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Interaction of Human Calpains I and II with High Molecular Weight and Low Molecular Weight Kininogens and Their Heavy Chain: Mechanism of Interaction and the Role of Divalent Cations†

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ABSTRACT: Calpain I prepared from human erythrocytes was half-maximally and maximally activated at 23 and 35 μM calcium ion, and two preparations of calpain II from human liver and kidney were half-maximally activated at 340 and 220 μM calcium ion and maximally activated at 900 μM calcium ion, respectively. High molecular weight (HMW) and low molecular weight (LMW) kininogens isolated from human plasma and the heavy chain prepared from these proteins inhibited calpain I as well as calpain II. The molar ratios of calpains to HMW kininogen to give complete inhibition of calpains were 1.4 for calpain I and 2.0 for calpain II, and those of calpains to heavy chain were 0.40–0.66 for calpain I and 0.85 for calpain II. LMW kininogen did not completely inhibit the calpains even with an excess amount of kininogen. The apparent binding ratio of calpain to HMW kininogen estimated from the disc gel electrophoretic analysis, however, was found to be 2:1, whereas those of calpain to LMW kininogen and of calpain to heavy chain were found to be 1:1. Calpains and kininogens failed to form complexes in the absence of calcium ion. In the presence of calcium ion, however, they formed the complexes, which were dissociable by the addition of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. The minimum concentrations of calcium ion required to induce complex formation between calpain I and kininogens and calpain II and kininogens were 70 and 100 μM, respectively. Some other divalent cations such as Mn^{2+}, Sr^{2+}, and Ba^{2+} were also able to induce the complex formation between calpains and kininogens. Calpain I was activated by these divalent cations except by Mn^{2+}, while calpain II was not activated by any of these divalent cations. These results indicated that the divalent cations required to activate calpains and the divalent cations required to induce the complex formation are not necessarily the same. The ionic radii of the effective divalent cations varied, ranging from 0.8 to 1.3 Å including calcium ion.

Calpain (calcium-dependent cysteine proteinase, EC 3.4.22.17) is known as a cytosolic proteinase widely distributed in mammalian and avian cells and has been isolated from various tissues (Murachi et al., 1981). Calpain has two distinct subclasses of the enzyme: calpain I, which requires low concentrations of Ca^{2+} (micromolar order) for activation, and calpain II, which requires high concentrations of Ca^{2+} (millimolar order) (De Martino, 1981; Yoshimura et al., 1983; Kitahara et al., 1984). These enzymes are composed of two different subunits, a large subunit with M_r 80 000 and a small subunit with M_r 30 000. Recently, amino acid sequences of calpain I of porcine, rabbit, and human and calpain II of rabbit were predicted by analyzing the base sequences of cDNA (Sakihama et al., 1985; Emori et al., 1986a,b; Aoki et al., 1986). Comparison of the amino acid sequences of rabbit calpains revealed that the small subunit of calpain I is identical with that of calpain II, whereas the large subunits of calpains I and II are different from each other, although they share some extent of homology (approximately 50%).

Calpastatin, a calpain-specific inhibitor, is also present in cytosol (Murachi et al., 1981). It was shown that the calpastatin formed a complex with calpain I in the presence of high concentrations of Ca^{2+} (millimolar order) and further that the calpain–calpastatin complex was easily dissociated by adding chelating reagents (Melloni et al., 1982).

On the other hand, Ohkubo et al. (1984) have isolated and characterized cDNA coding for human α_2TPI[1] and revealed that α_2TPI is identical with low molecular weight kininogen

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[1] Abbreviations: HMW kininogen, high molecular weight kininogen; LMW kininogen, low molecular weight kininogen; TPI, thiol proteinase inhibitor; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; β-ME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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(LMW kininogen) as judged from the amino acid sequence predicted from the cDNA structure. Further, Higashiyama et al. (1986a) demonstrated that the heavy chain of kininogens exhibited the highest inhibitory activity for papain and bound with 2 mol of the enzyme/mol of the heavy chain, while high molecular weight kininogen (HMW kininogen) and LMW kininogen inhibited only 1.4 mol of papain. Salvesen et al. (1986) reported that LMW kininogen contained three cystatin-like sequences designated domains 1, 2, and 3, and the activity of chicken calpain was inhibited by only domain 2, but the other two domains did not affect the activity.

In cases of tissue damage or breakdown of the blood cells, the release of calpains may occur, and the interaction of calpains with kininogens takes place as an inevitable and popular event. In this study, we demonstrate that kininogens or their heavy chain reversibly bind to the calpains in the presence of Ca^{2+}. We further report here the stoichiometry of the complex formation and the essential role of divalent cations for the interaction of calpains and kininogens.

Materials and Methods

Human livers and human kidneys obtained at autopsies were stored at −70 °C until use. Casein (Hammarsten grade) was a product of E. Merck, Darmstadt, Germany. Calcium chloride ($CaCl_2$) and EDTA (ethylenediaminetetraacetic acid) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and PMSF (phenylmethanesulfonyl fluoride) were obtained from Sigma Chemical Co., St. Louis, MO. Benzamidine chloride was a product of Tokyo Chemical Industry, Co., Ltd., Tokyo, Japan. DEAE-cellulose (DE-52) was purchased from Whatman, Kent, U.K., Sephacryl S-300 and Red Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-Bio-Gel A was from Bio-Rad, Richmond, CA. All other chemicals were of analytical grade.

Buffers. The following buffers were used in the chromatographic procedures for the purification of calpain II: 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol (β-ME), 1 mM benzamidine, 0.1 mM PMSF, and 0.15 M NaCl (buffer A); 20 mM imidazole hydrochloride buffer, pH 6.8, containing 5 mM EDTA, 1 mM EGTA, and 5 mM β-ME (buffer B); buffer B at pH 6.5 (buffer C).

