Review

The calpain-calpastatin system: structural and functional properties

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Introduction

In all animal cells, a soluble proteolytic system has been identified and found to be composed of a protease, named calpain, and a natural endogenous inhibitor protein, named calpastatin.[1-12] Several properties of this system suggest that it may have a highly specialized function specifically related to the general process of signal transduction and cell activation (for review see refs. 3, 4, and 12). This concept has been derived mostly from consideration on the intracellular localization of the calpain-calpastatin system, from its modulation by changes in Ca^{2+} concentration, and by the nature of the protein substrates of the proteinase.

However, several questions concerning the mechanism of activation and regulation of calpain, and the precise role of the different calpain isoforms, that are present in different amounts in single cell types, have not yet been solved.

The present contribution is intended as a general overview on each of these aspects, in an effort to provide a better understanding of the operation of the calpain-calpastatin system and of its physiological role in cell function.

Calpain structure

Animal calpains are expressed in two different isoforms classified on the basis of their different Ca^{2+} sensitivity as calpain I and calpain II.[1,3,5,13-15] In order to express proteolytic activity, calpain I requires micromolar Ca^{2+}, calpain II millimolar Ca^{2+}. In all cell types analyzed thus far, both isozymes are present, with the exception of circulating blood cells (erythrocytes, neutrophils, lymphocytes) in which only one type of the calpain I family has been found.[3,16-18] The absolute and relative amount of calpain differs from one cell to the other regardless of the presence of a single or of both isoforms.

All calpains are characterized by an heterodimeric structure composed of a large 80 kDa and of a small 30 kDa subunit.[3,4,12,19] In terms of function, the 80 kDa subunit contains the catalytic site, whereas the 30 kDa subunit probably exerts a regulatory function.

In the large subunit (*Figure 1*), four domains have been identified.[8,21,22] The N-terminal domain I might serve two distinct but correlated functions (see calpain activation). The removal of this domain promotes the conversion of the inactive proenzyme form (procalpain) to active enzyme (calpain) and the release of the active enzyme from its association with membranes. Domain II has a high degree of sequence homology with the typical cysteine proteinases, and thus contains the active site of calpain. Although at present the role of domain III still requires additional investigation, it appears to be involved in the binding of the natural inhibitor calpastatin or/and in the control of the function of domain II. Domains IV and IV′ of the small subunit[23] contain a calmodulin-like amino acid sequence that identifies the Ca^{2+} binding sites of the protease. Finally, domain V[23] has been postulated to exert a regulatory function since its removal increases calcium sensitivity, without affecting the quaternary structure of calpain or its ability to associate to membranes.[24]

It is reasonable to assume that other functions must still be attributed to single domains also in terms of binding sites for additional ligands recently discovered.[25-31] These functions should include the interaction of the proteinase with the natural activator (see calpain activation), and should clarify the exact distribution and function of the Ca^{2+} binding sites with respect to the conformational changes that are required for activation of calpain.

Thus, the implication is that evolution has provided calpain with a molecular architecture that is able to prevent the expression of the proteinase activity of domain II in an unregulated fashion. Calpain appears to possess a built-in self-regulation due to the presence of accessory domains that repress proteolytic activity and keep the enzyme, during the quiescent phase of the cell, in an inactive state. Upon binding with Ca^{2+} and with the activator, the enzyme is derepressed and

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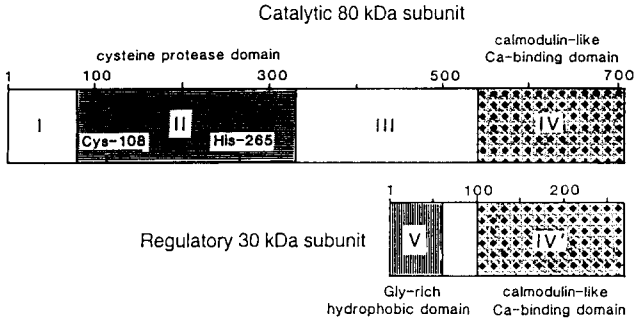

Figure 1 Molecular organization of calpain. The sequence shown is that of chicken muscle. Taken from Suzuki et al.[4]

becomes active through conformational changes that abolish interaction of accessory domains with the catalytic one.

A final consideration is that the structure of all 80 kDa and 30 kDa subunits examined thus far are highly homologous.[32] The slight differences in the sequence, among the different isozyme types, might be related to the different Ca^{2+} sensitivity that exists not only between calpains I and II but also among the same class of isozymes.

Structure of calpastatin

Calpastatin is the natural inhibitor protein of calpain,[1,3-5,33-37] is present in all animal cells containing the proteinase, and is characterized by a monomeric structure with a molecular mass of 105–110 kDa.

