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Modulation of rat brain calpastatin efficiency by post-translational modifications

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Abstract Calpains, the thiol proteinases of the calcium-dependent proteolytic system, are regulated by a natural inhibitor, calpastatin, which is present in brain tissue in two forms. Although both calpastatins are highly active on human erythrocyte calpain, only one form shows a high inhibitory efficiency with both rat brain calpain isozymes. The second calpastatin form is almost completely inactive against homologous proteinases and can be converted into an active one by exposure to a phosphoprotein phosphatase, also isolated from rat brain. Phosphorylation of the active calpastatin by protein kinase C and protein kinase A promotes a decrease in its inhibitory efficiency. The interconversion between the two inhibitor forms seems involved in the adjustment of the level of intracellular calpastatin activity on specific cell requirements.

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Key words: Brain; Proteolysis; Regulation; Calpain; Calpastatin; Phosphorylation-dephosphorylation

1. Introduction

All mammalian cells contain a Ca^{2+}-dependent proteolytic system, composed by one or more soluble thiol proteinase isoforms, named calpains, and by a specific calpain inhibitor, calpastatin [1–7]. Two calpain isozymes (μ-calpain and *m*-calpain) are well known forms identified in many cells and tissues, such as muscle, liver, kidney and brain [1–4,6]. Recently, the concept of a tissue-specific calpain form has also emerged [8], arising from the discovery of a 93 kDa calpain isozyme, exclusively localised in muscle cells. This proteinase form, however, was not well characterised, due to its very short life, also in transfected cells [7,8]. A function for this proteinase form in muscle cells is suggested by the correlation observed between the lack of its expression and a type of muscular dystrophy [9].

Regulation of calpain activity in the cells is accomplished by two factors; the first one is the $[Ca^{2+}]$, which must be increased, and the second one is due to the presence of calpastatin which controls translocation of calpain to plasma membrane and thereby the activation process of the proteinase [1–3,7].

The composition of rat brain Ca^{2+}-dependent proteolytic system is similar to that of the other tissues [4,10–14], containing both classical μ-calpain and *m*-calpain and their inhibitor, calpastatin. The functions of calpains in brain cells are particularly relevant on vesicle secretion at synaptic terminations and on long-term potentiation by degradation of cytoskeletal and neurofilament proteins, including calspectin, a spectrin-like molecule [10,12,14].

A number of laboratories are collecting evidences supporting the idea that calpains are involved in the aspecific degradation of intracellular proteins and structures and brain seems particularly susceptible to this proteolysis [for a review, see [15]]. From the digestion patterns obtained in a variety of experimental conditions, it has been postulated that calpain-induced cell damages can occur also in some pathological conditions [16–21] and particularly in postischemic tissues [10,12,14,22–25]. This hypothesis is further supported by the protective effects shown in vitro conditions by synthetic proteinase inhibitors [22,23].

Due to the fact that the Ca^{2+}-dependent proteolytic system also contains regulatory proteins, such as calpastatin and a calpain activator protein [3,26], expression of intracellular catalytic activity by these proteinases must take in account the level and the efficiency of these accessory proteins.

In this paper we studied the mechanism of regulation of this system in brain cells and identified two calpastatin forms with different inhibitory efficiency. The two forms are produced by phosphorylation–dephosphorylation process of a single protein. In addition, rat brain active calpastatin is very sensitive to degradation by *m*-calpain. Alteration of these processes could be responsible for the modification of the regulatory system, promoting abnormal intracellular activation of calpain.

2. Materials and methods

2.1. Materials

Human erythrocyte calpain was purified to homogeneity as previously described [28]. Histone III S, phosphatidylserine, HEPES and dioleylglycerol were purchased from Sigma Chemical Co. (USA), [γ-^{32}P]ATP from Amersham, UK.

