Properties of calpastatin forms in rat brain

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Abstract Four recombinant calpastatin forms, deduced from rat brain mRNAs and differing in the number of inhibitory repetitive domains from zero to four, were expressed and characterized for their inhibitory efficiency on μ- and *m*-calpain. Although the most effective one is a truncated calpastatin form composed of the N-terminal region (domain L) and a single inhibitory domain, all inhibitors are more active against μ-calpain, but are preferentially degraded and inactivated by *m*-calpain. The protein form composed exclusively of a domain L is deprived of any inhibitory activity but prevents inhibition of calpain by the other calpastatin forms, indicating that this calpastatin region could be relevant in the recognition of the proteinase. A calpastatin form having molecular properties similar to those of the recombinant truncated calpastatin, has also been found in rat brain. It does not derive from proteolysis of a higher molecular mass precursor. The expression of multiple calpastatin forms may be relevant for the specific modulation of the different calpain isozymes normally present in a single cell type.

Key words: Calpastatin; Calpain; Rat brain

1. Introduction

Intracellular calpain activity can be regulated by a natural protein inhibitor, named calpastatin [1–5]. The primary structure of calpastatin, deduced from rat liver cDNA [1], indicates that it is composed of five domains. The first, named L, is located at the N-terminal region of the molecule; the other four domains, characterized by a high degree of sequence homology, include the consensus sequence for calpain [6].

Recently we have demonstrated that rat brain contains five mRNAs for calpastatin, named RNCAST104, RNCAST103, RNCAST107, RNCAST23 and RNCAST110, which code proteins differing in length and in exon composition [7]. In contrast to the rat liver form, all rat brain calpastatins contain at the N-terminal region (domain L) an additional amino acid sequence deriving from exon 4 and two of these forms (RNCAST104 and RNCAST110 proteins) also contain a sequence derived from exon 6, which is absent in the liver protein. Three of them (RNCAST104, RNCAST103 and RNCAST107 proteins) contain four repetitive domains, whereas RNCAST23 protein is composed of a single inhibitory domain. The fifth mRNA (RNCAST110) codes a single putative domain L without inhibitory domains. The other major differences among these forms are the number and type of sites for post-translational modification, due, as indicated above, to the absence or presence of exons 4 and 6. It has been reported that in different tissues and mammalian species domain L of calpastatin is composed of conserved regions and variable peptide sequences resulting from alternative splicing [8]. However, a functional role for this domain has not been identified previously. The different regions that characterize the various rat brain calpastatin forms contain specific amino acid sequences susceptible of phosphorylation by protein kinase C together with PEST sequences that have been suggested to be correlated to the intracellular half-life of proteins [9].

In this paper, we describe the properties of recombinant rat brain calpastatins, expressed in *E. coli*, measuring their inhibitory activity and susceptibility to proteolytic degradation by μ- and *m*-calpain. We also propose a possible function for the truncated protein molecule containing exclusively the N-terminal region of calpastatin named domain L. Low-M_r calpastatin forms could be produced in the cell by proteolytic degradation of a high-M_r precursor or by expression of a mutated transcript containing an upstream stop codon. Here we explore the possibility that the calpain inhibitory activity found in rat brain and showing low molecular mass, as compared to the 'normal' calpastatins containing five domains, derives from the expression of a truncated form of calpastatin.

2. Materials and methods

2.1. Preparation of rat brain homogenate

Freshly collected rat brain was suspended in 3 volumes of 0.25 M sucrose, containing 1 mM EDTA, 1 mM 2-mercaptoethanol, proteinase inhibitors (E64, 0.1 mg/ml; pepstatin, 100 μM; antipain, 10 μM; phenylmethylsulfonyl fluoride, 1 mM; leupeptin, 100 μg/ml and calpain inhibitor I, 50 μM) and disrupted by using a Potter Elvehjem homogenizer. The particulate material was discarded by centrifugation at 10 000×*g* for 20 min; the clear supernatant was collected and immediately heated at 90°C for 3 min. All denatured proteins were removed by centrifugation.

