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Research Article

Changes in calpastatin localization and expression during calpain activation: a new mechanism for the regulation of intracellular Ca^{2+}-dependent proteolysis

M. Averna, R. De Tullio, P. Capini, F. Salamino, S. Pontremoli and E. Melloni*

Department of Experimental Medicine-Biochemistry Section and Centre of Excellence for Biomedical Research, University of Genoa, Viale Benedetto XV, 16132 Genoa (Italy), Fax: +39 010 518343, e-mail: melloni@unige.it.

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Abstract. The amount of calpastatin directly available in cytosol is under the control of [Ca^{2+}] and [cyclic AMP]. Prolonged calpain activation also promotes degradation of calpastatin. The fluctuation of calpastatin concentration in cell soluble fraction is accompanied by an initial decrease in calpastatin gene expression, followed by a fivefold increase in its expression when the inhibitor protein is degraded. This process can be conceptualized as a mechanism to regulate calpastatin availability in the cell. This conclusion is supported by the fact that calpain, the other component of this proteolytic system, undergoes changes in its levels of expression in a much more limited manner. Furthermore, this process can be observed both in cells exposed to different natural stimuli, or in other cell lines. Modification of calpastatin gene expression might represent a new tool for the in vivo control of the regulatory machinery required for the modulation of Ca^{2+}-dependent proteolysis.

Key words. Calcium-dependent proteolysis; calpain; calpastatin; calpastatin expression; enzyme regulation; calcium homeostasis.

Calpains, the proteases of the calcium-dependent proteolytic system, are involved in the regulation of cellular functions through the limited proteolysis of many target proteins [1–4]. The structure of the m-isoform of the classical calpain subfamily has been recently determined [5, 6], and shows features particularly adapted to the physiological role of this enzyme. In resting conditions, the classical μ- and m-calpains are located in the cytosol in an inactive form which undergoes a calcium-induced conformational transition required to align the essential residues located in the catalytic subdomains IIa and IIb [7, 8]. Although the precise events initiated by the binding of calcium and leading to the active state of this protease are not well defined, this mechanism represents a sensitive and effective regulation system which connects calpain activity to the intracellular [Ca^{2+}] changes induced by extracellular stimuli under precise cell conditions [8, 9].

The second regulatory agent is calpastatin, the natural inhibitor of the protease [10, 11]. Its primary structure indicates the presence of repeating inhibitory domains and an NH_2-terminal domain without inhibitory function [12–15]. Each inhibitory domain contains three subdomains that have precise functions as they interact with amino acid sequences present in both the catalytic and the regulatory subunit of calpain [16, 17]. Calpastatin tertiary structure is still unknown, however, in recent reports specific functional regions have been identified [18, 19]. Furthermore, multiple forms of calpastatin have been identified, mainly produced by alternative splicing of dif-

* Corresponding author.

ferent exons in the N-terminal region of the transcript [20–22]. The most abundant calpastatin forms are those composed, in addition to a variable N-terminal end, by four repetitive domains, but calpastatin forms having a lower number of repetitive units have also been identified [23]. The transcriptional changes in the N-terminal region lead to the production of calpastatin molecules having different putative posttranslational modification sites which might be responsible for determining calpastatin cell localization, as well as for modulation of its specificity and inhibitory efficiency [21, 24, 25]. Involvement of a calpastatin N-terminal domain in the regulation of calcium ion channel is also suggested [26]. These calpastatin properties are controlled by the coordinate action of protein kinase and protein phosphatase activities [27]: cyclic AMP (cAMP)-dependent protein kinase (PKA) supports the formation of calpastatin aggregates, decreasing the number of inhibitor molecules directly available for interaction with calpain. The reverse effect, which is mediated by a protein phosphatase, induces the release of calpastatin molecules from the aggregates. Furthermore, calpastatin can be degraded both in vitro and in in vivo conditions by calpains, producing an imbalance of this proteolytic system, leading to uncontrolled digestion of intracellular structures [15, 28–35].

Even if it is generally accepted that calpain activation occurs following an increase in intracellular free Ca^{2+}, it is also known that the same conditions promote formation of the calpain-calpastatin complex, preventing calpain translocation to the membrane, its activation and expression of catalytic activity. Because the same signal produces both calpain activation and inhibition, further mechanisms are required to facilitate the expression of calpain catalytic activity under intracellular conditions.

The mechanism described in this paper, together with previous experimental evidence [27], may represent a physiological answer to this problem. We now report that the amounts of available calpastatin at the moment of calpain activation are sufficiently low to allow such an activation process. In this paper we also describe a series of changes in the level of calpastatin expression which can be considered as a mechanism for continuously monitoring the amount of calpastatin in cells for the actual regulation of calpain activity.

