Association of calpastatin with inactive calpain: a novel mechanism to control the activation of the protease?

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Running title: Calpain-calpastatin complex in absence of Ca\(^{2+}\).

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Summary

It is generally accepted that the Ca\(^{2+}\)-dependent interaction of calpain with calpastatin is the most relevant mechanism involved in the regulation of Ca\(^{2+}\) induced proteolysis. We now report that a calpain-calpastatin association can occur also in the absence of Ca\(^{2+}\) or at very low Ca\(^{2+}\) concentration, reflecting the physiological conditions in which calpain retains its inactive conformational state. The calpastatin binding region is localized in the non-inhibitory L-domain region containing the amino acid sequences coded by exons 4 to 7. This calpastatin region recognises a calpain sequence located near the end of the DII domain. Interaction of calpain with those calpastatins lacking these sequences becomes strictly Ca\(^{2+}\)-dependent since in these conditions the transition to an active state of the protease is an obligatory requirement. The occurrence of the molecular association between Ca\(^{2+}\) free calpain and various recombinant calpastatin forms has been demonstrated by the following experimental results. Addition of calpastatin protected calpain from trypsin digestion. Calpain co-precipitated when calpastatin was immunoprecipitated. The calpastatin molecular size increased following exposure to calpain. The two proteins co-migrated in zymogram analysis. Furthermore calpain-calpastatin interaction was perturbed by protein kinase C (PKC) phosphorylation occurring at sites located at the exons involved in the association. At a functional level, calpain-calpastatin interaction at physiological concentration of Ca\(^{2+}\) represents a novel mechanism for the control of the amount of the active form of the protease potentially generated in response to an intracellular Ca\(^{2+}\) influx.

Introduction

Several reports have indicated that calpain can acquire different conformational states depending on the presence of low or high [Ca\(^{2+}\)] (1-9). Binding of Ca\(^{2+}\) to sites present in both the large and the small calpain subunits induces a conformational switch, which ultimately results in the rearrangement of the two catalytic subdomains into a functionally active catalytic site (3-8). This conformational modification is the limiting step in the calpain activation process and may be followed by an autoproteolytic degradation that removes the N-terminal region of domains I and V, stabilising a low Ca\(^{2+}\)-requiring form of the protease. The active enzyme form is regulated by calpastatin, the endogenous pertinacious inhibitor (10-12), which has a peculiar molecular structure consisting of one N-terminal (L)-domain and four repetitive inhibitory units (13-21), each one containing three highly conserved regions called A, B and C (22, 23). Sequence B contains the calpain consensus sequence; sequences A and C do not seem to be required for expression of the inhibitory activity, as indicated by experiments showing that a 27-residues peptide derived from the B sequence can interact and inhibit calpain (23-25). Although not required for inhibition, A and C sub domains can exert other functions, such as interaction with domains IV and VI in the presence of Ca\(^{2+}\) (26-28) or reduction of Ca\(^{2+}\) requirement when these isolated segments are added to the calpain assay mixtures (22). The conformational transition of calpain from the inactive to the Ca\(^{2+}\)-induced active state seems to be an obligatory step for the calpain-calpastatin association stabilized by interactions at many sites (29-33).

Barnoy et al. (34) have reported that an association between calpain and calpastatin can
occur within cells in the presence of physiological Ca\(^{2+}\) levels. This conclusion was reached on the basis of immunoprecipitation experiments carried out in cells homogenates prepared in the presence of 0.2 mM EDTA, a condition preserving the inactive calpain state. The fact that most of the earlier attempts were unsuccessful in identifying this association, could be due to a number of reasons including the use of high ionic strength media (35), inappropriate technical approaches and the lack of precise information on the molecular properties of the calpastatin preparations. This is a crucial point because calpastatin isolated from tissues or cell cultures might be a mixture of different forms deriving from alternative splicing or by proteolysis, showing also different inhibitory properties. It must also be recalled that calpastatin behaves as a high affinity substrate of calpain (36).

Calpain-calpastatin association in physiological conditions is also supported by our recent observations indicating that calpain undergoes a conformational transition following exposure to calpastatin, regardless of the presence of Ca\(^{2+}\) (37). By means of a monoclonal antibody capable to recognize changes in calpain conformation, we have demonstrated that such changes take place also in intact cells following stimulation with agonists that do not induce an increase in intracellular free Ca\(^{2+}\). These results indicate that association of calpastatin with calpain in the inactive Ca\(^{2+}\) free form, can occur in physiological intracellular conditions. Finally, since calpain-substrate complex can be formed even if the protease retains its inactive conformation (38, 39), it is reasonable to assume that also calpain-calpastatin association can take place in these conditions.

In the present paper we are reporting that the non-inhibitory region (L-domain) of calpastatin interacts with native micromolar and millimolar calpain at physiological concentrations of Ca\(^{2+}\). The specific exon sequences involved in such interaction have been identified in the region coded from exon 4 to 7. Phosphorylation by PKC at sites included in these sequences profoundly reduces this association. This finding provides further support to the physiological relevance of this process that may represent an additional mechanism for the regulation of calpain by controlling the rate and the extent at which the enzyme undergoes activation.

**Experimental Procedures**

**Materials**

Casein, BSA, trypsin, rabbit muscle aldolase, carbonic anhydrase, arachidonate, aprotinin, leupeptin, and dibutyryl cAMP were purchased from Sigma Aldrich. 4-(2-Aminoethyl)benzenesulfonylfluoride (AEBSF) was obtained from Calbiochem. Protein G-Sepharose, G25 gel, horseradish peroxidase-linked anti-mouse secondary antibody, ECL\(^{\text{TM}}\) detection system, Superdex\(^{\text{TM}}\) 75 10/300 GL column, Sephacyr S-300 gel and (γ\(^{32}\)P)ATP, and FITC-conjugated sheep anti-mouse antibody (Alexa 488) were obtained from Amersham Pharmacia Biosciences. DEAE 53 resin was obtained from Whatman Co.

