# Calcium-binding properties of human erythrocyte calpain

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The results presented provide more information on the sequential mechanism that promotes the $Ca^{2+}$-induced activation of human erythrocyte $\mu$-calpain under physiological conditions. The primary event in this process corresponds to the binding of $Ca^{2+}$ to eight interacting sites, of which there are four in each of the two calpain subunits. Progressive binding of this metal ion is linearly correlated with the dissociation of the proteinase, which reaches completion when all eight binding sites are occupied. The affinity for $Ca^{2+}$ in the native heterodimeric calpain is increased 2-fold in the isolated 80 kDa catalytic subunit, but it reaches a $K_d$ consistent with the physiological concentration of $Ca^{2+}$ only in the active autoproteolytically derived 75 kDa form. Binding of $Ca^{2+}$ in physiological conditions, and thus the formation of the 75 kDa subunit, can occur only in the presence of positive modulators. These are represented by the natural activator protein, found to be a $Ca^{2+}$-binding protein, and by highly digestible substrates. The former produces a very large increase in the affinity of calpain for $Ca^{2+}$, and the latter a smaller but still consistent decrease in the $K_d$ of the proteinase for the metal ion. As a result, both dissociation into the constituent subunits and the autoproteolytic conversion of the native 80 kDa subunit into the active 75 kDa form can occur within the physiological fluctuations in $Ca^{2+}$ concentration. The delay in the expression of the proteolytic activity with respect to $Ca^{2+}$ binding to native calpain, no longer detectable in the 75 kDa form, can be attributed to a $Ca^{2+}$-induced functional conformational change, which is correlated with the accessibility of the active site of the enzyme.

## INTRODUCTION

Calpain, the $Ca^{2+}$-dependent neutral proteinase, has been extensively investigated in recent years and characterized in several of its structural and functional properties [1–6]. Thus it has been established that the proteinase is present in the soluble fraction of all mammalian cells in multiple heterodimeric isoforms, containing a 80 kDa catalytically active subunit and a light 30 kDa regulatory one [7–10].

Owing to intramolecular constraints and an absolute requirement for $Ca^{2+}$ at concentrations exceeding the physiological range, calpain has been considered to be normally present in an inactive state. This peculiar condition has stimulated numerous investigations aimed at clarifying the mechanism(s) of calpain activation and also the cell site at which activation and activity occur [11].

Several reports have indicated that calpain exposed to high $Ca^{2+}$ concentration undergoes a rapid autoproteolytic process [12], resulting in the removal of a peptide from the N-terminus of both subunits and leading to the formation of a new calpain form with a $Ca^{2+}$ requirement much lower than that of the native proteinase form [13,14]. These results demonstrated that the N-terminal regions of both calpain subunits are involved in the maintenance of an inactive proteinase form. Accordingly, their removal by autoproteolysis could render the active site accessible to the substrate [15]. It should be remembered that a few calpain forms, although activated by $Ca^{2+}$, do not undergo proteolytic modification, indicating that in these cases a $Ca^{2+}$-induced reversible conformational transition operates in providing the accessibility of the active site [16]. In both activation mechanisms, the sequential events that lead to an active conformation of calpain involve, as the priming event, binding of $Ca^{2+}$ to specific binding sites probably localized in calmodulin-like regions present in each subunit [17,18].

