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Molecular and Catalytic Characterization of Intact Heterodimeric and Derived Monomeric Calpains Isolated under Different Conditions from Pig Polymorphonuclear Leukocytes†

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ABSTRACT: Evidence is presented of polymorphonuclear (PMN) cells derived from pig peripheral blood containing two molecular species of Ca^{2+}-dependent cysteine endopeptidases, calpains I and II, which require low and high concentrations of Ca^{2+}, respectively, for activation. Calpains I and II, purified from PMN homogenates, are heterodimers consisting of 83 plus 29 kDa and 80 plus 29 kDa subunits, respectively, which can be identified by using subunit-specific antibodies and which are identical with those of calpain species in other pig tissues and cells hitherto reported. However, a 70-kDa calpain can also be detected when pig PMN cells are disrupted by the nitrogen cavitation method under rather mild conditions, i.e., with minimal destruction of the lysosomes. Lines of evidence are presented showing that the 70-kDa species is devoid of the light subunit, that it is artificially derived from naturally occurring heterodimeric calpain I, and that the PMN cells before disruption contained no such monomeric form. The isolated 70-kDa calpain I, or monomeric artifact, requires only 1 μM Ca^{2+} for half-maximal activation, and it is less pH stable and much less heat stable than the parent heterodimeric calpain I. A possible mechanism for the production of this artifact is discussed.

Ca^{2+}-dependent cysteine endopeptidases (calpain, EC 3.4.22.17) and their specific endogenous inhibitor proteins (calpastatin) are known to be widely distributed in animal tissues (Murachi et al., 1981a; Murachi, 1983a,b). Two different types of calpain are known to exist, namely, calpains I and II, which show a low (micromolar) and high (millimolar) Ca^{2+} requirement for activation, respectively (Murachi et al., 1981b).

Calpains have been purified from various tissues of different animals [for reviews see Murachi (1983a,b)]. For some time previously, it was not clear whether calpain was a monomeric proteinase of approximately M_r 80 000 or a heterodimer of approximately M_r 110 000. The heterodimeric nature of an intact calpain molecule, either calpain I or II, was later established by chemical cross-linking experiments (Hatanaka et al., 1985), by the isolation of structurally related cDNA clones for the light (30 kDa)[1] and heavy (80 kDa) subunits (Sakihama et al., 1985; Ohno et al., 1984), and by the demonstration of the proteolytic digestibility of the light subunit during purification procedures (Mellgren et al., 1982). The extensive degradation of the light subunit during autolytic activation of a heterodimeric calpain has attracted much attention in view of the mechanism underlying the in vivo function of calpains (Inomata et al., 1985; Imajoh et al., 1986; DeMartino et al., 1986).

A monomeric calpain of 85 kDa was isolated from human PMN cells (Pontremoli et al., 1985; Pontremoli & Melloni, 1986). The authors reported that this 85-kDa monomer was the only molecular species of calpain they found in human PMN cells. This does not seem to be in accord with the general concepts hitherto accepted, i.e., that the intact form of calpain must be a heterodimer and that almost all animal tissues, with the rare exception of mammalian erythrocytes, contain two different genes for calpains I and II, respectively, both of which are also actually being expressed (Suzuki, 1987).

The present paper reports the existence of both calpains I and II of pig PMN cells in their respective heterodimeric forms

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[1] Abbreviations: PMN, polymorphonuclear; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate; psi, pounds per square inch; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPLC, high-performance liquid chromatography; kDa, kilodalton; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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and the isolation and characterization of these intact molecules as well as a smaller monomeric species of calpain I, which must be an artifact derivative formed in the initial phase of the purification procedures, i.e., during cell disruption.

Materials and Methods

Nomenclature. In the present paper, PMN calpain ID refers to intact, heterodimeric (83 plus 29 kDa) calpain I, IID to intact, heterodimeric (80 plus 29 kDa) calpain II, and IM to the derived, monomeric (70 kDa) calpain I. There was no evidence of the occurrence of calpain IIM or a derived, monomeric calpain II under the present experimental conditions.

