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CALPAIN DISSOCIATES INTO SUBUNITS IN THE PRESENCE OF CALCIUM IONS

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SUMMARY Calpain is a calcium dependent cysteine protease consisting of a catalytic 80K subunit and a regulatory 30K subunit. It has therefore been believed that calpain functions as a dimer. Here we have found that calpain dissociates into subunits in the presence of the Ca^{2+} required for the expression of activity and that the dissociated 80K subunit is enzymatically fully active. Moreover, the 80K subunit shows a calcium sensitivity identical to the activated form of calpain but not to the original control calpain. The results suggest that the activation of calpain corresponds to the dissociation into subunits in the presence of Ca^{2+} and that calpain functions as a monomer of the 80K subunit in vivo.

Calpain is a cysteine protease comprising an 80 kDa subunit (80K) and a 30 kDa subunit (30K). 80K contains a cysteine protease domain homologous to other cysteine proteases such as papain, and cathepsins B, L and H, and is thus regarded as the catalytic subunit. The function of 30K is not clear, but it is believed to be a regulatory subunit [1-3]. Quite recently we reexamined the conditions for dissociation and renaturation of calpain and found that 80K has full proteolytic activity equivalent to the untreated calpain species. The results suggested the possibility that calpain, which has been believed to function as a dimer, might act as a monomer in vivo [4]. We therefore examined the conditions for the dissociation of calpain into monomers and found that calpain dissociates into subunits in the presence of the calcium ion concentrations required for the expression of activity. The results described below suggest a novel mechanism for the activation of calpain in vivo distinct from that which has been believed thus far.

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MATERIALS AND METHODS

Materials

E-64 and leupeptin were purchased from Sigma and casein was obtained from Merck. Calpain was purified from rabbit skeletal muscle as described previously [5]. Purified calpain was dialyzed before use against 10 mM Tris-HCl, pH 7.5, containing 5 mM β-mercaptoethanol and 150 mM NaCl (Buffer A). Autolyzed calpain was prepared as described previously [6]. Briefly, m-calpain in buffer A was incubated for 5 min at 25℃ after the addition of 2 mM $CaCl_2$ and autolysis was stopped by the addition of 5 mM EDTA. Succinyl-leucyl-leucyl-valyl-tyrosyl-methylcoumarine amide (Suc-L-L-V-Y-MCA) was obtained from Peptide Institute Inc.

Gel filtration of calpain in the presence of Ca^{2+}

Purified m- or μ-calpain was pre-incubated in 200 μl buffer A containing 0.1% Triton X-100, 0.1～1mM $CaCl_2$, and E-64 (1000-fold molar excess over calpain) or 0.2 mM leupeptin for 5 min at 25℃. The sample was chromatographed on a Superose-6 column (10×300 mm, Pharmacia FPLC system) equilibrated with the same buffer but without E-64 or leupeptin. Peaks detected by absorbance at 280 nm were collected and analyzed by polyacrylamide gel electrophoresis with SDS (SDS/PAGE).

Activity assay for calpain and its subunit

Activities were measured with casein as a substrate as described before [6]. In the case of samples containing leupeptin, its concentration was reduced to below 0.1 μM by ultrafiltration (UFC3 LGC membrane, Millipore Ltd.) prior to activity assay. To analyze calpain activity by SDS/PAGE, the assay was stopped at intervals by the addition of 1/5 volume of SDS/PAGE loading buffer, 250 mM Tris-HCl, pH 6.8, containing 50% glycerol, 10% SDS and 2% β-mercaptoethanol, and the sample was analyzed by SDS/PAGE. Activities were also measured with Suc-L-L-V-Y-MCA as a substrate as described previously [7].

SDS/PAGE

Samples were mixed with a 1/5 volume of SDS/PAGE loading buffer, boiled for 5 min, and analyzed on 12% polyacrylamide gels according to Laemmli [8]. Gels were stained with Coomassie brilliant blue R250.

