Autolysis of human erythrocyte calpain produces two active enzyme forms with different cell localization

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Abstract The 80 kDa human erythrocyte calpain, when exposed to Ca^{2+}, undergoes autoproteolysis that generates a 75 kDa species, with an increase in Ca^{2+} affinity. It is demonstrated here that this proteolytic modification proceeds through an initial step producing a 78 kDa form which is rapidly converted to the 75 kDa one. In the presence of the calpain inhibitor E-64, the 78 kDa form accumulates and only small amounts of the 75 kDa polypeptide are formed. Following loading of erythrocytes with micromolar concentration of Ca^{2+}, in the presence of the ionophore A23187, the native 80 kDa calpain subunit is extensively translocated and retained at the plasma membrane, this process is accompanied by the appearance of only a small amount of the 75 kDa subunit which is released into the soluble fraction of the cells. Following exposure to μM Ca^{2+}, membrane-bound 80 kDa calpain is converted to the 78 kDa form, this conversion being linearly correlated with the expression of the proteinase activity. Taken together, these results demonstrate that the initial step in calpain activation involves Ca^{2+}-induced translocation to the inner surface of plasma membranes. In the membrane-bound form the native inactive 80 kDa subunit is converted through intramolecular autoproteolysis to a locally active 78 kDa form. Further autoproteolytic intermolecular digestion converts the 78 kDa to the 75 kDa form, no longer being retained by the membrane. This process generates two active forms of calpain, with different intracellular localisations.

Key words: Proteolysis; Calcium; Calpain; Activation process

1. Introduction

The Ca^{2+}-dependent proteinase, calpain, is normally localised in the cytosol of the cells [1–5], together with its natural inhibitor calpastatin. In this cell localisation, calpain is postulated to be inactive, due to the high requirement for Ca^{2+} [6–8], in the absence of which the enzyme retains a native conformation with an inaccessible active site. The transition from a high to low calcium requiring form results from autoproteolysis, which causes the removal of fragments at the N-terminal region of both 80 kDa catalytic and 30 kDa subunits [9–12]. The native catalytic subunit is then converted into a 75 kDa species, and the small subunit into a 18 kDa fragment. This new enzyme form expresses full catalytic activity at concentrations of calcium 50–100-times lower than that required by the native form, and has also been identified in red cells enriched with Ca^{2+} following exposure of cells to a ionophore. It seems therefore that autoproteolysis is an essential step that precedes the expression of catalytic activity. Evidence for the formation of intermediate calpain forms during its autoproteolytic process has been reported by Inomata et al. [13] and Hayashi et al. [14]. No information, however, has been provided concerning the kinetics of the generation of this intermediate or its catalytic properties.

Here, we have analyzed the kinetics of autoproteolysis of human red cell calpain, in order to obtain more information on the generation and on the function of the intermediate form between the native 80 kDa subunit and the 75 kDa autoproteolyzed subunit.

We now report that purified human erythrocyte calpain exposed to Ca^{2+} undergoes an autoproteolytic process which accumulates a 75 kDa polypeptide and involves during the early stages the transitory appearance of a 78 kDa form. Addition of E-64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane], a thiol-proteinase inhibitor, promotes considerable accumulation of the 78 kDa calpain form, accompanied by a decrease in the rate of formation of the 75 kDa polypeptide species, indicating that the first autolytic step is an obligatory intramolecular event. This conclusion is supported by the observation that, once bound to membranes, calpain undergoes autoproteolysis which leads to the accumulation of the 78 kDa form, which is capable of expressing catalytic activity and thus it can be considered as an active species of calpain.