Materials. Casein (Hammarsten grade), monoiodoacetic acid, and calcium chloride were obtained from E. Merck, Darmstadt, Germany; DEAE-cellulose (DE 52) was obtained from Whatman, Kent, U.K., and Ultrogel AcA 34 from LKB, Bromma, Sweden. Blue Sepharose CL-6B, an electrophoresis calibration kit containing low molecular weight proteins, and Ficoll-Paque were products of Pharmacia, Uppsala, Sweden. DEAE-Bio-Gel A was from Bio-Rad, Richmond, CA. The nitrocellulose membranes were from Schleicher & Schüll, Dassel, Germany, and peroxidase-conjugated affinity-purified goat anti-rabbit IgG was from Cappel, Cochranville, PA. All other reagents were of analytical grade and were products of Wako Pure Chemicals, Osaka, or Nakarai Chemicals, Kyoto, Japan. Distilled water was further purified with a Milli Q water purification system (Millipore Corp., Bedford, MA). The buffers used were as follows: buffer A, 20 mM Tris-HCl containing 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, pH 7.5; buffer B, buffer A supplemented with 50 mM NaCl.

Affinity-Purified Anti-Calpain IgG. Antisera were raised in rabbits, respectively, against the heavy subunits of pig calpains I and II and the light subunit of pig calpain. Affinity-purified, polyclonal antibody proteins were obtained by essentially the same methods as those described previously (Hatanaka et al., 1984; Yoshimura et al., 1984). The discriminative specificities of anti-calpain I heavy subunit IgG and anti-calpain II heavy subunit IgG had previously been established (Hatanaka et al., 1984; Yoshimura et al., 1984). The anti-calpain light subunit IgG prepared was found to be monospecific for the light subunit of both calpains I and II, showing no cross-reactivity with their heavy subunits. The light subunits of calpains I and II are known to be identical (Murachi, 1983a).

Isolation of PMN Cells. Landrace pigs, 6-month-old F_1 animals castrated 3 weeks after birth, were examined as to their health by means of both urinary tests using N-Multistix III reagent strips (Miles-Sankyo, Tokyo, Japan) and routine screening tests for serum enzymes and hematology. Blood was only collected from healthy animals. Fresh blood (5 L) was collected at a slaughterhouse and immediately mixed with EGTA (final concentration, 2.5 mM). PMN cells were isolated by Ficoll-Paque density centrifugation, followed by dextran sedimentation (Boyum, 1968). The PMN cells were washed twice in Hank's solution containing 2.5 mM EGTA in place of 2.5 mM $CaCl_2$ (hereafter referred to as the disruption buffer). They were finally suspended at 1×10^7 cells/mL in the disruption buffer. The average cell populations were as follows: PMN cells, 95.5%; eosinophils, 3.5%; monocytes, 0.3%, others, 0.7%.

Disruption of PMN Cells. To obtain cytosol preparations, the cells were disrupted either by pressure treatment according to the nitrogen cavitation method or by homogenization. For nitrogen cavitation, a cell disruption bomb (Parr Instrument Co., Moline, IL) was used at 4 °C under several different sets of conditions as to pressure and duration (Klempner et al., 1980). The degree of destruction of membranes and organelles was examined under a microscope, and the release of acid phosphatase from lysosomes was also monitored (Walloe, 1966). Thus, we chose 335 psi for 23 min as the best conditions and 475 psi for 23 min as more drastic conditions. After the pressure was released, the contents of the bomb were centrifuged at 105000*g* for 90 min at 4 °C to obtain a cell lysate. Homogenization of PMN cells was carried out gently with 4 volumes of buffer A in a Teflon-pestled glass homogenizer for 7 min at 4 °C. The homogenate was centrifuged at 105000*g* for 90 min at 4 °C. The supernatant obtained by either method was dialyzed against buffer B at 4 °C for 3 h and then used for the following experiments.

