Minireview

A novel aspect of calpain activation

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Abstract Calpain, a Ca^{2+}-dependent biomodulator, alters the properties of substrate proteins by cleaving them at a limited number of specific sites. Recent studies of the structure-function relationship of calpain and X-ray analysis of its Ca^{2+}-binding domain have revealed hitherto unknown features of the regulation of calpain activity. A novel dissociation/autolysis mechanism for the activation of calpain at the membrane is proposed, which incorporates recent findings from structure-function studies of calpain, and its implications are discussed.

Key words: Calpain; Protease; Proteolysis; Activation of calpain; Ca^{2+}; Biomodulator

1. Introduction

Calpain is a typical cytosolic cysteine protease that absolutely requires Ca^{2+} for activity [1–12]. It has attracted much attention as a fundamentally important cellular modulator implicated in a wide variety of biological phenomena related to Ca^{2+}. It might also play a role in various pathological states, including degenerative diseases of muscle and nerve, apoptosis, hypertension, cataract formation, rheumatoid arthritis, and Alzheimer's disease [8,9]. Two forms, μ- and m-calpain, are expressed ubiquitously in animal tissues and have been widely studied. Both are heterodimers, consisting of a homologous catalytic 80 kDa subunit and the identical regulatory 30 kDa subunit made up of distinct domains (Fig. 1). They are distinguished by the Ca^{2+} concentration required for in vitro activity: μ-calpain requires μM levels of Ca^{2+}, while m-calpain requires mM levels. Calpain cleaves target proteins in a restricted manner to modify their properties rather than digest the substrate proteins. Since calpain requires unphysiologically high Ca^{2+} levels, the activation or sensitization of calpain to Ca^{2+} is required before in vivo activity occurs. To answer the central question, What is the physiological function of calpain? it is essential to understand the regulatory and activation mechanisms of calpain. In this paper, our model for the activation of calpain is discussed, together with several recent findings that are important for discussion of the activation model.

2. Recent developments in the structure-function study of calpain

Recent studies have identified many calpain isoforms, indicating that calpain constitutes a large superfamily. The family members can be classified as typical calpains, which are further divided into ubiquitous and tissue-specific calpains, or atypical calpains (Fig. 1). Tissue-specific calpains were only recently discovered and their characterization as proteins and enzymes is still incomplete, although they are apparently more suitable than ubiquitous calpains to analyze the physiological function of calpain. Atypical calpains have a protease domain homologous to that found in the large subunit (CL, domain II), but lack other calpain domains, including the Ca^{2+} binding domain (domain IV). The analysis of atypical calpains, especially those from yeast and *Aspergillus*, should provide important clues to determine the physiological function of calpain in animals, since gene manipulation is much easier in these organisms [3].

The sequence identity of the protease domain (II) of calpain homologues to other cysteine proteases, although restricted to the immediate vicinity of the active site residues, suggests that II should be active without Ca^{2+}. If this is the case, Ca^{2+} is only necessary for structural purposes and not for catalysis directly. Various attempts to obtain an enzymatically active Ca^{2+}-independent fragment, by autolysis or the digestion of calpain with other proteases, have not been successful. However, a genetically engineered fragment expressed in *Escherichia coli*, which consisted essentially of domains I, II, and III, showed Ca^{2+}-independent activity corresponding to 44% of the original enzyme Ca^{2+}-dependent activity [13]. Thus, domain II, but not IV, is necessary and sufficient for protease activity.

Quite recent results of the X-ray structural analysis of the Ca^{2+}-binding domain (IV′) of the small subunit (CS) revealed five Ca^{2+}-binding EF-hand motifs, rather than the four predicted from the sequence of IV′ [14,15]. The presence of the most N-terminal EF-hand motif could not be predicted from the sequence. Furthermore, the fifth most C-terminal EF-hand motif does not bind Ca^{2+} but interacts with its counterpart in a second molecule, resulting in homodimer formation. These results suggest a novel model for the association of the two calpain subunits, in which the fifth EF-hand motifs in IV and IV′ interact with each other. In agreement with this model, calpain loses its activity and the ability to form a complex when 8–10 amino acid residues from the C-terminus of both subunits are cleaved by carboxypeptidase [16]. Studies of expressed mutants also indicated that removal of 22–25 C-terminal residues from IV′ eliminated the reconstitution ability of active calpain [17]. Thus, the fifth EF-hand motif in CL and CS is responsible for both heterodimer and homodimer formation.