**Isolation of calpain forms**

The single human erythrocyte calpain, belonging to the μ-isofrom class, was purified as described in (40). Rat brain μ- and m-calpains were purified as described in (41). Autoproteolized human erythrocyte calpain (75 kDa) was prepared as reported in (42). The 55 kDa form was obtained following incubation of native human erythrocyte calpain with 1 mM Ca\(^{2+}\) for 5 minutes. Following this treatment no protease activity was detectable, whereas in SDS-PAGE the major protein band in the stained gel showed a mass of approximately 55-60 kDa. This autolysis removes from the protease molecule both DI and DII domains (8).

**Preparation of native and recombinant calpastatins**

Native calpastatin isoforms were purified from rat brains and skeletal muscles as described in (43) and (41) respectively. The following recombinant rat brain calpastatins (18) were prepared: RNCAST104 (Gen/Bank accession number Y13588): containing the complete L-domain (exons 2-8) and four inhibitory units (exons 9-29); RNCAST23 (Gen/Bank accession number Y13591): containing the L-domain, lacking exon 6 and one inhibitory unit (exons 9-12); RNCAST110 (Gen/Bank accession number Y13590): corresponding to the free L-domain containing sequences coded by exons 2-7; RNCAST600: composed of the complete L-domain (exons 2, 4-8) and one inhibitory unit (exons 9-12); RNCAST300: composed of exon 8 and a single inhibitory unit (exons 9-12); Recombinant human calpastatin fragment, corresponding to a portion of human calpain inhibitor (GenBank/ accession number P20810) contains the exons 4-13. The oligonucleotides used to generate the expression clones were: for RNCAST300, the sense primer 5’-

TGGGATCCGAGAAGAAATCATTAACACCT  
ACTT containing a BamHI restriction site at the 5’ end; for RNCAST600, the sense primer 5’-
essential SH group without any change in the 100-fold excess idoacetamide to block the Human erythrocyte calpain was treated with a Calpain carboxymethylation Sephadex G25 column (1 x 17 cm), previously excess of reagents was removed by filtration on a calpain activity was almost completely lost, other functional protease properties (42). When the amount that inhibits one unit of calpain activity. One unit of calpastatin activity is defined as reported in (43). The specific activity of calpain forms used ranged α-amino groups under the specified conditions. One unit of calpain activity is defined as the amount that releases 1µM/h of free NH₂ groups (41). One unit of calpain activity is derived from amino acids 174 - 193 of RNCAST104) (GIKEGTIPPEYRKLLEKNEA, derived from the 20-residues peptide ATC containing an TGGAATTCTCACAAGGCGTCTATAGCATG GAG containing a BamHI restriction site at the 5′ end, and the antisense primer 5′-TGGATCCATGGCGTACAAAAACAGAACCT ATC containing an EcoRI restriction site at the 5′ end. The 20-residues peptide (GIKEGTIPPEYRKLEKNEA, derived from RNCAST104) corresponds to a portion of B-subdomain and was chemically synthesized. All RNCAST forms were expressed as GST-fusion proteins in E. coli (44). Monoclonal antibodies Monoclonal anti-calpain antibody (mAb 56.3) was prepared as described in (45). Monoclonal anti-calpastatin antibody (mAb 35.23) was produced as indicated in (44). Anti-phosphoserine monoclonal antibody (mAb) was purchased from Sigma Aldrich. Cell culture Jurkat cells (human T-lymphocyte line) were cultured at 37°C (5 % CO₂) with RPMI 1640 (Sigma Aldrich) growth medium containing 10 % foetal calf serum, 10 U/ml penicillin (Sigma Aldrich), 100 µg/ml streptomycin (Sigma Aldrich) and 4 mM L-glutamine. Methods Assay of calpain and calpastatin activity Calpain was assayed using human denaturated globin as substrate, following the liberation of free NH₂ groups (41). One unit of calpain activity is defined as the amount that releases 1µM/h of free α-amino groups under the specified conditions. The specific activity of calpain forms used ranged from 950 to 1050 units/mg. Calpastatin activity was assayed as reported in (43). One unit of calpastatin activity is defined as the amount that inhibits one unit of calpain activity. Calpain carboxymethylation Human erythrocyte calpain was treated with a 100-fold excess idoacetamide to block the essential SH group without any change in the other functional protease properties (42). When calpain activity was almost completely lost, excess of reagents was removed by filtration on a Sephadex G25 column (1 x 17 cm), previously equilibrated with 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA. Calpain digestion by trypsin Calpain (10 µg) was mixed with RNCAST600 (3 µg) or RNCAST300 (1.5 µg) in 50 µl of 50 mM sodium borate buffer pH 7.5, 0.5 mM 2-mercaptoethanol, containing alternatively 100 µM Ca²⁺ or 1 µM Ca²⁺ or 1 mM EDTA incubated for 30 minutes at room temperature with or without trypsin in a of 1000 to 1. To avoid calpain autoproteolysis in the course of the experiments carried out in the presence of Ca²⁺, carboxymethylated human erythrocyte calpain was used (42). At the end of the incubations, aliquots (10 µl) of the mixtures were collected and submitted to 10% SDS-PAGE (46). The proteins were then blotted (47) and immunostained with anti-calpain antibody mAb 56.3. Western Blotting At the end of the electrophoretic runs, proteins were transblotted from gels to nitrocellulose sheets as indicated in (47). The sheets were then incubated with the appropriate monoclonal antibody as specified elsewhere. The immunoreactive material was detected by a peroxidase conjugated secondary antibody (48) developed with an ECL® detection system. The immunoreactive bands were quantified with a Shimatsu CS9000 densitometer using a wavelength of 590 nm. Immunoprecipitation of calpastatin Recombinant calpastatins alone or mixtures of calpastatin and human erythrocyte calpain in a final volume of 50 µl of 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA were treated with 3 µg of anti-calpastatin mAb 35.23 at 4°C for 2 hours. Replacement of EDTA with Ca²⁺ to these mixtures is specified elsewhere. Protein G-Sepharose (15 µl) was then added to the samples and these mixtures were rotated end-over-end for 2 hours at 4°C. Sepharose beads were collected and washed three times with 0.1 ml of 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA. The beads were then suspended in 50µl of 60 mM Tris/HCl pH 6.8 containing 2% SDS, 2% 2-mercaptoethanol and 10% glycerol, and heated for 1 minute at 100°C. The insoluble particles were discarded by centrifugation, the clear solutions submitted to 12% SDS-PAGE and blotted. Calpain and calpastatins were immunodetected with anti-calpastatin mAb 35.23 or with anti-calpain mAb 56.3, and revealed by a peroxidase-linked secondary antibody. Size-exclusion experiments on Superdex™ 75 10/300 GL column
overnight at 25°C in the presence of 10 mM CaCl,

hours at 4°C and 125 Volts. At the end of the

1mM EDTA and 10 mM 2-mercaptoethanol for 2

Tris/HCl pH 8.0 containing 125 mM glycine,

The electrophoretic run was carried out in 25 mM

casein.