In the present study we have explored several still unknown aspects of the binding of $Ca^{2+}$ to native heterodimeric human erythrocyte calpain to isolated native 80 and 30 kDa subunits as well as to the autoproteolytically derived 75 kDa form. The results indicate that the binding of $Ca^{2+}$ to native heterodimeric calpain induces the dissociation of the oligomeric structure of the enzyme into its constituent subunits, each bearing four $Ca^{2+}$-binding sites. In the monomeric form the catalytic subunit has approximately twice the affinity of native calpain for $Ca^{2+}$, whereas after autoproteolytic conversion the increase is more than 20-fold. All the sequential mechanisms of calpain activation, primed by the binding of $Ca^{2+}$, are here shown to occur under physiological conditions, owing to the combined actions of the specific substrate and the natural activator protein. Both effectors significantly increase the affinity of the native 80 kDa subunit for $Ca^{2+}$, thus allowing its autoproteolytic conversion into the 75 kDa form at a physiological concentration of this metal ion. Taken together, these results contribute to the elucidation of the mechanism involved in $Ca^{2+}$-induced conformational and structural changes of $\mu$-calpain, required to provide the access of the substrate to the active site and hence to promote the activation of the proteinase in stimulated cells.

## MATERIALS AND METHODS

### Purification of human erythrocyte calpain

Human erythrocyte calpain was purified as previously reported [19].

### Assay of calpain activity

Calpain activity was routinely assayed, using human acid-denatured globin as substrate [20], in the presence of 1 mM $Ca^{2+}$.

Abbreviations used: E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

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One unit of calpain was defined as the amount of enzyme causing the release of 1 $\mu$mol of $NH_2$ groups per h.

### Carboxymethylation of calpain and preparation of 110 kDa calpain form

Purified native calpain (0.5 mg) was incubated at 25 °C in 0.25 ml of 50 mM sodium borate, pH 7.5 (buffer A), containing 0.1 mM EDTA; 1 mM (final concentration) iodoacetamide was then added to the protein solution at four 15 min intervals [21]. When the enzyme activity was less than 1 % of the original one, the reaction mixture was submitted to gel filtration on a Sephadex G-25 column (1 cm × 18 cm), previously equilibrated with buffer A, to remove the excess of iodoacetamide and the EDTA present in the sample.

### Preparation of 80 and 30 kDa calpain forms

Carboxymethylated calpain (0.5 mg) was treated with 75 $\mu$M $Ca^{2+}$ and loaded on a Superose 12 column (Pharmacia) previously equilibrated in buffer A containing 75 $\mu$M $Ca^{2+}$. The two eluted protein peaks, containing the two calpain subunits, were collected, concentrated and separately filtered on a Sephadex G-25 column (1 cm × 18 cm), previously equilibrated in buffer A, to remove all the $Ca^{2+}$. The presence of the 80 and 30 kDa calpain subunits in the two preparations was further determined by SDS/PAGE [8 % (w/v) gel].

### Preparation of the autoproteolysed 75 kDa calpain form

Purified native calpain (0.5 mg) was incubated for 1 min at 25 °C in buffer A containing 100 $\mu$M $Ca^{2+}$. The reaction was stopped by the addition of an excess of EDTA and the protein was carboxymethylated and filtered on a Superose 12 column as described above. The fractions containing the calpain subunit were concentrated and loaded on a Sephadex G-25 column. The presence of the 75 kDa calpain form in the preparation was established by SDS/PAGE.

### Binding of $Ca^{2+}$ to native 110 kDa calpain and to the isolated subunits

Samples of native calpain or of the isolated calpain subunits (derived from 50 $\mu$g of native calpain) were diluted in 0.4 ml of buffer A and put into the dialysis tubes. The bags were then transferred into vials containing 20 ml of buffer A and appropriate concentrations of $^{45}Ca^{2+}$ (specific radioactivity $3 \times 10^6$ c.p.m./$\mu$mol). After 4 h at room temperature, samples (20 $\mu$l) of the bag contents and of the equilibrating solutions were collected and counted for radioactivity in a $\beta$-counter. The equivalents of $^{45}Ca^{2+}$ bound to calpain were calculated from the differences between the counts present in each bag and in the equilibrating solution. The calpain-binding properties were analysed by using the equation $\nu = -K_d\nu/[Ca^{2+}] + \nu_{max}$, where $\nu$ is the equivalent of $Ca^{2+}$ bound to calpain, and $\nu_{max}$ the maximal binding capacity of the proteinase. The $K_d$ values were determined from the intercept on the abscissa corresponding to $\nu_{max}/K_d$.