2.2. Purification and assay of calpains and calpastatins

μ-Calpain and *m*-calpain were purified from rat brain and assayed as previously described [10]. One unit of enzyme activity is defined as the amount that releases 1 μmol/h of free α-amino groups under the specified conditions.

Calpastatin was assayed as previously described [11]. One unit of calpastatin activity is defined as the amount required to inhibit 1 unit of calpain activity.

2.3. Expression of calpastatins as fusion proteins in E. coli

Five cDNAs for calpastatin were obtained from rat brain mRNA by RT-PCR as reported previously [7]. The cDNA fragments coding for RNCAST104, RNCAST107, RNCAST110 and RNCAST23 calpastatins were amplified by using the sense primer 5′-TGGGATCCATGAGTACCACAGGAGCTAAGG, containing a *Bam*HI restriction site at the 5′ end and the antisense primer 5′-GCGAATTCAAAAGTCACCATCCACCAGC, containing an *Eco*RI restriction

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Abbreviations: GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction

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site at the 5′ end. Each insert was ligated into an *Eco*RI/*Bam*HI cut pGEX2T expression vector (Amersham-Pharmacia Biotech.) and *E. coli* TOP 10F′ cells were transfected by electroporation (Bio-Rad Gene Pulser). Recombinant plasmids were then sequenced by the dideoxynucleotide chain termination method [12] from both strands. Expression and purification of GST-calpastatins were carried out according to instructions from the manufacturer. Where indicated, GST-calpastatins were cleaved with thrombin [13] followed by isolation of recombinant calpastatins by affinity chromatography on glutathione-Sepharose columns.

2.4. Preparation of the anti-RNCAST23 monoclonal antibody (mAb)

To produce monoclonal antibodies against RNCAST23 calpastatin, GST-RNCAST23 fusion protein was coupled with hemocyanin [14], emulsified with complete Freund's adjuvant and injected intraperitoneally into mice. Two booster injections were administered at 10-day intervals; the third injection was done using isolated RNCAST23 calpastatin as an antigen. Three days after the last immunization the animals' spleen was harvested. All subsequent operations were carried out as reported [15]. Clones with the highest capacity to produce anti-RNCAST23 mAb were identified with a solid-phase radioimmunoassay, as previously described [16].

3. Results

To define the functional properties of the putative calpastatin proteins coded by mRNAs identified previously in rat brain [7], we expressed four of them in *E. coli* cells. Specifically, RNCAST104, RNCAST107, RNCAST23 and RNCAST110 were expressed, whereas RNCAST103 was not expressed due to its high similarity to the other two high M_r calpastatin forms. The recombinant proteins were recovered as fusion proteins with glutathione *S*-transferase (GST) and calpastatins were then isolated by digestion of the fusion protein with thrombin and further purified to homogeneity. The resulting protein solutions were analyzed in SDS-PAGE (Fig. 1) and, although the calculated molecular mass of the different calpastatin forms was lower than that shown on SDS-PAGE as reported previously [1], the differences in the apparent molecular mass were consistent with the length of the coding region of the cDNAs utilized for cell transfection.

In fact, RNCAST104 and RNCAST107 proteins showed the highest molecular mass, RNCAST23 (low M_r form)

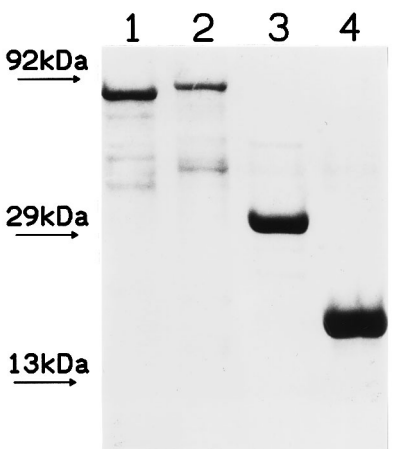

Fig. 1. SDS-PAGE of purified recombinant calpastatins. Recombinant calpastatins were expressed and purified as described in Section 2. Samples (3–7 μg) were submitted to 9% SDS-PAGE and Coomassie-stained. The arrows indicate the positions of molecular weight markers (glycogen phosphorylase, 92 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 13 kDa). Lane 1: RNCAST107; lane 2: RNCAST104; lane 3: RNCAST23; lane 4: RNCAST110.