The structure of calpastatins[38-41] consists of an N-terminal region (domain L) followed by 4 domains of approximately 140 residues each, characterized by a high degree of homology. An exception to this structure is represented by erythrocyte calpastatin in which domain L and 1 are missing.[42]

The four highly conserved regions represent four calpain interacting sites as indicated by the observation that each repetitive domain, produced by expressing cloned complementary DNA portions, possesses an identical inhibitory activity.[43,44] A similar conclusion has been reached on the basis of the observation indicating that calpastatin can be fragmented by calpain into small peptides, each one still retaining an almost complete inhibitory activity.[45-48] This result provides additional information concerning the existence of selective regions, between repetitive domains, each containing recognition sites for proteolytic attacks. Thus, in theory, one molecule of calpastatin should inhibit 4 molecules of calpain. However, several experimental results, obtained both with intact cells or in reconstructed systems, indicate that this potential inhibitory capacity of the natural intact protein molecule is not expressed with such stoichiometry.[17]

It is important to stress that the mode of inhibition of calpains by calpastatin is competitive and fully reversible, leading to fully active calpain and calpastatin, upon dissociation of the enzyme-inhibitor complex.[17,49-51] As in the case of calpains, each cell type seems to have its physiological or optimal amount of calpastatin with the result that in almost each cell, the level of the inhibitor is different and, conversely, a different calpain/calpastatin ratio is characteristic of the various cell types.

Table 1 Proposed events accompanying the primary and secondary function of calpain

	Primary function	Secondary (abnormal) function
Priming event	Increase in Ca^{2+} (within the physiological range)	Increase in Ca^{2+} (above the physiological range)
Site of calpain activation	Membranes (inner surface)	Membranes (inner surface), cytoskeleton or cytosol
Type of proteolytic modification	Highly specific (limited proteolysis of target proteins)	Non-specific (degradation of soluble and particulate protein substrates)
Function	Activation of a specific cell response	General protein catabolism (cell damage and death?)

Calpain substrates

Since the original discovery of calpain, cytoskeletal proteins have been recognized as the preferential substrates of the proteinase in all animal cells.[52-55] However, such identification has been obtained mostly in vitro at non-physiological concentrations of Ca^{2+}. It is therefore still to be established if degradation of these proteins occurs in vivo as part of the postulated primary or of the secondary or pathological function of calpain, as recently proposed by Murachi et al.[12] and schematically represented in *Table 1*.

According to these arbitrary definitions, the primary function should be correlated to physiological intracellular fluctuations in $[Ca^{2+}]$ with the result of a high degree of selectivity in calpain activity. Selectivity also should include recognition and limited proteolysis of a specific protein substrate, resulting, for example, in the activation of a specific enzyme, rearrangement of cytoskeletal protein interactions, membrane fusion, etc.

Secondary function should be correlated almost exclusively to pathological conditions characterized by an abnormally high increase in $[Ca^{2+}]$, resulting in an uncontrolled activation and loss in specificity of calpain function leading to a virtually indiscriminate degradation of cell proteins.

In our opinion, these points have not been considered sufficiently in the elaboration of the long list of protein substrates of calpain. Although cytoskeletal proteins in general are all potential substrates, calpain mediated signal transduction, induced by physiological fluctuations in $[Ca^{2+}]$ must involve a limited proteolysis of a restricted number of crucial target proteins.

This hypothesis is in accordance with the identifi-

cation in vivo of other potential substrates, including enzymes constitutively associated to the membranes such as Ca^{2+}-ATPase and NADPH oxidase,[56-59] or those that become associated to the membranes[60-63] during their activation process (protein kinase C). Moreover, receptor proteins, such as hormones[64-68] and growth factor receptors,[69-75] have also been reported to be susceptible to degradation by calpain.

An article of this length cannot be exhaustive of all the literature concerning the various calpain substrates described thus far. For this purpose, we refer to recent articles,[55,56] which contain a detailed list of the various potential substrates of calpain.

Another important problem, correlated with the nature of the substrates and with the so-called primary function of calpain, is the definition of the physiological role of the proteinase. At present, this role has been identified presumptively with the remodeling of cytoskeletal structures involved in cell division[55,77-80]; with the process of platelet aggregation[81-86] and granule exocytosis,[63,87,88] with the regulation of microtubule structure and function (for a review, see ref. 55), and of neuron development and differentiation.[89]

Limited proteolysis of PKC has been shown to be associated with the formation of the constitutively active fragment of the kinase, which becomes free to redistribute itself intracellularly.[60,61,63]

Moreover, recent experiments in our laboratory have indicated that calpain level and calpain activity are involved in cell commitment to terminal differentiation induced by HMBA.[90]

Also, the few examples reported in terms of the possible physiological role tend to illustrate the multiplicity of functions that can be accomplished by calpain in many fundamental biological processes. The understanding of the physiological role of this proteolytic system will contribute significantly to the comprehension of those mechanisms of signal transduction, which are linked to physiological fluctuation in the intracellular Ca^{2+} concentration that can result from external influx or mobilization from intra cellular stores.