RESULTS

Dissociation of calpain in $CaCl_2$

When m-calpain in buffer A was subjected to gel filtration, a single peak was obtained at an elution volume of 15 ml corresponding to a molecular weight of 120 kDa as estimated from the elution volumes of standard molecular weight marker proteins (Fig. 1-a). The peak gave two bands with molecular weights of 80K and 30K when analyzed by SDS/PAGE (Fig. 1-b), confirming that calpain is a heterodimer of 80K and 30K. The presence of 0.1% Triton X-100 in the gel filtration buffer did not change the elution profile. The m-calpain preparation pre-incubated in 1 mM $CaCl_2$ in the presence of E-64 or leupeptin to inhibit autolysis gave three peaks. Analyses of these peaks by SDS/PAGE showed that the first peak and the second peak eluting at 17 ml and 19 ml corresponded to 80K and 30K, respectively. The amounts in the 80K and 30K peaks were roughly equal on a molar basis. The minor bands below 80K in the peak eluting at 17 ml are autolyzed

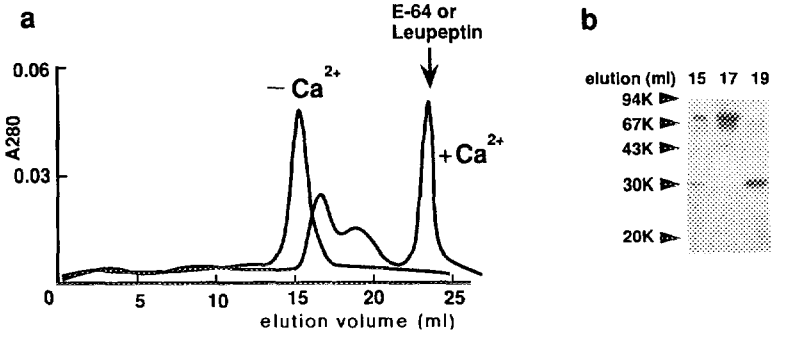

Fig. 1. Gel filtration of calpain in the presence of Ca^{2+}.
(a) Purified rabbit m-calpain (7nmol) was subjected to gel filtration in the presence of Triton X-100 after preincubation with or without 1 mM $CaCl_2$. SDS-PAGE analysis of the fractions eluted at 15, 17 and 19 ml is shown in (b).

products produced during preincubation even in the presence of E-64 or leupeptin. The third peak at 23 ml contained only the E-64 added to the sample. The results clearly indicate that m-calpian dissociates into subunits in the presence of 1 mM $CaCl_2$. In contrast, a single band comprising 80K and 30K was obtained upon gel filtration of m-calpain in the presence of 100 μM $CaCl_2$, where m-calpain is inactive. On the other hand, μ-calpain, which is active at 100 μM Ca^{2+}, dissociated into subunits under these conditions.

As shown in Fig. 2, a single proteinaceous band corresponding to an untreated calpian preparation was obtained upon gel filtration of the dissociated calpain solution after the removal of Ca^{2+} by the addition of EDTA. SDS/PAGE analyses of the protein fraction eluted at 15 ml indicated that it contained equimolar amounts of 80K and 30K. Taken together, these results clearly indicate that calpian dissociates into subunits in the presence of the Ca^{2+} concentration required for the expression of activity and that the dissociation is completely reversible and that dimeric calpain is reformed almost instantaneously upon the removal of Ca^{2+}.

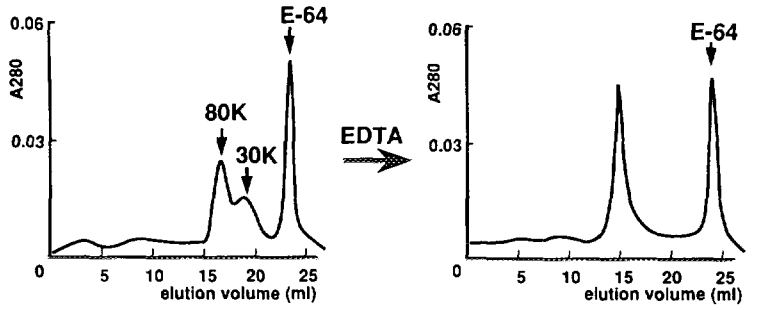

Fig. 2. Gel filtration profile of dissociated calpain after the removal of Ca^{2+}.
An excess amount of EDTA (final concentration 10 mM) was added to the dissociated calpain preparation prior to gel filtration. A single proteinaceous peak observed at 15 ml contained equimolar amounts of 80K and 30K by SDS-PAGE analysis (data not shown).