Assay of Calpain. Calpain activity was measured with alkali-denatured casein as substrate at a final concentration of 0.24% in 750 μL of the reaction mixture containing 25 mM β-ME, 100 mM glycerophosphate hydrochloride buffer, pH 7.5, 10 mM $CaCl_2$, and appropriate amounts of enzyme solution. The reaction was started by the addition of $CaCl_2$ solution. The mixture was incubated for 20 min at 30 °C, and the reaction was stopped by addition of 750 μL of 10% trichloroacetic acid. This solution was centrifuged at 1500*g* for 10 min. The absorbance of the supernatant at 280 nm was measured with a Hitachi spectrophotometer, Model 228. One unit of the enzyme was defined as the amount of enzyme that hydrolyzed casein resulting in an absorbance increase of 1.0 at 280 nm for 1 h.

Assay of Thiol Proteinase Inhibitor (TPI) Activity. The inhibitory activities of HMW kininogen, LMW kininogen, and the heavy chain of the kininogens for calpain I and calpain II were measured with casein as substrate. Calpain I (1.19 units) or calpain II (1.20 units) and increasing amounts of HMW kininogen, LMW kininogen, or the heavy chain were mixed in 300 μL of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA and 375 μL of 0.48% casein. The mixture was preincubated for 5 min at 30 °C, and the reaction was started by the addition of 75 μL of 5 mM $CaCl_2$ for calpain I and 10 mM $CaCl_2$ for calpain II. The following assay procedures are as described under Assay of Calpain.

Electrophoresis. Polyacrylamide slab gel electrophoresis with sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (1970). Disc gel electrophoresis in the absence of SDS was performed according to the method of Davis (1964).

Preparation of Calpain I from Human Erythrocytes. Calpain I, a low calcium-dependent cysteine proteinase, was purified from human erythrocytes by employing column chromatographies on DEAE-cellulose, Ultrogel AcA 34, Blue Sepharose, and DEAE-Bio-Gel A according to the method described by Hatanaka et al. (1983).

Preparation of Calpain II from Human Liver and Human Kidney. All purification procedures were performed at 4 °C unless otherwise specified. Calpain II from human liver and human kidney was purified by an almost identical method.

Step 1. After as much blood as possible was removed, human liver or human kidney was minced into small pieces and washed extensively with buffer A. The minced materials were then homogenized with 4 volumes of the above buffer for 10 min at 4 °C in a Waring blender. Each homogenate was centrifuged at 10000*g* for 1 h. The supernatant obtained from human kidney was subjected to the subsequent purification step without ammonium sulfate fractionation. On the other hand, the supernatant from human liver was brought to 33%–50% saturation of ammonium sulfate. The precipitate obtained was dissolved again in a minimum volume of cold buffer A.

Step 2. The sample was dialyzed overnight against the above buffer and applied to a DEAE-cellulose column (10 × 15 cm) equilibrated with the same buffer. The column was washed extensively with the same buffer until the absorbance at 280 nm decreased to a negligible level, and the proteins were eluted with a linear gradient of 0.15–0.5 M NaCl in a total volume of 6 L of the same buffer. Fractions containing calpain II activity were collected and brought to 60% saturation by slow addition of solid ammonium sulfate. After being gently stirred for 1 h, the suspension was centrifuged at 10000*g* for 30 min. The precipitate obtained was dissolved in a minimum volume of cold buffer B and dialyzed against buffer B containing 1 mM benzamidine and 0.1 mM PMSF.

Step 3. The dialyzate was subjected to gel filtration on a Sephacryl S-300 column (3.6 × 120 cm) equilibrated with the above buffer. Elution was performed with the same buffer. Fractions containing calpain II activity were collected and dialyzed overnight against buffer B.

Step 4. The dialyzate was applied to a DEAE-Bio-Gel A (1.6 × 22 cm) equilibrated with buffer B. The column was washed thoroughly with the same buffer. The proteins were eluted with a linear gradient of NaCl (0–0.4 M) in a total volume of 500 mL. Fractions containing calpain II activity were collected and dialyzed overnight against buffer C.

Step 5. The dialyzate was applied to a Red Sepharose column (1.8 × 20 cm) equilibrated with the above buffer. The column was washed thoroughly with the same buffer. The proteins were eluted with a linear gradient of KCl (0–1.0 M) in a total volume of 500 mL. Fractions containing calpain II activity were pooled and stored at 4 °C until use. This preparation was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA and 5 mM β-ME or 110 mM imidazole hydrochloride buffer, pH 7.3, containing 1 mM EGTA and 5 mM β-ME before use. Calpain II

preparations from human liver and kidney were finally purified about 95-fold with 35.0% yield and 52-fold with 8.6% yield, respectively, over the fractions containing calpain activity on DEAE-cellulose column chromatography.

The specific activities of calpain I from human erythrocytes and calpain II from human liver and kidney were 120, 94.7, and 72.7 units/mg of protein, respectively. Each purified calpain I and calpain II gave a single band on polyacrylamide disc gel electrophoresis. The subunit structures of the purified calpain I and calpain II were examined by SDS–polyacrylamide slab gel electrophoresis. Calpain I was composed of a large subunit of M_r 85000 and a small subunit of M_r 29000, while calpain II preparations from human liver and kidney were composed of a large subunit of M_r 75000 and three small subunits of M_r 30000, 25400, and 21600.

Purification of HMW Kininogen, LMW Kininogen, and Heavy Chain of Kininogens. Human HMW kininogen was purified to a homogeneous state by the procedures previously reported (Higashiyama et al., 1986a). LMW kininogen was also purified from fresh citrated human plasma by a newly developed procedure employing DEAE-Sephadex A-50, ammonium sulfate fractionation, DEAE-Sephacel, Red Sepharose, HA-Ultrogel, and butyl-Toyopearl (unpublished results), and the heavy chain was prepared from kinin-free LMW kininogen (α_2TPI$_2$) by the methods of Higashiyama et al. (1986a).

