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Phosphorylation of rat brain calpastatins by protein kinase C

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Abstract Calpastatin, the natural inhibitor of calpain, is present in rat brain in multiple forms, having different molecular masses, due to the presence of one (low Mr form) or four (high Mr form) repetitive inhibitory domains. Recombinant and native calpastatin forms are substrates of protein kinase C, which phosphorylates a single serine residue at their N-terminus. Furthermore, both low and high Mr calpastatins are phosphorylated by protein kinase C at the same site. These calpastatin forms are phosphorylated also by protein kinase A, although with a lower efficiency. The incorporation of a phosphate group determines an increase in the concentration of Ca^{2+} required to induce the formation of the calpain-calpastatin complex. This effect results in a large decrease of the inhibitory efficiency of calpastatins. We suggest that phosphorylation of calpastatin represents a mechanism capable to balance the actual amount of active calpastatin to the level of calpain to be activated.

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Key words: Calpastatin; Calpain regulation; Protein kinase C; Rat brain

1. Introduction

A variety of natural effectors are involved in the regulation of the calcium-dependent proteolytic system through two distinct processes, the first one promoting activation of calpain and involving interaction with phospholipids [1], nuclei and specific protein factors [2], the second one promoting inhibition by the association with the inhibitory protein calpastatin [3]. The formation of the enzyme-inhibitor complex occurs only in the presence of relatively high [Ca^{2+}], which induces a conformational change that discloses the catalytic site [4]. This reversible process is followed by the removal of peptide segments from the N-terminal region of calpain in two autoproteolytic steps, a process that further increases the affinity of calpastatin for the enzyme [5].

Recently, we have reported that the rat brain contains five different transcripts for calpastatin [6], some of which encoding the complete molecular structure, consisting of one N-terminal domain and four repetitive inhibitory domains (high Mr calpastatin). We have also identified truncated calpastatin forms, one having specifically the N-terminal domain associated with only one inhibitory domain (low Mr calpastatin) and another one consisting of the single and inactive N-terminal domain.

Expression of these recombinant forms [7] has revealed that the low Mr calpastatin is the most active form, showing an inhibitory activity approximately three times higher than that of the complete high Mr calpastatin form. Moreover, analysis of the properties of the isolated N-terminal domain has indicated that this region is involved in the specific recognition of calpain isozymes. The presence of multiple forms of calpastatin has been confirmed by the isolation from rat brain homogenates of a very active low Mr calpastatin together with high Mr inhibitor forms [7]. The concomitant expression of multiple calpastatin forms may be relevant to regulate separately μ-calpain and m-calpain that can be present in a single cell.

We have also established that calpastatin can undergo reversible post-translational modifications, producing changes in its capacity of calpain recognition and obviously in the inhibitory efficiency [8]. This modulatory mechanism has been suggested by the observation that calpastatin from many tissue homogenates is eluted by acidic ion exchange chromatography in two forms, both containing a mixture of high Mr and low Mr calpastatins. Treatment with protein kinases and phosphatases has indicated that both forms are interconvertible products of a reversible phosphorylation-dephosphorylation process [9]. In rat brain, phosphorylated calpastatin shows a considerably reduced inhibitory efficiency particularly against m-calpain [8], whereas in skeletal muscle, the same post-translational modification changes the specificity of the inhibitor.

We now report more detailed studies concerning the phosphorylation of calpastatin forms, purified from rat brain. These results indicate that phosphorylation occurs at the N-terminus of the protein molecule and results in the modulation of the affinity of calpastatin for the two homologous calpain isoforms. Identical results have been obtained with recombinant calpastatin forms. The generation of phospho and dephospho forms, with a different capacity to interact with calpain, could represent a mechanism to balance calpastatin and calpain during activation of the system.

2. Materials and methods

2.1. Purification and assay of calpains and calpastatins

Rat brain μ-calpain and m-calpain were purified and assayed as previously described [8]. One unit of enzyme activity is defined as the amount that releases 1 μmol/h of free α-amino groups under the specified conditions.

Rat brain calpastatins were purified and assayed as described [8]. One unit of calpastatin activity is defined as the amount required to inhibit one unit of enzyme activity.

2.2. Expression of calpastatins as fusion proteins in Escherichia coli

Five cDNAs for calpastatin were obtained from rat brain mRNA, amplified and ligated into the pGEX2T expression vector [6]. *E. coli* TOP 10F′ cells were transfected by electroporation. Expression and purification of GST-calpastatins were carried out as previously described [7].