Calpain activation

Calpain II (the millimolar [Ca^{2+}] requiring form) can become active following a number of different pathways, all of which may or may not occur in all animal cells.

The basic mechanism for activation is the conversion of the inactive proenzyme to an active enzyme form through an autoproteolytic digestion that removes a small peptide from both subunits (domain I or/and V) or from one or the other (for review, see refs. 3–5, 12, and 91). This modified protease form (*Figure 2* and *Table 2*) is active at lower concentration of Ca^{2+}, much more close to the physiological values. However, for autolysis to occur, high concentrations of Ca^{2+} are required and thus the problem of how to fulfill this initial requirement has to be solved.

A proposed biochemical mechanism that satisfies this requirement involves translocation from the cytosol of the inactive proenzyme in the presence of a concentration of Ca^{2+} significantly lower than that required for autolysis. Translocation results in the association to the inner surface of cell membrane, where interaction with phospholipid induces an enzyme conformation with higher sensitivity for Ca^{2+} that facilitates activation through the described autoproteolytic process (*Table 3*). Activated calpain, having lost the anchoring domain, is either released into the cytosol or remains, at least partially, associated to the membrane.[92] Since it is the interaction process by itself that increases sensitivity to Ca^{2+}, it is not surprising that one or two forms of calpains, which are not susceptible to autoproteolytic activation, can express proteolytic activity only so long as they remain in the membrane-bound form and return to an inactive state upon dissociation from the membrane surface.[18,93]

Although this autolytic activation process should be regarded as an obligatory step for calpain II type isozymes, we have shown that it also operates for those forms of calpain I for which the Ca^{2+} require-

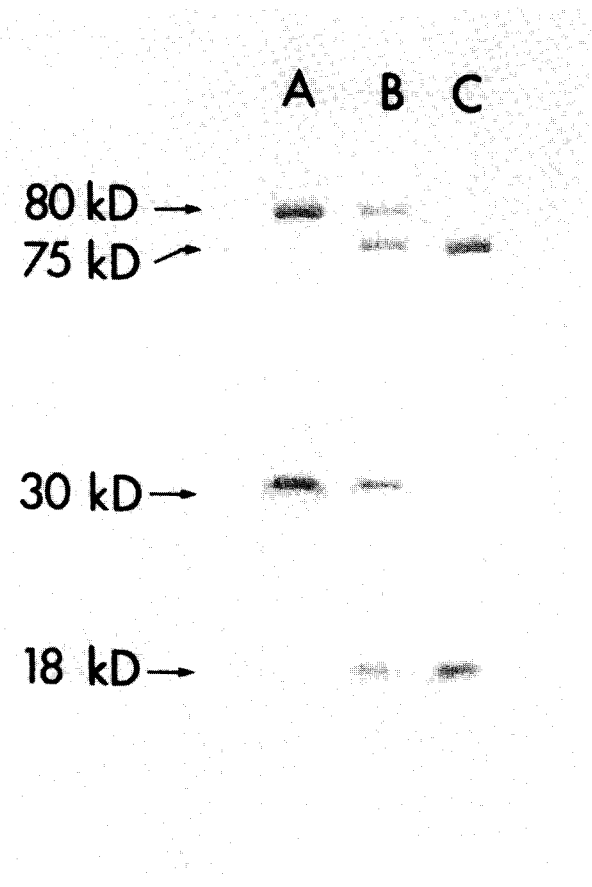

Figure 2 Typical electrophoretic pattern in the presence of sodium dodecyl sulfate indicating the changes in molecular properties of calpain occurring during the autolytic conversion from high to low Ca^{2+} requiring forms. For experimental details, see references 17 and 91. A, native calpain; B, mixture of native and activated calpain; C, activated calpain.

Table 2 Catalytic and molecular properties of procalpain and of calpain forms

Addition (preincubated with)	Activity assayed at μM Ca^{2+} (% of maximal)	Subunit composition (Kd)
CALPAIN I[a]		
None (EDTA)	0	80/30 (100%)
0.03 mM CA^{2+}, 1 min	50%	80/30 (50%) + 75/18 (50%)
0.1 mM Ca^{2+}, 1 min	100%	75/18 (100%)
CALPAIN II[a]		
None (EDTA)	0	80/30 (100%)
0.25 mM Ca^{2+}, 1 min	50%	80/30 (50%) + 76/18 (50%)
0.750 mM Ca^{2+}, 1 min	100%	76/18 (100%)