Coomassie Brilliant Blue and the bands were

under gentle shaking. The gels were stained with

on a 10% polyacrylamide gel, containing 1 mg/ml

2-mercaptoethanol and 10 mM EDTA and loaded

the two proteins were diluted in 60

(53). Briefly, calpain, calpastatin or mixtures of

Zymogram analysis was carried out as described in (43), using 50 µl of the clear supernatant.

Calpastatin activity was then assayed as described

in (43).

Zymography of mixtures of calpain and calpastatin

Zymogram analysis was carried out as described by Raser et al. (52) and modified by Simon et al. (53). Briefly, calpain, calpastatin or mixtures of the two proteins were diluted in 60 µl of 0.1 M Tris/HCl pH 6.8 containing 20% glycerol, 10 mM 2-mercaptoethanol and 10 mM EDTA and loaded on a 10% polyacrylamide gel, containing 1 mg/ml casein.

The electrophoretic run was carried out in 25 mM

Tris/HCl pH 8.0 containing 125 mM glycine, 1mM EDTA and 10 mM 2-mercaptoethanol for 2

hours at 4°C and 125 Volts. At the end of the electrophoretic run, the gel was incubated overnight at 25°C in the presence of 10 mM CaCl2 under gentle shaking. The gels were stained with Coomassie Brilliant Blue and the bands were

quantified by densitometric analysis using the Kodak Digital Science 1D Image Analysis Software.

Purification of protein kinases PKA and PKC and calpastatin phosphorylation

cAMP-dependent protein kinase (PKA) and Ca2+/phospholipid-dependent protein kinase (PKC) were purified from rat brain and assayed as described in (41). Calpastatin phosphorylation by PKA or PKC was carried out as reported in (54). Incorporation of γ-32P into calpastatin molecules was determined following 12% SDS-PAGE and autoradiography of the dried gels. The equivalents of γ-32P incorporated was measured using a Cyclone Storage Phosphor Screen system (Packard BioScience Company) and quantified utilising the version 03.10 Optiquant Acquisition & Analysis software (55).

Separation of calpastatin forms by ion-exchange chromatography

Freshly collected rat brain (2 g) were homogenised in 5 volumes of 50 mM sodium borate buffer pH 7.5, containing 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin, 10 µg/ml aprotinin and 2 mM AEBSF with a Potter-Evjem homogeniser. The crude homogenate was sonicated six times (10 seconds at 30 seconds intervals) and the particulate material was discarded by centrifugation at 25 000 x g. The clear supernatant was collected, heated at 100 °C for 3 min and the precipitated protein was discarded by centrifugation at 10 000 x g. The clear solution was loaded onto a DEAE 53 (1 x 5 cm) equilibrated in 50 mM sodium acetate 6.7, containing 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol. The column was washed with the equilibrating buffer and the absorbed proteins eluted with a NaCl gradient from 0 to 0.35 M. The flow rate was 0.5 ml/minute and fractions of 1 ml were collected.

Determination of intracellular calpain and calpastatin by confocal microscopy and fluorescence quantification

Control or stimulated Jurkat cells were fixed and permeabilized by Triton/paraformaldehyde method, as described in (56). Calpain and calpastatin intracellular localizations were determined by confocal microscopy, using anti-calpain mAb 56.3 or anti-calpastatin mAb 35.23 as primary antibody and a FITC-conjugated sheep anti-mouse as secondary antibody (Alexa 488 antimonie antibody Amersham Biosciences). The excitation/emission wavelengths were 488/522 nm and fluorescence intensity was quantified using the Laser Pix Software (BioRad Bioscience).
Results
Protection of calpain from trypsin digestion by recombinant calpastatins.

Although the role of the four inhibitory units has been well established, the physiological relevance of the N-terminal non-inhibitory region (L-domain) of calpastatin still remains uncertain. In an attempt to clarify this problem we have studied the interaction of calpain with two recombinant calpastatin forms: one (RNCAST600) containing the complete L-domain (exons 2-8) and a single inhibitor repetitive unit; a second one (RNCAST300), containing only the exon 8 of the L-domain and a single inhibitory unit. The two calpastatin forms were found to express an almost equally efficient inhibitory capacity on calpain activity at 100 µM Ca\(^{2+}\), indicating that the additional exons expressed in RNCAST600 are not relevant for its functional properties. Calpain was purified from human erythrocytes since these cells contain a single protease isoforms with specific catalytic requirements (9, 40). When the two calpastatin forms were added separately to calpain at 100 µM Ca\(^{2+}\), thus in conditions promoting the formation of the protease-inhibitor complex (2, 5, 11-13), calpain became completely resistant to trypsin digestion as indicated (Fig. 1A) by the persistence of the native 80 kDa calpain band. Calpain digestion with trypsin carried out in the absence of Ca\(^{2+}\) (Fig. 1B) generated a 40 kDa fragment corresponding to the N-terminal region of the protease and containing the DI and DII domains (57, 58).