Caseinolytic activity of the dissociated 80K species

The 80K subunits of μ - and m-calpains, μ 80K, and m80K were prepared by gel filtration of μ - and m-calpains in the presence of Ca^{2+} and leupeptin. The caseinolytic activities of μ 80K and m80K were compaired with those of μ - and m-calpains by following the SDS/PAGE patterns during hydrolysis. As shown in Fig. 3, hydrolysis of casein by μ - and m-calpains can be seen after 30 seconds of incubation under the conditions used. A similar pattern of casein hydrolysis was observed upon incubation with μ 80K and m80K. No significant differences in the patterns of casein hydrolysis were observed between the 80K subunits and the corresponding calpains under the conditions used. In other words, the 80K subunit and its parent calpain species have identical caseinolytic activities.

Properties of isolated 80K subunits

The calcium sensitivity of calpain increases significantly upon autocatalytic modification of the N-terminal regions of both subunits [9]. Since this autocatalytic modification is observed in the presence of Ca^{2+} prior to the expression of protease activity, this step has been regarded as activation [10,11]. In order to analyze the correlation between this activation step and dissociation into subunits, the calcium sensitivity of 80K derived from both native calpain and autolyzed activated calpain was examined. The calcium sensitivity of m80K derived from native m-calpain was much higher than that of native m-calpain and coincided with that of autolyzed activated m-calpain as shown in Fig.4. N-terminal sequence analysis of the 80K preparation used showed N-terminus to be blocked, suggesting that the N-terminal region is not modified autocatalytically [12]. Furthermore, an N-terminally modified m80K preparation, i.e. truncated m80K, isolated from autolyzed m-calpain showed the same calpain sensitivity as native 80K.

In summary, the 80K preparation showed essentially the same calcium sensitivity as that of autolyzed activated calpain regardless of modification in the N-terminal region. Some kinetic parameters of m80K and truncated m80K in comparison with

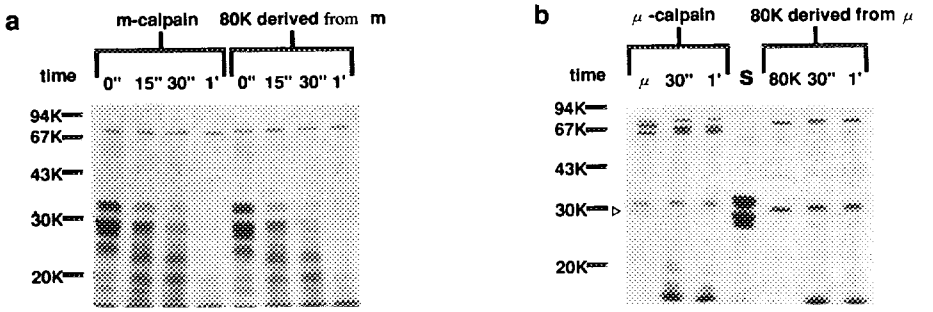

Fig. 3. Time course of caseinolysis by calpain and its 80K subunit. Casein (4.5 μ g) and calpain (50 μ g) or 80K were incubated with 1 mM $CaCl_2$ in 10 mM Tris-HCl, pH 7.5, containing 1 mM β-mercaptoethanol at 4℃. Equimolar amounts of calpain and corresponding 80K were used. The reaction was terminated by adding excess EDTA and SDS-PAGE loading buffer at intervals and the mixtures were analyzed by SDS-PAGE. (a): m-calpain and 80K derived from m-calpain, (b): μ -calpain and 80K derived from μ -calpain. Lane S : casein used as a substrate. Open triangle indicates the existence of 30K.