2.2. Purification of calpains from rat brain

Freshly collected brains from five rats (10 g) were suspended in 5 volumes of 0.25 M sucrose containing 1 mM EGTA and 0.5 mM β-mercaptoethanol and disrupted by using a Potter Elvehjem homogenizer. Cells were lysed by sonication (4 bursts of 10 s each) and the particulate material was discarded by centrifugation at $100\,000\times g$ for 20 min. The clear supernatant (homogenate) was collected and loaded onto a DE-53 column (10 ml) equilibrated in 50 mM sodium acetate buffer, pH 6.7, containing 0.1 mM EGTA and 0.5 mM β-mercaptoethanol (Buffer A). The absorbed proteins were eluted by a salt gradient from 0 to 0.35 M NaCl. The peaks containing calpain activities were separately collected, concentrated, dialyzed against 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EGTA, 0.5 mM

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Abbreviations: PKA, cAMP-dependent protein kinase; PKC, Ca^{2+}-phospholipid-dependent protein kinase

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β-mercaptoethanol (Buffer B) and 0.3 M NaCl, and loaded onto a Phenyl Sepharose CL 4B column (1×10 cm) equilibrated in the same dialysis buffer. This hydrophobic chromatography is crucial to remove any residual calpastatin activity which is not retained by the resin. The fractions containing the calpain activity were concentrated by ultrafiltration and loaded onto a Sephadex G-200 column (2×120 cm) equilibrated in Buffer B.

2.3. Purification of calpains from rat skeletal muscle

μ-Calpain and *m*-calpain were purified from rat skeletal muscle following the procedure described in [27].

2.4. Purification of calpastatins from rat brain

Rat brain homogenates were prepared as described for calpains purification, then heated at 90°C for 3 min. The separation of the calpastatin forms has been achieved setting a very reproducible procedure using a 5-ml column packed with DE-cellulose, equilibrated in Buffer A, in a BioRad FPLC system. The absorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.35 M, then 0.6 M NaCl was applied. Calpastatins were further purified following the procedure previously described for skeletal muscle calpastatins [27].

2.5. Assay of μ-calpain, m-calpain and calpastatin activities

Calpains were routinely assayed as previously reported [27]. One unit of enzyme activity is the amount that releases 1 μmol/h of free α-amino groups under the specified assay conditions using acid-denaturated bovin globin as substrate.

Calpastatin activity was routinely assayed as reported in [27] using human erythrocyte calpain, which has been shown to be the calpain isoform most sensitive to calpastatin inhibition.

2.6. Purification of brain phosphatase

Rat brain homogenates were prepared in Buffer A containing 50 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, as described for calpains purification. The suspensions were centrifuged and applied on a DE-52 column (1×10 cm) equilibrated in the same buffer. The absorbed proteins were eluted by a salt gradient from 0 to 0.4 M NaCl. The fractions containing phosphatase activity were collected, concentrated to 2 ml by ultrafiltration and loaded on a Sephadex G200 column previously equilibrated in 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA. The eluted fractions containing the phosphatase activity were collected and used. The procedure resulted in a purification of approximately 1150 fold.

2.7. Preparation of [^{32}P]histone

Histone III S (1 mg) was incubated in 1 ml of sodium borate, pH 7.5, containing 10 μM [^{32}P]ATP (5×10^7 counts/min), 5 mM $MgCl_2$, 50 μg of phosphatidylserine, 1 μg dioleylglycerol and 50 U of purified PKC. The mixture was incubated at 30°C for 30 min and loaded on a Sephadex G100 (2×100 cm) previously equilibrated with 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA. The fractions containing [^{32}P]histone were collected, concentrated to 1 ml and the resulting solution used as source of labelled histone (10^7 counts/min/mg).

2.8. Assay of phosphoprotein phosphatase

The enzyme was assayed using [^{32}P]histone as substrate by the procedure described in [29] and modified as follows. The mixture contained, in a final volume of 0.25 ml of 50 mM sodium acetate, pH 6.0, [^{32}P]histone III S (50 000 counts/min) and the appropriate amount of enzyme. One unit of phosphatase activity was defined as the amount causing the release of 1 nmol of phosphate groups from [^{32}P]histone in 30 min at 37°C, in the specified conditions.