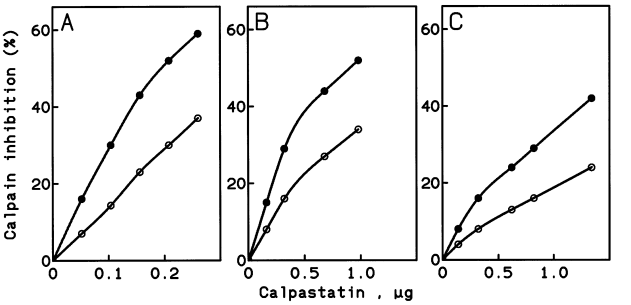

Fig. 2. Inhibitory efficiency of recombinant calpastatins on homologous μ-calpain and *m*-calpain. Purified rat brain μ-calpain (●) or *m*-calpain (○) were incubated with the indicated amounts of the different calpastatins in the standard conditions described in Section 2. A, B and C refer to RNCAST23, RNCAST104 and RNCAST107, respectively.

showed an M_r of approximately 30 kDa, and RNCAST110 an M_r of approximately 15 kDa.

These calpastatin forms were then tested for their inhibitory efficiency on homologous μ- and *m*-calpain. As shown in Fig. 2A, the RNCAST23 calpastatin was the more efficient form in inhibiting both proteinases, although with a higher effectiveness on μ-calpain. RNCAST104 and RNCAST107 (Fig. 2B,C) were also more active on μ-calpain, whereas RNCAST110, the form deprived of inhibitory domains, was completely inefficient against both calpain forms (data not shown). At molar basis, RNCAST23 calpastatin was 2.78 or 3.95 times more active as compared to RNCAST104 and RNCAST107, respectively.

In order to verify the hypothesis that domain L may be involved in the recognition of the calpain molecule by calpastatin, we measured the effect of RNCAST110 protein on the inhibitory efficiency of the active calpastatin forms. As shown in Fig. 3A, addition of RNCAST110 protein resulted in a progressive decrease of the inhibition promoted by RNCAST104, RNCAST107 and RNCAST23 on μ-calpain; 65–75% of the inhibitory activity was abolished at the highest concentrations of RNCAST110 protein used. The effect on *m*-calpain was much less pronounced (Fig. 3B), indicating that RNCAST110 has a higher affinity for μ-calpain. Although such a protein has not yet been identified in any tissue, it can be postulated that its expression may be required to reduce the actual level of calpain inhibitor activity exerted by the excess of calpastatin that has been reported to be normally present in the cell [17].

It is known that calpastatin from different sources can be degraded by calpain isoforms and that *m*-calpains can cause inactivation of the inhibitor following the formation of the enzyme-inhibitor complex [11]. We then analyzed if the active recombinant calpastatins were equally sensitive to degradation by homologous calpains. As shown in Fig. 4A, μ-calpain was ineffective in promoting inactivation of the recombinant calpastatin forms. On the contrary, exposure to *m*-calpain promoted a progressive inactivation of the calpastatin forms; approximately 50% of the original inhibitory activity of all three calpastatins was lost within one hour.

To establish if multiple calpastatin forms were expressed in rat brain, crude extracts from this tissue were prepared in the presence of a mixture of protease inhibitors, immediatly heated at 90°C to avoid, as much as possible, the proteolysis of native calpastatin and submitted to SDS-PAGE. The gel