Experimental Procedures

Materials

Ca^{2+} ionophore A23187, leupeptin, phenylmethylsulfonyl fluoride (PMSF), phorbol-12-myristate-13-acetate (PMA), nerve growth factor (NGF) and forskolin were purchased from Sigma. Anti-calpastatin monoclonal antibody (mAb) 35.23, which binds the N-terminal region of this protein, was produced as described [36]. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse secondary antibody was obtained from Amersham Biosciences. Anti-Protein Kinase Cα antibody was purchased from Santa Cruz Biotechnology. Anti-Phospho-CREB (Ser^{133}) antibody was provided from Cell Signaling Technology (Beverly, MA, USA). Diethylaminoethyl cellulose (DE53) was obtained from Whatman. Hydroxyapatite (HA)-Ultrogel was purchased from Amersham Biosciences.

Human erythrocyte calpain was purified as reported previously [37], and one unit of calpain activity is defined as the amount causing the production of 1 µmol of acid-soluble-NH_2 groups under conditions reported previously [38].

Methods

Cell cultures

Human pheochromocytoma PC12 cells were purchased from the American Type Culture Collection (A.T.C.C.) (Rockville, MD, USA) and grown as described previously [39]. Murine erythroleukemia (MEL) cells were obtained and cultured as specified previously [40]. PC12 cells cultured in RPMI medium (which contains 0.42 mM $CaCl_2$) were exposed to 10 nM Ca^{2+}-ionophore A23187 for different times of incubation. These conditions were previously set up to promote changes in intracellular Ca^{2+} homeostasis not affecting of cell properties such as viability, rate of growth and sensitivity to differentiating agents even after 10 days of calcium ionophore treatment [41].

Immunofluorescence confocal microscopy and calpastatin fluorescence quantification

PC12 cells grown on glass slides were fixed and permeabilized by the Triton/paraformaldehyde method, as described in [36]. Cells were then treated with 7 µg/ml anti-calpastatin mAb 35.23 diluted in phosphate-buffered saline (PBS) containing 5% (v/v) fetal-calf serum. After incubation for 3 h at room temperature, cells were washed three times in PBS and treated with 8 µg/ml FITC-conjugated sheep anti-mouse (Amersham Biosciences) secondary antibody for 1 h. Staining of chromatin was carried out by incubating fixed cells with 2 µg/ml propidium iodide for 5 min [42]. Cells were mounted on coverslips with FluoroGuard antifade reagent (Bio-Rad Laboratories) before analysis. Images of the samples were collected the same day by confocal microscopy using a Bio-Rad MRC1024 instrument (krypton/argon laser) on a Nikon Diaphot 200. The excitation/emission wavelengths were 488/522 nm for fluorescein-labeled antibodies and 488–568/605 nm for propidium iodide-stained chromatin. Quantification of fluorescence was obtained with Laser Pix Software (Bio-Rad Microscience).

Levels of calpastatin activity in PC12 cells

PC12 cells were cultured in Roswell Park Memorial Institute (RPMI) medium (which contains 0.42 mM $CaCl_2$) in 75-cm^2 flasks. After each experiment, confluent cells were washed with phosphate saline buffer and harvested with 3 ml of 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EGTA and 0.5 mM β-mercaptoethanol (buffer A). Cell suspensions were lysed by sonication (four bursts of 10 s each) in the presence of 0.1 mg/ml leupeptin and 2 mM PMSF. The particulate material was discarded by centrifugation at 100,000 × g for 15 min, and the clear solution (crude extract) was heated at 100 °C for 3 min and centrifuged at 100,000 × g for 10 min. The soluble material was collected and loaded onto a 5-ml column packed with DE53 equilibrated in buffer A.

The absorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.35 M, and calpastatin activity was measured using human erythrocyte calpain [38] and human acid denatured globin as substrate [43]. Human erythrocyte calpain was chosen because it is equally sensitive to all calpastatin forms, regardless of their sources. Levels of calpastatin were calculated from the area underlying the activity peaks. One unit of calpastatin activity is defined as the amount of inhibitor required to inhibit one unit of human erythrocyte calpain.

Immunoblot analysis

PC12 cells, or MEL cells (1×10^5) were lysed in electrophoresis buffer and submitted to 12% SDS-polyacrylamide gel electrophoresis (PAGE) as described by [44].

Proteins were then transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. Membranes were probed with a specific anti-calpastatin monoclonal antibody 35.23 [36], or with an anti-PKCα antibody, or with an anti-Phospho-CREB (Ser^{133}) antibody followed by a peroxidase-conjugated secondary antibody as described by [45] and then developed with an enhanced chemiluminescence detection system (Amersham Biosciences).

Immunoreactive bands were quantified by scanning analysis with a Shimadzu CS-900 densitometer.