Unexpectedly, in these conditions RNCAST600 prevented calpain degradation as well as the accumulation of the 40 kDa peptide, still produced however in the presence of RNCAST300. The increase in ionic strength by addition of 0.15M NaCl (Fig. 1B lane 4) or the presence of low (1µM) Ca\(^{2+}\), a value close to physiological conditions, (Fig. 1C) did not modify the protective effect of RNCAST600. Identical results were obtained by substituting the sodium borate buffer with HEPES or TRIS/HCl at the same concentration (data not shown). Thus, in conditions resembling the physiological state, in terms of salt and Ca\(^{2+}\) concentrations, and in which calpain is present in the inactive, Ca\(^{2+}\) free form, the protective effect shown by RNCAST600 can be attributed to the presence of the non-inhibitory region (L-domain).

Identification of calpain calpastatin interaction by immunoprecipitation.
These observations were considered as an indication that the L-domain, present in RNCAST600 but not in RNCAST300, could interact with inactive free Ca\(^{2+}\) calpain form. Further evidence for the occurrence of this association was obtained by immunoprecipitation experiments. As shown in Fig. 2, when a mixture of calpastatin RNCAST600 and of calpain was added to an immobilized anti-calpastatin antibody in the presence of EDTA, the resin retained calpain (Fig. 2, lane 4) even though both the antibody and Protein G-Sepharose showed no affinity for the protease (Fig. 2, lane 2). In the presence of RNCAST300, no co-immunoprecipitation was observed (Fig. 2, lanes 5 and 6); this confirming that L-domain is required for interaction between calpastatin and calpain in its inactive state. Identical results were obtained in the presence of 1 µM Ca\(^{2+}\) (data not shown).

Molecular requirements for the interaction of calpastatin with calpain in its inactive free Ca\(^{2+}\) form.
The structural requirements for such interaction were explored using a number of recombinant calpastatins differing in the type of exons expressed in the L-domain. These inhibitor forms were tested on their efficiency in protecting calpain from trypsin digestion and in their ability to co-immunoprecipitate with calpain. As shown in Table I, protection from trypsin digestion and co-immunoprecipitation was observed only with those calpastatins containing exons from 4 to 7. Calpastatin forms lacking these regions, such as RNCAST300 or the 20-peptide residue derived from the B subdomain (24), resulted to be totally unable to interact with inactive calpain. These observations are in agreement with previous reports indicating that association of calpain to calpastatin in dividing myoblast cells and in myoblast differentiation does not require changes in intracellular physiological [Ca\(^{2+}\)] (34). Our results, however, provide additional information by demonstrating that specific determinants present in the L-domain of calpastatin, identified in exons from 4 to 7, are directly involved in the molecular association between the protease and its natural inhibitor.

Calpain-induced changes in molecular size of calpastatin in physiological like conditions.
A further approach for establishing calpain-calpastatin interaction involved the evaluation of the changes in the gel chromatography elution volume of calpastatin induced by the addition of calpain. As shown in Fig. 3A, in the absence of Ca\(^{2+}\), RNCAST600, normally emerging in the column included volume, appeared, when calpain...
was present, in fractions close to the void volume, indicating an increase in its molecular size. Addition of 0.15M NaCl and 1µM Ca$^{2+}$, to reconstitute a physiological-like condition, does not affect the changes in calpastatin elution volume (Fig. 3B). The calpain induced changes in molecular size of calpastatin has also been studied using the gel distribution method, previously described by Ackers (49). This procedure is based on the partition of a macromolecule between an aqueous phase and a gel phase. Thus, an increase in the amount of a macromolecule in the aqueous phase is an indication of a corresponding increase in its molecular size. We have observed that the amount of calpastatin RNCAST600 present in the aqueous phase is significantly increased by the addition of calpain (Fig. 3C, bars B-D). These results were not modified by the presence of Ca$^{2+}$ at concentrations from 0 to 2 µM (bars E-H of Fig. 3C). The truncated 75 kDa calpain form showed calpastatin interacting properties identical to the 80 kDa native protease form (cf. bar D and I of Fig. 3C), whereas the inactive 55 kDa calpain form, autolytically produced from the 75 kDa form, with the removal of the DII domain did not retain the calpastatin interaction capacity (Fig. 3C, bar J). These data are in agreement with those reported in Fig. 1 showing that calpastatin protects from trypsin digestion a region of calpain near to the end of DII domain. A number of controls have been carried out to establish the requirements and the specificity of this protein-protein interaction (Fig. 3D). We have found that other proteins such as BSA, carbonic anidrase, or aldolase (Fig. 3D, bars C-E and F-H) were unable to replace calpain or interfere with the effect produced by calpain. On the contrary, addition of 0.5 M NaCl (Fig. 3D, bar J) prevented completely the effect of calpain, indicating that electrostatic charges are involved in the formation or stabilization of the complex.

Interaction of calpastatin with inactive calpain in zymography.

The formation of a calpain-calpastatin complex has been studied using the zymography technique, a procedure that allows the separation of free from associated proteins and the quantification of the protease activity. As shown in Fig. 4A, the intensity of the calpain band was largely reduced if RNCAST600 was present in the starting sample. Similar results were obtained with µ- and m-calpain, with the autolyzed 75 kDa calpain form, and with other recombinant or native calpastatin forms. As expected, RNCAST300 resulted to be totally ineffective (Fig. 4A).

Since calpain and calpastatin migrated in positions very far one from the other, the disappearance of calpain activity band in the zymogram can be ascribed to the formation of a complex preserved during the electrophoretic run. Since identical findings has been obtained with different calpain isoforms or with different inhibitor preparations, regardless of their sources, it can be concluded that interaction of calpastatin with inactive calpain is a general process, not due to the peculiar property of a single calpain isoforms.

To identify directly the complex in the running gel, 0.5 cm slices were cut, and, the concomitant presence of calpain and calpastatin was established by immunoblotting following the elution of the protein. As shown in Fig. 4B, in addition to small amounts of free calpain and calpastatin, localized in their typical position, the largest quantity of both proteins were identified in a single gel slice, indicating that they had co-migrated as a complex, during the electrophoretic run.

Affinity of calpastatin for inactive and active calpain.