[a] Human erythrocyte calpain I or rat skeletal muscle calpain II were preincubated in the specified condition and then assayed for activity in the presence of 1 or 20 μM Ca^{2+}, respectively. The subunit composition was determined as in *Figure 3*.
For experimental details, see Pontremoli et al.[17,30,31]

Table 3 Activation of erythrocyte calpain at 1 μM [Ca^{2+}], in the presence of cell membrane fractions

Addition	Activation (conversion of procalpain to calpain) (% of maximal)
mM Ca^{2+}	100%
Plasma membrane (inside out vesicles)	68%
Stripped membranes deprived of cytoskeletal protein	65%
Phospholipid vesicles	60%

Erythrocyte calpain was incubated with the indicated membrane fraction for 1 min in the presence of 1 μM Ca^{2+}. The activation was calculated by the appearance of catalytic activity measurable at 1 μM Ca^{2+}. The various membrane fractions were prepared from human erythrocytes. For experimental details, see Pontremoli et al.[100]

ment, although in the μM range, is still too high with respect to the physiological values.[3]

This translocation-activation mechanism is particularly interesting because it represents a site-directed activation of a proteinase within a crucial cell compartment. Thus, proteins involved in the cytoskeleton-plasma membrane interaction, transmembrane or intrinsic membrane proteins, enzymes translocated to the surface of membranes or to the cytoskeleton array, can all become calpain substrates. Aside from other considerations, it is interesting to speculate that joint translocation to structural elements of the cell of both the proteinase and the substrate could be visualized as one of the mechanisms that confers the required specificity for calpain activity.

A second mechanism of activation that appears to be restricted to those calpain forms with a requirement for Ca^{2+} above 50 μM (including all calpain II and few calpain I isoforms) involves interaction of the inactive proenzyme with a low molecular weight activator protein (*Figure 3*) associated to the cell cytoskeleton proteins.[28,31] Following association with the activator, procalpain undergoes autoproteolytic conversion to a low Ca^{2+} requiring form that dissociates from the activator becoming a soluble, fully active protease (Pontremoli, S., et al., unpublished observation).

Interaction between calpain and its activator requires a low (μM) concentration of Ca^{2+} which suggests its occurrence in vivo by means of a translocation process essentially identical to the one that promotes translocation of procalpain to the inner surface of the membrane.

Although the presence of a calpain activator has been described in several cells,[25,26,28,31] it has not been considered thus far as an important component in the regulation of calpain. We have suggested previously, and again propose here, that interaction with the activator protein is an important mechanism in activation of calpain.

In terms of mode of interaction, the activator binds to calpain in a molar ratio of approximately 1/1; binding of the activator is Ca^{2+} dependent, occurs at a site different from that of calpastatin, and abolishes inhibition by calpastatin[29,31] without displacing the inhibitor from the enzyme molecule (*Figure 4*).

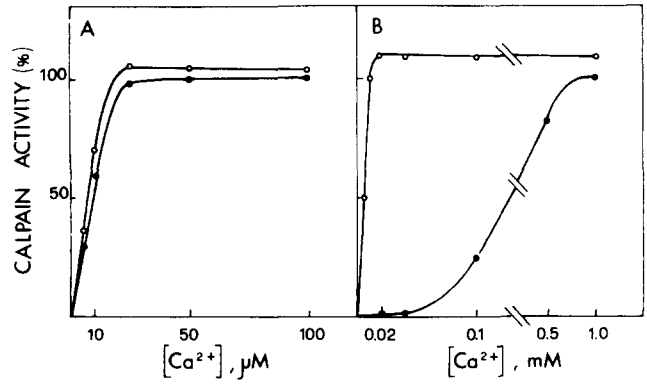

Figure 3 Effect of the natural activator protein on the catalytic properties of calpain I (A) and II (B). Rat skeletal muscle calpain I and II were assayed at the indicated [Ca^{2+}] in the absence (closed symbols) or in the presence (open symbols) of the activator protein added in equal amounts to both proteinase. For experimental details, see Pontremoli et al.[31]