Ca^{2+}-ATPase activity in PC12 cells

Membranes from PC12 cells cultured in the presence of 10 nM Ca^{2+}-ionophore for different times were prepared, and Ca^{2+}-ATPase activity was assayed as described in [46].

Levels of PKC activity in MEL cells

MEL cells (40×10^6) were treated with PMA 100 ng/ml for 4 and 24 h. After incubation the cells were lysed by sonication in 2 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM leupeptin, 2 mM PMSF and then centrifuged at 100,000 × g for 10 min. The supernatant was collected and loaded onto HA-Ultrogel and PKC activity was determined on 100 µl of the eluted fractions as previously described [47].

Levels of µ- and m-calpain activity in PC12 cells

PC12 cells were cultured in RPMI medium in 75-cm^2 flasks. At the end of each treatment, when the cells were confluent, they were lysed and processed as for the preparation of calpastatin crude extracts with the following modifications: crude extracts were directly loaded on a Source 15Q column (3 ml) equilibrated in buffer A. Proteins were eluted with a linear gradient of NaCl from 0 to 0.4 M. Aliquots of each fraction were used to assay calpain activity. The levels of µ- and m-calpain were calculated from the area underlying the activity peaks.

Levels of calpain and calpastatin mRNA in PC12 or MEL cells

Total RNA was isolated from PC12 cells, or MEL cells (1×10^6 each), by extraction with guanidium thiocyanate, and aliquots (5 µg RNA/20 µl) were reverse transcribed as described [48]. Equal amounts (1 µl) of each sample were coamplified in the presence of primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense primer 5′-ACGACCCCTTCATTGACC-3′ and antisense primer 5′-TGCTTCACCACCTTCTTG-3') and of primers for calpastatin and calpains. The oligonucleotides for calpastatin were sense primer for PC12 cells 5′-CTGGCATCTCCCAGAATGAG-3′ and antisense primer 5′-TCAAAAGTCACCATCCACCAGC-3′; sense primer for MEL cells 5′-TGTGCCTCGGGCCTCCATGTGC-3′and antisense primer 5′-TGGAGCTGCTGCATGGGTGCTTGGAG-3′. The oligonucleotides for µ-calpain were sense primer 5′-GATGGAGCTACCCGCACAGAC-3′, antisense primer 5′-GTGGAGGGCAC CACCACATAC-3′; the oligonucleotides for m-calpain were: sense primer 5′-GCCACCCGCACAGACATC-3′, antisense primer 5′-AAGGTGGAAGGCACGAGAATG-3′. Polymerase chain reaction (PCR) conditions were: for calpastatin, 22 cycles of 94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s; for calpains, 92°C for 2 min, 22 cycles of 92 °C for 10 s, 55 °C for 30 s, 68 °C for 3 min and 68 °C for 6 min. PCR products were separated on 1% agarose gel stained with ethidium bromide, and the amount of amplified products was determined by densitometric analysis using the Kodak Digital Science 1D Image Analysis Software. Messenger RNA (mRNA) levels were quantitatively determined with the method described by Chelly et al. [49] following the modification reported in [50].

Results

Effect of intracellular calcium increase on calpastatin localization

In order to identify changes in calpastatin levels and localization, PC12 cells were stimulated with Ca^{2+}-ionophore in controlled conditions affecting neither cell growth nor sensitivity to differentiating agents. The limited increase in Ca^{2+} ions produced in these conditions could mimic the primary alteration in tissues of hypertensive rats [28].

As shown in figure 1, in resting PC12 cells, calpastatin, detected with a specific monoclonal antibody, is localized in aggregates which are close to the nucleus (fig. 1A). The cytosolic fluorescence is very poor at the level of background, indicating that almost all the calpastatin molecules are confined in these structures and are not available for calpain inhibition. Following 2 days of treatment (fig. 1B) the aggregates are still present, but a significant fluorescence is now detectable in the soluble fraction, suggesting a redistribution of calpastatin as soluble free molecules. After 8 days of incubation in the presence of Ca^{2+}-ionophore (fig. 1C), the aggregates are decreased in number and intensity, and calpastatin is mostly localized in the cytosol. In these conditions, a modification of cell shape also occurs, probably because the differentiation process is triggered by the elevated [Ca^{2+}]. Hence, activation and regulation of Ca^{2+}-dependent proteolysis are linked processes both induced by the same signal, such as an intracellular increase in Ca^{2+}.

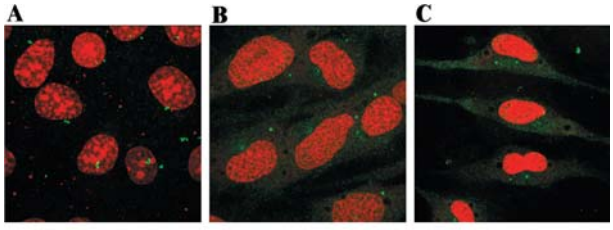

Figure 1. Effect of an increase in intracellular free calcium on calpastatin localization in PC12 cells. PC12 cells were cultured in the absence (*A*) or presence of 10 nM Ca^{2+}-ionophore A23187 for 2 days (*B*) or 8 days (*C*). After treatment, calpastatin localization was determined by confocal microscopy using anti-calpastatin mAb 35.23 as described in the 'Experimental Procedures' section.

