting both μ- or m-calpain isolated from rat brain. μ-Calpain and m-calpain were purified from rat brain, as previously described [24].

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2.2. Preparation of homogenates from LAN-5 cells

LAN-5 cells were cultured in RPMI medium containing 15% foetal calf serum in 75 cm^2 flasks. Confluent cells were exposed to 0.25 μM Ca^{2+}-ionophore A23187 for different times of incubation or in the presence of different Ca^{2+} concentrations. If not specifically indicated, the experiments were performed in RPMI medium, which contains 0.42 mM Ca^{2+}, without any Ca^{2+} addition. After incubation, cells were rapidly washed three times with phosphate saline buffer, and harvested with 3 ml of 50 mM sodium borate buffer pH 7.5 containing 0.1 mM EGTA and 0.5 mM β-mercaptoethanol (buffer A). Cell suspensions were sonicated (four bursts of 10 s each) in the presence of 0.1 mg/ml leupeptin and 2 mM PMSF. The particulate material was discarded by centrifugation at 100 000×*g* for 15 min. Finally, the clear solution was heated for 3 min at 90°C, centrifuged and the supernatant (heated homogenate) was used as indicated.

2.3. Preparation of homogenates from erythrocytes of normotensive and hypertensive rats

Erythrocytes from normotensive and hypertensive rats were lysed in three volumes of buffer A containing 0.1 mg/ml leupeptin and 2 mM PMSF and centrifuged at 100 000×*g* for 15 min. The supernatants were heated at 90°C for 3 min, centrifuged at 100 000×*g* for 15 min and the soluble materials were submitted to sodium dodecyl sulfate (SDS)–PAGE as described below.

2.4. Identification of calpastatin species on SDS–PAGE

Aliquots (500 μg) of heated homogenates prepared from LAN-5 or rat erythrocytes (see above) were submitted to 10% SDS–PAGE. At the end of the electrophoretic run, the gel was washed to remove SDS, and then cut in 0.3 cm slices [24]. Each slice was extracted with 0.3 ml of buffer A for three times and the solutions obtained from each sample were pooled. This procedure recovers almost completely the protein loaded into the gel. Finally, calpastatin activity was assayed on 200 μl of the recovered solutions using human erythrocyte calpain, being this enzyme highly sensitive to native and post-translational modified calpastatins [24]. Consequently, the inhibition measured in these conditions reflects the actual amounts of calpastatin present in the assay mixture. One unit of calpastatin is defined as the amount required to inhibit one unit of enzyme activity. One unit of enzyme activity is defined as the amount that releases 1 μmol/h of free α-amino groups under the specified conditions.

2.5. Radioimmunoassay of calpastatin on SDS–PAGE slices

Aliquots of the solution obtained following SDS–PAGE extraction were used to determine calpastatin concentration with a solid-phase radioimmunoassay [32]. A 96 well plate was coated with 100 μl of each sample of extracted material and was incubated overnight at 4°C. The solution was removed, and the wells were blocked with 5% skim milk powder dissolved in phosphate saline buffer. After three washings, 100 μl of monoclonal antibody (mAb) 35.23 specific for the L-region of calpastatin [20] was added to each well and incubated for 2 h. After three washes with phosphate saline buffer, an anti-mouse iodinated antibody was used to detect the formation of calpastatin–mAb complex.

2.6. N-Terminal sequence analysis of 15 kDa calpastatin

SDS–PAGE-extracted 15 kDa calpastatin was lyophilised, suspended in water containing 20% acetonitrile and loaded on the Beckman LF 3000 protein sequencer to determine primary structure.

3. Results

To identify the in vivo modifications of calpastatin which occur following calpain activation, we have used as model LAN-5 neuroblastoma cells, because neuronal cells seem highly sensitive to damages induced by the calcium-dependent proteolytic system.

As shown in Fig. 1, following addition of A23187 to the cell medium, total calpastatin activity, directly assayed on lysates previously heated to inactivate calpain, increases 1.3–1.4-fold during the first 30 min of incubation. At longer times, it progressively decreases, reaching values corresponding to 40% of the original total activity, at the end of the incubation (120 min). Addition of the synthetic calpain inhibitor I [31] prevents almost completely the disappearance of calpastatin activity, but it has little or no appreciable effect on the increase of inhibitory activity observed at shorter times of incubation. It can be speculated that the biphasic behaviour of calpastatin is the result of two sequential processes generating first free inhibitory domains [24] followed by a non-specific degradation which completely inactivates the inhibitor protein.