Immunoelectrophoretic Blot Analysis (Western Blot Analysis). Polyacrylamide slab gel electrophoresis in the presence of 0.1% SDS was performed according to the method of Laemmli (1970) with 12% resolving and 3% stacking gels. The proteins were transferred to nitrocellulose membranes and then analyzed according to the method of Towbin et al. (1979). Protein staining was performed with Amido black, while immunostaining was carried out with the respective antibodies by the method of Hawkes et al. (1982) using *o*-dianisidine as the substrate for peroxidase.

HPLC Analysis. A Shimadzu Model LC-6A HPLC apparatus (Shimadzu Corp., Kyoto, Japan) equipped with a 7.5 × 300 mm column of TSK G-3000SW XL gel (Toyo-Soda, Co., Tokyo, Japan) was used. To approximately 25 mL of each cytosolic preparation obtained from PMN cells was added solid ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation and then dissolved in 0.2 mL of buffer B. All the procedures were carried out at 4 °C. A 20-μL aliquot of each solution was applied to the column, and then HPLC was performed with buffer B at a flow rate of 0.5 mL/min and room temperature. The effluent from the column was collected in fractions every 24 s (approximately 200 μL/tube), and aliquots of each fraction were subjected to immunological dot-blot analysis as described below.

Dot-Blot Analysis Using Anti-Calpain IgG. For each fraction from the HPLC column, a set of three dot-blot tests was conducted using anti-calpain I heavy subunit IgG, anti-calpain II heavy subunit IgG, and anti-calpain light subunit IgG. An Advantec Model DP-96 dot plate apparatus (Advantec Toyo Kaisha, Tokyo, Japan) was employed. One plate had 96 wells; into each well was placed a 65-μL aliquot of a fraction. Then the contents of the wells were transferred to a nitrocellulose membrane (7.5 × 11 cm) by applying a negative pressure across the membrane according to the method described in the manufacturer's manual. Immunostaining of the membrane with anti-calpain IgGs was conducted in the same way as for Western blot analysis (see above). Reflectometric quantification of the staining was performed with a Joyce-Loebl Model Chromoscan-3 densitometer (Joyce-Loebl, Gateshead, U.K.), which permitted automatic scanning at a wavelength of 530 nm and at a speed of 30 mm/s with an aperture of 0.3 × 5 mm. The pen-recorded data were further computer-processed so as to obtain the integral intensity of the staining of a given dot. For such processing, the dots on the membrane that exhibited no visible staining were used as blanks.

Purification of Calpains from PMN Cells. Purification of calpains from PMN cells was carried out by the method of Kitahara et al. (1984), which included chromatographies on DEAE-cellulose, Ultrogel AcA 34, Blue Sepharose CL-6B, and DEAE-Bio-Gel A. In some instances, calpain IM was

found to be eluted at a position slightly different from that for calpain ID, but the difference was not large enough to necessitate the use of any specific new methods. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Assaying of Calpain Activity. Calpain activity was determined with casein as the substrate. Each incubation mixture, in a final volume of 1.0 mL, contained 0.4% casein, 100 mM imidazole hydrochloride buffer, pH 7.5, 5 mM cysteine, and 0.1 mM $CaCl_2$ for calpain I (M or D) or 5.0 mM $CaCl_2$ for calpain II. After incubation for 30 min at 30 °C, the reaction was terminated by adding 1 mL of 5% trichloroacetic acid. Acid-soluble products were determined colorimetrically by the method of Ross and Schatz (1973), for which 0.4 mL of the filtrate was diluted with the reagents to a final volume of 2.8 mL, and then the absorbance at 750 nm was read against a blank. The mixture after reaction without $CaCl_2$ was used as the blank. One unit of calpain was defined as the amount of enzyme that caused an increase in absorbance at 750 nm of 1.0 under the above conditions. For determining the Ca^{2+} requirements of different calpain species, Ca^{2+}-EGTA buffers were prepared as described previously (Yoshimura et al., 1983). An association constant of 5.05×10^{-6} M was used for calculation of ionized calcium concentrations (Harafuji & Ogawa, 1980).