The zymogram analysis was also utilized to compare the affinity of calpastatin for calpain in its inactive (free Ca$^{2+}$) form and in its active state. The two calpain conformations were obtained by exposing the protease to EDTA or to high [Ca$^{2+}$]. As shown in Fig. 5, the disappearance of the calpain band as a function of calpastatin concentration, showed a behaviour (Fig. 5, inset) comparable to that of calpain inhibition measured in the presence of 1mM Ca$^{2+}$. Thus, RNCAST600 associates with similar affinity to calpain regardless of its conformational state. Although RNCAST300 did not interact with calpain in the zymogram analysis, it still expressed an inhibitory capacity at high [Ca$^{2+}$].

Role of the non-inhibitory L-domain on the calpain-calpastatin interaction.

The interaction of calpastatin L-domain with calpain is further supported by the observation showing that the addition of the free L-domain (RNCAST110), totally deprived of calpain inhibitory activity, can decrease the inhibitory efficiency of RNCAST600 at high [Ca$^{2+}$] (Fig. 6). Thus, the L-domain present in the two calpastatin forms can compete for the same binding site on calpain. In fact, RNCAST300, lacking the L-domain, is insensitive to the competitive effect of RNCAST110.

Altogether, the results herewith reported suggest a novel function of the L-domain in driving the
interaction between calpain and calpastatin. To explore the existence of mechanisms controlling the ability of the L-domain to associate to calpain, we have studied the effect of calpastatin phosphorylation since, as previously reported (41, 54), calpastatin can be phosphorylated by PKA and by PKC. The consensus sequences for such kinases, identified with NetPhos 2.0 Server software, are almost completely confined into the L-domain.

Effect of calpastatin phosphorylation on its interaction with inactive and active calpain.
RNCAST600 was found to be phosphorylated by both kinases (Fig. 7A) and that only the PKC phosphorylated form was significantly reduced in its capacity to interact with inactive calpain (Fig. 7B) and in its inhibitory efficiency on active calpain form (Fig. 7C). To define the regulatory role of this post translational modification, we have compared PKC phosphorylation with the L-domain exon composition in various calpastatin forms. As shown in Table II, a high degree of incorporation occurred only in those calpastatin forms containing the exon 6 which is present in the calpastatin L-domain region, interacting with Ca²⁺ free inactive calpain. Thus, the insertion of negative charges at these sites abolishes calpain-calpastatin interaction, further indicating the electrostatic nature of the calpastatin-inactive calpain association.

Phosphorylation by PKA, occurring at exon 8 located outside of the interacting region, does not affect the formation of the complex (Fig. 7B and C).

Phosphorylation of calpastatin appeared to take place also in “in vivo”, as demonstrated by the fact that total rat brain calpastatin can be separated into two major peaks following ion exchange chromatography (Fig. 8A). Both peaks contain a 70 kDa calpastatin together with small amounts of a 30 kDa form more represented in peak 1. With anti-phosphoserine antibody, only peak 2 was found to contain a phosphorylated calpastatin species (Fig. 8B), which resulted unable to interact with inactive Ca²⁺ free calpain form (Fig. 8C). As expected, the isolated non-phosphorylated calpastatin, present in the peak 1, retained its full interacting capacity (Fig. 8C). Although no information is available on the nature of the protein kinases involved in “in vivo” phosphorylation of calpastatin, these experiments are suggesting that in physiological conditions calpain-calpastatin association might be a highly regulated process.

“In vivo” calpain-calpastatin interaction.
This conclusion becomes more relevant upon quantification of the fraction of the total calpain molecules that associate with calpastatin in intact cells. For this purpose we have utilized a monoclonal antibody having the highest affinity for the conformation that calpain can acquire following association with calpastatin (37). As an experimental model, we have used Jurkat cells, stimulated with arachidonate, known to commit Jurkat cells to apoptosis through a transient early phase not involving appreciable changes intracellular free [Ca²⁺] (60, 61). To obtain an elevation of intracellular [Ca²⁺], cells were stimulated with the Ca²⁺-ionophore A23187. The results reported in Fig. 9 demonstrate that in unstimulated cells calpain is poorly stained by the mAb 56.3 and almost no calpastatin is detectable in the cytosol, being in these conditions largely confined in perinuclear aggregates (59). Following a brief exposure to arachidonate, calpastatin appears in consistent amounts in cytosol, concomitantly with a significant increase in calpain-mediated fluorescence, revealing the formation of the calpain-calpastatin complex. As compared to arachidonate, stimulation with the Ca²⁺-ionophore caused an almost two fold higher level of cytosolic calpastatin with no increase in calpain mediated fluorescence. These results not only link the formation of the complex to the availability of calpastatin but also demonstrate that a very large fraction of cytosolic calpain molecules are associated with the inhibitor.

Discussion
In the present paper we are demonstrating that the non-inhibitory region (L-domain) of calpastatin, can interact with the DII domain of calpain in its inactive calcium free form. This association, requiring the presence of the ammino acid sequence coded by exons from 4 to 7, has been demonstrated using different experimental approaches including: protection from trypsin digestion, immunoprecipitation and gel chromatography. In addition, a direct demonstration of the complex formation was obtained in zymography. This interaction shows a high degree of specificity and is stabilized by electrostatic charges localized in the interacting regions. In this respect of significant relevance is the negative effect produced by the introduction of negative phosphate groups in exon 6 of calpastatin L-domain.

Our observations are providing new information at a molecular level on such interaction, previously suggested to occur by Barnoy et al (34).
in developing muscle, and are defining its physiological role. Furthermore, our results are not in contrast with the recognized essential role of Ca^{2+} ions in promoting calpain-calpastatin association (12, 27-33). On the contrary, they clarify the physiological role of the non-inhibitory L-domain of calpastatin. Related to this conclusion is the observation that calpastatin lacking this N-terminal region, can associate and thereby inhibit the protease only in its active state. A peculiar aspect of the interaction between calpastatin and inactive calpain is its regulation by phosphorylation mechanisms. In the following model are represented the different steps of this new regulatory mechanism that we are proposing.