### Purification of rat brain calpain activator

Rat brain was homogenized in 5 vol. of buffer A containing 0.1 mM EDTA and 1 mM PMSF, and cells were disrupted with a Potter–Elvehjem homogenizer followed by sonication (four bursts of 10 s each). The particulate material was discarded after centrifugation at 10000 ***g*** for 20 min and the clear supernatant was heated at 90 °C for 3 min. The denatured proteins were discarded after centrifugation and the clear supernatant was loaded on a Source 15Q column (Pharmacia) previously equilibrated in the same buffer. The calpain activator protein was not retained by the column and emerged during washing. The fractions containing the activator activity were collected and concentrated by precipitation with 80 %-satd. $(NH_4)_2SO_4$. The precipitated proteins were collected, dissolved in buffer A, dialysed and then loaded on a butyl-agarose column (1 cm × 5 cm) previously equilibrated with buffer A. The activator protein activity was eluted from the column immediately after the washed proteins, indicating a weak interaction of the activator protein with the hydrophobic resin. The fractions containing the activity were pooled, concentrated by ultrafiltration and subjected to gel chromatography on a Sephadex G-200 column previously equilibrated with buffer A. The rat brain calpain activator protein shows on gel filtration a molecular mass of approx. 20 kDa. The activator activity was assayed by adding appropriate amounts of the fractions collected to the calpain assay mixture, in the presence of 1 $\mu$M $Ca^{2+}$.

### Preparation of S-nitrosylated 80 kDa calpain subunit

Calpain (0.5 mg) was incubated in sunlight with sodium nitroprusside in sodium acetate buffer, pH 5.5, as previously described [22]. After 15 min, when the remaining enzyme activity was less than 10 % of original, the incubation mixture was stopped by adjusting the pH to 7.5, and 75 $\mu$M $Ca^{2+}$ (final concentration) was added. The resulting solution was loaded on a Superose 12 column previously equilibrated in buffer A containing 75 $\mu$M $Ca^{2+}$. The eluted protein was collected, treated with 0.1 mM EDTA and incubated overnight at 0 °C in the presence of 1 mM dithiothreitol to re-activate the proteinase. The incubation mixture was concentrated and filtered on a Sephadex G-25 column (1 cm × 18 cm) previously equilibrated with buffer A.

## RESULTS

### Effect of $Ca^{2+}$ on the oligomeric structure of human erythrocyte calpain

Purified human erythrocyte calpain, subjected to HPLC–gel-permeation chromatography (Figure 1), emerged as a single peak with an apparent molecular mass of 110 kDa. After addition of 100 $\mu$M $Ca^{2+}$ to the chromatography buffer, two protein peaks were eluted with elution volumes corresponding to the molecular masses of the two constituent calpain subunits, 80 and 30 kDa. The presence of the two calpain subunits in the two protein peaks has been established by SDS/PAGE (see Figure 1, inset). These results indicate that the dimeric calpain structure undergoes dissociation after binding of $Ca^{2+}$ to the proteinase molecule. The correlation between $Ca^{2+}$ concentration and extent of dissociation has been established by adding increasing $Ca^{2+}$ concentrations to the column equilibration buffer in the chromatographic analysis shown in Figure 1 and by measuring the change in molecular mass (from 110 to 80 kDa) of calpain. The results (Figure 2) indicated that dissociation of calpain proceeds with a $K_{0.5}$, the [$Ca^{2+}$] required for 50 % dissociation, of approx. 18 $\mu$M, reaching completion at [$Ca^{2+}$] higher than 50 $\mu$M.