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Abbreviations: PKC, protein kinase C; PKA, protein kinase A; EDTA, ethylene diamine tetraacetate; GST, glutathion *S*-transferase

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2.3. Isolation of rat brain protein kinase A (PKA) and protein kinase C (PKC)

PKA and PKC from rat brain were assayed and purified as previously described [8]. The final PKC preparation resulted to be a mixture of PKC isozymes, composed by 2/3 of α-PKC and 1/3 of β-PKC. δ-isozyme was present only in trace amounts. One unit of protein kinase activity was defined as the amount causing the incorporation of 1 nmol of ^{32}P into histone type IIIS under the specified conditions [8].

2.4. Phosphorylation of native and recombinant calpastatins by PKC and PKA

Purified native or recombinant calpastatin were incubated at 30°C for 30 min with purified rat brain PKC in 50 mM sodium borate, pH 7.5, containing 5 mM $MgCl_2$, 10 μM ATP, 0.4 μCi [$\gamma^{32}P$]ATP, 10 μg phosphatidylserine, 0.2 μg diacylglycerol and 0.5 mM $CaCl_2$. Under these conditions, the reaction is almost linear for the first 5–10 min and substrate phosphorylation is completed in approximately 20–25 min [8]. The reaction was stopped by heating at 90°C for 3 min, the mixture was centrifuged at 50 000 × *g* for 10 min and the supernatant, containing phosphorylated calpastatin, was collected. Phosphorylated calpastatin was then isolated by affinity chromatography on a Sepharose column containing immobilized calpain under the conditions specified below. ^{32}P incorporation into calpastatin was measured on an aliquot of the eluted protein by counting in a scintillation counter and expressed as mol of ^{32}P/mol of protein.

The phosphorylation of calpastatins by PKA was carried out in the same conditions replacing diacylglycerol, phosphatidylserine and calcium with 20 μM cAMP [9].

2.5. Affinity chromatography of calpastatin onto a carboxy-methylated calpain-immobilized column

Erythrocyte calpain was purified and inactivated with iodoacetamide as previously described [10]. A calpain-Sepharose column was prepared by coupling carboxy-methylated calpain (0.4 mg) with CNBr Sepharose (Amersham Pharmacia Biotech) following the manufacturer's instructions and equilibrated in 50 mM sodium borate buffer pH 7.5, containing 0.5 mM β-mercaptoethanol and 1 mM $CaCl_2$. Calpastatin was loaded onto the column and eluted with 50mM sodium borate buffer pH 7.5, containing 0.5 mM β-mercaptoethanol and 0.1 mM ethylene diamine tetraacetate (EDTA).

2.6. Identification of phosphorylated amino acid residues

Phosphorylated native or recombinant calpastatin was digested overnight with trypsin (ratio 100:1) in 50 mM sodium borate buffer, pH 8.5, containing 5% CH_3CN. After digestion, the sample was lyophilized, suspended in 50 μl of H_2O and the pH was acidified by the addition of 0.1% trifluoroacetic acid. The tryptic peptides were separated by HPLC using a C-18 reverse phase chromatographic column (1 × 100 mm) equilibrated in distilled water containing 0.1% trifluoroacetic acid. The elution of the tryptic peptides was carried out by a linear gradient of CH_3CN (from 0 to 70%). To identify the radioactive peptides, an aliquot (0.01 ml) of each eluted fraction was applied onto a nitrocellulose sheet which was then submitted to autoradiography. This analysis revealed only one ^{32}P-labelled peak. Fractions corresponding to the labelled peak were collected, lyophilized and the amino acid sequence of the labelled peptide was determined with a Beckman L3000 sequencer [11].

3. Results

3.1. Phosphorylation of native and recombinant low Mr calpastatin

We have previously reported [6] that rat brain contains a mRNA encoding a form of calpastatin having the N-terminal domain and a single inhibitor repeat (low Mr form). The recombinant protein, expressed in *E. coli*, showed not only calpastatin activity against both μ- and m-calpain but also a much higher inhibitory efficiency as compared to the other recombinant calpastatin forms. A similar low Mr calpastatin form has been isolated from rat brain tissue and possesses the identical functional properties of that expressed in *E. coli*.