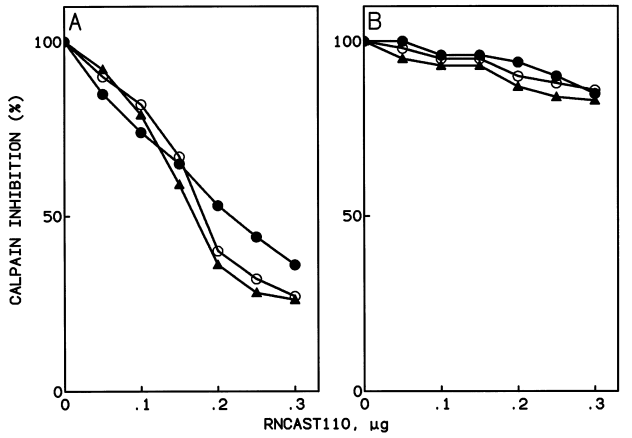

Fig. 3. Effect of RNCAST110 recombinant protein on the inhibition of μ-calpain and *m*-calpain by RNCAST107, RNCAST104 and RNCAST23 calpastatins. Purified rat brain μ-calpain (A) or *m*-calpain (B) were incubated, as described in Section 2, in the presence of purified RNCAST23 (●), RNCAST104 (○) or RNCAST107 (▲) calpastatin in an amount capable to promote 50% of proteinase inhibition. The indicated quantities of RNCAST110 protein were also added to the incubation mixtures. The calpain inhibition is expressed as percentage of the original value, measured in the absence of RNCAST110 protein.

was then cut in 2-mm slices, proteins were eluted from each slice and assayed for the presence of calpastatin activity. Two peaks of calpain inhibitor activity were identified (Fig. 5), the first one showing a molecular mass identical to that of the high-M_r recombinant calpastatins (RNCAST104 or RNCAST107), the second one having the mass of the low-M_r recombinant calpastatin RNCAST23. Identical results were obtained using different tissue extracting media, supplemented with a variety of protease inhibitors with or without the heat step (data not shown), indicating that a low-M_r calpain inhibitor is constitutively expressed in rat brain. To establish the molecular nature of this calpain inhibitor form, its reactivity with an anti-RNCAST23 monoclonal antibody was assayed in a solid phase radioimmunoassay. As shown in Fig. 5, a peak of immunoreactivity, showing an electrophoretic migration corresponding to that of the recombinant RNCAST23 calpastatin, was detected, indicating a high degree of similarity between the form coded by the RNCAST23 transcript and the low-M_r calpastatin identified in rat brain.

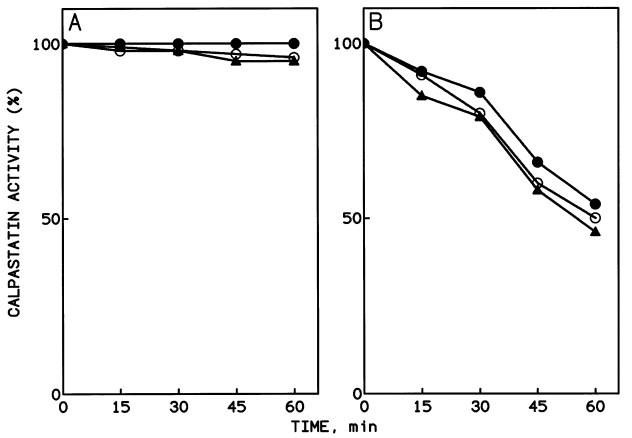

Fig. 4. Inactivation of calpastatins by μ-calpain and *m*-calpain. Rat brain μ-calpain (A) or *m*-calpain (B) were incubated with equimolar amounts of recombinant calpastatins in the conditions specified previously [10]. At the indicated times, the incubation mixtures were heated at 90°C for 2 min to inactivate calpain and the residual activities of RNCAST23 (●), RNCAST104 (○), RNCAST107 (▲) calpastatins were assayed as described in Section 2.

4. Discussion

Evidence is found that the calpain family, in addition to the ubiquitous forms, is composed of many other members including tissue specific calpains [18]. Recently, genomic sequences coding for calpain-like proteins, lacking the calmodulin domain, have also been identified [19,20]. It is difficult to conceive that regulation of multiple calpain forms, with different molecular characteristics and specificity, could be accomplished by a single intracellular protein inhibitor form, namely calpastatin. The presence of different partial transcripts for calpastatin has been described in several mammalian tissues, indicating that multiple forms of the natural protein calpain inhibitor, produced by alternative splicing or derived from mutations [8,21], could provide specific calpain inhibitory equipment of the cell. Moreover, post-translational modifications [10] could also be involved in modulating each calpastatin form in both efficiency and calpain target selectivity. We have recently reported the identification of five mRNAs in rat brain, coding calpastatin forms having different primary structures in the N-terminal domain, followed by a number of inhibitory repeats from zero to four [7].