2. Materials and methods

2.1. Purification of human erythrocyte calpain

Human erythrocyte calpain was purified as previously described [15], modified as follows: packed red cells 50 ml were lysed in 5 vols. of water containing 1 mM EGTA; the membranes were discarded by centrifugation at 25000×*g* for 10 min and the supernatant was treated with 125 g (wet powder) of DE 32 previously extensively washed with 50 mM sodium acetate, pH 6.7, containing 0.1 mM EGTA (buffer A) and stirred for 10 min at 5°C. The resin was collected on a Buckner funnel, washed with 1 l of buffer A and transferred on a glass column (2.5×15 cm). The absorbed proteins were eluted in a single step with 0.2 M NaCl dissolved in buffer A. The fractions containing calpain activity were collected, precipitated in 50% saturated ammonium sulfate and centrifuged at 25000×g for 10 min. The pellet was suspended in buffer A and dialyzed for 4 h in the same buffer solution. The dialyzed material was then loaded onto a column (2.5×3 cm) of Source 15Q (Pharmacia) previously equilibrated in buffer A. The proteins were eluted with a linear gradient of 0–0.3 M sodium chloride. The fractions containing calpain activity were collected, dialyzed for 4 h against sodium borate (50 mM, pH 7.5) containing 0.1 mM EGTA (buffer B). To the dialyzed material was added sodium chloride at a final concentration of 0.3 M and the solution loaded onto a column (1×10 cm) containing Phenyl-Sepharose CL 4B (Pharmacia) equilibrated with buffer B, containing 0.3 M NaCl. The resin was washed and the bound proteins were

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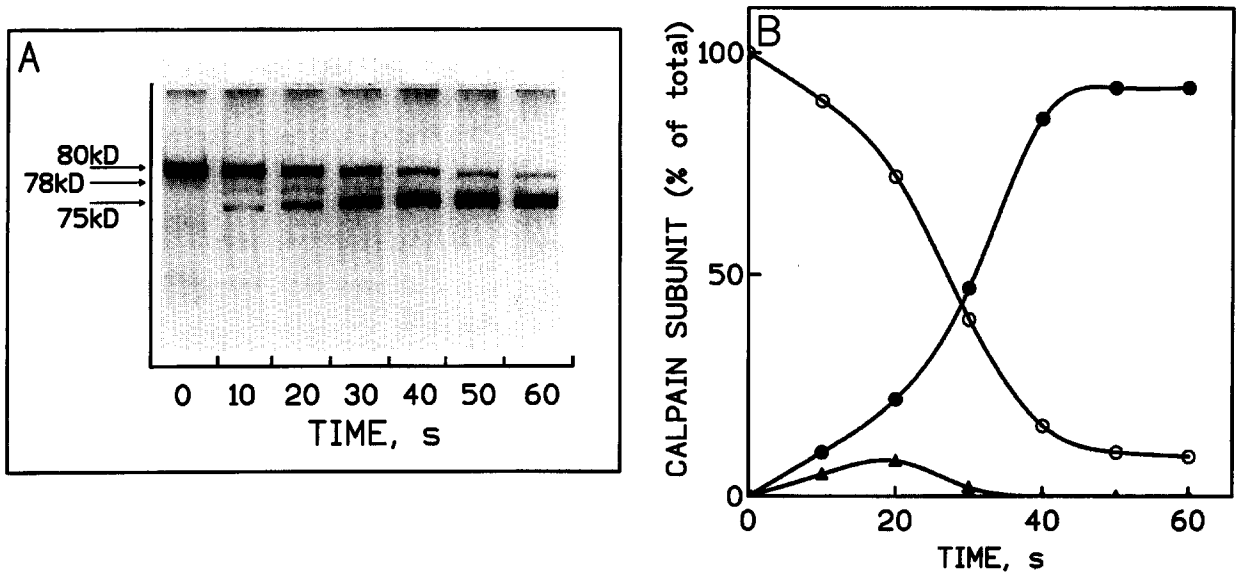


Fig. 1. SDS-PAGE of calpain following exposure to Ca^{2+}. (A) Purified human erythrocyte calpain (50 μg) was incubated in 0.2 ml of 50 mM sodium borate buffer, pH 7.5, in an ice bath with 100 μM Ca^{2+}. At the indicated times aliquots (20 μg) of the incubation mixture were collected, and heated at 100°C for 2 min in the presence of 5% 2-mercaptoethanol and 2% SDS. The solution was then subjected to SDS-PAGE. The protein bands were stained with Coomassie brilliant blue and destained in 7% acetic acid and 10% ethanol. (B) The amounts of single calpain subunits were calculated by scanning the destained gel. (○) 80 kDa calpain subunit; (●) 75 kDa form; (▲) 78 kDa form.

eluted by washing with buffer B without added salt. This step completely separated calpain from its inhibitor, calpastatin, which was not retained by the resin. The fractions containing calpain activity were concentrated by ultrafiltration and loaded onto a Sephadex G-200 column (1.5×120 cm) previously equilibrated with buffer B. This new procedure reduces the time required to obtain the purified enzyme and significantly increases (approx. 2-fold) the yield.