Determination of Free Calcium. Distilled and double-deionized water which showed more than 18 MΩ cm was used. Calcium–EGTA (Ca^{2+}–EGTA) buffers (Yoshimura et al., 1983) were prepared by adding various amounts of $CaCl_2$ to a mixture of 110 mM imidazole hydrochloride buffer, pH 7.3, containing 1 mM EGTA and 5 mM β-ME. A dissociation constant of 5.5×10^{-6} M (Harafuji & Ogawa, 1980) was used for calculation of the free calcium concentration.

Determination of Protein Concentration. Protein concentrations were determined by employing $E^{1\%}_{280nm}$ values of 7.0 for HMW kininogen (Kato et al., 1981), 7.8 for LMW kininogen (Müller-Esterl & Fritz, 1984), 7.8 for the heavy chain of kininogens, and 7.2 for calpain I and calpain II (Higashiyama et al., 1986b).

Complex Formation of Calpains and Kininogens. A constant amount (7.5×10^{-11} mol) of kininogens and the heavy chain and varying amounts of calpains I and II were used for this experiment. These proteins were dialyzed for 3 days against 110 mM imidazole hydrochloride buffer, pH 7.3, containing 1 mM EGTA and 5 mM β-ME before use. The complex formations between calpain I or II and kininogens or the heavy chain were performed at room temperature. After 15-min incubation, aliquots from the reaction mixtures were subjected to disc gel electrophoresis in order to analyze the complex formations.

Protein Quantification on Disc Gel by Densitometric Analysis. Protein quantification on the gels was carried out by the use of a dual-wavelength TLC scanner CS-900 (Shimadzu) with a scanning speed of 80 mm/min and a chart speed of 96 mm/min at 650 nm. To analyze densitometric tracings, we measured the height (cm) of the peaks from the top to the base line.

Results

Calcium Requirements for the Activity of Calpain I and Calpain II. Calcium requirements of calpain I and two calpain II preparations were determined in the Ca^{2+}–EGTA buffer system. Calpain I was half-maximally and maximally activated at 23 and 35 μM Ca^{2+}, respectively. On the other hand, the calpain II preparations from human liver and kidney were half-maximally activated at 340 and 220 μM Ca^{2+} and maximally at 900 μM Ca^{2+}, respectively.

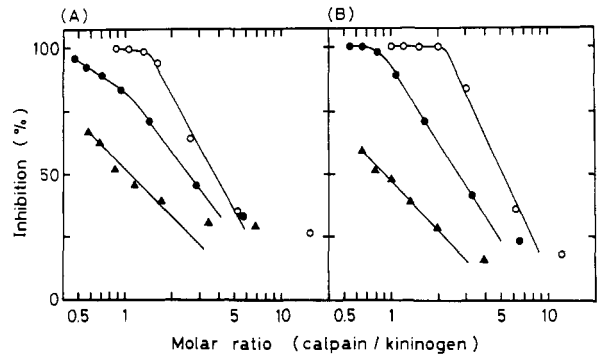

FIGURE 1: Inhibition of caseinolytic activity of calpains by HMW and LMW kininogens and the heavy chain: (A) inhibition of calpain I; (B) inhibition of calpain II. Calpain I from human erythrocytes and calpain II from human liver were preincubated with various amounts of HMW and LMW kininogens and the heavy chain at 30 °C for 5 min. The reaction was started by adding $CaCl_2$ and incubated at 30 °C for 15 min. Caseinolytic activity was measured at 280 nm. (○) HMW kininogen, (▲) LMW kininogen, and (●) heavy chain

Inhibitory Effects of Kininogens and Heavy Chain on Calpain I and Calpain II. The inhibitory effects of kininogens and heavy chain on the activity of calpain I (1.19 units, 1.44×10^{-10} mol) and calpain II from human liver (1.20 units, 1.66×10^{-10} mol) were examined. Among two kininogens and the heavy chain, HMW kininogen had the strongest inhibitory activity toward the two calpains. The order of the inhibition capacity of kininogens and the heavy chain to both calpain I and calpain II was HMW kininogen, heavy chain, and LMW kininogen as shown in Figure 1. On the other hand, with respect to the susceptibilities of calpain I and calpain II toward HMW kininogen and the heavy chain, calpain II was more sensitive than calpain I, but toward LMW kininogen, both calpains were almost compatible. The molar ratios of calpains to HMW kininogen to give complete inhibition were 1.4 for calpain I and 2.0 for calpain II, and those of calpains to heavy chain were 0.40–0.66 for calpain I and 0.85 for calpain II, but LMW kininogen did not completely inhibit calpain I and calpain II even with a sufficient amount of the kininogen.

Complex Formation of Calpains with Kininogens and Heavy Chain. To characterize the interaction of calpains with kininogens and heavy chain, various amounts of calpains were incubated with a constant amount (7.5×10^{-11} mol) of kininogen or heavy chain at room temperature for 15 min, and the complex formation was analyzed by disc gel electrophoresis in the absence of SDS.

Figure 2A shows complex formation between calpain I and kininogens or heavy chain in the presence of 5 mM calcium ion. The complexes, which were formed in calpain to kininogens or heavy chain molar ratios of 0.5:1 and 1:1, migrated as two or three bands with slow mobilities (see lines), leaving certain amounts of kininogens or heavy chain at the original positions. In a molar ratio of 2:1, the amount of the complexes increased, and kininogens and heavy chain mostly disappeared. Further, in a molar ratio of 3:1, degradation products originated from kininogens, heavy chain, and/or complexes increased, especially in the reaction mixtures of calpain I and kininogens.

The complex formation with calpain II was carried out in the presence of 10 mM calcium ion. As shown in Figure 2B, the electrophoretic patterns of the complexes formed with calpain II are very similar to those of the complexes formed with calpain I, but in the reaction mixtures many degradation products are observed through the molar ratios examined.

