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Modulation of the Calpain Autoproteolysis by Calpastatin and Phospholipids

E. Melloni, M. Michetti, F. Salamino,* R. Minafra, and S. Pontremoli

*Institute of Biological Chemistry, University of Genoa, Viale Benedetto XV, 1- 16132 Genoa, Italy; and *Institute of General Physiology and Biological Chemistry, University of Sassari, Sassari, Italy*

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The Ca-induced autoproteolysis calpain proceedes through the sequential formation of two forms of active enzyme with molecular masses of 78 kD and 75 kD, respectively. The autolysed calpains are produced by the cleavage of the peptide bond between Ser^{15}-Ala^{16} and then between Gly^{27}-Leu^{28}. Calpastatin reduces with high efficiency the transition from 78 kD to 75 kD calpain forms. At higher concentration also the first autolytic event is blocked. The data are consistent with the presence of two calpain forms with different susceptibility to calpastatin. Furthermore, calpain, once bound to phospholipid vesicles, undergoes autoproteolysis which preferentially accumulates the 78 kD species. These data provide new information on the activation process of calpain, indicating that a Ca-induced conformational change is the triggering event, followed by the appearance of the active 78 kD calpain which can be considered the preferential form of calpain at the membrane level.

Native calpain is present in various cell types in an inactive proenzyme which is converted in an active form throughout an autoproteolytic process, triggered by the binding of calcium ions to the calmodulin like domain of the proteinase (1-5). Although no information is at present available on the nature of the signal induced by binding of Ca^{2+}, it is well established that the first detectable structural modification consists in the removal of a peptide fragment from the N-terminal region of catalytic subunit (6-8). The autolysed proteinase form still retains its dependency by calcium, although with a much lower requirement.

Previously (9), we have shown that the autoproteolytic conversion of the 80 kD inactive to the active calpain species proceeds through the formation of two different active forms, having molecular mass of 78 kD and 75 kD, respectively. It has been also established that the 78 kD form is produced preferentially on the inner surface of the cell membranes through an intramolecular digestion and that it remains associated to the membrane until its conversion to the 75 kD calpain species, produced by intra- or intermolecular autoproteolysis. This low Mr species, once formed, is released into the intracellular space. Consistently, the two active calpain forms are not only produced by different enzyme mechanisms, but probably represent a distinct site directed activation for the proteinase.

In this paper we report the characterization of the two active forms with respect to the sites of cleavage. Moreover, calpastatin, the natural calpain inhibitor, has been shown to preferentially inhibit the formation of the 75 kD subunit with a concomitant accumulation of the 78 kD species. Also phospholipids have been found to favour the accumulation of this intermediate calpain form, without affecting the overall digestion process.

Taken together, these data demonstrate that activation of human erythrocyte calpain occurs through a multistep process in which various proteinase forms are produced with different catalytic properties.

MATERIALS AND METHODS

Purification of human erythrocyte calpain. Human erythrocyte calpain was purified as previously reported (9).

Assay of calpain activity. Calpain activity was routinely assayed, using human acid denatured globin as substrate (10).

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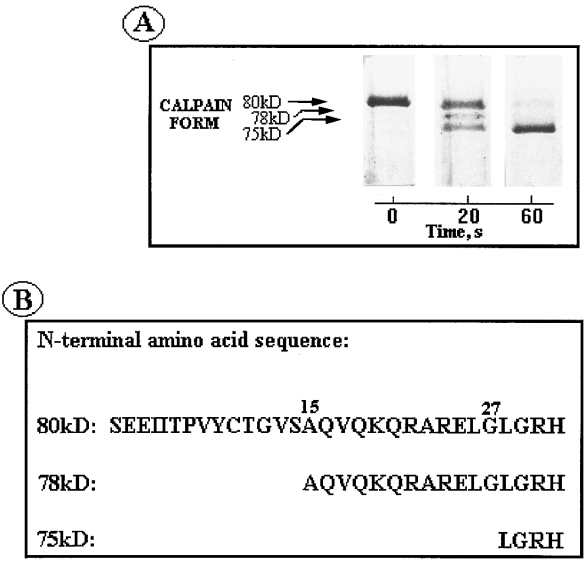

FIG. 1. Sites of cleavage recognized by calpain during the production of the 78 kD and 75 kD calpain forms. A. Purified calpain, 20 μg, was incubated, in an ice bath, with 0.5 ml of 50 mM sodium borate buffer, pH 7.5, with 100 μM Ca^{2+}. At the indicated times, aliquots of the incubation mixture, corresponding to 5 μg, were collected and submitted to 8% SDS-PAGE. The gel was stained with coomassie brilliant blue and destained by free diffusion in 7% acetic acid and 10% ethanol. B. The autolysed forms were prepared as described in Methods, their N-terminal sequences were determined automatically by a Beckman LF 3000 Protein Sequencer and compared with the calpain sequence published by Sorimachi (22).