Amino Acid Composition. Reduced and carboxymethylated calpains IM, ID, and IID (approximately 10 μg each) were added to 0.2 mL of constant-boiling HCl, sealed under reduced pressure, and then hydrolyzed at 110 °C for 24 h. Amino acid analysis was carried out with a TSK Model CCP 8000 amino acid analysis system (Toyo-Soda). Separate determinations were not carried out for half-cystine or tryptophan.

Results

Immunological Identification of Heterodimeric and/or Monomeric Calpain Molecules in PMN Cells. Typical results of Western blot analysis are shown in Figure 1. When the cell lysate was obtained by nitrogen cavitation at 335 psi (Figure 1A), anti-calpain I heavy subunit antibody stained one single band of 70 kDa, while anti-calpain II heavy subunit antibody stained one single band of 80 kDa. Anti-calpain light subunit antibody stained a band of 29 kDa. With the supernatant of the homogenate (Figure 1B), the calpain I heavy subunit band appeared at a position corresponding to 83 kDa, while other bands did not change in position, compared with disruption by nitrogen cavitation at 335 psi. The lysate obtained on nitrogen cavitation at 475 psi (Figure 1C) showed a somewhat intermediate picture: two bands, 83 and 70 kDa, appeared for calpain I. These results indicate that the original form of the heavy subunit of calpain I in PMN cells must have been of 83 kDa and that it can undergo breakdown to varying degrees into a 70-kDa protein depending upon the conditions used for disruption of the cells. Such breakdown does not apparently occur in the heavy subunit of calpain II. Since the light subunits of calpains I and II are identical (Murachi, 1983a), the results shown in Figure 1 do not indicate whether or not disappearance of the light subunit of calpain I has taken place concomitantly with the breakdown of its heavy subunit.

Chromatographic Characterization of PMN Cell Calpains. The changes in subunit structures that occurred with the disruption of PMN cells were further characterized by chromatographic analysis. Figure 2 is a photograph showing the results of dot-blot analysis of consecutive series of fractions eluted from the HPLC column. The positions of the strongly stained dots are the elution positions of the respective antigens from the HPLC column. More quantitative data are shown in Figure 3. The appearance of the heavy and light subunits at an identical elution position means that they constitute a heterodimer, while the peak lacking the light subunit represents a monomeric form. Thus, nitrogen cavitation at 335 psi yielded one heterodimer with calpain II antigenicity at position 12 and one monomer with calpain I antigenicity at position 15 (Figure 3A). The homogenate gave heterodimers with calpain I and II antigenicity at positions 11 and 12, respectively (Figure 3B). The lysate obtained on nitrogen cavitation at 475 psi yielded three peaks at positions 11, 12, and 15, which correspond, respectively, to heterodimeric calpain I, heterodimeric calpain II, and monomeric calpain I (Figure 3C). It should be noted that the peak corresponding to the calpain light subunit at position 11 is significantly higher for the homogenate than for the 475-psi lysate, whereas that at position 12 shows almost no difference, suggesting the partial disappearance of the light subunit in the 475-psi lysate. It is therefore concluded that the nitrogen cavitation yielded monomeric calpain I, which was no longer accompanied by the light subunit.