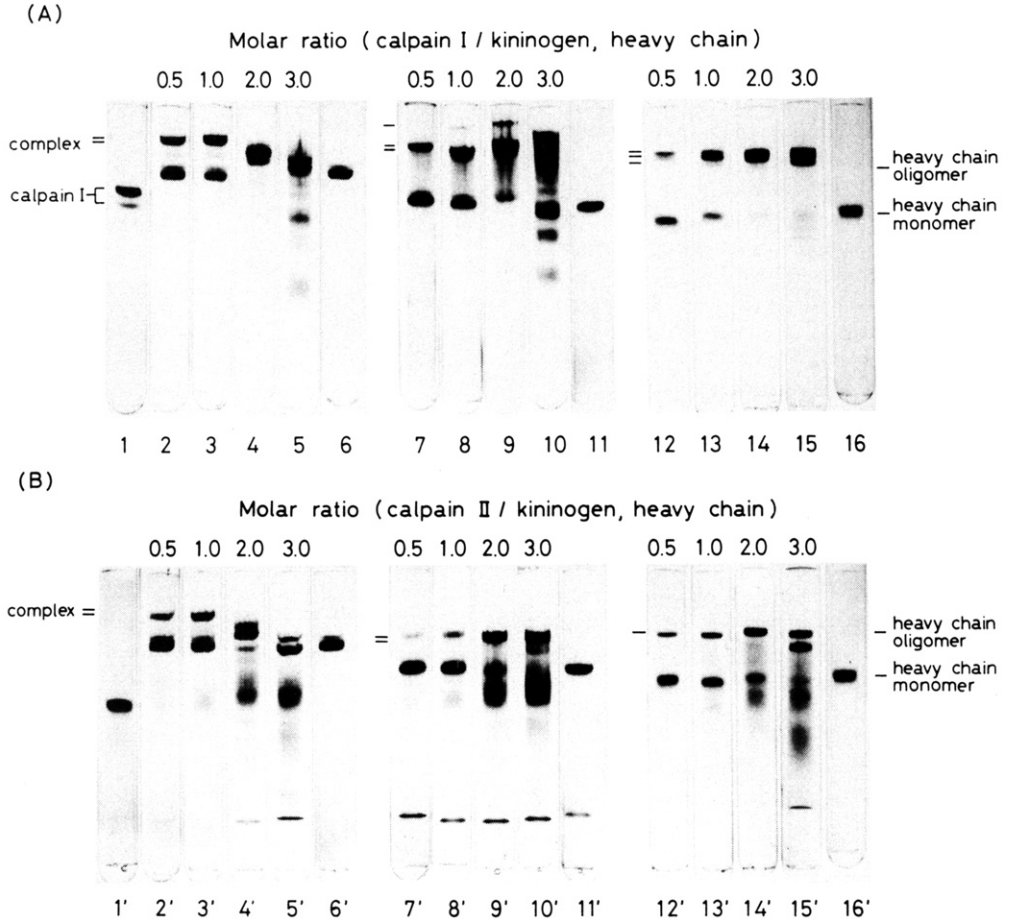

FIGURE 2: Disc gel electrophoretic analysis of complex formation between calpains and HMW and LMW kininogens or heavy chain in various molar ratios: (A) complex formation between calpain I and kininogens or heavy chain; (B) complex formation between calpain II and kininogens or heavy chain. Calpains I and II were applied to gels 1 and 1′, HMW kininogen to gels 6 and 6′, LMW kininogen to gels 11 and 11′, and heavy chain to gels 16 and 16′. Reaction mixtures of calpain I and HMW kininogen, calpain I and LMW kininogen, and calpain I and heavy chain were respectively applied to gels 2–5, 7–10, and 12–15 in calpain to kininogens or heavy chain molar ratios of 0.5:1, 1:1, 2:1, and 3:1. In the same manner, the reaction mixtures of calpain II and HMW kininogen, calpain II and LMW kininogen, and calpain II and heavy chain were applied to gels 2′–5′, 7′–10′, and 12′–15′ in the molar ratios of 0.5:1, 1:1, 2:1, and 3:1. The amount of kininogens or heavy chain applied to each gel was 7.5×10^{-11} mol in the presence of 5 mM $CaCl_2$ for calpain I and 10 mM $CaCl_2$ for calpain II.

Since the calpains are very sensitive to autodigestion, the bands of calpains are not observed through the experiments under a sufficient amount of calcium ion (5 and 10 mM).

Effects of Calcium Ion on Complex Formations between Calpains and Kininogens or Heavy Chain. The calcium requirement for complex formation was examined with various calcium concentrations. The experiment was performed in a calpain to kininogen or heavy chain molar ratio of 1:1 at room temperature with an incubation time of 15 min.

When the calcium concentration was changed from 18 to 200 μM, calpain I formed complex with HMW kininogen and LMW kininogen at calcium concentrations of 70 μM or higher (Figure 3A), while it formed the complex with heavy chain from 33 μM calcium ion, although the complexes formed at low calcium concentrations were faint. Calpain II also formed the complexes with kininogens at a calcium concentration of 100 μM or higher (Figure 3B), and it formed the complex with heavy chain from 70 μM calcium ion. It was noticed that, in calcium concentrations as low as 18–200 μM, no degradation products appeared, and calpains were not autodigested even when in an excess amount. Moreover, in a calpain to LMW kininogen or heavy chain molar ratio of 1:1, both components, calpain I and LMW kininogen (gel 10), calpain II and LMW kininogen (gel 8′), calpain I and heavy chain (gel 14), and calpain II and heavy chain (gel 12′), disappeared almost completely at calcium concentrations of 200 or 70 μM, indicating that the 1:1 molar ratio is the equivalent binding ratio. However, in the calapins and HMW kininogen system, about half the HMW kininogen remained at the original position at 200 μM calcium concentration. This indicates that the equivalent binding ratio of calpains to HMW kininogen is more than 1:1, most probably 2:1, referring to the data in Figure 1.