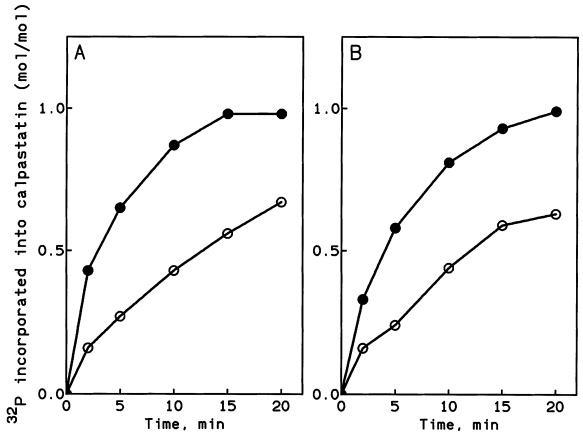

Fig. 1. Phosphorylation of native and recombinant low Mr calpastatin by PKC and PKA. Purified native (A) or recombinant (B) low Mr calpastatin were incubated with PKC (●) or PKA (○) and [γ-^{32}P]ATP as described in Section 2. At the indicated times, the reactions were stopped by heating at 90°C for 3 min and the mixtures were centrifuged at 50 000 × *g* for 10 min. The clear supernatants were then filtered through a calpain-Sepharose column equilibrated with 50 mM sodium borate, pH 7.5, containing 1 mM Ca^{2+}. Calpastatin was retained by the resin and then eluted with the same buffer containing 0.1 mM EDTA. ^{32}P incorporation into calpastatin was measured on an aliquot of the eluted protein by counting in a scintillation counter and expressed as mol of ^{32}P/mol of protein.

To determine if phosphorylation is a mechanism for the control of calpastatin's efficiency and selectivity, we have incubated the native rat brain and recombinant low Mr calpastatins with PKC, also purified from rat brain. As shown in Fig. 1, both proteins incorporate ^{32}P in an amount reaching a ratio of 1 mol of phosphate per mol of protein. In comparative analysis, PKA resulted to be less efficient in promoting calpastatin phosphorylation.

To identify the calpastatin region in which phosphorylation occurs, the inhibitor was incubated for 30 min with PKC. In these conditions, described in Fig. 1, the phosphorylation reaction was completed. Phospho-calpastatin was recovered from the incubation mixture by heating at 90°C for 3 min followed by affinity chromatography on a Sepharose column containing immobilized carboxy-methylated calpain (see Section 2). The [^{32}P]calpastatin was then collected and digested with trypsin. The tryptic peptides were separated by HPLC using a C18-reverse phase column and an aliquot of the eluted material was analyzed for its radioactivity content. Only one peptide peak was found radioactive and fractions containing the labelled material were collected, pooled and utilized to determine the amino acid sequence. As shown in Fig. 2, the amino acid sequence identified in both native and recombinant inhibitors corresponds to a segment present at the N-terminus of calpastatin. Furthermore, the identified sequence is consistent with a PKC phosphorylation site [12].

3.2. The effect of phosphorylation on inhibitory properties of low Mr calpastatins

To establish the effect of phosphorylation on the inhibitory properties of native and recombinant low Mr calpastatins, their phosphorylated forms were analyzed for their capacity to inhibit homologous μ-calpain and m-calpain. As shown in Fig. 3, unphosphorylated native and recombinant low Mr calpastatins show an inhibitory activity against rat brain μ-

Calpastatin type	labelled peptide
Recombinant low M_r calpastatin	IE*SK
Native low M_r calpastatin	IE*SK
(N-terminal sequence) MSTTGAKAVRIESKQ	

*, labelled residue

Fig. 2. The site of phosphorylation by PKC in recombinant and native low Mr calpastatin. Recombinant or native low Mr calpastatin was incubated with PKC as described in the legend to Fig. 1 and in Section 2. The reaction was stopped after 30 min of incubation, when phosphorylation was completed, by heating at 90°C for 3 min. The sample was centrifuged at 50 000×*g* for 10 min and the supernatant, containing phosphocalpastatin, was collected. Phosphocalpastatin was recovered by affinity chromatography on calpain-Sepharose (Section 2.5) and then digested with trypsin (Section 2.6). The tryptic peptides were separated by C-18 reverse phase chromatography and the radioactive peptide, identified as described (Section 2.6), was sequenced.

calpain and m-calpain, although with a higher efficiency with respect to the former. Following phosphorylation, a significant decrease in the efficiency of the inhibitors against both proteinase isozymes occurs. Phosphorylated native and recombinant low Mr calpastatins express less than 1/2 of their potential activity on μ-calpain and are almost completely inactive on m-calpain. These data provide additional evidence supporting the role of the N-terminal region of the molecule in conferring the specificity and efficiency to the entire inhibitor molecule.