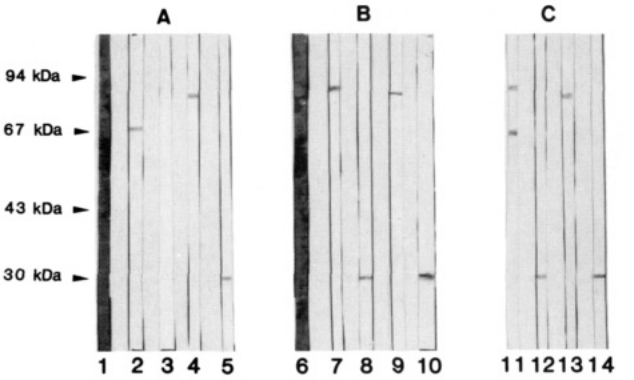

FIGURE 1: Immunoelectrophoretic blot analysis of PMN cell cytosol preparations. (Lanes 1, 2, 4, and 5) Lysate obtained on nitrogen cavitation at 335 psi; (lane 3) washings with Hank's solution of PMN cells before disruption; (lanes 6–10) homogenate; (lanes 11–14) lysate obtained on nitrogen cavitation at 475 psi. Protein staining was performed with Amido black for lanes 1 and 6. The antibodies used for immunostaining were anti-calpain I heavy subunit IgG for lanes 2, 7, and 11; anti-calpain II heavy subunit IgG for lanes 4, 9, and 13; and anti-calpain light subunit IgG for lanes 3, 5, 8, 10, 12, and 14. The amounts of the proteins applied to each lane were 1 μg for lanes 1–4, 6–9, and 11–13; and 2 μg for lanes 5, 10, and 14. Note that PMN cell washings (lane 3) contained no light subunit protein and that the staining intensities of the light subunit proteins increased with increasing amounts of the sample proteins applied (lanes 8 vs 10 and 12 vs 14), suggesting that the blot analysis was conducted in a good sensitivity range. The migration positions of marker proteins are indicated by arrows: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

Purification of Calpains from PMN Cells Disrupted under Different Conditions. The monomeric calpain I was purified from the PMN cell lysate obtained on nitrogen cavitation at 335 psi. From the same lysate, calpain II was also purified. As a control, calpains I and II were purified by using the same chromatographic method from the homogenate of PMN cells. The final products were subjected to Western blot analysis. As shown in Figure 4A, calpain I isolated from the nitrogen cavitation lysate gave only one protein band of 70 kDa, whereas calpain I isolated from the homogenate gave two bands of 83 and 29 kDa. Calpain II isolated from the nitrogen cavitation lysate gave two bands of 80 and 29 kDa. Although not shown, calpain II isolated from the homogenate gave the same bands as those observed for calpain II. Parts B–D of Figure 4 show the immunological identity of each of the protein bands that were visualized on protein staining (Figure 4A). From these results, it was concluded that the calpain