To better define this process, we have determined the changes in calpastatin localization by measuring the fluorescence levels both in aggregates and in the cytosol. As shown in figure 2A, in untreated cells calpastatin fluorescence is almost completely confined in aggregates. Following treatment with Ca^{2+}-ionophore (fig. 2B), total fluorescence increases progressively, after 2 days of incubation reaching values approximately four- to fivefold higher compared with control. This increase is due to the appearance of comparable amounts of calpastatin in cytosol. After 8 days of treatment (fig. 2C), the aggregates are reduced to very low levels, with the concomitant further increase in cytosolic calpastatin (sevenfold). The large increase in fluorescence observed following Ca^{2+}-ionophore incubation can be ascribed to the release of calpastatin as free molecules in cytosol. Moreover, Western blot analysis indicates that after 2 days of treatment, native calpastatin protein level is unaffected, whereas after 8 days of incubation (fig. 2D) the amount of high molecular weight calpastatin is decreased to ~ 60% of its original value. These data indicate that calpastatin degradation occurs concomitant with a prolonged calpain activation, as previously observed [34].

Expression of calpastatin in Ca^{2+}-enriched cells

We have also analyzed whether these changes in the level of available calpastatin are correlated to modifications in the transcription rate for this protein. As shown in figure 3A, following 2 days of incubation with Ca^{2+}-ionophore,

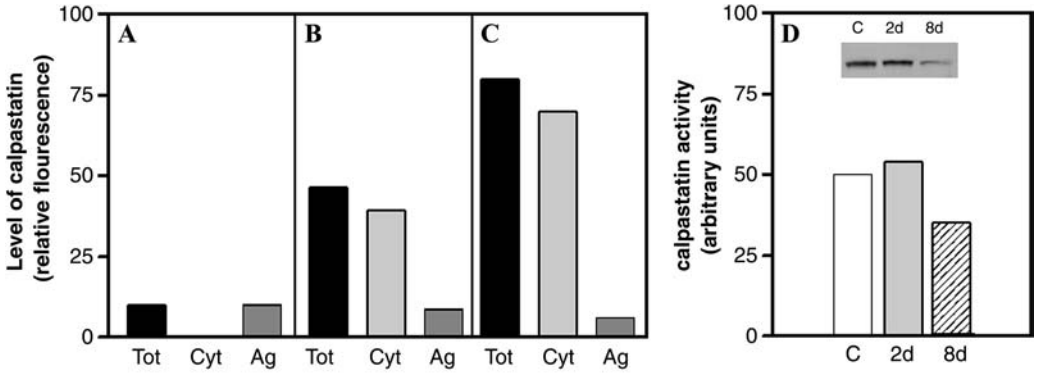

Figure 2. Measurement of calpastatin distribution and activity in PC12 cells following exposition to calcium ions. PC12 cells were cultured in the absence (*A*) or presence of 10 nM Ca^{2+}-ionophore A23187 for 2 days (*B*) or 8 days (*C*). The levels of total (Tot), cytosolic (Cyt) and aggregated (Ag) calpastatin fluorescence were quantified as described in 'Methods'. (*D*) PC12 cells cultured in the absence (C) or in the presence of 10 nM Ca^{2+}-ionophore A23187 for 2 (2d) or 8 days (8d) were lysed, and calpastatin activity was determined after ion-exchange chromatography as described in 'Methods'. Inset: Levels of calpastatin protein from PC12 cells determined by immunoblot.

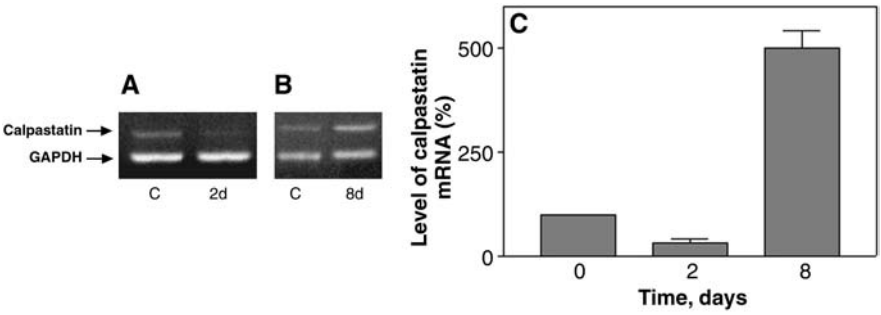

Figure 3. Effect of an increase in intracellular free calcium on calpastatin mRNA levels in PC12 cells. Equal amounts of cDNA (1 µl) from PC12 cells untreated (C) and cultured for 2 (2d) (*A*) or 8 days (8d) (*B*) in the presence of 10 nM Ca^{2+}-ionophore A23187 were submitted to 22 cycles of amplification. The primers used for GAPDH and calpastatin are indicated in 'Methods'. PCR products were separated on 1% agarose gel stained with ethidium bromide. (*C*) mRNA levels determined by densitometric analysis of the gel. The results obtained are the mean ± SD of three separate experiments.