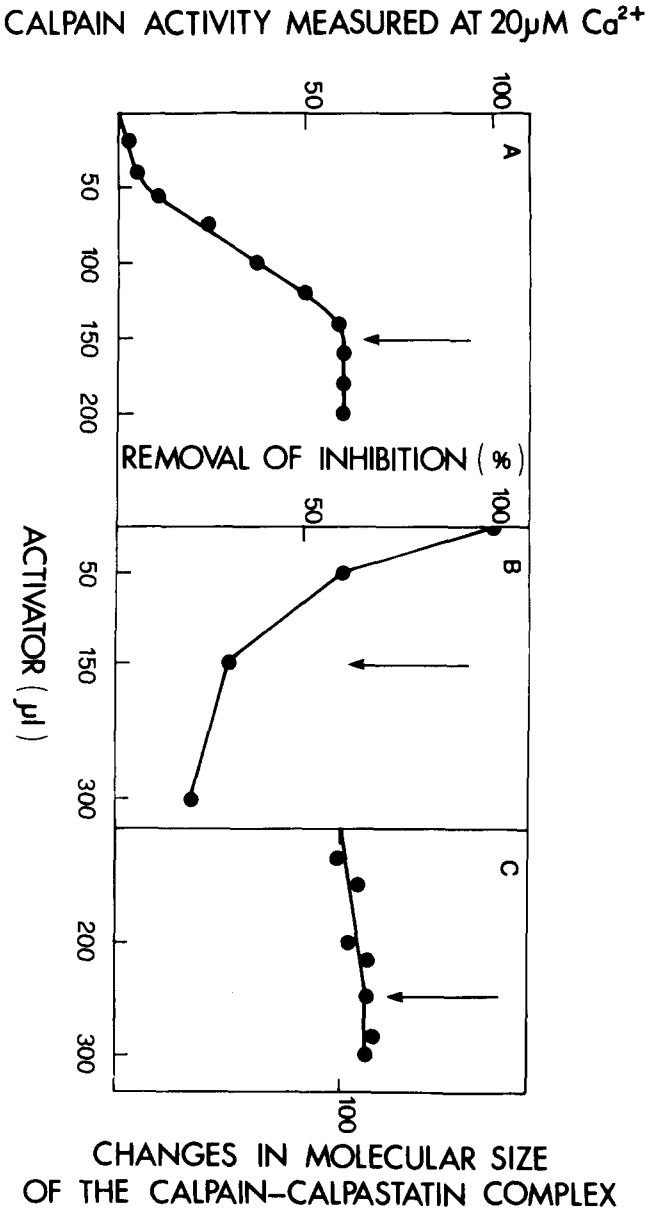

Figure 4 (A) Effect of increasing concentrations of the activator protein on calpain activity measured at 5 μM [Ca^{2+}]. Rat skeletal muscle calpain II was assayed at μM Ca^{2+} in the presence of the indicated amounts of the activator protein. For experimental details, see Pontremoli et al.[31] The arrow indicates the equimolarity between calpain and the activator. (B) Effect of the activator protein on the inhibition of calpain by calpastatin. Rat skeletal muscle calpain II was assayed at 500 μM Ca^{2+} in the presence of an amount of calpastatin that produces 90% inhibition. Purified activator protein was then added in the indicated amounts and calpain activity was assayed. For experimental details, see Pontremoli et al.[31] The arrow indicates the equimolarity between calpain and the natural activator. (C) Effect of the activator complex on the calpain II-calpastatin complex. The variations in the molecular size of the calpain-calpastatin complex were evaluated on the basis of changes in the gel-distribution pattern[102] of the complex following addition of the indicated amounts of the activator protein. For experimental details, see Pontremoli et al.[31]

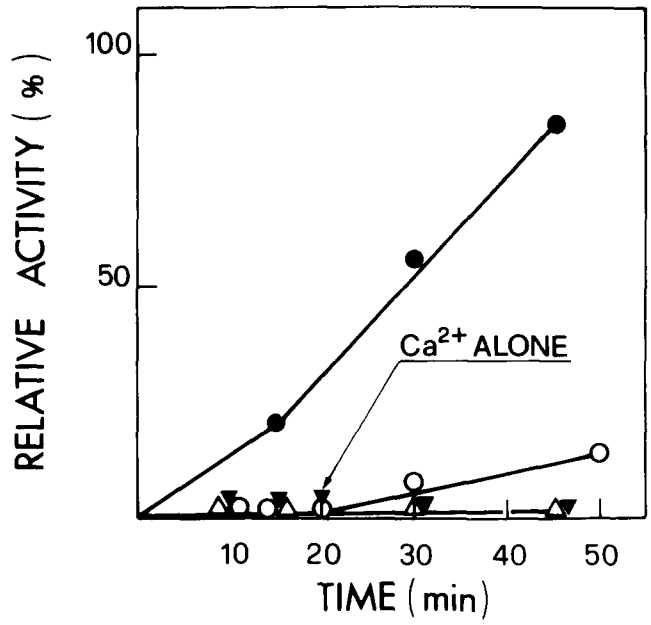

Figure 5 Effect of substrate on the conversion, at 1 μM Ca^{2+}, of erythrocyte procalpain to active calpain. Erythrocyte calpain was incubated with 1 μM Ca^{2+} in the absence (▼) or in the presence of saturating amount (2 mg/ml) of denatured human globin (●), casein (○), or bovine serum albumin (△) (BSA). At the time indicated, aliquots of the mixtures were collected and assayed for calpain activity in the presence of 1 μM Ca^{2+}. For experimental details, see Pontremoli et al.[17]

A third mechanism is that suggested by the experimental data reported in *Figure 5* which indicate that formation of an autolyzed form of calpain constitutively active at μM Ca^{2+} can also occur in the presence of a highly digestible substrate and of micromolar amounts of Ca^{2+} (*Figure 5*).