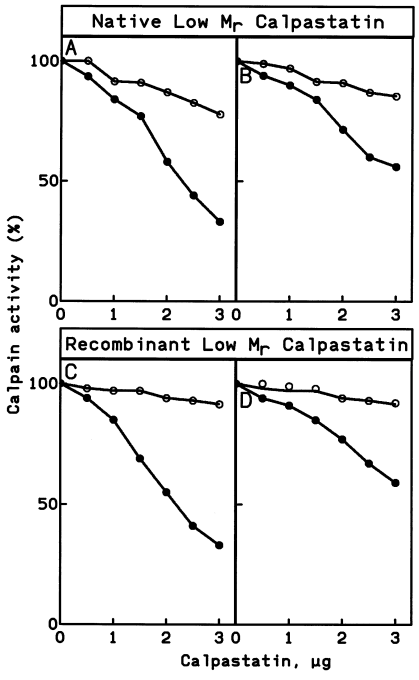

Fig. 3. The effect of phosphorylation on the inhibitory efficiency of low Mr calpastatins. Rat brain-purified μ-calpain (A, C) or m-calpain (B, D) were incubated with the indicated amount of unphosphorylated (●) or phosphorylated (○) native or recombinant low Mr calpastatin for 10 min at 37°C in sodium borate buffer, pH 7.5, containing 100 μM Ca^{2+} or 1 mM Ca^{2+} in the case of μ-calpain or m-calpain, respectively. The reaction was stopped by addition of trichloroacetic acid (7.5% final concentration) and the samples were centrifuged at 50 000×*g* for 10 min. The calpastatin activity was assayed in the supernatant using μ-calpain and m-calpain purified from rat brain. Data are expressed as percentage of the residual calpain activity.

3.3. The effect of phosphorylation on formation of the calpain-calpastatin complex

The decrease in calpastatin inhibitory activity generated by phosphorylation is also enhanced by the increase in the Ca^{2+} concentration required for the formation of the calpain- inhibitor complex. This has been demonstrated by the following experiment in which immobilized μ-calpain was used to assay the enzyme-inhibitor complex in the presence of increasing [Ca^{2+}] and with dephosphorylated or phosphorylated calpastatin molecules. As shown in Fig. 4, unphosphorylated low Mr calpastatin was not retained by the resin in the absence of Ca^{2+}. In the presence of 5 μM Ca^{2+}, only a few molecules of calpastatin were retained, whereas at 50 μM Ca^{2+}, all calpastatin molecules remained associated to the resin. Under the same experimental conditions, the phosphorylated calpastatin required a 10-fold higher concentration of Ca^{2+} to be retained by the resin through the formation of the complex with calpain. Identical results were obtained using recombinant calpastatin (data not shown).

Taken together, these results indicate that phosphorylation modulates the inhibitory properties of calpastatin by changing both the efficiency and affinity for calpain.

3.4. Phosphorylation of high Mr recombinant calpastatins

The rat brain contains mRNA encoding high Mr calpastatin forms, differing one from the other by the presence of peptides deriving from specific exones [6]. We have expressed RNCAST104 and RNCAST107, containing four repetitive domains together with the L-domain. The two calpastatin forms were then analyzed for their susceptibility to phosphorylation by PKC. As shown in Fig. 5A, high Mr calpastatins

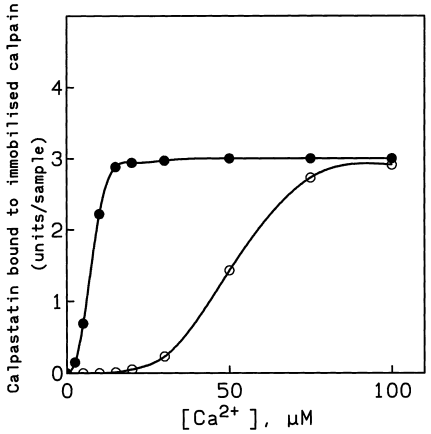

Fig. 4. The effect of phosphorylation on the formation of the calpain-calpastatin complex. Unphosphorylated (●) or phosphorylated (○) native calpastatin was loaded on a calpain-Sepharose column (see Section 2) in the presence of the indicated concentrations of calcium. The retained inhibitor was eluted with the equilibrating buffer containing 0.1 mM EDTA instead of calcium. The amount of calpastatin retained by the immobilized calpain was determined assaying the inhibitory activity eluted from the column.