2.2. Assay of calpain activity

Calpain activity was routinely assayed, using human denatured globin as a substrate [16], in the presence of 1 mM Ca^{2+}. Where indicated the concentration of Ca^{2+} was changed, according to the aims of the experiments. One unit of calpain activity is defined as the amount of enzyme that causes the release of 1 μmol of free amino groups.

2.3. Preparation of calpain antibody

The anti-calpain monoclonal antibody (mAb 56.3) was prepared as previously described [17].

3. Results

When purified human erythrocyte calpain is exposed to 100 μM Ca^{2+}, the native 80 kDa catalytic subunit is rapidly converted into the 75 kDa species (Fig. 1). During the initial few seconds of incubation, a 78 kDa protein band also becomes detectable. As shown in Fig. 1B, concomitantly with the disappearance of the 80 kDa subunit, the 75 kDa form progressively accumulates, whereas the 78 kDa form transitory appears after 10–20 s of incubation. This indicates that the intermediate calpain form is produced at very early times, but does not accumulate in the mixture, probably being rapidly degraded to the 75 kDa calpain form. To characterize better the kinetics of formation of this intermediate calpain form, we have exposed purified human erythrocyte calpain to increasing calcium concentrations in the absence or presence of the substrate (Fig. 2). Maximal accumulation of the 78 kDa form is observed at Ca^{2+} concentration between 70 and 100 μM, corresponding to those promoting maximal activation

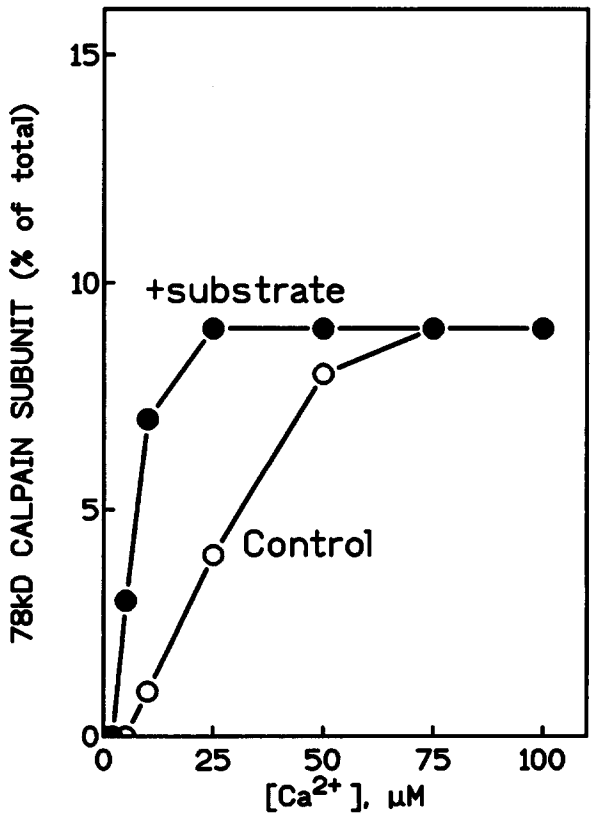
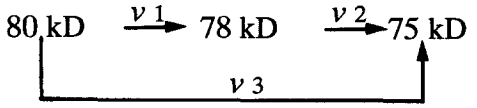


Fig. 2. Effect of substrate on the formation and accumulation of the 78 kDa calpain form. Aliquots of 5 μg of purified human erythrocyte calpain were incubated with the indicated [Ca^{2+}] in the absence or presence of 200 μg/ml of human denaturated globin for 20 s in an ice bath; calpain subunits were separated by SDS-PAGE and the destained slab gels were scanned.