2.9. Isolation of rat brain PKA and PKC

Rat brains (10 g) were homogenized in 4 volumes of 10 mM HEPES, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA and 10 mM β-mercaptoethanol. The soluble fraction was recovered following centrifugation at 100 000×*g* for 15 min and submitted to ion exchange chromatography on DE-cellulose as previously reported [30]. The eluted fractions were assayed for PKA and for PKC activity [30]. The fractions containing PKA (52–73) or PKC activity (79–137) were pooled and submitted to affinity chromatography on threonine–Sepharose, as described in [31]. The fractions containing the protein kinase activities were collected, concentrated to 2 ml and then loaded onto a Sephadex G200 column (1.2×12 cm) equilibrated in 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA. The fractions containing protein kinase activities were pooled, concentrated and used as source of PKA or of PKC. One unit of protein-kinase activity was defined as the amount causing the incorporation of 1 nmol of ^{32}P into histone type III S, in the conditions specified in [30].

3. Results

3.1. Separation of calpastatin from rat brain

Rat brain extracts were submitted to ion exchange chromatography, in mild acid conditions, using a 5 ml column, containing DE-53 resin. The eluted calpastatin activity was assayed using human erythrocyte calpain, previously shown to be very sensitive to inhibition by calpastatin, independently from the source of the inhibitor. As shown in Fig. 1A, calpastatin activity, present in rat brain extracts, was fractionated in two distinct peaks, the first one emerging at approximately 0.09–0.1 M NaCl and the second one at 0.2 M NaCl. The presence of two peaks almost equally represented was not due to the concomitant elution of calpain; in fact, the same pattern has been obtained with homogenates previously heated at 90° for 3 min, a treatment which completely inactivated calpain activity (data not shown). The fractions containing the two peaks of inhibitory activity, named calpastatin I and II, respectively, were separately collected and these proteins were further purified, accordingly to the procedure described in Section 2. In all these steps, calpastatin activity was routinely assayed using the human erythrocyte calpain.

3.2. Inhibitory efficiency of rat brain calpastatins

The efficiency of the two calpastatin forms was then tested on μ- and *m*-calpain, also purified from rat brain (Fig. 1B,C). Calpastatin I inhibited both calpain isoforms (Fig. 1B), showing a slightly higher efficiency for the μ-isozyme. Calpastatin II, however, expressed very poor inhibitory activity against both brain proteinases (Fig. 1C), although it was able to inhibit the human erythrocyte calpain, with an efficiency comparable to that of calpastatin I. To verify that these differences in activity between the calpastatin I and II are not due to artifacts of the experimental procedures, the two inhibitors were also tested on μ- and *m*-calpain purified from rat skeletal muscle [27]. The results obtained were very similar to those shown in Fig. 1B,C (data not shown), indicating that calpastatin II is a low active protein inhibitor probably representing a reservoir to be utilized in specific cell conditions.

3.3. Effect of a rat brain phosphoprotein phosphatase on the properties of rat brain calpastatins

This hypothesis implies the possibility that calpastatin II might be converted in an active form, similar to calpastatin I. This idea is supported by the evidence that, by exposing calpastatin II to the action of a phosphoprotein phosphatase also isolated from rat brain, the chromatographic properties of this inhibitor form were modified (Fig. 2A), becoming similar to that of calpastatin I. In parallel, following the treatment with the phosphatase, a large increase in inhibitory efficiency has been observed (Fig. 2B). With this procedure, calpastatin II has been converted into a form showing molecular and functional properties similar to those of calpastatin I. Exposure of calpastatin I to the same treatment had no effect on its molecular or inhibitory properties (data not shown). Taken together, these data suggest the existence of an inter-