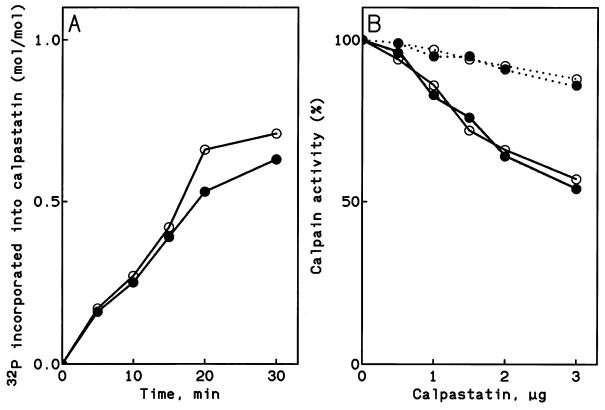

Fig. 5. Phosphorylation of recombinant high Mr calpastatins by PKC. (A) Recombinant RNCAST104 and RNCAST107 calpastatins were incubated with PKC for the indicated times and in the conditions described in Section 2 and in the legend of Fig. 1. The radioactivity associated to RNCAST 104 (●) and to RNCAST107 (○) is expressed as mol of ^{32}P/mol of protein. (B) The inhibitory efficiency of RNCAST104 calpastatin (●) and RNCAST107 calpastatin (○), unphosphorylated (solid lines) or phosphorylated (dotted lines), were determined as reported in Section 2 and in the legend to Fig. 3, using rat brain μ-calpain.

are phosphorylated by PKC at a lower extent as compared to the low Mr forms (see Fig. 1). Only 0.5 equivalents of ^{32}P have been incorporated under the same conditions in which phosphorylation of low Mr calpastatin was completed. Phosphorylation of both high Mr calpastatins results in a decrease in their inhibitory efficiency (Fig. 5B).

Furthermore, analysis of the tryptic peptides containing the labelled phosphate groups has indicated also for the two high Mr calpastatins that the phosphorylation catalyzed by PKC occurs at the level of the same serine residue located within the PKC target sequence near the N-terminal domain, as for the low Mr calpastatin (data not shown).

4. Discussion

It is well-documented that calpastatin is the primary negative modulator of classical calpain forms [13–15]. However, it has not yet completely been defined (i) how calpain interacts with calpastatin, (ii) how calpastatin can discriminate between calpain isozymes and finally, (iii) how calpain can escape the calpastatin inhibition.

The first difficulty in providing final experimental responses to these questions arises from the fact that both proteins, enzyme and inhibitor, are localized in the same cell compartment. This situation is rather unusual since normally the proteinase inhibitors are localized in cell regions that must be protected and not were the proteinases that are supposed to be active. For instance, cystatins, the lysosomal proteinase inhibitors, are preferentially cytosolic proteins and their targets, cathepsins, are confined in lysosomes. This condition does not apply to the calpain-calpastatin system in which calpastatin is participating in the regulation of the calpain activity in the same subcellular compartment. On the basis of this assumption, however, selective mechanisms must be operative in order to assure a continuous appropriate level of active inhibitor and proteinase forms in order to provide a controlled and selective degree of proteolysis.

The N-terminal region of calpastatin seems particularly relevant for the overall function of the protein. This domain, expressed by us as an isolated protein [8], although resulting completely deprived of inhibitory activity, competes with the other calpastatin forms and displaces the proteinase from the complex with the inhibitory calpastatins. The role of this protein appears to be more relevant, since it contains also the site of phosphorylation by PKC.

Phosphorylation of calpastatin results in a decrease in the inhibitory efficiency suggesting that phosphorylated calpastatin can act as a reservoir of inhibitor molecules. The correlation between the PKC-mediated signal transduction and calpastatin phosphorylation remains to be elucidated, but it suggests the existence of a pathway linking the calpain activation and regulation to external cell stimuli.

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