This mechanism[16,17,50] has never received great attention, probably due to the slow rate at which the autoproteolytic conversion occurs.

However, the rate of the process should not represent a limitation to its physiological role; on the contrary, this mechanism could explain some pathological lesions that presumably occur as a result of repeated slow activation of calpain. Moreover, it should be considered that this mechanism represents the only one capable to promote activation of calpain in the cytosolic compartment of the cell at low [Ca^{2+}].

At the end of this brief description of the possible activation mechanisms of calpain, proposed thus far by different groups,[3-5,12] we want to emphasize that the basic mechanism for regulation of calpain still remains that represented by the fluctuation in Ca^{2+} concentration, which can also promote a selective site-directed activation of the proteinase at the level of structural elements of the cell.

Intracellular operation of the calpain-calpastatin system

The calpain-calpastatin system is itself a potentially auto-regulated system being composed of a protease and of its natural inhibitor.

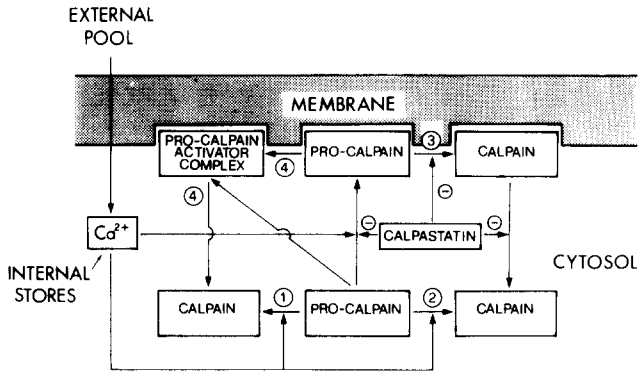

Figure 6 Proposed model for calpain activation. Procalpain indicates the native form of the proteinase; calpain indicates the active form. Numbers in parentheses refer to single activating steps promoted by: 1) high (abnormal) increase in the concentration of Ca^{2+}; 2) presence of a digestible substrate at relatively low [Ca^{2+}]; 3) interaction with membrane phospholipids; and 4) association to the natural protein activator. The symbol ⊖ refers to the action of calpastatin that can result in preventing both calpain activity and autoproteolytic activation. Furthermore, calpastatin competes with membranes for binding of native calpain.[92]

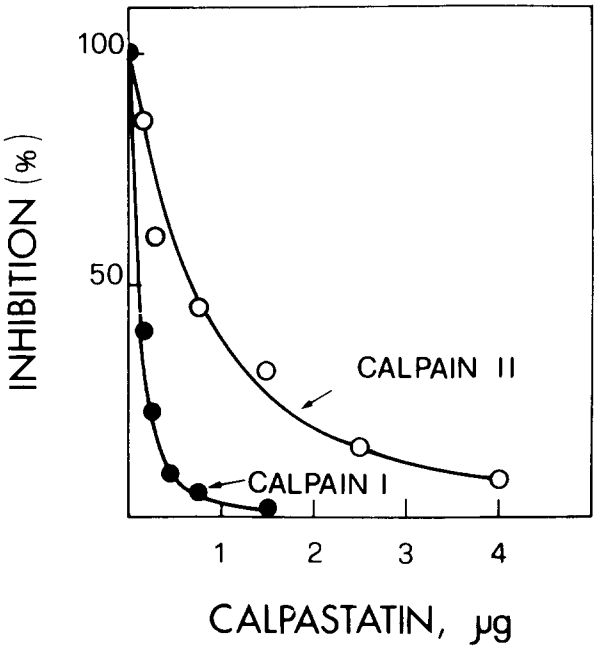

Figure 7 Effect of calpastatin on the activity of rat skeletal muscle calpain I and II. Rat skeletal muscle calpain I was assayed in the presence of 0.1 mM Ca^{2+} and of the indicated amounts of purified rat skeletal muscle calpastatin. Calpain II was assayed in the same conditions with the exception that calcium concentration was raised to 1 mM. With both calpain forms, the concentration of Ca^{2+} used had previously been established to promote maximal interaction between the proteinase and the inhibitor. For experimental details, see Pontremoli et al.[101]

However, since both components are localized predominantly in the same cytosolic compartment, a first question concerns the problem of how calpain can escape calpastatin control.