Dissociation of Complex. To examine if the complex formation is reversible or not, 20 mM EGTA was added to the reaction mixture in which the complex was formed. All the complexes formed in various molar ratios were clearly dissociated, and several bands of kininogens, heavy chain, and calpains appeared accompanied with some amount of degradation products. Typical dissociation patterns of the complexes are shown in Figure 4. Figure 4A corresponds to the dissociation patterns of the complexes shown in Figure 2A (gels 1–5) and Figure 4B to those in Figure 2B (gels 1′–6′). As shown in Figure 4, the complexes are completely dissociated, and HMW kininogen appears in most of the patterns of Figure 4. Calpain I is also observed in the patterns of gels 2–4 in Figure 4A and calpain II in the patterns of gels 7–10 in Figure 4B. The dissociation patterns from the complexes in higher molar ratios (2:1 and 3:1) contain several broad bands with faster mobilities, which seem to be of degradation products.

Effects of Various Divalent Cations on Activities of Calpain I and Calpain II. The effects of various divalent cations on

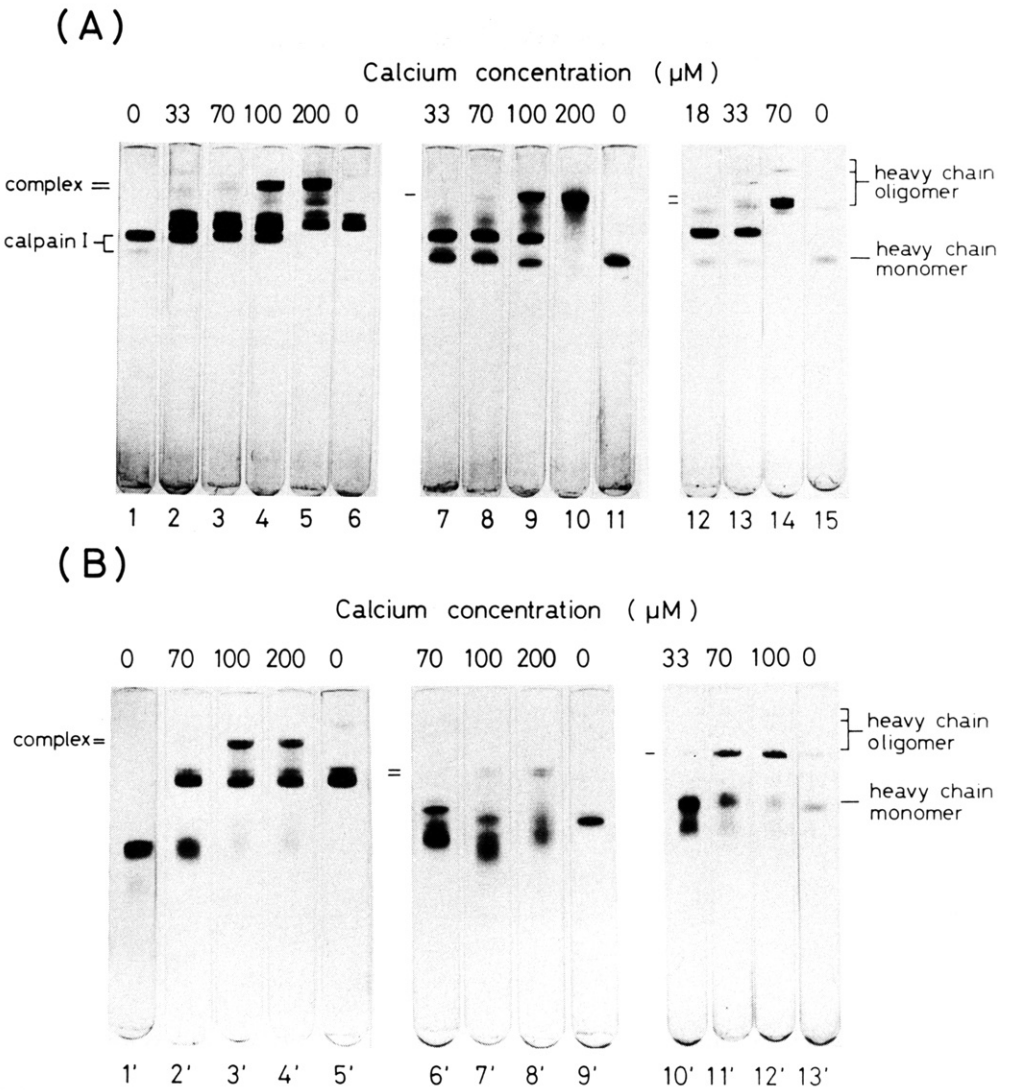

FIGURE 3: Disc gel electrophoretic analysis of complex formation between calpains and kininogens or heavy chain in various calcium concentrations: (A) complex formation between calpain I and kininogens or heavy chain; (B) complex formation between calpain II and kininogens or heavy chain. Calpains I and II were applied to gels 1 and 1′, HMW kininogen to gels 6 and 5′, LMW kininogen to gels 11 and 9′, and heavy chain to gels 15 and 13′. Reaction mixtures of calpain I and HMW kininogen, calpain I and LMW kininogen, and calpain I and heavy chain were respectively applied to gels 2–5, 7–10, and 12–14 in a calpain to kininogens or heavy chain molar ratio of 1:1 with different calcium concentrations as indicated above each gel column in Figure 5A. In the same manner, reaction mixtures of calpain II and HMW kininogen, calpain II and LMW kininogen, and calpain II and heavy chain were applied to gels 2′–4′, 6′–8′, and 10′–12′ in a calpain II to kininogens or heavy chain molar ratio of 1:1 with different calcium concentrations as indicated above each gel column in Figure 5B. The amount of the proteins applied to each gel was 7.5×10^{-11} mol.