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Abbreviations: CL, calpain large subunit (80 kDa); CS, calpain small subunit (30 kDa)

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3. Necessity of calpain activation

The Ca^{2+} concentrations required for half-maximal activity of calpain (K_a) measured in vitro usually range from 5–50 μM for μ-calpain, and 200–1000 μM for m-calpain. Nevertheless, both calpains seem to be active at physiological Ca^{2+} concentrations of 100–300 nM, suggesting that more than Ca^{2+} alone is required for calpain activity in vivo. In fact, activator proteins and factors that increase the Ca^{2+} sensitivity of calpain have been described. Most recently, an activator protein specific for μ-calpain, isolated from rat brain, was sequenced [18]. This activator is a Ca^{2+}-binding protein that forms a 1:1 complex with μ-calpain, but not with m-calpain, and increases its Ca^{2+} sensitivity 10 fold without affecting the V_{max} value. Another activator specific for m-calpain is found in skeletal muscle. It also forms a 1:1 complex with m-calpain and lowers the K_a value from 400 μM to 15 μM by facilitating autolysis [19]. These two activators bind to the membrane in a Ca^{2+}-dependent manner, suggesting that they function on the membrane.

Phospholipids, especially acidic phospholipids, greatly reduce the Ca^{2+} concentration necessary for autolysis and thus for activity [20,21]. In their presence, μ-calpain becomes fully active at μM or lower Ca^{2+} concentrations, whereas phospholipids are insufficient to activate m-calpain at physiological Ca^{2+} concentrations. Although some other activators and factors including DNA have been reported [22], their nature and physiological implications and the mechanism of activation are still very poorly understood.

Autolysis, which modifies the N-terminal regions of both subunits, increases the Ca^{2+}-sensitivity of calpain significantly [2,23]. While it is quite clear that autolysis lowers the K_a value of both μ- and m-calpain, a critical problem in autolytic activation is that a higher Ca^{2+} concentration is required to initiate autolysis [24]. The calpain cascade, in which μ-calpain activates m-calpain at near μM levels of Ca^{2+}, is interesting but inconsistent with previous findings [24]. Arguments still remain over how autolysis occurs in vivo and whether autolysis is necessary to raise Ca^{2+} sensitivity or for calpain activity. Molinari et al. reported the hydrolysis of Ca^{2+}-ATPase and band 3 protein when calpain remains intact [25]. It should be mentioned that the autolysed forms of μ-calpain show distinct substrate specificities [26]. To summarize the recent results, autolysis does not seem to be necessary for the expression of protease activity, but it has an important effect on the dissociation and thus the activation of calpain.

4. Dissociation and activation

Dissociation and reassociation studies of calpain have revealed that calpain dissociates into subunits in the presence of Ca^{2+} and that the dissociated CL retains full enzymatic activity [27,28]. Studies of the expression of CL have indicated that it is fully active without the co-expression of CS [13,29]. In most cases, CL expressed without CS forms a homodimer. In some cases, however, CL seems to exist as a monomer. CL and CS, and thus IV and IV′, are unstable and tend to form aggregates, but become stable after dimer formation. CL and CS will form homodimers, at least when only one is present. CL is fully active enzymatically whether or not it forms a dimer. Taken together, the dissociation of calpain into subunits implies its activation and dissociated CL should be regarded as an active species in vivo. However, the enzymatic characterization of CL as an active species in vivo is inadequate and the exact Ca^{2+} concentration required for dissociation has not yet been determined.