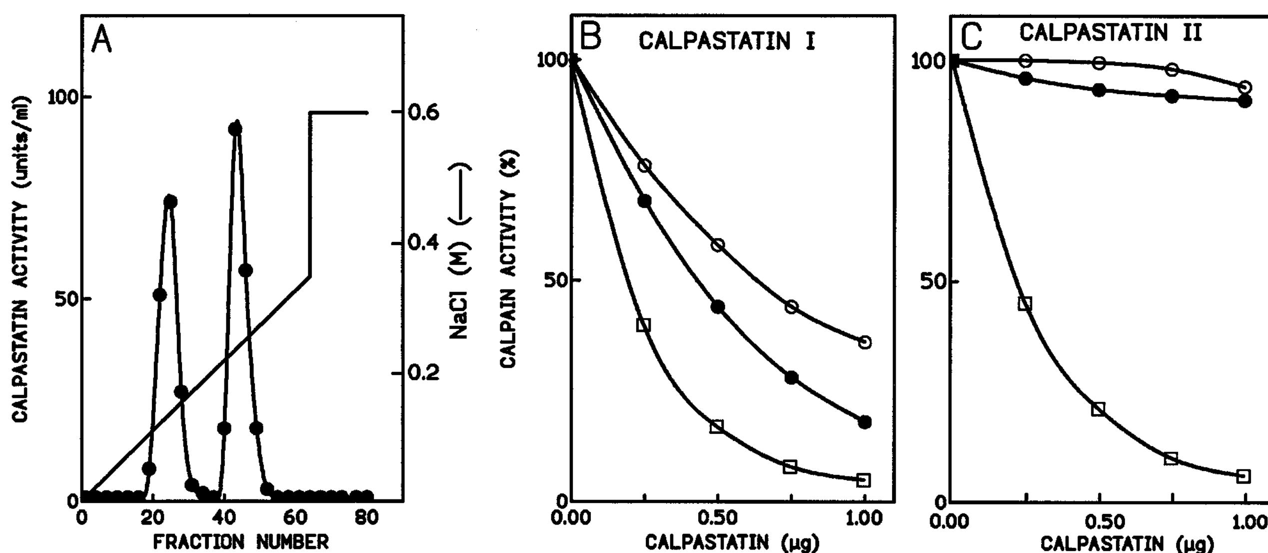

Fig. 1. Properties of the two calpastatin forms from rat brain. A: Rat brain homogenate was fractionated by ion-exchange chromatography, as described in Section 2. The absorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.35 M, and then the column was washed with the same buffer containing 0.6 M NaCl. The eluted fractions were assayed for calpastatin activity using human erythrocytes calpain, in the conditions specified in Section 2. B,C: The separated calpastatin I and II forms were further purified, as described in Section 2. Rat brain μ-calpain (○), and *m*-calpain (●) were then assayed in the presence of the indicated amounts of purified calpastatin I or calpastatin II (see Section 2). The results are expressed as percentage of the calpain activity measured in the absence of any inhibitor. For comparison, inhibition of human erythrocyte calpain by the two calpastatins, determined in the same conditions, is also shown (□).

conversion process between the two calpastatins, promoted by a dephosphorylation–phosphorylation process.

3.4. Phosphorylation of rat brain calpastatin I

To establish if calpastatin I could be phosphorylated, we have isolated the two major rat brain protein kinases, PKA and PKC. Calpastatin I was then incubated with these purified kinases for 30 min and the mixtures were submitted to a SDS-PAGE. At the end of the electrophoretic runs, SDS was removed and the gels were then cut in 2 mm slices. Each slice was collected and the proteins were eluted in 50 mM borate buffer, pH 7.5, containing 0.1 mM EDTA, following mechan-