Purification of human erythrocyte calpain inhibitor. Calpastatin was purified by the procedure previously reported (11), modified as follows: the dialysed material was loaded onto a column (2.5 $\times$ 3 cm) of Source 15Q (Pharmacia) previously equilibrated with sodium acetate buffer 50 mM, pH 6.7, containing 0.1 mM EDTA. The protein was eluted with a linear gradient 0-0.3 M sodium chloride. The fractions containing calpastatin activity were pooled, dialysed against sodium borate buffer 50 mM, pH 7.5, containing 0.1 mM EDTA and applied to a column (0.5 $\times$ 6 cm) of Threonine Sepharose (Pharmacia) equilibrated with the same buffer. The resin was washed and the bound proteins were eluted with a linear gradient of sodium chloride from 0 to 0.2 M dissolved in the same buffer (50 + 50 ml). The fraction containing calpastatin activity were concentrated to 2 ml on Amicon YM10 membrane.

Assay of calpastatin activity. Calpastatin activity was routinely assayed using purified calpain and appropriate amounts of inhibitor source (11). One unit of calpastatin was defined as the amount required to inhibits 1 unit of erythrocyte calpain.

Preparation of 78 kD calpain form. Purified calpain (20 μg) was incubated for 1 min in ice bath in 50 mM sodium borate buffer, pH 7.5, in the presence of 50 μM E-64 and 75 μM Ca^{2+}. The incubation was stopped by the addition of 8% TCA, centrifuged and the pellet was washed with acetone and resuspended in 10% CH_3CN in H_2O.

Preparation of 75 kD calpain form. Purified calpain (20 μg) was incubated in 50 mM sodium borate buffer, pH 7.5, containing 100 μM Ca^{2+} for 1 min at 25°C. The reaction was stopped by the addition of 8% TCA, centrifuged and the pellet was washed as above described.

Preparation of phospholipids vesicles. Aliquots (corresponding to 50 μg) of the following phospholipids: Phosphatidylcoline, Phosphatidyl inositol, Phosphatidyl ethanolamine, Phosphatidic acid and Phosphatidyl serine were dried under nitrogen flux and the dried film was sonicated in 1 ml of 50 mM sodium borate buffer, pH 7.5 (5 burst of 10 s each).

RESULTS

In order to provide definitive demonstrations on the number and on the type of the calpain species produced during the activation process (6-9, 12-16), native human erythrocyte calpain was exposed to Ca^{2+} and the products of the autoproteolytic digestion were evaluated at different times of incubation. As shown in Fig. 1A, a transient appearance of a 78 kD species

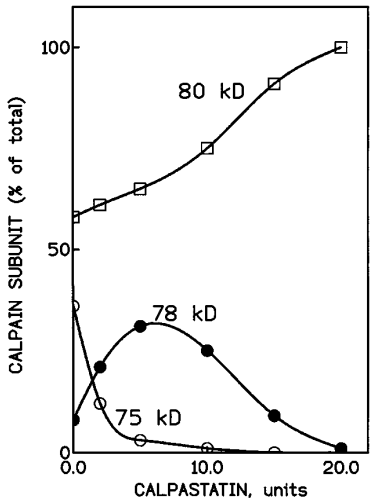

FIG. 2. Effects of increasing concentrations of calpastatin on the calpain autoproteolysis. Purified calpain (5 μg) was incubated, in ice bath, with 0.125 ml of 50 mM sodium borate, pH 7.5, in the presence of 100 μM Ca^{2+} and in the absence or presence of the indicated amounts of purified human erythrocyte calpastatin. The incubation mixtures were stopped after 20 s with 1 mM EDTA and submitted to 8% SDS-PAGE. The amount of each calpain form were determined by using scanning of the stained gels in CS-9000 Shimadzu scanner.

is evident at the early times of incubation, followed by the massive formation of the 75 kD form. No other intermediate digestion products could be detected in these conditions. This conclusion received further support by evaluation of the sites of cleavage of the native proteinase. As shown in Fig. 1B, the 78 kD form is produced by the cleavage of the Ser^{15}-Ala^{16} peptide bond, whereas the 75 kD form is generated by the successive hydrolysis of the peptide bond between Gly^{27} and Leu^{28}. No other sequences could be identified, indicating that the two autolysed calpain species are discrete forms of the proteinase, produced during its activation process.

In order to provide a better understanding of the molecular events occurring during this activation process, calpastatin, the natural inhibitor of the proteinase, was added to the activation mixture to analyze its effect on the rate of the formation of the intermediates. As shown in Fig. 2, in the presence of increasing amounts of calpastatin, the formation of the 75 kD calpain form is progressively reduced, concomitantly with a large increase in the accumulation of the 78 kD species. At higher concentrations of the inhibitor, the formation of the 78 kD is progressively reduced and, at the highest concentration employed, no autoproteolysis occurs.