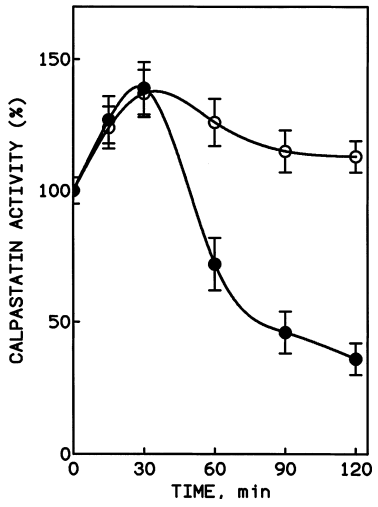

Fig. 1. Effect of calcium loading in LAN-5 cells on levels of calpastatin activity. LAN-5 cells were preincubated for 1 h at 37°C in the absence (filled circles) or in the presence of 50 μM calpain inhibitor I (unfilled circles). The cells were then treated with 0.25 μM Ca^{2+}-ionophore for the indicated times. After incubation, heated homogenates were prepared as described in Section 2. Before the assay of calpastatin activity, an aliquot (0.5 ml) of heated homogenates was rapidly filtered through a Superdex G25 column (1×10 cm) in order to remove the synthetic low M_r proteinases inhibitors. Protein was collected in a single fraction (approximately 2.5 ml) and calpastatin activity was then assayed as described in Section 2 using human erythrocyte calpain.

To better explore the possible in vivo calpain-mediated proteolysis of calpastatin, we have analysed calpastatin molecular species present in LAN-5 cells, by submitting the heated homogenates to SDS–PAGE. As shown in Fig. 2A, in untreated LAN-5 cells, two major peaks of calpastatin activity were detected. The first one has a molecular weight of approximately 70–75 kDa and the second one of approximately 30 kDa. Both forms are recognised in Western blot by an anti-calpastatin antibody raised against the L-domain of rat brain calpastatin (Fig. 2A, inset). Based on the electrophoretic mobility, and on the recognition by anti-calpastatin antibody, the first calpastatin form corresponds to the native entire calpastatin molecule and the second form is similar to a 30 kDa calpastatin form, which is expressed in rat brain [20] and consisting of the L-domain and a single inhibitory domain. Preincubation of LAN-5 cells with the synthetic calpain inhibitor I (Fig. 2A, unfilled circles) has no effect on the nature and on the levels of calpastatin, indicating that these forms are constitutively expressed also in these cells. Following 30 min of incubation in the presence of ionophore A23187 (Fig. 2B), calpastatin is now recovered in four major peaks. Two of these forms (70 kDa and 30 kDa) correspond to those expressed in untreated cells; the other two (50 kDa and 15 kDa) are new species presumably resulting from the deg-