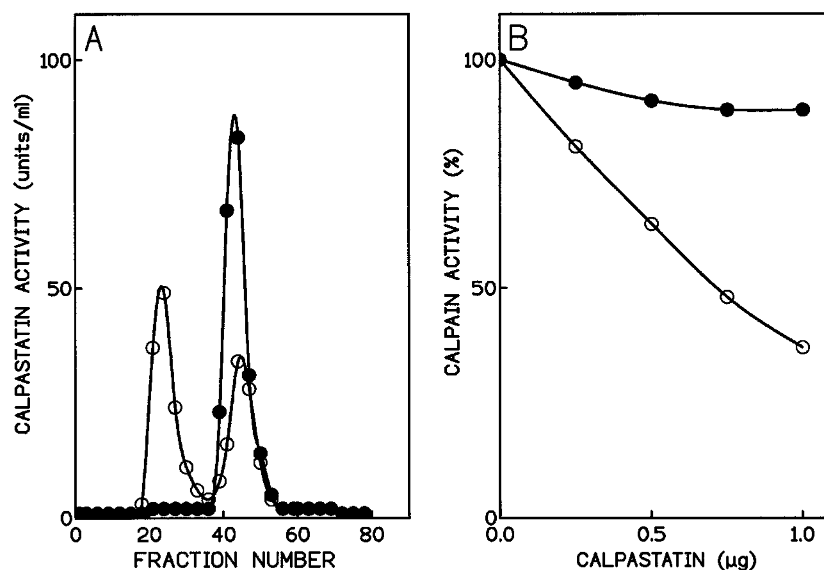

Fig. 2. Change in properties of calpastatin II following treatment with brain phosphoprotein phosphatase. A: Calpastatin II was isolated from rat brain as described in Section 2 and in the legend to Fig. 1A. The phosphoprotein phosphatase was purified from rat brain following the procedure described in Section 2. Calpastatin II was incubated with rat brain phosphoprotein phosphatase in sodium acetate buffer, pH 6.0, at 37°C for 30 min, then the reaction was stopped by heating at 90°C for 3 min. The solution was submitted to DE-53 chromatography in the same conditions used in Fig. 1A. The elution profile of untreated calpastatin II (●) and that of phosphatase-treated calpastatin II (○) are shown. B: The inhibitory efficiency of untreated calpastatin II (●) and of phosphatase-treated calpastatin II (○) were assayed using purified rat brain *m*-calpain, in the conditions specified in Section 2.