### Binding of $Ca^{2+}$ to native calpain and to isolated constituent subunits

To find out more about the relationship between calpain and $Ca^{2+}$ ions, additional experiments were performed to define the number and the affinity of the $Ca^{2+}$-binding sites in the dimeric

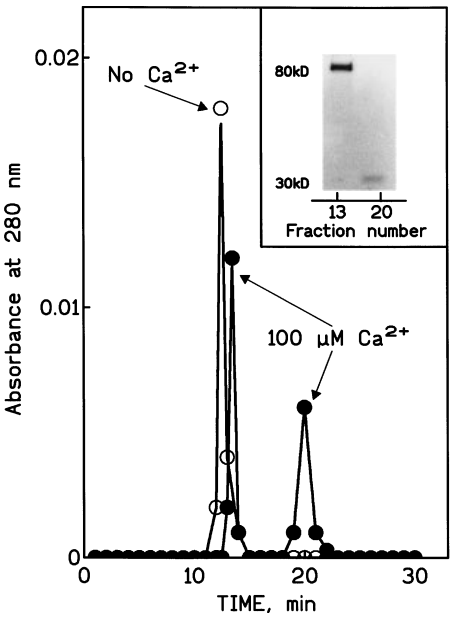


**Figure 1 Effect of $Ca^{2+}$ on the heterodimeric structure of calpain**

Carboxymethylated human erythrocyte calpain (20 $\mu$g) was loaded on a Superdex 75 column (Pharmacia) previously equilibrated with buffer A in the presence of 0.1 mM EDTA (○) or in the presence of 75 $\mu$M $Ca^{2+}$ (●). Fractions containing the two peaks were pooled and analysed by SDS/PAGE [8% (w/v) gel]. The gel was stained with Coomassie Brilliant Blue and destained in 7% (v/v) acetic acid and 10% (v/v) ethanol. In the inset the resulting protein bands are shown (abbreviation: kD, kDa).

110 kDa proteinase form, as well as in the isolated 80 and 30 kDa subunits. As shown in Figure 3, native calpain, incubated with increasing concentrations of $^{45}Ca^{2+}$, bound a maximum of approx. eight equivalents of $Ca^{2+}$, with a $K_d$ of approx. 25 $\mu$M. All eight binding sites were independent and showed similar affinities. Because both calpain subunits contain putative $Ca^{2+}$-

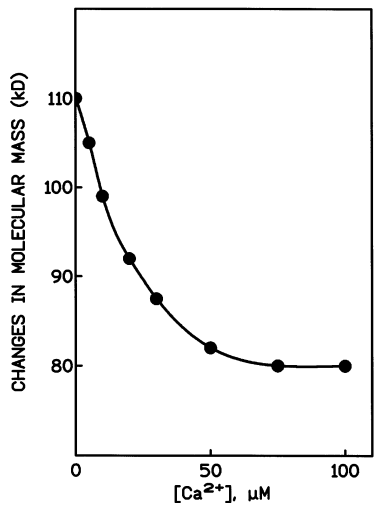


**Figure 2 $Ca^{2+}$ requirement for the dissociation of heterodimeric calpain**

Carboxymethylated calpain (20 $\mu$g) was subjected to gel chromatography, as described in the legend to Figure 1, in the presence of the indicated $Ca^{2+}$ concentrations. Changes in molecular mass were evaluated by the changes in elution time of the major protein peak.