calpastatin mRNA is decreased approximately threefold compared with control values, indicating that its expression is largely repressed. It is important to point out that these experimental conditions also promote a large increase in cytosolic calpastatin levels (see fig. 1B). However, following 8 days of treatment (fig. 3B), the amount of calpastatin mRNA is increased approximately fivefold with respect to untreated PC12 cells (fig. 3C).

Taken together, these data suggest that calpastatin gene expression is somehow related to the availability of this protein in the cytosol. Thus, under conditions causing the large calpastatin consumption observed after 8 days of exposition to Ca^{2+}-ionophore, a fivefold increase in the rate of calpastatin mRNA synthesis occurs (fig. 3C).

Changes in calpain expression under conditions promoting its activation

Thus, in order to establish whether these changes in calpastatin expression are part of a complex regulation process or are an adaptation to alterations in calpain levels, we have also investigated modifications occurring in the expression of both µ- and m-calpain. We have observed that while in PC12 cells m-calpain mRNA levels are unchanged after either 2 or 8 days of Ca^{2+}-ionophore treatment (fig. 4), the amount of µ-calpain mRNA is slightly decreased (25–30%) after 2 days, and then increases (up to 60%), with respect to control values, following 8 days of treatment. Although this increase in µ-calpain expression could be attributed to the high rate of utilization of this proteinase occurring under these conditions, these modifications seem less significant than those observed for calpastatin.

Calpastatin degradation implies that calpain is active under these experimental conditions. To verify this hypothesis, we at first investigated the intracellular degradation of specific calpain substrates such as the plasma membrane Ca^{2+}-ATPase [46]. As shown in figure 5, the level of active Ca^{2+}-ATPase decreases as a function of the incubation time, confirming that calpain becomes activated. This fact is further supported by the observation that under these conditions, µ-calpain is consumed, its activity reduced up to 40% with respect to untreated cells following 8 days of exposure to Ca^{2+}-ionophore (fig. 6). In contrast, m-calpain activity remains unchanged.

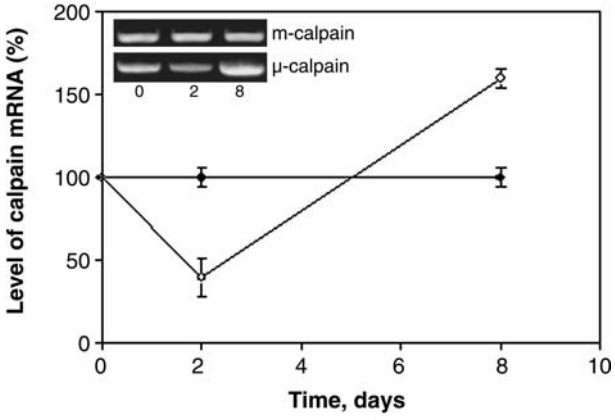

Figure 4. Effect of an increase in intracellular free calcium on µ- and m-calpain mRNA levels in PC12 cells. Equal amounts of cDNA (1 µl) from PC12 cells cultured in the presence of 10 nM Ca^{2+}-ionophore A23187 for the indicated times were submitted to 22 cycles of amplification. The primers used for µ- (○) and m-calpain (●) are indicated in 'Methods'. PCR products were separated on 1% agarose gel stained with ethidium bromide, and mRNA levels were determined by densitometric analysis of the gel. The amount of GAPDH transcript coamplified with µ- or m-calpain was the same for all the samples considered.