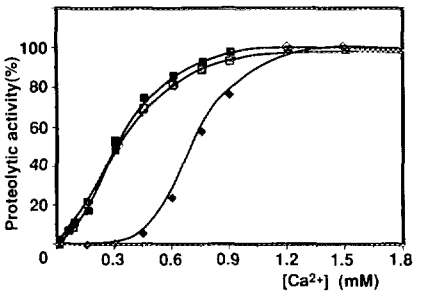

Fig. 4. Ca^{2+} requirements of calpain and its 80K subunit.
The proteolytic activity of rabbit m-calpian, 80K derived from m-calpain, and truncated 80K isolated from autolyzed m-calpain was measured following incubation at 30℃ for 20 min at various $CaCl_2$ concentrations as described [6]. ◆: native m-calpain ,◇: autolyzed activated m-calpain, ▩: 80K derived from m-calpain, □: truncated 80K derived from autolyzed m-calpain. The activity of native m-calpian with 2 mM $CaCl_2$ was taken as 100%.

native m-calpain are summarized in Table 1. Various properties, except for calcium sensitivity, are essentially the same for native m80K, truncated m80K, and native m-calpain. Thus m80K and m-calpain seem to have the same protease activity. This is partly supported by the fact that both showed the same pattern of casein hydrolysis as analyzed by SDS/PAGE (see Fig. 3). The presence of 30K affects only the calcium sensitivity of 80K. In fact, preliminary results indicate that the addition

Table 1. Comparison of some kinetic parameters of three calpain species

	native m-calpain	m80K	truncated m80K
Optimum pH	**7.5**	**7.5**	**7.5**
Ca^{2+}concentration for 50% activity (μ M)	**600**	**300**	**300**
Kinetic Parameters for α -Casein			
Km **(μ M)**	**9.3**	**9.3**	**9.3**
Vmax (μ mol/mg/min)	**5.1**	**4.9**	**4.9**
for Suc-L-L-V-Y-MCA			
Km **(μ M)**	**600**	**620**	**620**
Vmax (nmol/mg/min)	**250**	**238**	**238**
Inhibition by E-64	**Yes**	**Yes**	**Yes**

Native m-calpain, m80K, and truncated m80K were isolated as described [6]. The protease activity was measured at 10℃ for 5 min for casein and at 4 ℃ for 20 min for Suc-L-L-V-Y-MCA.

of the 30K subunit to native 80K, reduces the calcium sensitivity to that of untreated m-calpain.

DISCUSSION

The molecular properties of calpain have been studied only in the absence of Ca^{2+}. In the presence of Ca^{2+}, calpain forms aggregates and precipitates, which hampers various analyses including physicochemical measurements. As reported in a previous paper [4], we established the conditions for the denaturation and renaturation of calpain and obtained a fully active 80K subunit. In the present study the conditions were further improved to prevent calpain from forming aggregates and precipitates in the presence of Ca^{2+}. Under conditions where Triton X-100 was incorporated, it became possible to analyze the molecular properties of calpain in the presence of Ca^{2+} even at higher protein concentrations. In fact, without Triton X-100, calpain forms aggregates upon the addition of Ca^{2+} and a chromatogram such as that shown in Fig. 1 cannot be obtained. Triton X-100 does not affect the reformation of calpain and a dimer of 80K and 30K is formed upon the removal of Ca^{2+}.

The fact that the dissociated 80K subunit possesses the same proteolytic properties as native calpain or the dimeric form, except in terms of calcium sensitivity, is very important. The calcium sensitivity of the 80K subunit is identical not to that of native calpain but to autolyzed activated calpain. Although the results described in this paper were obtained in vitro, calpain appears to dissociate into subunits in vivo when the intracellular Ca^{2+} concentration increases and functions as a monomer of the 80K subunit. Both calpain subunits contain a calmodulin-like Ca^{2+} binding domain in the C-terminal region [1-3]. In other words, calpain can be regarded as a calmodulin-dependent enzyme. Calmodulin usually associates with calmodulin-dependent enzymes and activates them when it binds Ca^{2+} and its hydrophobic domain becomes exposed to the surface [13-16]. In the case of calpain, however, the dissociation into subunits occurs upon formation of a Ca^{2+}-"calmodulin" complex. The effects of the formation of the Ca^{2+}-calmodulin complexes of each subunit are apparently opposite to each other, although calpain contains two calmodulin-like domains whereas ordinary calmodulin-dependent enzyme systems contain only one [17-19].