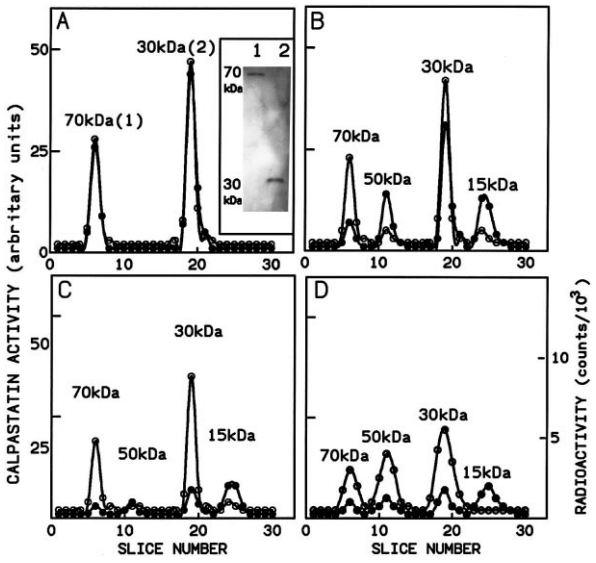

Fig. 2. Identification of calpastatin species in LAN-5 cells. A: LAN-5 were incubated for 1 h in the absence (filled circles) or in the presence of 50 μM synthetic calpain inhibitor I (unfilled circles), then heated homogenates were prepared as described in Section 2. Calpastatin species were separated by SDS–PAGE and their activity was assayed using 200 μl of extracted protein as described in Section 2. Inset: The two peaks of calpastatin activity extracted from the gel were collected and separately submitted to another SDS–PAGE. A Western blot analysis was performed to detect calpastatin protein using mAb 35.23. B and C: Identification of calpastatin species in calcium-enriched cells. Cells incubated as in A were exposed to 0.25 μM Ca^{2+}-ionophore A23187 for 30 min or 120 min, respectively, then lysed. Calpastatin species were separated and assayed as in A. Filled circles indicate the incubations performed in the absence of calpain inhibitor, unfilled circles those containing calpain inhibitor I. D: Immunoidentification of calpastatin species in calcium-enriched cells. Aliquots (100 μl) of the material extracted from each gel slice obtained in C were submitted to a solid radioimmunoassay using anti-calpastatin mAb 35.23 (see Section 2). Unfilled symbols refer to radioactivity recovered in each well, filled symbols to calpastatin activity.

radation of the native forms. This hypothesis is supported by the effect of the synthetic calpain inhibitor I, which, once added to cell incubation medium, prevents almost completely the appearance of both 50 kDa and 15 kDa fragments. At longer times of incubation (Fig. 2C), the four calpastatin forms are still present although in significantly lower amounts; in these conditions, more than 60–65% of calpastatin is inactivated. Again, addition of synthetic calpain inhibitor I largely prevents the disappearance of native calpastatin forms. To better characterise these degraded fragments, we have analysed their ability to interact with the anti-calpastatin antibody directed against their calpastatin L-domain [21] (Fig. 2D). The first three calpastatin forms are recognised quantitatively by a solid-phase radioimmunoassay, indicating that these fragments contain the N-terminal domain and a different number of repetitive inhibitory domains. However, the form having the lowest molecular mass (15 kDa) is unable to interact with the antibody and corresponds to a free repetitive inhibitory domain.

Further molecular analysis of this SDS–PAGE-extracted 15 kDa calpastatin form on FPLC, carried out by gel filtration on a Superdex 75 column, has revealed that together with a 15 kDa protein some other fragments with lower mass are

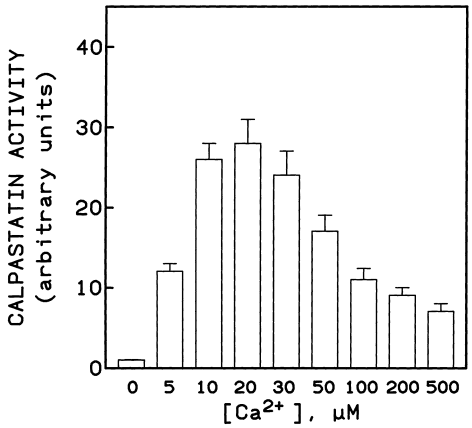

Fig. 3. Accumulation of intracellular 15 kDa calpastatin form as a function of increasing Ca^{2+} concentrations. LAN-5 cells were incubated as described in Section 2 for 120 min at 37°C in the presence of 0.25 μM ionophore and the indicated [Ca^{2+}]. [Ca^{2+}] was established by the addition to the medium of appropriate amounts of EGTA [38]. Each sample was processed as reported in Section 2 and in Fig. 2. Calpastatin activity was measured using human erythrocyte calpain.

present (data not shown). The N-terminal amino acid sequence, carried out on this peptide mixture, resulted to be: Pro-Asp/Glu-Pro. This sequence is repeated three times in rat brain calpastatin [20] and the sizes of the putative fragments formed are in agreement with the molecular masses of the active calpastatin fragments obtained from LAN-5 cells. This selective cleavage of calpastatin shows a functional significance, since the isolated free inhibitory domain displays a from 4- to 7-fold higher inhibitory efficiency against both homologous μ- and m-calpains, suggesting the degradation process is involved also in an amplification of the cell inhibitory capacity.

In order to establish if the in vivo biphasic behaviour of calpastatin digestion is due to the action of one or the other calpain isozyme, we have analysed the formation and the disappearance of the 15 kDa fragment in the presence of increasing Ca^{2+} (Fig. 3). At [Ca^{2+}] between zero and 20 μM, an