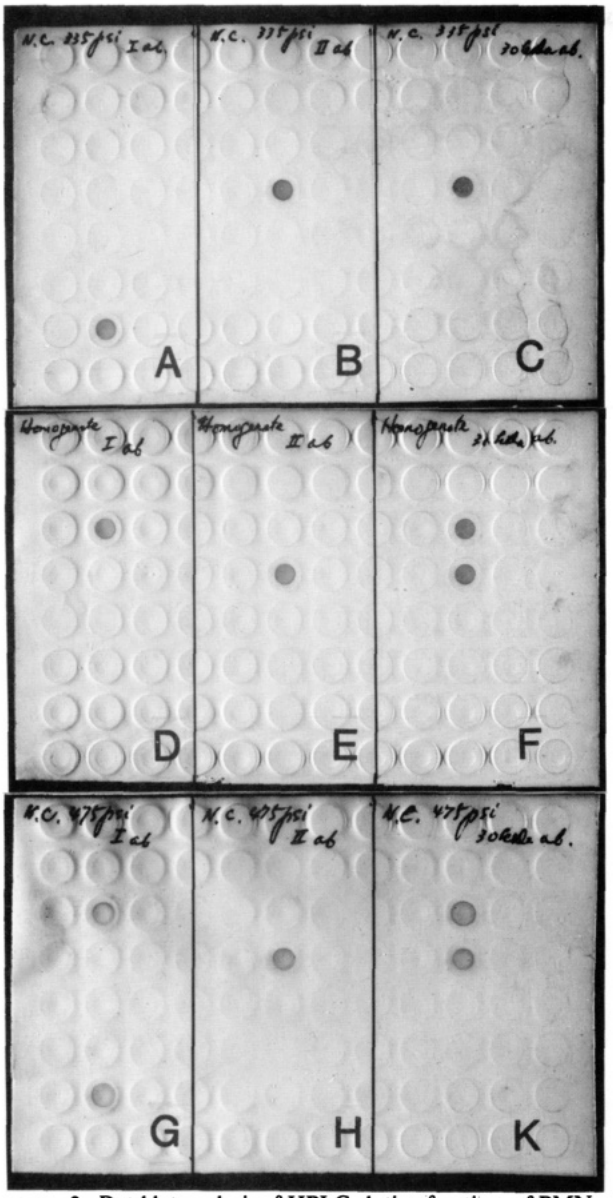

FIGURE 2: Dot-blot analysis of HPLC elution fractions of PMN cell cytosol preparations using anti-calpain antibodies. The nine panels (A–H, K) represent nine individual experiments conducted in parallel. A total of 24 elution fractions collected at 24-s intervals was analyzed for each of the three different cytosol preparations and with each of the three different antibodies. Panels A–C are for the lysate obtained on nitrogen cavitation at 335 psi, panels D–F for the homogenate, and panels G, H, and K for the lysate obtained on nitrogen cavitation at 475 psi. Immunostaining was carried out with anti-calpain I heavy subunit IgG in panels A, D, and G; anti-calpain II heavy subunit IgG in panels B, E, and H; and anti-calpain light subunit IgG in panels C, F, and K. Aliquots (65 μL each) of the fractions eluted from the HPLC column were placed into the wells of the Advantec dot plate apparatus, transferred to a nitrocellulose membrane, and then immunostained. In each panel, the top left well is for fraction 1, which emerged from the HPLC column immediately after initiation of a chromatographic run. Fraction numbers, or position numbers for the dots, increase from the top to the bottom and from the left to the right in each panel. For example, three 65-μL aliquots of elution fraction number 9 for the 335-psi lysate were placed, respectively, at the top and second from the left in each or panels A–C.

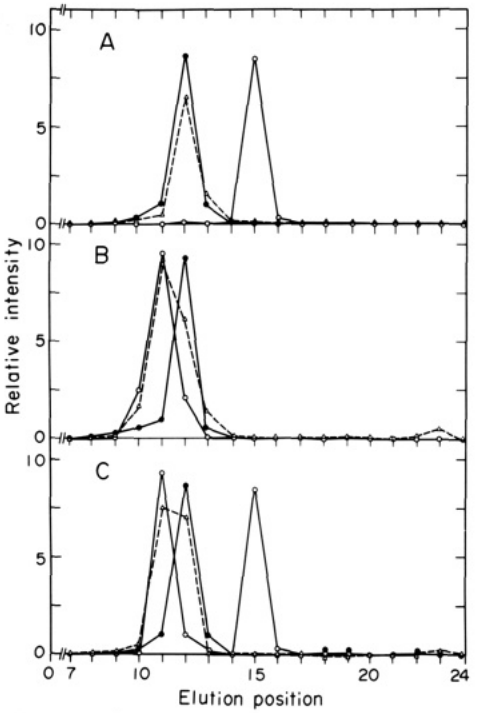

FIGURE 3: Location of calpain antigenicities associated with the HPLC elution fractions of PMN cell cytosol preparations. The graphs represent reflectometric determinations made on anti-calpain-immunostained dots, shown in Figure 2. (Panel A) Lysate obtained on nitrogen cavitation at 335 psi; (panel B) homogenate; (panel C) lysate obtained on nitrogen cavitation at 475 psi. Three different antibodies were used: (○) anti-calpain I heavy subunit IgG; (●) anti-calpain II heavy subunit IgG; (△) anti-calpain light subunit IgG. The elution positions (abscissa) correspond to the respective HPLC elution fractions collected every 24 s. The integral intensities of the immunostaining of the dots on a nitrocellulose membrane (ordinate) are expressed in arbitrary units.