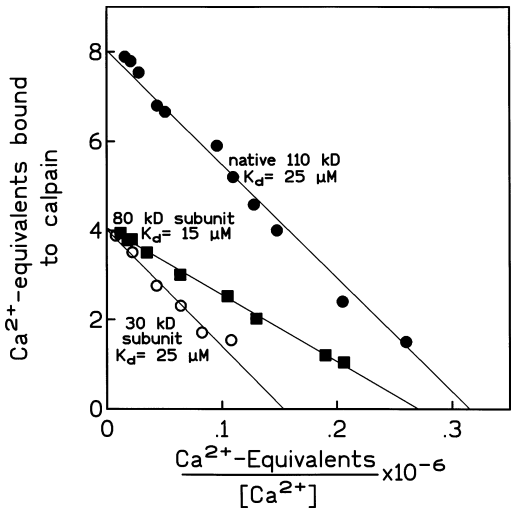


**Figure 3 Binding of $Ca^{2+}$ to native calpain and to its isolated subunits**

Carboxymethylated native 110 kDa calpain, 80 kDa subunit and 30 kDa subunit were prepared as described in the Materials and methods section. The binding of $Ca^{2+}$ was determined by equilibrium dialysis (see the Materials and methods section). After 4 h, aliquots (20 $\mu$l) of the bag contents and of the equilibrating buffers were collected and the radioactivity was counted. The equivalents of $Ca^{2+}$ bound to calpain, and the $K_d$ values, were determined as described in the Materials and methods section.

binding sites in the calmodulin-like domain, similar experiments were performed on the isolated calpain subunits. As shown in Figure 3, each subunit bound four equivalents of $Ca^{2+}$ with $K_d$ values of approx. 15 $\mu$M for the 80 kDa subunit and 25 $\mu$M for the 30 kDa polypeptide. These results indicate that the eight binding sites measured on the 110 kDa native calpain molecule

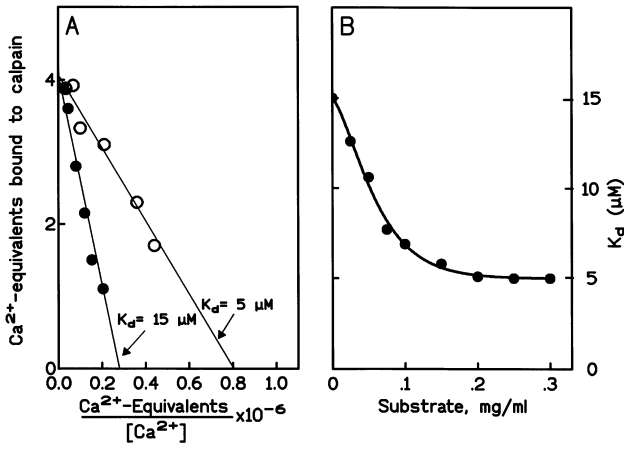


**Figure 4 Effect of substrate on the binding of $Ca^{2+}$ to the 80 kDa catalytic calpain subunit**

(**A**) Carboxymethylated 80 kDa calpain was prepared as described in the Materials and methods section. $Ca^{2+}$-binding experiments were performed as described in the Materials and methods section, in the absence (●) or the presence (○) of 0.2 mg/ml human denatured globin [23] added to the enzyme solutions. The $K_d$ values were determined as described in the Materials and methods section. (**B**) $Ca^{2+}$-binding experiments on carboxymethylated 80 kDa calpain were performed as described for (**A**), with the addition of the indicated amounts of globin to the enzyme solutions. After 4 h, 20 $\mu$l of the bag contents and of the equilibrating buffers were counted for radioactivity. The results are expressed as changes in $K_d$ as a function of the substrate concentrations.