The proteins corresponding to rat brain calpastatin mRNAs have now been expressed in *E. coli* and characterized at a functional level. It seems particularly relevant that the more efficient form is the calpastatin containing a single in-

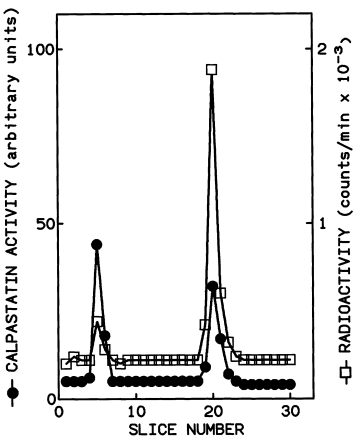

Fig. 5. Calpastatin forms present in rat brain. An aliquot of rat brain homogenate, prepared as described in Section 2 and corresponding to 50 μg of protein, was submitted to SDS-PAGE. After the electrophoretic run, the gel was washed twice (5 min each) in 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol and 20% methanol, to remove the excess of SDS, and a third time with the same buffer solution, without methanol. The gel was then cut into 0.2-cm slices which were homogenized in 0.3 ml of the last gel washing buffer. The gel was removed by centrifugation and the clear supernatants were collected. In order to obtain a complete protein elution the extraction procedures were repeated three times. Finally the solutions obtained from a single slice were combined and aliquots were used for the assay of calpastatin activity on μ-calpain (●) or for the presence of calpastatin immunoreactive molecules by a solid-phase radioimmunoassay, using a monoclonal antibody directed against RNCAST23 recombinant protein (□).

hibitory domain (low-M_r form) as compared to those containing the four repeats (high-M_r forms). These findings suggest that only one inhibitory domain is operating with high efficiency in each calpastatin molecule, the other repeats being silent or much less active. Moreover, the physical and chemical parameters, evaluated with the PHYSCHEM program on each recombinant calpastatin, reveal that the active calpain inhibitors can be considered stable proteins, showing a comparable instability index of approximately 37–48 [22], whereas the RNCAST110 protein, that contains only the N-terminal region of calpastatin and lacks inhibitory domains and has an instability index over 60, is predicted as potentially instable.

The competition found between the RNCAST110 protein and the other recombinant calpastatins may provide new suggestions concerning the mechanism of formation of the enzyme-inhibitor complex, as well as the possible role of this moiety of the calpastatin molecule in the modulation of its inhibitory activity. The highest specificity of this calpastatin N-terminal fragment for μ-calpain is in agreement with the observation that the inhibitory efficiency of all the rat brain recombinant calpastatins is lower on *m*-calpain which is, on the contrary, the form that promotes proteolytic inactivation of all calpastatin isoforms.

Digestion and inactivation of calpastatin by *m*-calpain could be related to a postulated 'pathological function' of calpain, resulting from an indiscriminate activation of the proteinase facilitated by a decrease in the intracellular level of calpastatin [23]. A number of reports suggest that calpain is involved in many pathological disorders [5,24,25]. A multiplicity of calpastatin forms may be required to maintain proper control on the catalytic activity of different calpain isoforms expressed by cells and hence preventing the onset of irreversible functional damages. We have reported previously that in rat skeletal muscle [26] and in rat brain [10] a phosphorylation/dephosphorylation process is involved in the modulation of calpastatin efficiency and selectivity. The identification of multiple calpastatin mRNAs in rat brain and the evidence that at least two of these forms are expressed represents a further amplification of the number of the calpastatin types that could be produced in a single cell, resulting in a more accurate regulation of proteinase activity.

It will be of interest to establish if multiple forms of calpastatin are also present in other tissues and if the question whether the expression of the calpastatin forms is correlated to a specific function of calpain played a role in these tissues.

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