and expression of catalytic activity of native calpain [18]. Addition of substrate has no effect on the accumulation of the 78 kDa subunit, although it reduces approx. 3–4-fold the concentration of Ca^{2+} promoting the maximal accumulation of the intermediate calpain form. According to the data reported, the kinetics of autoproteolysis, and hence of activation, of calpain can be explained by the model:



which considers that the 80 kDa native subunit is first converted at a rate v_1 into the 78 kDa form, then further digested at a rate v_2 into the more stable 75 kDa form. Moreover, the 75 kDa form might be produced directly in a single step, without the formation of an intermediate. In both cases, v_1 must be lower than v_2 or alternatively lower than the sum (v_2+v_3). If no direct 80 kDa→75 kDa conversion occurs the formation of the 78 kDa calpain form becomes the limiting step in calpain activation. In order to discriminate between these two possibilities, we have studied the effect of the thiol-proteinase inhibitor E-64 on the Ca^{2+}-induced autoproteolysis of human erythrocyte calpain. As shown in Fig. 3A, in the presence of increasing amounts of E-64, the level of the 75 kDa subunit decreases with a concomitant increase in the 78 kDa calpain form. We have also observed (Fig. 3B) that at low concentrations of Ca^{2+}, considerable accumulation of the 78 kDa calpain form still occurs, whereas the formation of the 75 kDa polypeptide is almost completely inhibited. These findings indicate that the formation of the 78 kDa species is almost completely insensitive to the inhibition by E-64, whereas the generation of the 75 kDa form is almost completely inhibited. Furthermore, since in these experiments the formation of the 78 kDa form occurs in the absence of the accumulation of the 75 kDa species, it can be concluded that the appearance of the 78 kDa form represents the first autoproteolytic event that initiates the process of calpain activation. On the basis of the effect induced by E-64, it is also conceivable to suggest that the 80 kDa→78 kDa conversion is carried out via intramolecular digestion, whereas the 78 kDa→75 kDa conversion may also be the result of intermolecular degradation.

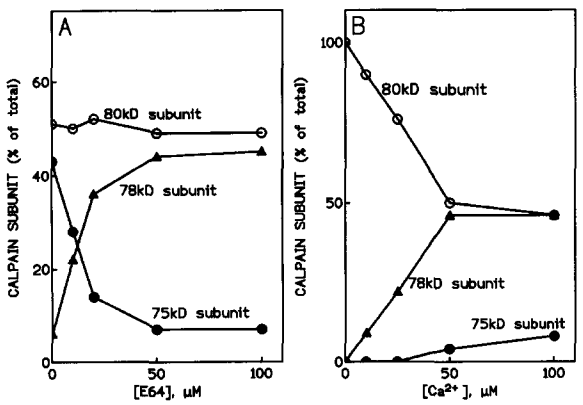


Fig. 3. Effect of E-64 on the Ca^{2+}-induced autolysis of human erythrocyte calpain. (A) 5 μg of human erythrocyte calpain were incubated in an ice bath in 50 mM sodium borate, pH 7.5, in the presence of 200 μg/ml of substrate and increasing E-64 concentrations. After 20 s the incubation mixtures were treated and scanned as reported in Fig. 1. (B) Aliquots of human erythrocyte calpain were incubated for 20 s in an ice bath in the presence of 200 μg/ml of substrate and 100 μM E-64 at the indicated Ca^{2+} concentration. The incubation mixtures were subjected to electrophoresis and scanned.