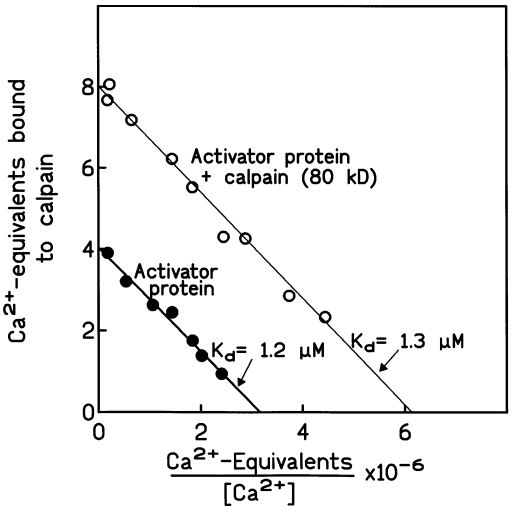


**Figure 5 Effect of the activator protein on the binding of $Ca^{2+}$ to the 80 kDa catalytic calpain subunit**

The $Ca^{2+}$-binding properties of the calpain activator were determined with the same equilibrium dialysis as used for calpain. Samples (5 $\mu$g) of the activator were diluted in 0.4 ml of buffer A in the absence (●) or the presence of 20 $\mu$g of 80 kDa calpain form (○), put into a dialysis bag and equilibrated with the indicated concentration of $^{45}Ca^{2+}$. After 4 h, aliquots (20 $\mu$l) of the bag contents and of the equilibrating buffers were collected and counted for radioactivity. The equivalents of $Ca^{2+}$ bound to calpain, and the $K_d$ values, were determined as described in the Materials and methods section.

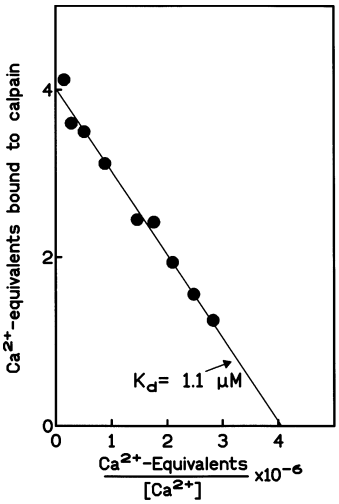


**Figure 6 Binding of $Ca^{2+}$ to the 75 kDa calpain form**

Autoproteolysed 75 kDa calpain subunit was prepared as described in the Materials and methods section. Samples derived from 50 $\mu$g of native calpain were subjected to equilibrium dialysis in the presence of the indicated $^{45}Ca^{2+}$ concentrations. After 4 h, aliquots (20 $\mu$l) of the bag contents and of the equilibrating buffers were collected and counted for radioactivity. The equivalents of $Ca^{2+}$ bound to calpain, and the $K_d$ values, were determined as described in the Materials and methods section.

correspond to the sum of two sets of four binding sites, equally distributed on both subunits, and that dissociation of the heterodimer produces a significant increase in the affinity for $Ca^{2+}$ of the 80 kDa catalytic subunit.

### Effect of substrate and of the activator protein on the affinity for $Ca^{2+}$ of the isolated calpain catalytic subunit

It has previously been established that calpain decreases the $[Ca^{2+}]$ required for the expression of catalytic activity in the presence of a highly digestible substrate [23], as well as after interaction with its natural activator protein [24]. On the basis of these observations, we have explored the effects of the substrate and of the activator on the properties of the $Ca^{2+}$-binding sites of the 80 kDa subunit. As shown in Figure 4(A), the addition of a highly digestible substrate did not modify the total number of available $Ca^{2+}$-binding sites, whereas it promoted a 3-fold increase in affinity with a change of the $K_d$ from 15 to 5 $\mu$M. This effect was dose-dependent (Figure 4B) and became maximal at 0.2 mg/ml substrate concentration, corresponding to that required by the enzyme for expression of the maximal activity.

As regards the calpain activator, we first established that this polypeptide was itself a $Ca^{2+}$-binding protein, containing four $Ca^{2+}$-binding sites (Figure 5, ●) with a $K_d$ of 1.2 $\mu$M. Maximal effects on calpain were observed when the activator was mixed with the proteinase in a ratio of 0.25:1 (w/w), roughly corresponding to a 1:1 complex. With this mixture, containing the 80 kDa calpain subunit and equimolar activator protein (Figure 5, ○), eight equivalents of $Ca^{2+}$ were bound, with a $K_d$ of 1.3 $\mu$M. The total number of eight binding sites represents the sum of the two sets of four binding sites present in calpain and in the activator protein molecule. Because all the binding sites revealed the same affinity for $Ca^{2+}$, it can be concluded that the activator protein induces a more than 10-fold increase in affinity for $Ca^{2+}$ of the 80 kDa calpain subunit. Furthermore, when the native 110 kDa calpain is incubated with $Ca^{2+}$ in the presence of the activator protein, its dissociation into the constituent subunits occurs at $Ca^{2+}$ concentrations between 1 and 2 $\mu$M (results not shown). The activator protein represents the natural effector that allows the overall calpain activation process to operate at physiological $Ca^{2+}$ concentrations.