This question is particularly relevant when one considers that Ca^{2+}, which is required for activation of

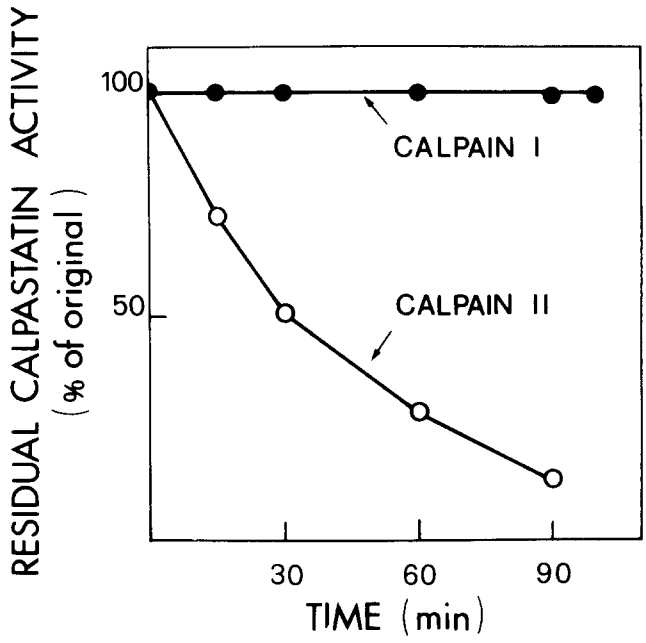

Figure 8 Degradation of rat skeletal muscle calpastatin by homologous calpains. Purified rat skeletal muscle calpastatin was incubated with homologous calpain I or calpain II, in a ratio of 1:0.8 (on a unit basis), in the presence of 0.1 mM calcium; a concentration at which calpain II is inhibited 30–35%. At the times indicated, aliquots of the incubation mixtures were collected, heated for 2 min at 90° C to inactivate calpain, and calpastatin activity was assayed. As indicated in the text, calpain I degrades calpastatin to still active fragments[101]; this explains the observed preservation of full inhibitory capacity. Calpain II promotes calpastatin degradation to inactive fragments; thus, explaing the progressive loss of inhibitory efficiency. For experimental details, see Pontremoli et al.[101]

calpain, also promotes formation of the enzyme inhibitor complex. Thus, due to the localization of both components in the same cell compartment, the signals that promote activation of calpain must also promote a selective localization of the proteinase in a different cell compartment. In this respect, translocation from the cytosol could fulfill, on a spatial basis, this requirement. Furthermore, translocation exerts a dual effect since it induces association of procalpain to the membrane phospholipids, as well as interaction of the proteinase with the cytoskeleton associated natural activator.

Thus, in an oversimplified model (see *Figure 6*) the mechanism of operation of the calpain-calpastatin system could be initially represented by a steady state situation in which calpain at physiological intracellular [Ca^{2+}] remains in a non-operational state. Increase in Ca^{2+} concentrations within the range of physiological fluctuation would then promote activation in accordance with one or the other described mechanisms. Obviously, this simple scheme does not consider all the aspects of the problem. Among others, two represent probably the most important ones.

A first one is that concerning the exact role of calpastatin; the second one is that related to the existence of the same or of a different mechanism of activation