Table I: Effect of Divalent Cations on Activity of Calpain I and Calpain II[a]

	calpain I from erythrocytes			calpain II from liver			calpain II from kidney		
	cation (mM)			cation (mM)			cation (mM)		
	5	10	complex formation	5	10	complex formation	5	10	complex formation
Ca^{2+}	100	*b*	+	100	100	+	100	100	*b*
Mg^{2+}	0	0	−	3.8	7.6	−	0	0	*b*
Ni^{2+}	9.6	9.7	−	7.6	11.0	−	3.3	8.3	*b*
Zn^{2+}	0	0	−	0	0	−	*b*	*b*	*b*
Mn^{2+}	3.4	4.9	+	0	3.8	+	0	0	*b*
Sr^{2+}	103.2	105.1	+	0	3.8	+	0	0	*b*
Ba^{2+}	44.4	91.9	+	3.8	7.6	+	0	0	*b*

[a] Figures are expressed in percent activity, taking as 100% when the activity was measured at 5 mM Ca^{2+} for calpain I and 10 mM Ca^{2+} for calpain II. All the cations were supplied as the chloride form. [b] Not determined.

the activities of calpain I from human erythrocytes and calpain II from human liver and kidney are shown in Table I. Calpain II was not activated by Mg^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Sr^{2+}, and Ba^{2+} even at 10 mM concentration. On the other hand, calpain I was fully activated at 5 and 10 mM Sr^{2+} and activated 44% and 92% at 5 and 10 mM Ba^{2+}.

Effects of Various Divalent Cations on Complex Formation between Calpains and Heavy Chain. Disc gel electrophoretic patterns of the complexes between calpains and heavy chain in the presence of various divalent cations are shown in Figure

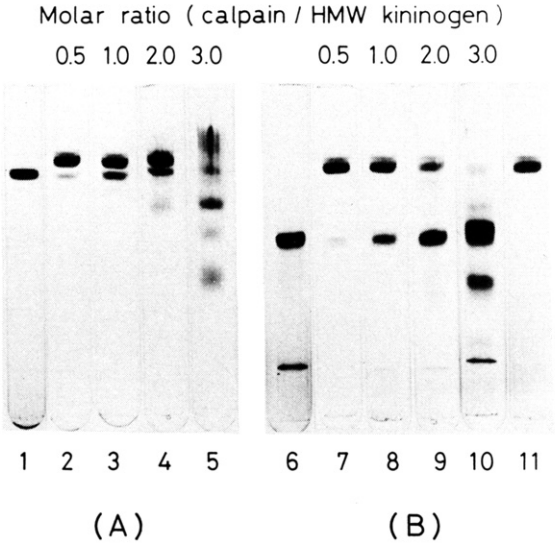

FIGURE 4: Disc gel electrophoretic analysis of the dissociation of the complexes formed between calpains and HMW kininogen. Reaction mixtures of calpain I and HMW kininogen and of calpain II and HMW kininogen were incubated in calpain to HMW kininogen molar ratios of 0.5:1, 1:1, 2:1, and 3:1 in the presence of 5 mM $CaCl_2$ for calpain I and 10 mM $CaCl_2$ for calpain II. After complex formation, 20 mM EGTA was added to each reaction mixture, and the mixture was subjected to disc gel electrophoresis. Calpain I and calpain II were applied to gels 1 and 6 and HMW kininogen to a gel 11. The reaction mixtures of calpain I and HMW kininogen and of calpain II and HMW kininogen in molar ratios of 0.5:1, 1:1, 2:1, and 3:1 were applied to gels 2–5 and to gels 7–10, respectively. The amount of kininogen applied to each gel was 7.5×10^{-11} mol.

5. To know the effect of divalent cations on the complex formation, several divalent cations (Mg^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}) at 100 μM and 10 mM were added to the reaction mixtures of calpains and heavy chain. In the experiment using 100 μM divalent cations, calpains I and II formed the complexes with heavy chain in the presence of Ca^{2+} but did not form the complexes in the presence of other cations (Figure 5A,C). However, they formed the complexes in the presence of Mn^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} at 10 mM concentration (Figure 5B,D). In this experiment, the complexes formed between calpain I and heavy chain consisted of three bands (Figure 5A,B), while those between calpain II and heavy chain gave a single band on polyacrylamide disc gel electrophoresis (Figure 5C,D).

Panels A–D of Figure 5 show the relationship between the ionic radii of the divalent cations and the ability to induce the complex formation. The complexes formed are semiquantitatively represented as the absorption intensities (height of the peaks) of the bands scanned with a densitometer. These data indicate that the ionic radii to induce the complex formation ranged from 0.8 to 1.3 Å. The order of intensity to induce the complex formation was $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+}$ in the calpain I and heavy chain system and $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mn^{2+}$ in the calpain II and heavy chain system at 10 mM concentration.

Discussion

The study of the interaction between calpain and its cytosolic inhibitor calpastatin has provided an explanation for the