### Binding of $Ca^{2+}$ to the autoproteolytically derived 75 kDa calpain form

According to previous observations [3,4,6], only the autoproteolysed 75 kDa calpain subunit should be considered the intracellular active calpain species, showing catalytic activity at physiological $Ca^{2+}$ concentrations. To verify that the profound changes in the $Ca^{2+}$ requirement accompanying the conversion of the 80 kDa into the 75 kDa subunit were due to a large increase in the affinity for $Ca^{2+}$, the $Ca^{2+}$-binding properties of the autoproteolytically generated calpain form were determined. As shown in Figure 6, the 75 kDa proteinase form bound a maximum of four equivalents of $Ca^{2+}$ with a $K_d$ of 1 $\mu$M, one order of magnitude greater than that of the sites present in the native 80 kDa subunit.

### Correlation between binding of $Ca^{2+}$ and expression of the catalytic activity of calpain

To define the relationship between the binding of $Ca^{2+}$ and the expression of proteolytic activity, the correlation of the kinetics of binding of the metal ion with the appearance of the catalytic activity of calpain was analysed. As shown in Table 1, in native calpain or in the isolated native 80 kDa catalytic subunit, the $[Ca^{2+}]$ to saturate half of the $Ca^{2+}$-binding sites was significantly lower than that necessary to promote $V_{max}/2$. In contrast, this difference was not detectable with the 80 kDa form associated with the activator protein or with the 75 kDa form; in these cases

**Table 1 Comparison of $Ca^{2+}$-binding properties and catalytic efficiencies of the different forms of human erythrocyte calpain**

Uncarboxymethylated native calpain forms were assayed with saturating amounts of human denatured globin as substrate (see the Materials and methods section), in the presence of increasing concentration of $Ca^{2+}$, to determine the $[Ca^{2+}]$ required to promote $V_{max}/2$ (results are means ± S.D. for four separate experiments). The calpain forms were prepared as described in the Materials and methods section. Data for $K_d$ are taken from Figures 3 and 6. The calpain-to-activator protein ratio for the last row was 1/0.25 (w/w), corresponding to a 1:1 molar ratio.

| Calpain form | $K_d$ ($\mu$M) | $[Ca^{2+}]$ required to promote $V_{max}/2$ ($\mu$M) |
|---|---|---|
| 110 kDa | 25 | 40 ± 4 |
| 80 kDa | 15 | 32 ± 4 |
| 75 kDa | 1 | 0.9 ± 0.06 |
| 80 kDa + activator protein | 1.2 | 1.0 ± 0.06 |

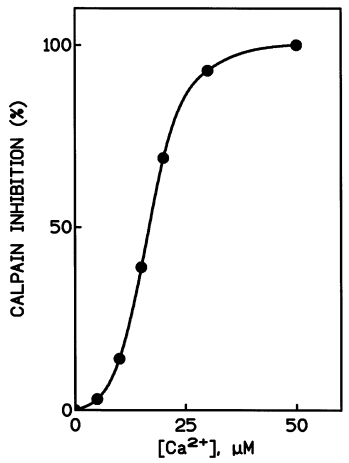


**Figure 7 Effect of $Ca^{2+}$ on the inhibitory efficiency of E-64**

Native calpain (20 $\mu$g) was incubated in the presence of 30 $\mu$M E-64 in buffer A for 5 min in an ice bath with 0.1 mM EDTA or in the presence of the indicated $Ca^{2+}$ concentration. The reaction was stopped by the addition of 0.1 mM EDTA and the incubation mixtures were separately loaded on a Sephadex G-25 column (0.5 cm × 12 cm) previously equilibrated in buffer A. The eluted fractions (250 $\mu$l each) were assayed as described in the Materials and methods section in the presence of 1 mM $Ca^{2+}$. Results are expressed as percentages of the amount of enzyme activity recovered after gel chromatography of the same amount of calpain treated in 0.1 mM EDTA without E-64.