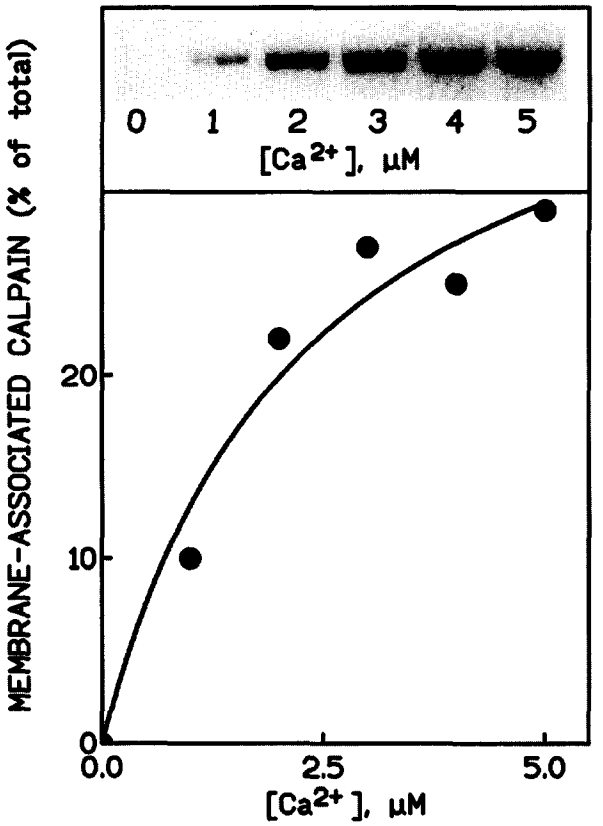


Fig. 4. Translocation of calpain on plasma membranes. Human erythrocytes were incubated at 37°C in isotonic medium with the calcium ionophore A23187 in the presence of the indicated concentrations of Ca^{2+}. After 30 min the cells were collected, lysed by hypotonic shock in 1 mM EGTA and the membranes were spun down by centrifugation. Aliquots of 0.5 mg of the membranes were subjected to SDS-PAGE followed by immunoblotting. Immunoblotting was performed using the anticalpain mAb 56.3; protein bands were detected with horseradish peroxidase conjugated goat anti-mouse IgGs.

However, these conclusions leave open the question of whether the catalytic activity is also expressed by 78 kDa calpain and if this form has any physiological relevance. We have approached this problem by loading human erythrocytes with low concentrations of Ca^{2+}, in the presence of the ionophore A23187. Under these conditions (Fig. 4) an appreciable amount of calpain is recovered in association with the plasma membrane. Maximal binding (25–30% of total) is obtained following treatment with 2–3 μM Ca^{2+}. The plasma membranes isolated from treated erythrocytes were found to contain only the 80 kDa calpain form (Fig. 4, inset). Only small amounts (less than 5%) of the 75 kDa calpain form were identified in the soluble fraction of the cells (data not shown). This finding can be explained on the basis of previous results [18–20] indicating that the 75 kDa form no longer has its membrane 'anchoring regions'. The calpain containing membranes were incubated in the presence of 1 μM Ca^{2+} and aliquots were collected and subjected to SDS-PAGE, to iden-