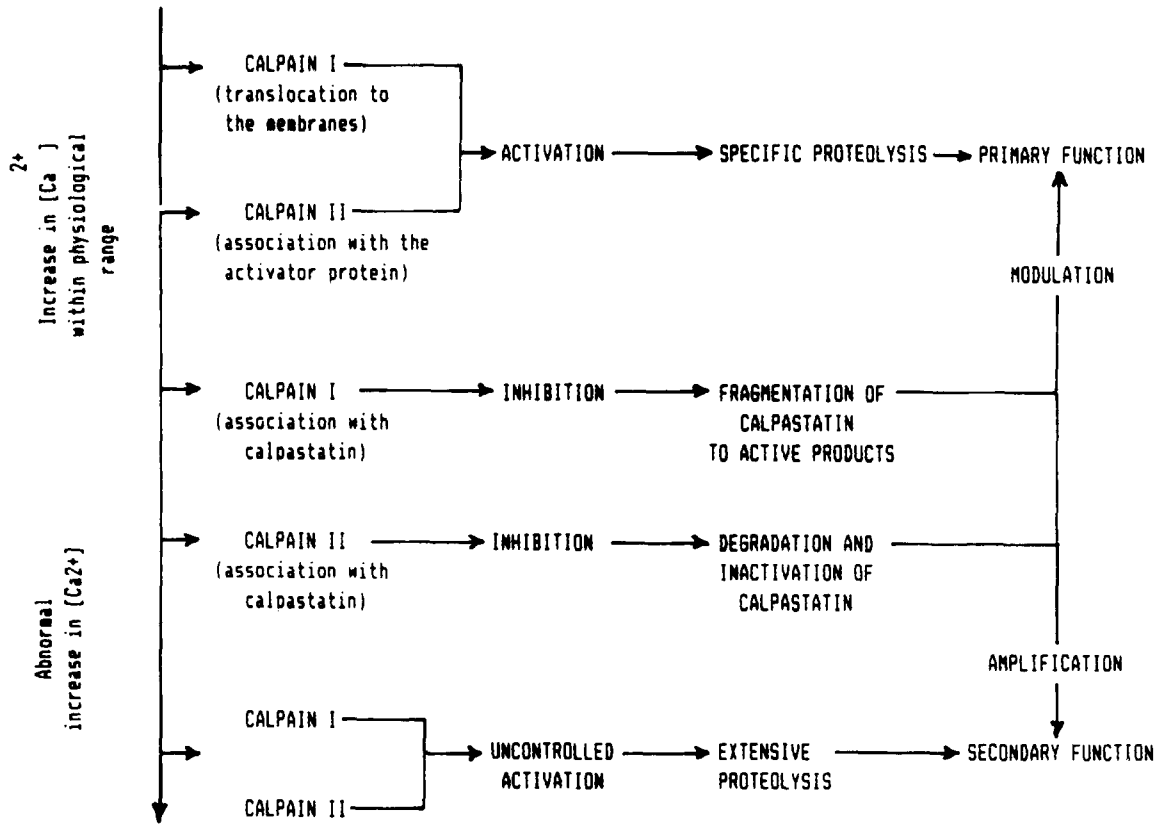

Figure 9 Proposed model for the operation of the calpain–calpastatin system.

for calpain I and calpain II especially in cells where the two isoforms are present.

As far as the first problem is concerned and on the basis of the structural properties previously described, calpastatin should inhibit four equivalents of calpain per mole.

Furthermore, as far as the intracellular localization of the inhibitor is concerned, there are reports that indicate its presence in association with plasma membrane or with intracellular organelles.[5,94,95]

Based on the preceding, calpastatin should be able to express a large inhibitory capacity even when present in an amount lower than that of calpain, and might be localized at sites at which calpain is translocated in order to undergo activation. Both conditions should make it impossible for the protease to ever be active. Nevertheless, when intracellular Ca^{2+} is raised, calpain becomes active as demonstrated on the basis of different experimental parameters.[96–99]. Moreover, calpastatin, as mentioned before, can be degraded by calpain so the mechanism by which the inhibitor can modulate calpain activity remains obscure.

In studies performed with rat skeletal muscle cells that contain both calpain isoforms, the following observations were obtained: calpain I is highly sensitive to inhibition by calpastatin (*Figure 7*), is not stimulated by the endogenous activator proteins (see *Figure 3*), and degrades calpastatin only to active fragments. Calpain II, however, is highly sensitive to activation by the stimulatory protein (see *Figure 4*), degrades calpastatin to complete inactivation (*Figure 8*), and expresses proteolytic activity at concentrations of Ca^{2+} lower than those required for inhibition by calpastatin (see *Figures 7* and *8*).

On the basis of these observations, we speculate that regulation of the calpain-calpastatin system proceeds according to a sequence of events represented in *Figure 9*. A first assumption is that in several types of cell, activation by external stimuli involves a transient rise in intracellular Ca^{2+} concentration which is kept within physiological range.

In these conditions, calpain I is activated through translocation to the membranes and eventually becomes a soluble constitutive active proteinase that participates in the regulation of cell activity by expressing its primary highly selective function. Interaction with calpastatin, located in the cytosol or associated with cell membrane, can also occur particularly if Ca^{2+} concentration tends to increase, and at this stage the calpain-calpastatin system becomes susceptible to strict regulatory control.

In order to ascribe a physiological role to calpain II, we suggest that this isoform also undergoes activation if the fluctuation in $[Ca^{2+}]$ reaches a concentration sufficient to promote its interaction with the natural activator.

Thus, we can postulate that both calpain isoforms are involved in the expression of the physiological pri-

mary function of the calpain-calpastatin system provided that their activation occurs within physiological fluctuations in Ca^{2+} concentration.

However, if the intracellular concentration of the metal ion becomes abnormally high due to pathological conditions, then the calpain-calpastatin system loses its physiological function and acquires the property of an uncontrolled proteolytic power that can only participate to an indiscriminate general catabolism of intracellular proteins.

When these abnormal or pathological conditions occur, it is likely that calpain II activity also becomes predominant because it can promote degradation of calpastatin; thus destroying the potential regulatory capacity of the cell.

All these considerations partially derive from the difficulty in ascribing a precise physiological role to an intracellular proteinase (calpain II) that, for its activity, depends on a concentration of Ca^{2+} that can never be reached in normal conditions.

Since the existence in the cell of a mechanism that converts the native proenzyme to active enzymes with much higher sensitivity to Ca^{2+} has been proved experimentally, it is reasonable to assume that both the activities of calpain I and II participate in crucial steps correlated with the expression of specific cell functions.

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