The autolysis of calpain only modifies the N-terminal region of both subunits. Our unpublished results indicate that removal of the N-terminal region of CL, but not of CS, facilitates the dissociation of calpain into subunits. Assuming that

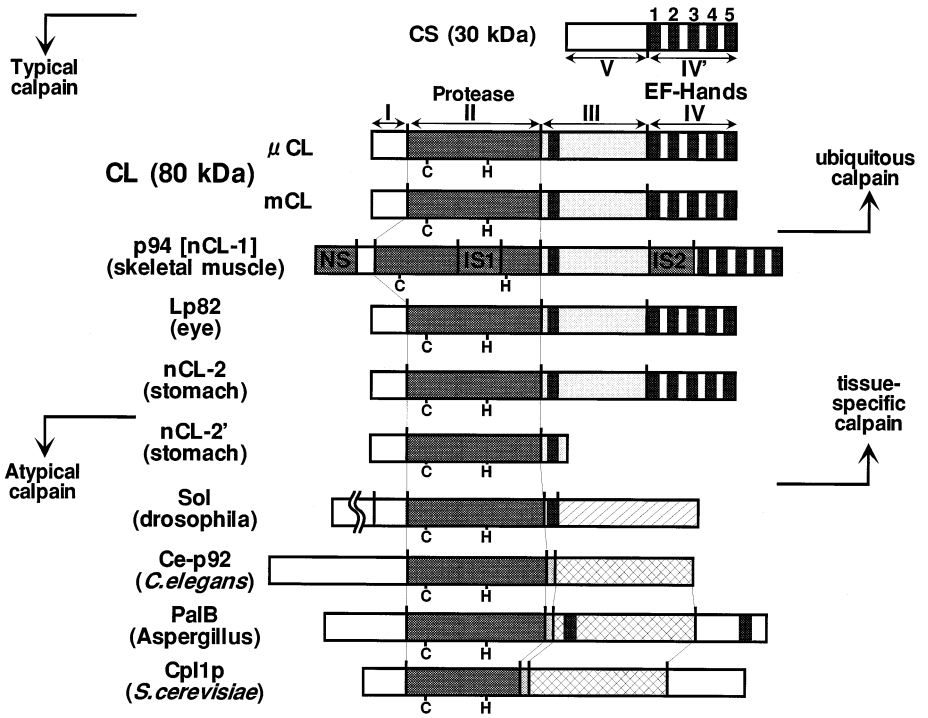

Fig. 1. Some typical members of the calpain superfamily.

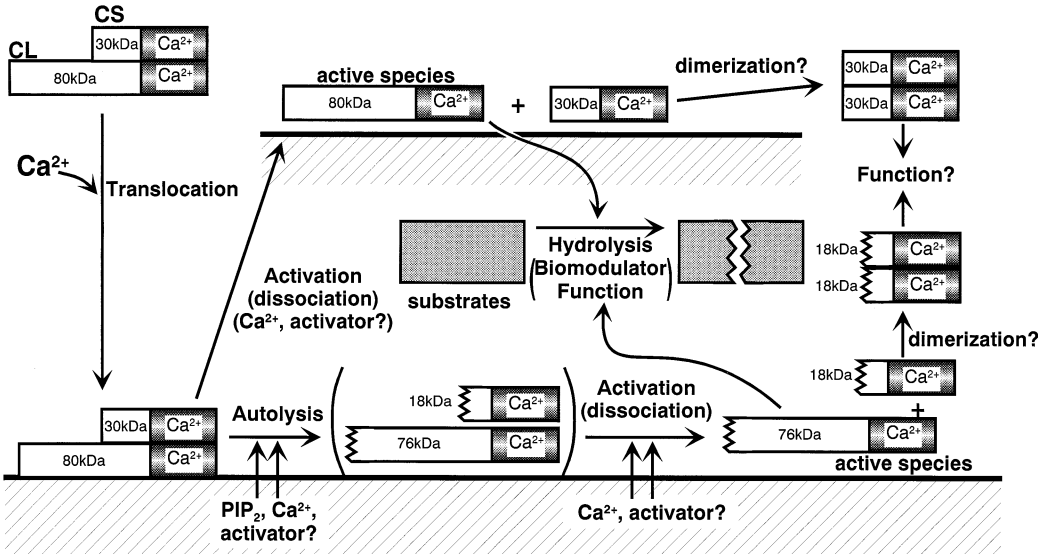

Fig. 2. Dissociation/autolysis mechanism for activation of calpain. For details see text.