Effect of different stimuli on calpastatin expression and localization

To determine whether these changes in calpastatin expression and localization also take place in cells exposed to different stimuli, PC12 cells were treated with NGF, their natural differentiation inducer [39] which also promotes intracellular calcium fluctuations [51]. As shown in figure 7A, following 6 days of NGF exposure, calpastatin mRNA is largely decreased (60–70%) in parallel

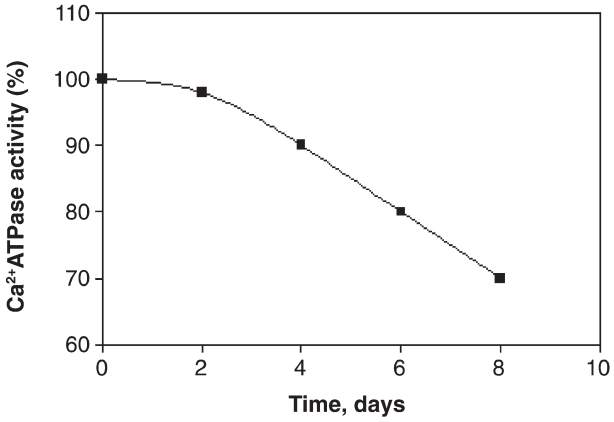

Figure 5. Levels of Ca^{2+}-ATPase activity in PC12 cells cultured in the presence of Ca^{2+}-ionophore. PC12 cells were cultured in the presence of 10 nM Ca^{2+}-ionophore A23187 for the indicated times. After incubation, cells were lysed and membranes were prepared as described in [46]. Ca^{2+}-ATPase activity was measured through the formation of its labeled acyl-phosphate intermediate visualized following SDS-PAGE, using Cyclone Storage Phosphor screen (Packard).

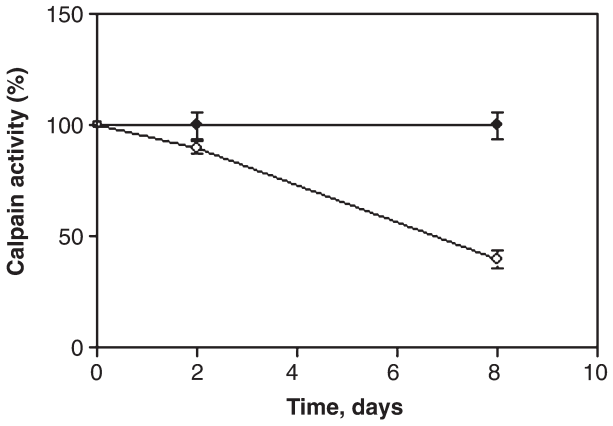

Figure 6. Levels of μ- and m-calpain activity in PC12 cells cultivated in the presence of Ca^{2+}-ionophore. PC12 cells were cultivated in the presence of 10 nM Ca^{2+}-ionophore A23187 for the indicated times. After incubation, confluent cells were lysed and processed as described in 'Methods'. μ- (○) and m-calpain (●) levels were determined after ion-exchange chromatography, measuring the area underlying the activity peaks. The results obtained are the mean ± S.D. of three separate experiments.

with the appearance of this protein in the cytosol, as indicated by the increase in total calpastatin fluorescence (fig. 7B). These observations further support the idea that the level of free calpastatin molecules is directly related to the rate of its gene expression.

To further analyze this correlation in another cell model, we treated MEL cells with PMA, which is known to induce both calpain-mediated PKC down regulation through an increase in intracellular [Ca^{2+}] [41, 52]. As shown in figure 8A, the level of PKCα is 80% reduced within 4 h of incubation, with 90% recovery following 24 h of exposure to PMA (fig. 8C, open bars).

The disappearance of PKCα blocked cell growth, which is 80% recovered when the level of the kinase is also restored (fig. 8C, filled bars). The level of calpastatin mRNA changes following an identical kinetics: at four hours of PMA exposure, it is 50–60% reduced, returning after 24 h of incubation approximately to 100% (fig. 8B, C, gray bars).

Involvement of cAMP on calpastatin expression in PC12 cells

We have previously reported that calpastatin phosphorylation is involved in the regulation of specificity and efficiency [24–25]. A PKA-mediated phosphorylation was found to promote calpastatin aggregation. To further

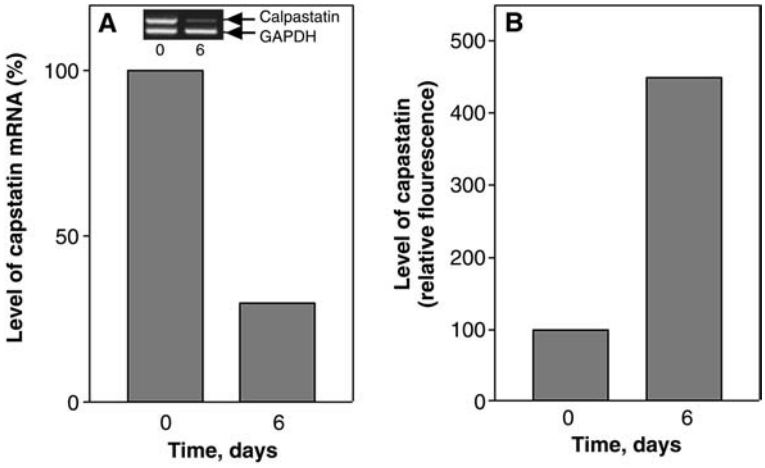

Figure 7. Effect of NGF treatment on levels of calpastatin mRNA and on calpastatin fluorescence. (*A*) Calpastatin mRNA levels from PC12 cells, treated for the indicated times with 100 ng/ml NGF, were determined after 22 cycles of amplification as previously described, by measuring the ethidium bromide intensity of the amplified fragments separated by agarose electrophoresis (inset). (*B*) Calpastatin fluorescence in PC12 cells treated for the indicated times with 100 ng/ml NGF was determined as described in 'Methods'.