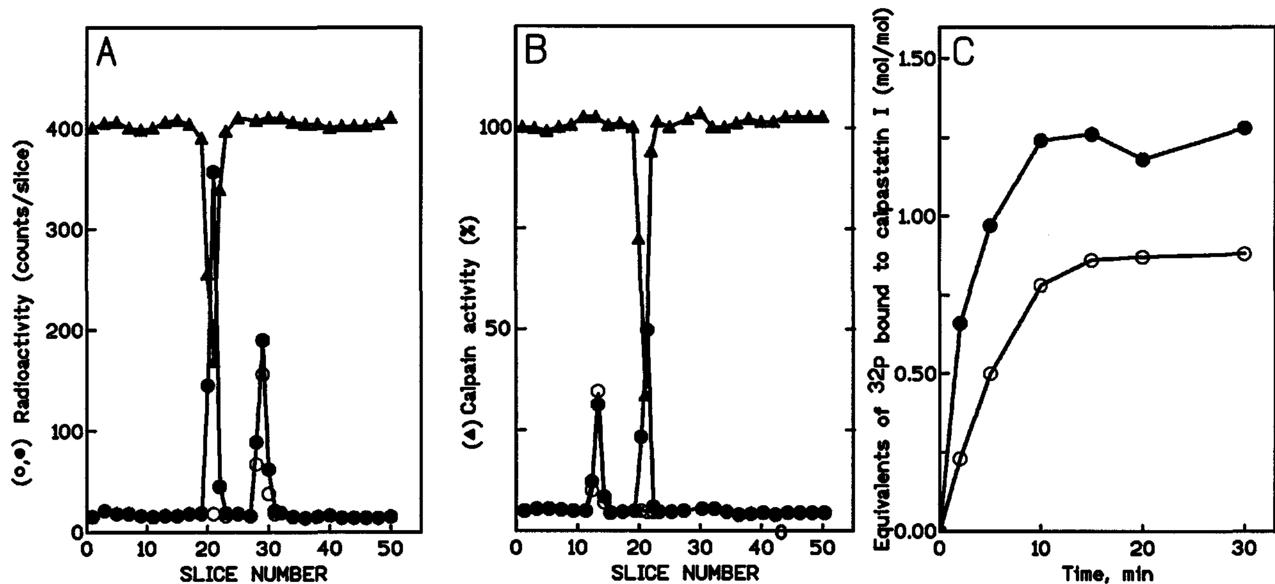

Fig. 3. Phosphorylation of calpastatin I with PKA or PKC. Calpastatin I was incubated with purified PKA in the presence of cAMP and [γ^{32}P]ATP (A) or with PKC in the presence of phospholipids and diacylglycerol (B), as described in Section 2. To the incubations, the same volume of a solution containing 4% SDS and 10 mM β-mercaptoethanol (final concentrations) was added, and the mixtures heated at 100°C and submitted to SDS-PAGE. At the end of the electrophoretic run, the gel was removed, washed with 50 mM borate buffer, pH 7.5, containing 20% methanol and 5 mM β-mercaptoethanol and cut in 2 mm slices. The protein present in each slice was eluted as described in Section 2 and the resulting solutions were divided in two aliquots; the first one was used for the determination of the radioactivity content (●) and the second one for the measurements of the inhibitory capacity on human erythrocyte calpain activity (▲). As control, protein kinases were incubated as above without calpastatin and the mixtures were submitted to SDS-PAGE. The gels were cut in 2 mm slices and the radioactivity was determined (○). C: Calpastatin I was incubated with purified PKC (●) or PKA (○) as above and, at the times indicated, aliquots of the incubation mixtures were collected, heated at 90°C for 2 min to inactivate the kinases and centrifuged at 50000×g. To the clear solutions, Ca^{2+} ions were added to a final concentration of 1 mM and the mixtures loaded onto 1-ml column filled with Sepharose-immobilized carboxymethyled human erythrocyte calpain [27] and equilibrated with 50 mM sodium borate buffer, pH 7.5, containing 1 mM Ca^{2+}. The column was washed with 3-column-volume of the same buffer and the retained calpastatin was eluted substituting in the buffer the Ca^{2+} ions with 1 mM EDTA. The eluted protein, the calpastatin activity and the associated radioactivity were determined as described in Section 2. The incorporation of phosphate groups into calpastatin was expressed as mol of ^{32}P per mol of inhibitor.

ical disruption of the gel samples. The clear solutions were collected and divided into two aliquots; the first one was counted, and the second one was used to assay calpastatin activity. As shown in Fig. 3A, under the peak of calpastatin activity, a peak of radioactivity was also detected, indicating that calpastatin I was phosphorylated. Both PKC (Fig. 3A) and PKA (Fig. 3B) are capable to phosphorylate calpastatin in in vitro conditions. Incubation of calpastatin with PKC promotes the incorporation of 1.2–1.3 equivalents of ^{32}P per calpastatin molecule; exposure to PKA induces the incorporation of 0.75–0.8 equivalents of ^{32}P per calpastatin molecule (Fig. 3C).

Taken together, these results confirm that phosphorylation and dephosphorylation of calpastatin can occur in cell-free system; the different incorporation of phosphate groups, observed with PKA and PKC, can be explained on the basis of different accessibility of the phosphorylable sites.

3.5. Degradation of brain calpastatin I by homologous calpain

To obtain an abnormal intracellular activation of calpain, a modification of the mechanism of regulation must occur. Due to the fact that, following the formation of the calpain–calpastatin complex, the inhibitor can be slowly degraded [32], prolonged activation of the proteinase may result in extensive digestion of this regulatory component. To verify the susceptibility of brain calpastatin I to degradation by homologous calpain, we promoted the formation of the enzyme–inhibitor complexes and we measured the possible disappearance of calpastatin activity. As shown in Fig. 4, calpastatin I was almost completely resistant to μ-calpain, whereas it was very quickly inactivated by *m*-calpain. To better characterize this process, we tested calpains isolated from rat skeletal muscle. Also when incubated with the proteinases from this tissue, calpastatin I resulted preferentially degraded by *m*-calpain, although with less efficiency, as compared with the same isozyme from brain (Fig. 4).