These data indicate that the two autolytic steps, leading to activation of calpain are differently sensitive to the inhibitory effect of calpastatin, and that 80 kD calpain form expresses a lower affinity for the inhibitor molecule than the autolysed forms. Furthermore, these data provide demonstration that Ca^{2+} is an essential effector for those conformational changes that must be induced in all calpain forms for the binding of calpastatin. However the affinity for the inhibitor, very low in the native conformation of the proteinase, progressively increases as the N-terminal is first partially and then completely removed.

In a previous paper (9), we have observed that calpain, once bound to membrane following cell loading with Ca^{2+}, undergoes activation throughout its conversion into the 78 kD species. To provide new information on the nature of the membrane components active in this process, calpain was incubated with calcium in the presence of a mixture of phospholipids, in a composition similar to that present in erythrocyte membranes. As shown in Fig. 3A, in these

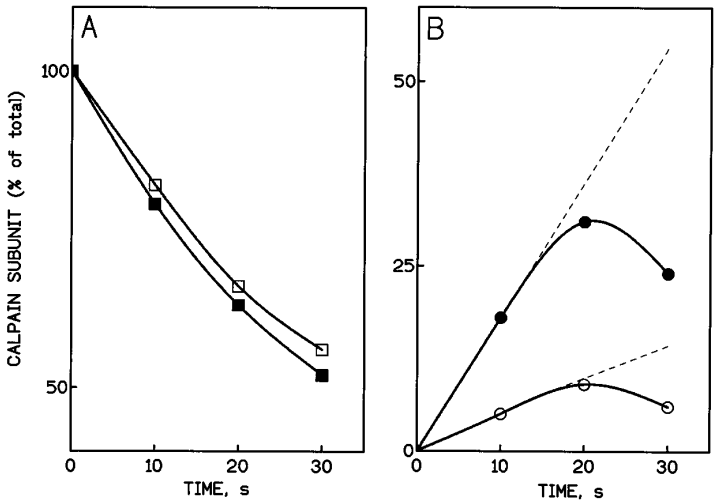

FIG. 3. Effect of phospholipids on the autolysis of calpain. Purified calpain (20 μg) was incubated in ice bath with 0.5 ml of sodium borate buffer 50 mM, pH 7.5, in the presence (unfilled symbols) or absence (filled symbols) of 50 μg/ml phospholipid vesicles. At the indicated time, aliquots (100 μl) were collected and submitted to 8% SDS-PAGE. The amounts of 80 kD (A) and of 78 kD (B) calpain species were calculated by scanning the stained gels.

conditions the rate of disappearance of the native calpain subunit 80 kD is only slightly affected whereas (Fig. 3B) the accumulation of the 78 kD species is increased more than 4 times, presumably as a result of stabilising effects induced by the association to the phospholipid vesicles.

DISCUSSION

The data presented in this paper are consistent with a model shown in Fig. 4 in which, together with previous observations (6-9, 12-17), new and more extensive information is collected.

The model illustrates the sequential formation of three active proteinase species following binding of Ca^{2+} to calpain. The first one retains the intact native structure in which however the active site becomes accessible, as demonstrated by the intramolecular autoproteolysis and by the binding of calpastatin, although with very low affinity. The second and the third active

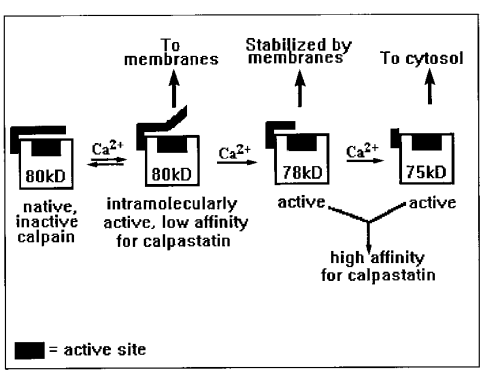

FIG. 4. Proposed model for the activation of calpain.

form have been structurally modified and can be considered to be able to express full catalytic activity and to bind calpastatin with high affinity.

The data obtained and reported herewith contribute not only to a better understanding of the general activation mechanism of calpain; they infact strongly suggest that the 78 kD form represents the activity form of calpain at the membrane level. The effect of phospholipids promoting accumulation of this form is a further experimental evidence obtained in support of this conclusion. Consistently, the presence of the 75 kD form on the soluble fraction of the cell (9) can be visualised as an indication for a different site-directed activation for this calpain species.

In conclusion, our data suggest that, within transient and physiological increase in [Ca^{2+}], the preferential form of active calpain occurs at the membrane level, an hypothesis which is in support of previous observations indicating that transmembrane proteins appear to be the preferential substrates of calpain (18-21).

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