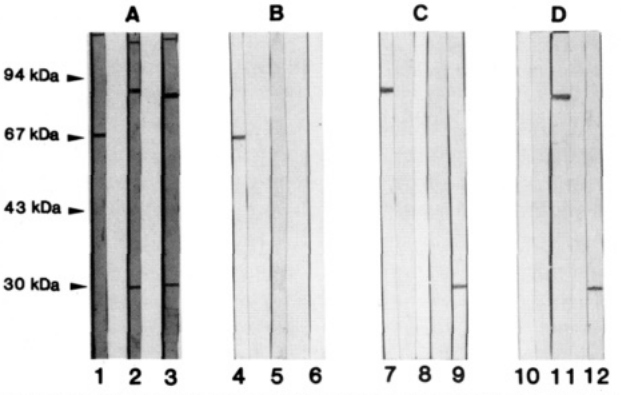

FIGURE 4: Purities and antigenicities of isolated PMN calpains IM, ID, and IID. PMN calpains were purified according to the protocols in Table I. To each lane was applied 1 μg of purified protein: (lanes 1 and 4–6) IM; (lanes 2 and 7–9) ID; (lanes 3 and 10–12) IID. After SDS–polyacrylamide gel electrophoresis and transblotting of the migrated proteins onto a nitrocellulose membrane, the membrane was stained with Amido black for lanes 1–3 and with anti-calpain antibodies for lanes 4–12. The antibodies used were anti-calpain I heavy subunit IgG for lanes 4, 7, and 10; anti-calpain II heavy subunit IgG for lanes 5, 8, and 11; and anti-calpain light subunit IgG for lanes 6, 9, and 12. The same marker proteins as those in Figure 1 were used.

I species isolated from the 335-psi lysate was a monomeric 70-kDa protein, designated IM, while the calpain I species isolated from the homogenate was a heterodimeric 83 plus 29 kDa protein, designated ID. The calpain II species isolated from both the nitrogen cavitation lysate and the homogenate was heterodimeric, consisting of 80- and 29-kDa subunits and was called IID. Since there was no appreciable protein staining other than that of the bands mentioned above, the final products were estimated to be of more than 90% purity.

The protocols for the purification of calpains IM and IID from the nitrogen cavitation lysate and calpains ID and IID from the homogenate are summarized in Table I. The observed purification levels (748–20 200-fold) were not very different from those reported earlier for calpains from pig

Table I: Purification of PMN Calpains IM, ID, and IID from Pig PMN Cells

method for cell disruption	molecular species of calpain	purification step[a]	total protein (mg)	total activity (units)	specific activity (units/mg of protein)	purification (x-fold)
nitrogen cavitation at 335 psi	IM	1	2280	(22.6)[b]	(0.01)[c]	1
		2	7.3	(22.6)[b]	(3.10)[c]	310
		3	0.44	22.6	51.4	5140
		4	0.14	20.2	144	14400
		5	0.084	17.0	202	20200
	IID	1	2280	119	0.05	1
		2	35	134	3.83	77
		3	2.08	101	48.6	972
		4	0.52	80.8	155	3100
		5	0.28	70.8	253	5060
homogenization	ID	1	2470	(777)[b]	(0.31)[c]	1
		2	87.6	(777)[b]	(8.96)[c]	29
		3	7.50	777	104	335
		4	1.46	278	190	613
		5	0.34	78.9	232	748
	IID	1	2470	149	0.06	1
		2	39.6	149	3.76	63
		3	2.42	120	49.6	827
		4	0.61	95.5	157	2617
		5	0.30	83.8	279	4650

[a] Steps: 1, crude extract (supernatant); 2, DE-52 chromatography; 3, Ultrogel AcA 34 chromatography; 4, Blue Sepharose CL-6B chromatography; 5, DEAE-Bio-Gel A chromatography. [b] The total activity was assumed to be equal to the value determined after Ultrogel AcA 34 chromatography since the total activities of these fractions could not be directly determined due to the presence of calpastatin. [c] The value was obtained from the deduced total activity.