At nanomolar concentration of Ca^{2+}, typical of resting cells, calpain retains its inactive conformation and is unable to undergo activation at the plasma membrane. In these conditions, the equilibrium $\mathcal{Q}$ is shifted to the right, accumulating inactive calpain in cytosol. Following a rapid rise in $[\text{Ca}^{2+}]_i$, a large fraction of this cytosolic inactive calpain could undergo activation. To avoid such potential massive activation of calpain the concentration of the inactive Ca^{2+} free protease must be maintained at very low level. This can be accomplished, even at nanomolar physiological [Ca^{2+}], through the association of the still inactive calpain with the cytosolic calpastatin (equilibrium $\mathcal{Q}$). Thus the cytosolic calpastatin pool acquires a fundamental role in the control of the number of calpain molecules susceptible to activation. The equilibria $\mathcal{Q}$ and $\mathcal{Q}_1$ are indicating that also the cytosolic calpastatin pool is highly regulated through phosphorylation–dephosphorylation processes. This model is also supported by the experimental data reported in Fig. 9 showing that calpastatin mobilization is always accompanied by the formation of the calpain-calpastatin complex in cells stimulated with agonists that do not promote changes in intracellular Ca^{2+} homeostasis.

All these observations indicate that the calpastatin L-domain exerts a crucial role in the regulation of calpain activation, being the site of interaction with calpain in its inactive state. Hence, the alternative splicing processes shown to occur in the L-domain (9, 16, 18, 19) acquire more relevant biochemical significance, being the mechanism by which calpastatin can acquire a different capacity to interact with inactive calpain.

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References
Legends to Figures.

Fig. 1. Effect of recombinant RNCAST300 and RNCAST600 calpastatins on the digestion of calpain by trypsin.

Human erythrocyte calpain was purified and carboxymethylated (CM-) with iodoacetamide as described in (42). Recombinant calpastatin RNCAST300 and RNCAST600 were expressed and purified as described in Materials.

(A) CM-calpain (10 µg) was incubated at room temperature in 50 µl of sodium borate buffer, pH 7.5, containing 100 µM Ca²⁺ and 0.5 mM 2-mercaptoethanol without (lane 1) or with 0.01 µg of trypsin (lane 2-4) in the presence of 3 µg of RNCAST600 (lane 3) or of 1.5 µg of RNCAST300 (lane 4). The digestion was stopped by the addition of 2% SDS and heating at 100°C (see Methods). Aliquots (10 µl) were submitted to 10% SDS-PAGE and then protein was blotted as reported in Methods. Calpain was stained with the anti-calpain mAb 56.3.

(B) CM-calpain (10 µg) was incubated at room temperature as in (A), substituting Ca²⁺ with 1 mM EDTA, without (lane 1) or with 0.01 µg of trypsin (lane 2-5) in the presence of 3 µg of RNCAST600 (lane 3 and 4) or of 1.5 µg of RNCAST300 (lane 5). In lane 4, 0.15M NaCl was added to the incubation mixture to increase the ionic strength close to the physiological range. SDS-PAGE and calpain staining were carried out as in (A). Carboxymethylation does not alter the digestion pattern of calpain (data not shown).

(C) CM-calpain (10 µg) was incubated at room temperature as in (A) in the presence of 1 µM Ca²⁺, without (lane 1) or with 0.01 µg of trypsin (lane 2-4) in the presence of 3 µg of RNCAST600 (lane 3) or of 1.5 µg of RNCAST300 (lane 4). SDS-PAGE and calpain staining were carried out as in (A).

Figure 2. Evidence for calpain-calpastatin interaction revealed by co-immunoprecipitation in the absence of Ca²⁺.

RNCAST600 (0.5 µg) was immunoprecipitated with anti-calpastatin mAb 35.23 and the immunoprecipitated (IP) material was solubilized as described in Methods. Following SDS-PAGE calpastatin was detected by immunoblotting using the anti-calpastatin mAb 35.23 (lane 1). A control, human erythrocyte calpain (1 µg) was submitted to immunoprecipitation with anti-calpastatin mAb 35.23. The IP material was solubilized and submitted to SDS-PAGE as described in Methods. Following SDS-PAGE calpain was detected by immunoblotting using the anti-calpain mAb 56.3 (lane 2). RNCAST600 (0.5 µg) was mixed with human erythrocyte calpain (1 µg) and immunoprecipitated as above. The immunoprecipitated material was solubilized and divided in two aliquots. Both samples were submitted to SDS-PAGE. In lane 3, calpastatin was immunoblotted with anti-calpastatin mAb 35.23, and in lane 4 calpain was immunoblotted with mAb 56.3. RNCAST300 (0.25 µg) was mixed with human erythrocyte calpain (1 µg) and immunoprecipitated as above. The IP material was solubilized and divided in two aliquots. Both samples were submitted to SDS-PAGE and calpastatin immunoblotted with anti-calpastatin mAb 35.23, (lane 5) and calpain with mAb 56.3 (lane 6). A pure sample of human erythrocyte calpain (0.5 µg) was submitted to SDS-PAGE and immunoblotted with anti-calpain mAb 56.3 to establish the migration of the protease (lane 7). IgHC refers to the Immunoglobulin Heavy Chain; IgLC to the Immunoglobulin Light Chain.

Figure 3. Changes in molecular size of calpastatin following exposure to calpain in the absence or in the presence of physiological [Ca²⁺].

(A) Gel chromatography in the absence of Ca²⁺. RNCAST600 alone (10 µg, unfilled circles) or with 48 µg of human erythrocyte calpain (filled circles) was diluted in 50 µl (final volume) of 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA and 0.5 mM 2-mercaptoethanol and the solutions loaded on a Superdex™ 75 10/300 GL column, previously equilibrated with the same buffer. The flow rate was 0.5 ml/min and proteins were eluted with the equilibrating buffer. Fractions of 0.5 ml were collected. Aliquots (30 µl) of each fraction were collected, heated for 1 minute at 100°C and assayed for calpastatin activity as described in (43).