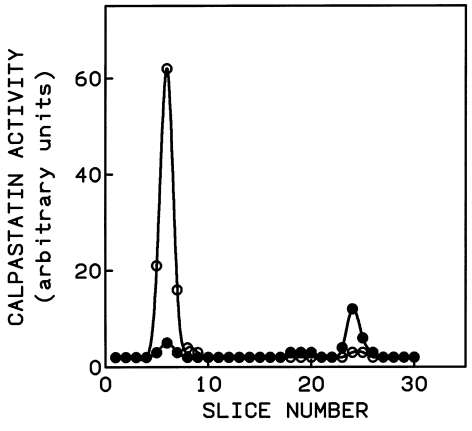

Fig. 4. Identification of calpastatin species in erythrocytes of normotensive and hypertensive rats. Heated homogenates prepared from erythrocytes of normotensive and hypertensive rats were submitted to SDS–PAGE and after the electrophoretic run, the gel was processed as described in Section 2. Filled circles: hypertensive rats; unfilled circles: normotensive rats.

increase in the amount of the 15 kDa fragment is observed, whereas, as expected, at [Ca^{2+}] higher than 20 μM, a progressive loss of this low molecular weight calpastatin form occurs. Altogether, these observations indicate that the two phases of calpastatin degradation process are mediated by different calpain isozymes. Specifically, μ-calpain is active at lower [Ca^{2+}] and is preferentially involved in the generation of the 15 kDa fragment, whereas m-calpain, requiring much higher [Ca^{2+}], is involved in a non-specific calpastatin digestion which causes the loss of inhibitory activity.

In vivo fragmentation of calpastatin has been identified also in red cells of hypertensive rats. As shown in Fig. 4, when calpastatin from erythrocyte lysates of normotensive rats is analysed in SDS–PAGE as above, a single calpastatin form has been detected showing a migration corresponding to a molecular mass of approximately 75 kDa. However, in cells from hypertensive rats, the native 75 kDa calpastatin is largely reduced and only partially replaced by 10–15 kDa still active calpastatin fragments. These data indicate that conservative fragmentation and degradation of calpastatin occur in cells in which calcium concentration increases for longer times, as it happens in erythrocytes of these hypertensive animals.

4. Discussion

In this paper, we demonstrate that degradation of calpastatin, previously observed in vitro [22–24], occurs in vivo following a prolonged activation of calpains. In the early phase, the process seems to be operating in order to promote an amplification of the inhibitory effect of calpastatin, required to block all activated calpain molecules generated in these conditions. This salvage is produced by the preferential action of μ-calpain, which both in vitro and in vivo conservatively degrades calpastatin promoting the formation of free calpastatin repetitive domains. The amplification process derives not only from an increase in the number of polypeptides showing calpain inhibitory activity, but also by the fact that the isolated, naturally generated repetitive fragments are more active than the native entire calpastatin molecule.

The second phase, in which calpastatin is further and completely degraded, is carried out by m-calpain and occurs at higher [Ca^{2+}].

The direct involvement of calpain in this digestion is supported by previously in vitro experiments and also by the in vivo effect of the synthetic calpain inhibitor I, which prevents both fragmentation and digestion of calpastatin. In vivo degradation of calpastatin by calpain is also reported in apoptotic cells [33] although no evidence is provided on the formation of free inhibitory domains during this process. The existence on calpastatin molecules of sites specifically recognised by proteinases has also been demonstrated exposing in vitro native calpastatin to thrombin, which produces a fragmentation similar to that observed with μ-calpain (data not shown), or with caspase which promotes the degradation of calpastatin during cell apoptosis [34,35]. Calpain-mediated calpastatin degradation is also reported in post-mortem bovine muscle [36]. This digestive process could be interpreted as an amplification of the inhibitory capacity in the cells, being a single calpastatin molecule able to produce more than one free inhibitory domain.

The conservative degradation of calpastatin occurs only at low [Ca^{2+}], indicating that μ-calpain is preferentially involved in this process. The non-specific digestion accompanied by loss of inhibitory activity requires longer times of incubation or high [Ca^{2+}], suggesting the involvement of m-calpain, as indicated also by the effect of these proteinases in in vitro experiments [37].

Furthermore, fragmentation and degradation of calpastatin has been detected in red cells of hypertensive rats, which contain a calpain form particularly effective in degrading calpastatin [24]. Disappearance of calpastatin activity requiring large increases in [Ca^{2+}] or during longer times of incubation seems related to the triggering of the 'pathological role of calpains', a situation in which the regulatory machinery and the proteinase component of the calcium-dependent proteolytic system become unbalanced. The easier calpain activation occurring after calpastatin degradation seems involved in the production of cell damages such as cytoskeleton degradation, ion pump digestion, changes in shape and volume of the cells, identified in different pathological conditions. Hence, the identification of 15 kDa calpastatin fragment may characterise conditions preceding the onset of this pathological role of calpain and may be important to establish other situations in which the proteinase can produce cell damages.

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