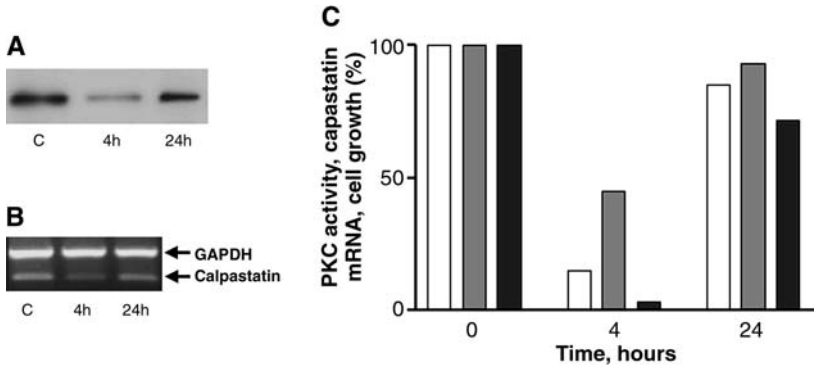

Figure 8. Changes in calpastatin expression in MEL cells treated with PMA. (*A*) MEL cells (1×10^5 cells) untreated (*C*) or treated with 100 ng/ml PMA for 4 (4 h) or 24 hours (24h) were lysed in SDS-PAGE buffer and submitted to Western blot analysis to detect PKCα protein (see 'Methods'). (*B*) Equal amounts of cDNA (1µl) from MEL cells untreated (*C*) or treated with 100 ng/ml PMA for 4 (4 h) or 24 h (24 h) were submitted to 22 cycles of amplification and separated on 1% agarose gel stained with ethidium bromide. The primers used for GAPDH and calpastatin are indicated in 'Methods'. (*C*) PKC activity (open bars) and calpastatin mRNA levels (gray bars) and cell growth (filled bars) were determined on MEL cells untreated or treated for the times indicated with 100 ng/ml PMA. The levels of PKC activity were measured as described in 'Methods'. Quantification of calpastatin mRNA was determined by densitometric analysis, as previously reported. Cell growth was calculated as indicated in 'Methods'.

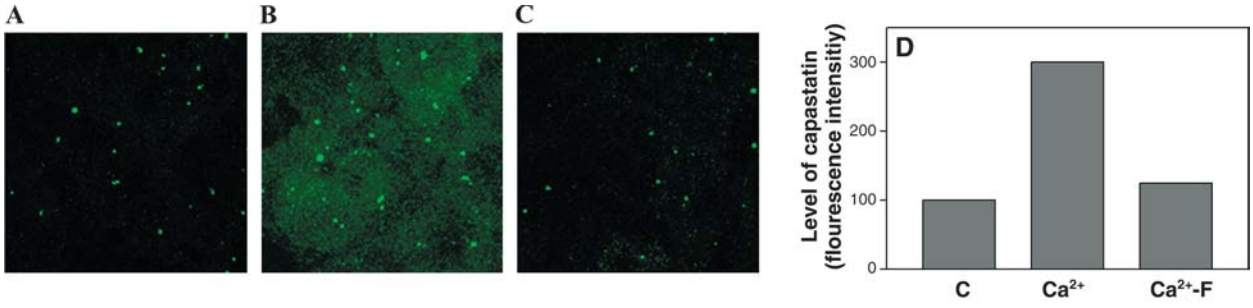

Figure 9. Effect of forskolin incubation on calpastatin localization in PC12 cells. PC12 cells were incubated in the presence of 20 µM forskolin for 1 day (*A*), or in 10 nM Ca^{2+}-ionophore A23187 for 2 days (*B*), or in 10 nM Ca^{2+}-ionophore A23187 for 2 days and then with 20 µM forskolin for 1 day (*C*). (*D*) Calpastatin fluorescence was determined in PC12 cells cultured in the presence of 20 µM forskolin for 1 day (*C*), or in the presence of 10 nM Ca^{2+}-ionophore A23187 for 2 days (Ca^{2+}), in the presence of 20 µM forskolin for 1 day after exposure for 2 days to 10 nM Ca^{2+}-ionophore A23187 (Ca^{2+}-F). Levels of calpastatin fluorescence were calculated as described in 'Methods'.

characterize this process, we have incubated PC12 cells with forskolin, known to stimulate intracellular cAMP production [53]. As shown in figure 9A, forskolin does not induce changes in calpastatin localization when incubated with untreated cells; however, it promotes calpastatin reaggregation in PC12 cells treated with Ca^{2+}-ionophore (compare fig. 9B and C).