(B) Gel chromatography in the presence of 1µM Ca²⁺ and 0.15 M NaCl. To reflect more closely the physiological conditions, to the protease solution and to the equilibrating buffer 0.15M NaCl and 1µM Ca²⁺ were added. Chromatography and assay of calpastatin activity were carried out as in (A)
(C) Gel distribution analysis. RNCAST600 (0.5 µg) alone or together with the addition indicated elsewhere were added in a final volume of 0.5 ml to 0.5 ml of packed gel (Sephacryl S-300). The distribution of calpastatin between the aqueous phase and the gel fraction was determined as described in Methods. The bars refer to: A, RNCAST600 alone; B-D, RNCAST600 in the presence of increasing amount of calpain (C, 1.5, 2.3, 4.6 µg respectively); E-F, RNCAST600 in the presence of 1 and 2 µM Ca²⁺ respectively; G-H, RNCAST600 in the presence of 2.3 µg of calpain (as in bar C) and 1 or 2 µM Ca²⁺; I-J, RNCAST600 in the presence of 75 kDa and 55 kDa autolyzed calpain forms (75kDa C, 55kDa C), respectively. The total activity of RNCAST600 used in each experiment was taken as 100%. Assay of calpastatin activity was carried out as in (A). The values reported are the arithmetical means of four different experiments.

(D) Specificity of the interaction process. RNCAST600 (0.5 µg) alone or together with the indicated additions was determined as in (C) and in Methods. The bars refer to: A, RNCAST600 alone; B, RNCAST600 in the presence of 2.3 µg of calpain (C); C-E, RNCAST600 in the presence of bovine serum albumin (BSA), carbonic anidrase (CA) or rabbit muscle aldolase (Ald), respectively; F-H, same mixtures used in C-E containing also 2.3 µg of calpain (C); I-J, a mixture of RNCAST600 and calpain (as in B) in the presence of 0.15 M and 0.5 M NaCl respectively. Assay of calpastatin activity was carried out as in (A). The values reported are the arithmetical means of four different experiments.

Figure 4. Characterization of calpain-calpastatin interaction by zymography.
(A) (Upper part). The calpain isoforms isolated from the indicated sources and the erythrocyte autolyzed 75 kDa form (1 µg) were incubated without (-) or with (+) calpastatin (0.25 µg) in the presence of EDTA as specified in Methods. The mixtures were then submitted to zymography. The calpain activity was revealed following overnight exposure of the gel to 10 mM Ca²⁺ at 25°C and staining with Comassie brilliant blue (see Methods) (53).
(Lower part). Calpastatin forms (1 µg) isolated from the indicated sources and recombinant RNCAST300 were mixed with the same amount of human erythrocyte calpain and submitted to zymography as described above. The calpain activity was revealed as previously described.

(B) Human erythrocyte calpain alone (3 µg), calpastatin RNCAST600 alone (0.7 µg) and a mixture of these two proteins were submitted to zymography as described in (A) and in Methods. In the inset the representative images of the three zymogram analysis are shown. To identify the calpain-calpastatin complex, at the end of the electrophoretic run, the unstained gels were cut in 20 slices (0.5 cm each). Proteins were extracted from each slice by gentle shaking for 5 hours in 0.3 ml of distilled water. Aliquots (100 µl) of these solutions were subjected to 10% SDS-PAGE and immunoblotted with anti-calpain mAb 56.3 or with anti-calpastatin mAb 35.23. The immunoreactive bands (from slice 3 to 17) were detected and quantified as reported in Methods.

1: immunostaining with mAb 56.3 of the electrophoretic run containing calpain (3 µg) alone;
2: immunostaining with mAb 35.23 of the electrophoretic run containing RNCAST600 (0.7 µg) alone;
3a: immunostaining with mAb 56.3 of the electrophoretic run containing a mixture of calpain (3 µg) and RNCAST600 (0.7 µg);
3b: immunostaining with mAb 35.23 of the electrophoretic run containing a mixture of calpain (3 µg) and RNCAST600 (0.7 µg).

Figure 5. Efficiency of the calpain-calpastatin interaction in the presence and in the absence of Ca²⁺.
Human erythrocyte calpain (1.1 µg) alone or mixed with increasing amounts (from 0 to 0.25 µg) of RNCAST600 was submitted to zymography as described in Fig. 4 and in Methods. Calpain activity in each band (shown in the inset) was identified and quantified (unfilled circles) as described in Methods. The band intensity observed in the absence of calpastatin was taken as 100% of calpain activity. Alternatively, human erythrocyte calpain (0.25 µg) was assayed in the presence of 1 mM Ca²⁺ with increasing amounts (from 0 to 0.60 µg) of RNCAST600 (filled circles) or of RNCAST300 (from 0 to 0.03 µg, unfilled squares). The values reported are the arithmetical means of five different experiments ± SD.

Figure 6. Effect of calpastatin L-domain (RNCAST110) on the inhibition of calpain by RNCAST600 and RNCAST300 in the presence of Ca²⁺.
Human erythrocyte calpain (0.25 µg) was assayed with 1 mM Ca²⁺ in the presence of 0.06 µg of RNCAST600 (unfilled circles) or 0.03 µg of RNCAST300 (filled circles) with increasing amounts of RNCAST110 (from 0 to 0.1 µg).
The calpain activity measured in the absence of calpastatin was taken as 100%. The results are the arithmetical means of five different experiments ± SD.

**Figure 7. Effect of calpastatin phosphorylation on calpain-calpastatin interaction.**
(A) Phosphorylation of RNCAST600 by PKA and PKC. RNCAST600 (6.3 µg) was phosphorylated by PKA and PKC as described in Methods. Aliquots (0.6 µg) of the two mixtures were submitted to 12% SDS-PAGE and γ-32P incorporation was detected and quantified as described in Methods. The amount of phosphate groups incorporated in calpastatin was approximately 1.4-1.6 ± 0.3 equivalents per mol of inhibitor. (B) Interaction of phosphorylated calpastatin with inactive calpain. The native and the two phosphorylated calpastatin forms (0.25 µg) were mixed with 1 µg of human erythrocyte calpastatin and submitted to zymography in the presence of 1 mM EDTA. Calpain activity was revealed following overnight exposure to 10 mM Ca2+ and staining of the gels. (C) Interaction of phosphorylated calpastatin with active calpain. Human erythrocyte calpain (0.25 µg) was assayed in the presence of 1 mM Ca2+ with increasing amounts of native (unfilled circles), PKA-phosphorylated (unfilled squares) or PKC-phosphorylated (filled squares) RNCAT600. The calpain activity detected in the absence of RNCAST600 was taken as 100%. The values reported are the arithmetical means of five different experiments.