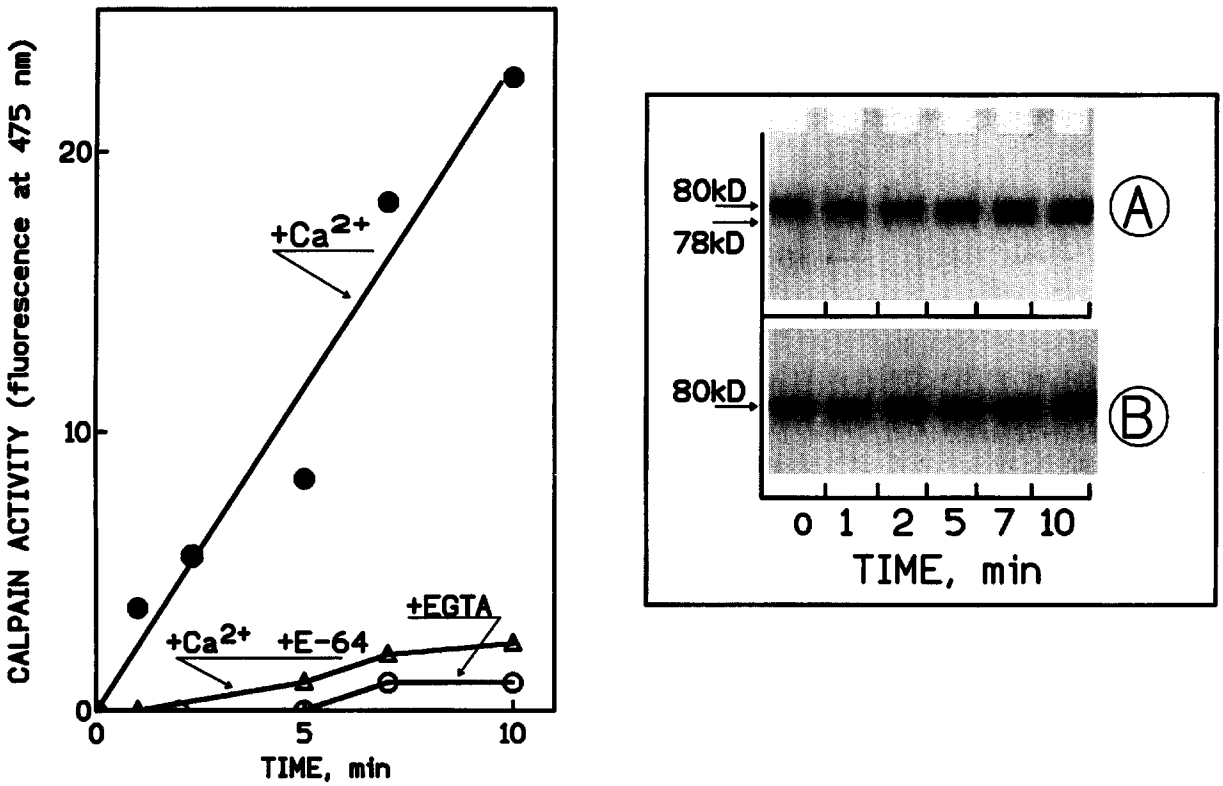


Fig. 5. Expression of catalytic activity and autolysis of membranes associated calpain. Human erythrocyte calpain was incubated in an ice bath in 50 mM sodium borate, pH 7.5, in the presence of 100 μM Ca^{2+} (A) or 1 mM EGTA (B) in the absence or presence of E-64. At the indicated times aliquots of incubation mixtures corresponding to 5 μg of calpain were collected and subjected to 8% SDS-PAGE. At the same times aliquots of incubation mixtures corresponding to 3 μg of calpain were collected and assayed for proteolytic activity in the presence of 1 μM Ca^{2+} as reported in Section 2.

tify the calpain species present, or assayed for calpain activity at 1 μM Ca^{2+} in the absence or presence of E-64.

As shown in Fig. 5, following exposure to calcium, in addition to the native 80 kDa subunit, only the 78 kDa form becomes detectable. This process is completely blocked by the presence of EGTA, indicating the absolute requirement for Ca^{2+} (Fig. 5, inset). The appearance of the 78 kDa species is linearly correlated with the expression of proteolytic activity which is completely inhibited by the addition of EGTA or E-64. These data represent the first demonstration that membrane-associated inactive calpain is activated by μM Ca^{2+} through autoproteolytic digestion which accumulates a locally active 78 kDa form.

4. Discussion

This paper provides new information concerning the mechanism of activation of human erythrocyte calpain through its translocation to the inner surface of plasma membranes and the formation of an early and crucial intermediate species.

Careful analysis of the autoproteolytic process, which calpain undergoes in the presence of calcium, has revealed that the formation of the 75 kDa calpain form occurs in two steps. The first one involves the conversion of the native 80 kDa proteinase form into a 78 kDa form through an intramolecular autoproteolytic event followed by a second step which generates the well known 75 kDa fragment. This second step is sensitive to inhibition by E-64, suggesting that the initial intramolecular digestion has now been replaced by an intermolecular process that reveals the accessibility of the active site. Loading of erythrocytes with calcium results in the translocation of one third of the native soluble enzyme to the inner surface of plasma membranes. The membrane-associated 80 kDa calpain is inactive and only following conversion into the 78 kDa form does catalytic activity become also detectable at low Ca^{2+} concentration.

These findings are consistent with a model suggesting that activation of the proteinase requires two sequential events: first, translocation to the inner surface of the plasma membrane and thus interaction with the anchoring activating cofactors [19–21], and second, autoproteolytic digestion, producing the 78 kDa form. This digestion apparently exposes the active site for interaction with exogenous substrates, also promoting the second autolytic event which generates the 75 kDa calpain form. This active calpain species, having lost most of the membrane-anchoring segments, is recovered in the cytosolic fraction.

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