To establish whether cAMP is involved in the control of calpastatin expression together with its cellular localization, we tested whether cAMP-response element-binding protein (CREB) is activated in the experimental conditions employed. To analyze this possibility, we used MEL cells exposed to PMA in which (see fig. 8) a large change in calpastatin transcription rate occurs. As shown in figure 10, it clearly appears that after 4 h of incubation with PMA, CREB becomes phosphorylated to a large extent indicating active transcription of cAMP-related genes. It is reasonable to think that under these conditions the calpastatin gene could be expressed at a higher rate, producing the accumulation of inhibitory molecules. Hence, the cAMP signal can have a dual role depending on the level of cytosolic free [Ca^{2+}]: it can induce calpastatin aggregation through PKA activation, or it can promote calpastatin synthesis through CREB activation.

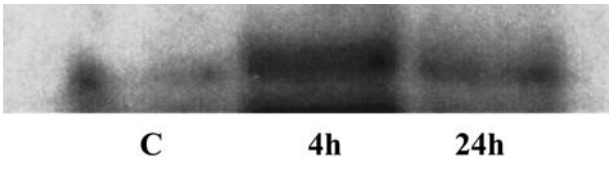

Figure 10. CREB phosphorylation in MEL cells treated with PMA. MEL cells untreated (1×10^5 cells) (C) or treated with 100 ng/ml PMA for 4 (4 h) and 24 hours (24 h) were lysed in SDS-PAGE buffer and submitted to Western blot. Phospho-CREB transcription factor was detected using the anti-Phospho-CREB (Ser133) antibody as described in 'Methods'.

Discussion

Regulation of intracellular calcium-dependent proteolysis is accomplished primarily by changes in [Ca^{2+}] and also by calpastatin, the natural protein inhibitor of calpain. However, it is now emerging that calpastatin undergoes posttranslational modifications and changes in cellular localization which affect both specificity and inhibitory capacity [1–4, 24, 25]. A number of protein kinases and phosphatases are involved in such processes, directing calpastatin to its appropriate cell function through the insertion or removal of phosphate groups in specific protein regions. We now report that calpastatin synthesis is also directly related to the levels and cellular distribution of the inhibitor.

Our observations indicate that calpastatin expression is somehow linked to the inhibitory capacity required by the cell. In resting cells, when no Ca^{2+}-dependent proteolysis is required, calpastatin is almost completely unavailable for calpain inhibition and is associated in large aggregates, probably representing intracellular storage. Calpastatin becomes available in the cytosol only in response to an increase in calcium ions. This shift in localization is fully reversible and under the control of cAMP-mediated phosphorylation. The increase in free calpastatin molecules in the cytosol is directly correlated to a decrease in its expression. Hence, calpastatin redistribution in the soluble fraction of the cell not only represents an increase in the inhibitory capacity in this cellular compartment, but is also related to calpastatin gene expression. An abundance of the inhibitor molecules seems the signal for reduction of its expression. However, if calpastatin is degraded due to prolonged calpain activation, the inhibition in the synthesis of its mRNA is removed and transcription is increased up to fivefold. A similar situation has been observed in the kidney of hypertensive rats, in which degradation of calpastatin is associated with high levels of expression [50]. These findings suggest that calpastatin cytosolic levels are somehow monitored to control the expression of this protein. When calpastatin is released from storage aggregates, its expression is decreased; when calpastatin is consumed to protect the cell from calpain digestion, its expression is significantly enhanced. Posttranscriptional and posttranslational calpastatin modifications are required to prevent damage to cell structures that uncontrolled calpain activation might produce. The intracellular level of cAMP plays a fundamental role in this modulation because it induces calpastatin removal from the cytosol through activation of PKA. Activation of CREB observed under conditions of active calpastatin synthesis also suggests possible involvement of the cAMP signal in regulation of calpastatin gene expression. Probably these two processes do not occur simultaneously, since calpastatin synthesis is triggered in the presence of high [Ca^{2+}], when the inhibitor molecules are degraded. The imbalance in the regulatory system due to calpastatin degradation occurring when calpain is activated for a long time can cause disruption of essential cell structures, a phenomenon known as pathological calpain function.

All the other components of this proteolytic system, such as μ- and m-calpain, do not undergo changes in expression comparable to those observed for calpastatin. Thus, the extent of fluctuation in calpastatin expression and the physiological conditions under which it occurs indicate that this process is a further regulatory mechanism required to define the actual inhibitory capacity of the cell.

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