As described, the calcium sensitivity of calpain increases significantly when the N-terminal region of the 80K subunit and/or the 30K subunit is modified by autolysis [9]. In the case of μ-calpain, modification of the N-terminal region of 80K precedes that of 30K [20]. In the case of m-calpain, however, modification of 30K occurs first [21,22]. If the activation step corresponds to the modification of the N-terminal region of the subunits, the activation mechanisms of μ- and m-calpains are apparently different. As the various properties of μ- and m-calpains are quite similar, it is strange that the activation mechanisms for μ- and m-calpains are significantly different. According to our model for the activation of calpain summarized in Fig. 5, modification of the N-terminal region of the 80K and/or 30K subunits facilitates the dissociation of calpain leading to activation. We presume that the activation of calpain, namely the increase in Ca^{2+} sensitivity, is fundamentary controlled by the dissociation of the 30K subunit which is governed by Ca^{2+} concentration. Activation is not the primary consequence of modification

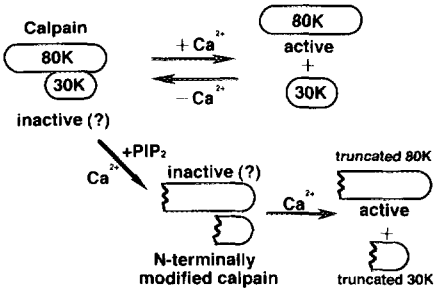

Fig. 5. Model for calpain activation.
Calpain dissociates into subunits in the presence of Ca^{2+} and reassociates upon the removal of Ca^{2+}. Modification of the N-terminal regions of 80K and/or 30K accelerates dissociation and phosphatidylinositol-bis-phosphate lowers the Ca^{2+} concentrations for dissociation.

of the N-terminal region of 80K and/or 30K. This is supported by the fact that 80K and N-terminally modified 80K have the same protease activities including calcium sensitivity. Furthermore it has been shown that phospholipids such as phosphatidylinositol bisphosphates, PIP2, lower the Ca^{2+} concentration required for autolysis and accelerate the activation of calpain [23-26]. The effect of phospholipids on the activation of calpain can also be interpreted in terms of the acceleration of calpain dissociation by lowering the Ca^{2+} concentration for autocatalytic modification.

The Ca^{2+} concentrations required for calpain dissociation and for the expression of protease activity of dissociated 80K seem to be quite close, although a precise analysis is now in progress. μ-Calpain becomes fully active at physiological Ca^{2+} concentrations, $10^{-7}\sim10^{-6}$ M Ca^{2+}, in the presence of PIP2. m-Calpain, however, requires mM levels of Ca^{2+} for the expression of activity in vitro and PIP2 alone cannot reduce the required Ca^{2+} concentration to physiological levels. Clearly some other factor is required to explain the dissociation of m-calpain at the physiological Ca^{2+} level. According to the model presented in Fig.5, the 30K subunit regulates the Ca^{2+} sensitivity of the 80K subunit. Is this the only function of the 30K subunit? Is modification of 80K and/or 30K required for dissociation? Where and how does the dissociation of calpain into subunits occur in vivo? These as well as other questions are most important and interesting as future issues to understand the physiological functions and structure-function relationship of calpain.

In summary, calpain dissociates into subunits in the presence of Ca^{2+} yielding a dissociated catalytic 80K subunit that is enzymatically fully active and whose calcium sensitivity is identical to that of the autolyzed active form. These totally unexpected but very important results will have a significant effect on current studies on calpain as well as on cellular calcium signalling.

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