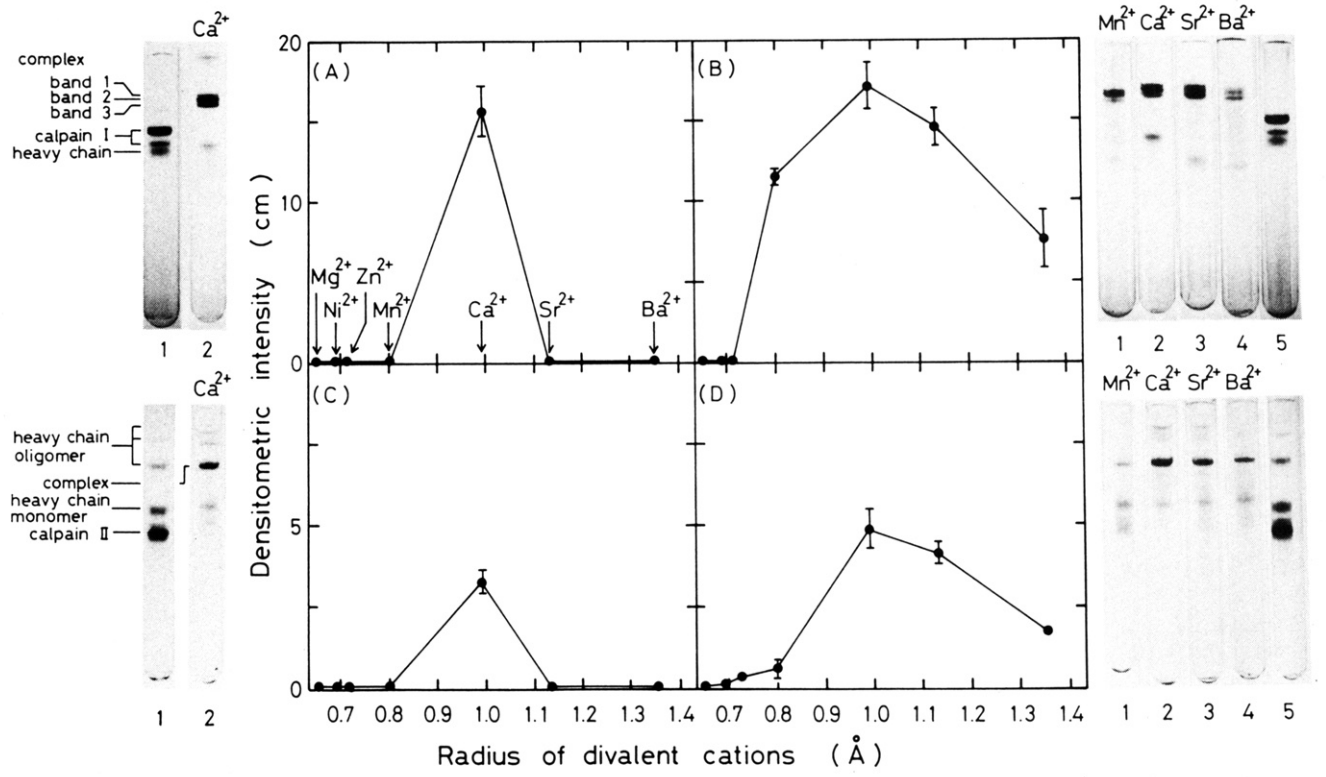

FIGURE 5: Effects of various divalent cations on complex formation between calpains and heavy chains: (A and B) complex formation between calpain I and heavy chain in the presence of 100 μM and 10 mM divalent cations, respectively; (C and D) complex formation between calpain II and heavy chain in the presence of 100 μM and 10 mM divalent cations. After 15-min incubation of calpain I or II with heavy chain, aliquots were applied to polyacrylamide disc gel electrophoresis. The gels were stained with Coomassie brilliant blue R-250 and subjected to densitometric analysis. The absorption peaks (height) of the band(s) for the complex(es) were plotted against ionic radii of divalent cations. The bands for complex in (A) and (B) consist of three (bands 1, 2, and 3). The densitometric intensities of the triplet bands are totaled and expressed as one absorption peak. Since the complex band and an oligomer band of the heavy chain in (C) and (D) have the same migration rate, densitometric intensity of the complex is expressed as a difference between the intensity of the measured bands and that of the oligomer band. (A) Gel 1 is a pattern in the presence of Ca^{2+} and gel 2 without cation. Other cations had no effect for forming complex at concentrations of 100 μM. (B) Gels 1–4 are patterns in the presence of Mn^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}, and gel 5 is without cation. (C) Gel 1 is a pattern in the presence of Ca^{2+} and gel 2 without cation. (D) Gels 1–4 are patterns in the presence of Mn^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}, and gel 5 is without cation.

regulation of the intracellular protein degradation system (Murachi et al., 1981; Melloni et al., 1982). However, in the presence of divalent cation the interaction of calpains and extracellular inhibitors, kininogens, has been reported only a few times (Higashiyama et al., 1986a; Schmaier et al., 1986; Salvesen et al., 1986). Accordingly, it is very important to know the differences of the effects of divalent cations on the complex formation of calpains with kininogens and their derivatives and on the activation of calpains.

As to the calcium concentration needed to induce the complex formation with kininogens, calpain I required at least 70 μM and calpain II 100 μM, and for complex formation with heavy chain, calpain I required 33 μM and calpain II 70 μM calcium ion. This difference of calcium requirements between calpain I and calpain II is not substantial, referring to that for the activation of two calpains. In addition, it should be noticed that at this level of calcium concentration of 70–100 μM calpain II is not sufficiently activated. The latter phenomenon was further confirmed by the experiment using other divalent cations. As shown in Table I and Figure 5, Mn^{2+} could induce the complex formation between calpain I and heavy chain even if calpain I was not activated. Likewise, three divalent cations, Mn^{2+}, Sr^{2+}, and Ba^{2+}, were also able to induce the complex formation at 10 mM without activation of calpain II. With respect to the activation of calpain II, Kubota et al. (1984) reported that purified calpain II from fresh human placenta could be fully activated by 10 mM Sr^{2+} but not by 10 mM Ba^{2+}. On the other hand, like our data, Suzuki et al. (1979) also reported that purified calpain II from skeletal muscle from human cadavers was not activated by Sr^{2+} and Ba^{2+} at 10 mM concentration. This discrepancy is thought to depend on the time up to getting human organs. The preparation of calpain II from fresh placenta possessed an intact small subunit (M_r 30000), while our preparations from liver and kidney possessed degradated small subunits (M_r 30000, 25400, and 21600). It can be postulated that the activation of calpain II with Sr^{2+}, Ba^{2+}, and Mn^{2+} except for Ca^{2+} depends on the molecular conservation of small subunits. Further, the above data strongly suggest that the divalent cation to activate calpain and the cation to induce the complex formation might be separate cations and act on different parts of calpain. In addition, since we observed that the isolated large subunit (catalytic subunit) of calpain I could combine with the heavy chain of kininogens (unpublished data), the binding site of calpain should be located in the large subunit.