CL is an active species, the previous results of autolysis studies, which show that the N-terminal modification enhances the Ca^{2+} sensitivity of calpain, can be interpreted in terms of the dissociation of N-terminally modified CL from CS [28]. Dissociation could be a consequence of autolysis. Since dissociated intact CL is fully active, however, autolysis is not a prerequisite for activation. Autolysis is important for activation in terms of dissociation, but it is not sufficient. Rather, true activation requires dissociation into subunits.

Our model for the activation of calpain, which combines the results of dissociation studies with a previous membrane activation theory, is summarized in Fig. 2. Calpain translocates to the membrane when it binds to Ca^{2+}. This induces conformational changes in calpain and exposes hydrophobic regions, probably ascribed to IV and IV′ on the surface of the molecule. The Ca^{2+} concentrations required for the conformational changes are significantly lower than those required for its dissociation into subunits and the expression of activity. On biological membranes, the dissociation of calpain into subunits occurs in the presence of Ca^{2+}, membrane phospholipids, or activator(s). Membrane phospholipids decrease the Ca^{2+} concentration required for autolysis, resulting in the modification of CL, which eventually causes the dissociation of μ-calpain under physiological conditions. Dissociated CL hydrolyzes substrates, either at the membrane or after its liberation into the cytosol. The model has another advantage; dissociation into subunits might facilitate the release of dissociated CL from the membrane. Indeed, autolysed CL is found only in the cytosol [30]. The mechanism for the dissociation of calpain into subunits without autolysis is currently hypothetical and awaits further studies.

This dissociation model introduces an additional mechanistic step for the activation of calpain, but the earlier controversy over Ca^{2+} concentration still remains, at least as far as m-calpain is concerned. In the case of m-calpain, none of the conditions or factors reported so far activate m-calpain at physiological Ca^{2+} concentrations. Thus, a sophisticated mechanism is probably essential for the autolysis and dissociation of m-calpain, which involves activators or factors that significantly reduce the Ca^{2+} requirement. Translocation to the membrane might be one of the important steps necessary to sequester calpain from its endogenous inhibitor, calpastatin.

Tissue-specific calpains, identified quite recently, appear to exist by themselves and their counterpart proteins corresponding to CS have not yet been identified. Their protein levels are very low in contrast to their relatively high mRNA levels. The dissociation mechanism suggests that tissue-specific calpains exist in an active dissociated form, resulting in a very low protein level.

Dissociated CL or CS, or their autolysed forms, have not yet been identified in vivo. The detection of dissociated CL or CS, which should be possible by using specific antibodies raised against a dissociated single subunit, will provide clues to answer important questions: where, how, and to what extent does the dissociation of calpain occur? Dissociation without autolysis might be a reversible process, whereas autolysis-involved dissociation should be irreversible. Recently identified homologues of CS are found as a dimer and have a specific function [31], suggesting that CS might also form a dimer after its dissociation from CL, and have a function distinct from protease activity. Likewise, the possibility that CL also forms a homodimer after its dissociation from CS also exists, but this is open to debate. The Ca^{2+}-induced dissociation of calpain is observed with both μ- and m-calpain. Furthermore, our unpublished studies indicate that a homodimer of IV and IV′ formed in the absence of Ca^{2+} dissociates in the presence of Ca^{2+}, although the exact Ca^{2+} concentration necessary for dissociation has not been determined.

There are, however, inconsistent results [17]. Both subunits of μ- and m-calpains are co-precipitated in Ca^{2+} by monoclonal antibodies directed against a single subunit [32]. The X-ray structural analysis of IV′ suggests dimer formation both in the presence and absence of Ca^{2+} [14,15]. The fact that dissociation occurs in Ca^{2+} is very important [28,33], although Ca^{2+}-induced aggregation makes conclusive experiments on this point very difficult. Apparently, more precise analysis of the Ca^{2+}-induced dissociation is essential.

5. Perspectives

In this review, we briefly summarize the current knowledge of the structure and function of calpain and propose a novel dissociation/autolysis mechanism for its activation at the membrane. In order to unravel the physiological function of calpain, knowledge of the activation mechanism is most important, as is understanding of the effect of specific calpain inhibitors [12] and studies using antibodies specific for certain species of calpain during activation. It is hoped that the proposed activation model stimulates discussion on the activation mechanism, leading to understanding of the physiological function of calpain.

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