a good correlation between the binding of $Ca^{2+}$ and the expression of catalytic activity was observed. These results suggest that the expression of activity in native calpain is preceded by a conformational change that leads to autoproteolysis and hence to a marked modification in the accessibility of the active site. This assumption is further supported by the observation that an irreversible inhibitor, such as *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), known to react with the essential SH group at the active site, was inactive when tested with native calpain in the absence of $Ca^{2+}$ and became effective in the presence of increasing concentrations of $Ca^{2+}$ (Figure 7).

## DISCUSSION

It has beeen known for many years that the addition of $Ca^{2+}$ to native calpain triggers a series of events that lead to the formation of a new proteinase form, still retaining an absolute $Ca^{2+}$ dependence but capable of expressing proteolytic activity at physiological concentrations of the metal ion [3,4,6]. With almost all calpain isoforms, of the effects induced by $Ca^{2+}$ the most relevant corresponds to an autoproteolytic digestion that removes the N-terminal region of both subunits. In a few calpain isoforms the effect of $Ca^{2+}$ is limited to a fully reversible transition to an active conformational state without any proteolytic modification of the primary structure. The results presented here provide a better understanding of the role of $Ca^{2+}$ on the activation process of calpain and specifically on the effect of $Ca^{2+}$ on the oligomeric organization of the proteinase, as well as on the kinetics of binding of $Ca^{2+}$ and on the nature of effectors capable of affecting the characteristics of such kinetics. We have also defined how binding of the metal ion to native calpain and to the isolated catalytic subunit is related to the expression of catalytic activity. It has been observed that $Ca^{2+}$ induces dissociation of the calpain heterodimer with a $K_{0.5}$ of approx. 18 $\mu$M. Native calpain binds eight equivalents of $Ca^{2+}$ with a $K_d$ of approx. 25 $\mu$M, with four on each of the two subunits. In its monomeric form, the catalytic 80 kDa subunit approximately doubles its affinity for $Ca^{2+}$, indicating that dissociation and binding of $Ca^{2+}$ are correlated processes. These observations are consistent with those previously reported [6,7], indicating that the function of the small (30 kDa) subunit is involved in maintaining those constraints that prevent uncontrolled expression of catalytic activity by the 80 kDa subunit. Additional changes in the $Ca^{2+}$ sensitivity of the 80 kDa subunit are accomplished by the presence of a highly digestible substrate [23], which promotes a significant increase in the affinity for $Ca^{2+}$.

A stronger positive effect on the affinity of the calpain binding sites is promoted by the activator protein [23,24], which is itself a $Ca^{2+}$-binding protein capable of modifying more than 10-fold the $K_d$ of the calpain binding sites. Thus, in the presence of the activator protein, the catalytic subunit can bind $Ca^{2+}$ at physiological concentrations and can be converted into the active enzyme form, corresponding to the 75 kDa calpain species. The autolysed calpain form shows the highest $Ca^{2+}$-binding affinity, corresponding to that observed for the native subunit in the presence of the activator. Furthermore, in the isolated 75 kDa subunit, binding of $Ca^{2+}$ is linearly correlated with the expression of catalytic activity, whereas in both native heterodimeric protein and the isolated 80 kDa subunit, binding of $Ca^{2+}$ precedes the appearance of catalytic activity. Thus dissociation of the heterodimeric structure, followed by conversion into a stable 75 kDa subunit, is induced by the binding of $Ca^{2+}$ to native calpain; this sequential mechanism can take place under physiological conditions owing to the presence of a specific calpain activator protein.

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