Table I and Figure 5 showed another evidence that not all divalent cations could induce the complex formation and further, among the cations that could induce the complex formation, there exists an order of intensity: in the calpain I and heavy chain system the order was $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+}$, while in the calpain II and heavy chain system the order was $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mn^{2+}$, suggesting that calpain II prefers divalent cations with relatively large ionic radii as compared to calpain I.

The molar ratio of calpain II to HMW kininogen to give complete inhibition was 2.0 as shown in Figure 1. The number was also confirmed by the experiments with papain as target protease (Higashiyama et al., 1986a; Sasaki et al., 1986). However, Salvesen et al. (1986) reported that calpain exhibited the affinity to bind only with domain 2 of the heavy chain of LMW kininogen. The heavy chain in the present study also showed nearly one to one stoichiometry (0.85:1) for binding with calpain II, and as to the LMW kininogen, complete inhibition of calpains was not obtained even in excess amounts of the kininogen. Since three molecules of HMW and LMW kininogens and heavy chain share the identical mass structure of heavy chain (Kato et al., 1976; Kitamura et al., 1983; Takagaki et al., 1985), how could we explain this conspicuous discrepancy of binding ratios between them? In the previous study (Sasaki et al., 1983), we reported that α_2TPI (kinin-free LMW kininogen) well inhibited calpain (CANP), and a recent study (Higashiyama et al., 1986a) also revealed that degradation products such as kinin-free LMW kininogen (α_2TPI) and kinin-free and fragments 1 and 2 free HMW kininogen exhibited higher inhibition capacity than LMW and HMW kininogens and the heavy chain showed the highest binding ratio of 2.0. These data suggest that the affinity or inhibition capacity of the kininogens and their derivatives containing the heavy chain moiety not only depends on their primary structure of the heavy chain but is also strongly affected by the other factors as higher order structures modified with light chain, fragments 1 and 2, and disulfide bridge(s). It would be also certain that the binding capacity is largely affected by the difference of the target proteinase.

The relationship between the inhibition capacity and the complex formation was analyzed on disc gel electrophoresis. As shown in Figure 2, in calpains and kininogens or heavy chain systems, most HMW and LMW kininogens and heavy chain disappeared in a molar ratio of 2:1, forming the complexes. These data are not necessarily compatible with the results obtained in the inhibition assay (Figure 1). This incompatibility appears mostly due to the autodigestion of the calpains, which did not combine with the counterpart inhibitors, especially in calpains and LMW kininogen or heavy chain systems under sufficient calcium ion. In fact, as shown in Figure 3, in a molar ratio of 1:1, calpain and LMW kininogen (gel 10) and calpain and heavy chain (gel 14) almost completely disappeared, forming the complexes at calcium concentrations of 200 and 70 μM, respectively, without producing degradation products, and in the HMW kininogen and calpain system approximately half the amount of the HMW kininogen remained unreacted. These data suggest that HMW kininogen forms the complex with calpain of 2:1 molar ratio and LMW kininogen and heavy chain form the complexes of 1:1 molar ratio (Figure 3).

In physiological and pathological conditions, the interaction of calpains with kininogens may occur when calpains are released from the tissues. Since calpains are widely distributed in the tissues and cells (Murachi et al., 1981), inflammation, immune diseases such as allergy, autoimmune diseases, malignant tumors, all of which bring about tissue damage, could be the cause to release the calpains. As shown in this study the interactions of calpains with kininogens, which occur in high calpain to kininogen molar ratios, are usually accompanied by autodigestion of calpains and cleavage of kininogens. In addition, as we have reported recently (Higashiyama et al., 1986b), this cleavage of kininogens involves the liberation of Lys-bradykinin, indicating that the interaction occurs not only for inhibiting the proteolytic activity of calpains but also for induction of kinin liberation, which is followed by the increase in permeability of the peripheral vessels. This may facilitate the migration into tissues of phagocytes that serve as scavengers for the repair of damaged tissues. This possible process is of interest as an extracellular function of the intracellular cysteine proteinase calpain.

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Registry No. Ca, 7440-70-2; Mn, 7439-96-5; Sr, 7440-24-6; Ba, 7440-39-3; calpain, 78990-62-2.

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Processivity of the DNA Polymerase α–Primase Complex from Calf Thymus

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ABSTRACT: The processivity of the DNA polymerase α–primase complex from calf thymus was analyzed under various conditions. When multi-RNA-primed M13 DNA was used as the substrate, the DNA polymerase α–primase complex was found to incorporate 19 ± 3 nucleotides per primer binding event. This result was confirmed by product analysis on sequencing gels following DNA synthesis on poly(dT)·$(rA)_{10}$. The processivity depends strongly on the assay conditions but does not correlate with enzymic activity. Lowering the concentration of Mg^{2+} ions to less than 2 mM increases the processivity to 60. Replacing Mg^{2+} by 0.2 mM Mn^{2+} results in 90 nucleotides being incorporated per primer binding event. Neither the presence of ATP nor the addition of noncognate deoxynucleotide triphosphates affects the processivity of the DNA polymerase α–primase complex. Lower processivity was induced by lowering the reaction temperature, by adding spermine, spermidine, or putrescine, in the presence of the antibiotics novobiocin and ciprofloxacin, by adding *Escherichia coli* single-stranded DNA binding protein, or by adding calf thymus topoisomerase II and RNase H. Three single-stranded DNA binding proteins from calf thymus, including unwinding protein 1, do not affect processivity to any significant extent. Freshly prepared DNA polymerase α–primase complex exhibits in addition to its processivity of 20 further discrete processivities of about 55, 90, and 105. This result suggests that further subunits of the polymerase α–primase complex are necessary to reconstitute the holoenzyme form of the eukaryotic replicase.

Catalysis by polymerizing enzymes can be described in terms of two limiting cases. First, after the incorporation of one monomeric building block, the polymerase might dissociate from the substrate. This kind of synthesis has been designated as distributive. Second, after initiation, the polymerase might continue until it has reached the end of the substrate. This

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