**Figure 8. Interaction between calpain and “in vivo” phosphorylated calpastatin.**
(A) Rat brain calpastatin forms were separated by ion-exchange chromatography as described in Materials. Calpastatins present in the eluted fractions were identified following the inhibition of human erythrocyte calpain activity. The two peaks, designated as peak 1 and peak 2, were separately collected and concentrated. (B) Samples (25 µl) of the two calpastatin peaks were submitted to 12% SDS-PAGE, blotted and proteins revealed with both anti-calpastatin mAb 35.23 (anti-CAST) or with an anti-phosphoserine antibody (anti-Ser-P). (C) Human erythrocyte calpain (one unit) was mixed with one unit of calpastatin peak 1 or 2 and submitted to zymography in the presence of EDTA. Calpain activity was detected following substrate digestion and staining of the gels.

**Figure 9. Identification and quantification of the calpain/calpastatin complex in “in vivo” conditions.**
(A) Jurkat cells (2 x 10^5), untreated and stimulated with 100 µM arachidonate or with 1 µM A23187 Ca2+-ionophore were fixed and permeabilized as described in Methods and in (56). Cells were then loaded with anti-calpain mAb 56.3 or with anti-calpastatin mAb 35.23 antibodies and the immuno-complexes were revealed with a secondary Alexa 488 antimouse antibody (see Methods). Fluorescence intensity was then measured with a BioRad confocal microscope and quantified with the Laser-Pix software. The results of the quantifications are shown in (B). The open bars refer to calpain fluorescence, the filled bars to calpastatin. The arrow indicates the calpastatin perinuclear localization in control cells.
Table I. Correlation between the exons composition of calpastatin L-domain and the ability to form a complex with calpain in its inactive conformation.

<table>
<thead>
<tr>
<th>Calpastatin form</th>
<th>Exons composition of L-domain</th>
<th>Number of inhibitory domains</th>
<th>Protection of calpain from trypsin digestion (%)</th>
<th>Co-immunoprecipitation (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNACAST104</td>
<td>2,4-8</td>
<td>4</td>
<td>90 ± 8</td>
<td>100</td>
</tr>
<tr>
<td>RNCAST23</td>
<td>2,4,5,7,8</td>
<td>1</td>
<td>88 ± 8</td>
<td>90</td>
</tr>
<tr>
<td>RNCAST600</td>
<td>2,4-8</td>
<td>1</td>
<td>96 ± 5</td>
<td>100</td>
</tr>
<tr>
<td>Human calpastatin fragment</td>
<td>4-8</td>
<td>1</td>
<td>85 ± 10</td>
<td>90</td>
</tr>
<tr>
<td>RNCAST110</td>
<td>2,4-8</td>
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<td>70 ± 12</td>
<td>85</td>
</tr>
<tr>
<td>RNCAST300</td>
<td>8</td>
<td>1</td>
<td>5 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>B subdomain</td>
<td>--</td>
<td>--</td>
<td>0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*a/* The exons expressed in the L-domain were established from the amino acid sequence of each protein and compared with the known sequences (reviewed in 2). The schematic drawing at the top of the table represents the structure of calpastatin showing also the four repetitive domains (1 to 4) and the subdomains contained in each unit (capital letters). The numbers in parenthesis refer to the exons contained in each calpastatin domain.

*b/* The recombinant calpastatin forms were prepared as described in Materials.

*c/* The number of repetitive inhibitor units were established from the amino acid sequence of each protein and compared with the known sequences (9, 13, 18).

*d/* Calpain was incubated in the presence of 1 mM EDTA with the indicated recombinant calpastatin forms as described in Methods and in the legend to Fig. 1. Protection of calpain from trypsin digestion was determined measuring the percentage of native 80 kDa calpain band present in the incubation mixtures after 60 minutes of exposure to trypsin.

*e/* Calpain mixed with the indicated recombinant calpastatin forms were added to the immobilized anti-calpastatin antibody, as described in Methods and in the legend to Fig. 2. The values refer to the relative amounts of calpain retained by the gel beads, compared to the total amount added, evaluated by western blotting following SDS-PAGE.

n.d. = not detectable.
Table II. Correlation between exon composition of calpastatin L-domain and susceptibility to phosphorylation by PKC.

<table>
<thead>
<tr>
<th>Calpastatin form&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Exon in L-domain&lt;sup&gt;b&lt;/sup&gt;</th>
<th>&lt;sup&gt;c&lt;/sup&gt;32P incorporation&lt;sup&gt;c&lt;/sup&gt; (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNCAST300</td>
<td>8</td>
<td>0.11</td>
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<tr>
<td>RNCAST600</td>
<td>2,4,5,6,7,8</td>
<td>1.56</td>
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<tr>
<td>RNCAST 23</td>
<td>2,4,5,7,8</td>
<td>0.44</td>
</tr>
<tr>
<td>RNCAST107</td>
<td>2,4,5,7,8</td>
<td>0.42</td>
</tr>
<tr>
<td>RNCAST104</td>
<td>2,4,5,6,7,8</td>
<td>1.65</td>
</tr>
</tbody>
</table>

<sup>a</sup> The recombinant calpastatin forms were prepared as described in Methods.

<sup>b</sup> The exons expressed in the non-inhibitory L-domain have been established comparing the amino acid sequences of these proteins with known sequences (9).

<sup>c</sup> <sup>32</sup>P incorporation was determined as described in Methods.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

Calpain inhibition (%) vs. RNCAST110, µg

RNCAST300  RNCAST600
Figure 7

Figure 7
Figure 8
Figure 9
Association of calpastatin with inactive calpain: a novel mechanism to control the activation of the protease?
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