The high susceptibility of calpastatin to degradation by homologous *m*-calpain and the interconversion between the two calpastatin forms may represent the basis for the production of cellular conditions suitable for uncontrolled activation of calpain.

4. Discussion

Many reports are suggesting that brain intracellular structures are particularly susceptible to calpain degradation, [15–25,33–35]. To produce these effects, calpain must escape to a complex mechanism of modulation, which the evolution has developed around the proteinase. In fact, the first level of regulation is intrinsic to calpain molecules, which contain intramolecular constraints capable to prevent the expression of catalytic activity [28], modulating the accessibility of the active site and stabilizing inactive conformations. The second level is represented by the natural inhibitor, which controls the num-

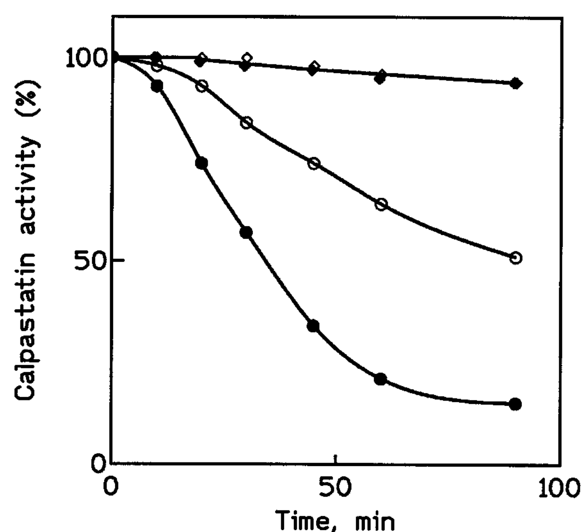

Fig. 4. Degradation of calpastatin I by calpains. Calpastatin I (7 units) was incubated with the same units of rat brain *m*-calpain (●), rat brain μ-calpain (♦), rat skeletal muscle *m*-calpain (○) or rat skeletal muscle μ-calpain (◇) in 0.7 ml of 50 mM sodium borate buffer, pH 7.5, containing 100 μM Ca^{2+} or 1 mM Ca^{2+}, in the case of μ- or *m*-calpain, respectively. Rat skeletal muscle calpains were purified following the procedure described in [27]. At the times indicated, aliquots of the incubation mixtures (corresponding to 0.5 calpastatin units) were collected, heated to 90°C for 2 min to inactivate the proteinases and centrifuged at 50000×*g* for 10 min. The clear solutions were collected and assayed for their content of calpastatin activity. The results are expressed as percentage of calpastatin activity recovered following the same treatments, without exposure to proteinases.

ber of the calpain molecules that must be activated, as well as the expression of catalytic activity [5,36].

In this paper, we are providing evidence on the molecular bases through which the Ca^{2+}-dependent proteolytic system can be involved in aspecific degradation of brain cell structures. Using a very sensitive calpain form, we have demonstrated that rat brain contains, in addition to the well-known calpastatin, a second form which is very poorly active on the homologous calpains. This new form is produced by phosphorylation of the active calpastatin and it may be considered as a reservoir to be activated by dephosphorylation. We have also isolated from rat brain a phosphoprotein phosphatase which catalyzes the conversion of the inactive calpastatin into the active form. Calpastatin I phosphorylation is promoted by isolated PKA or PKC, both capable to introduce approximately 1 equivalent of phosphate per molecule of calpastatin.

Interconversion between the two calpastatin forms has been observed also in other rat tissues where the process has been considered as a tool required to change the selectivity of calpastatin for one or the other calpain form [27,37]. In brain, however, this process could be visualised as the mechanism that fits the actual inhibitory activity to the cell requirement.

The high sensitivity of calpastatin to degradation by *m*-calpain, and particularly by the homologous proteinase, seems a peculiar property of brain tissue. The loss of this regulatory component of the system, together with the interconversion process, might produce further and uncontrolled activation of the proteinases and aspecific degradation of cell structures, a condition named 'pathological role' of calpain.

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