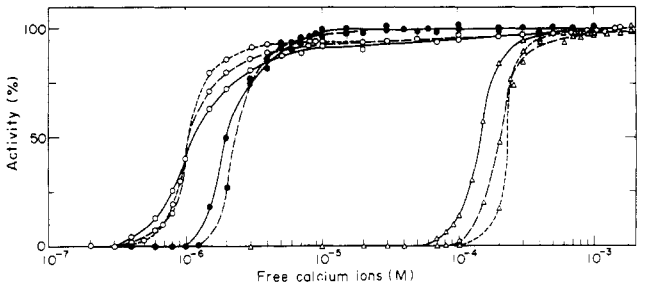

FIGURE 5: Ca^{2+} requirements of PMN calpains IM, ID, and IID. The numbers of calpain samples prepared on different dates were three for calpain IM (○), two for ID (●), and three for IID (△).

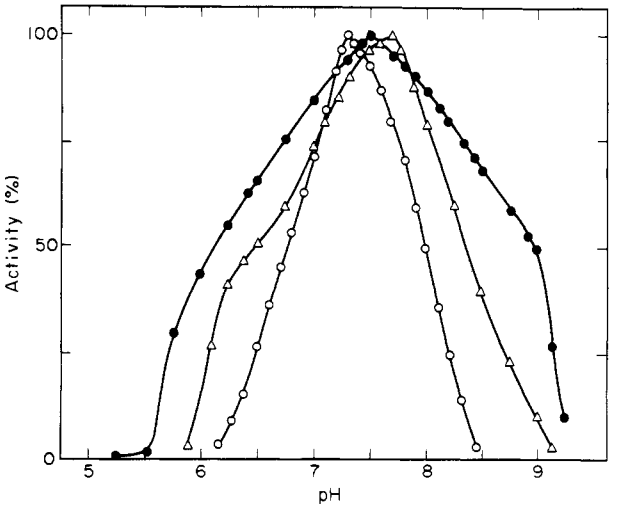

FIGURE 6: Effect of pH on the activities of PMN calpains IM, ID, and IID. The incubation mixture contained 3 μg of purified calpain IM (○), 6 μg of ID (●), or 6 g of IID (△). Acetate and Tris–acetate buffers (0.1 M), adjusted to the given pHs, were used.

erythrocytes and kidney (Kitahara et al., 1984). The specific caseinolytic activities of the final products (202–279 units/mg of protein) were also in a range comparable with those reported for pig calpains I and II (Kitahara et al., 1984). It should be noted here that the specific activities of calpains IM and ID were not very different, but they were always lower than that of calpain IID. Such a difference between calpains I and II is a well-established fact (Yoshimura et al., 1983; Kitahara et al., 1984). Table I also shows that the yield of calpain IM from the nitrogen cavitation lysate was significantly lower than that of calpain ID from the homogenate.

Since the calpain IID species purified from two different sources were shown to be indistinguishable, only calpain IID isolated from the homogenate was used in the following experiments.

Ca^{2+} Requirements. The Ca^{2+} requirements of calpains IM, ID, and IID were compared. As shown in Figure 5, calpain IM was activated at a lower Ca^{2+} concentration than that required by calpain ID or IID; it was half-maximally activated at approximately 1 μM free Ca^{2+}. Calpain ID was half-maximally activated at approximately 2 μM and fully activated at 10 μM free Ca^{2+}. Calpain IID was half-maximally activated at 200 μM and maximally activated at 1 mM free Ca^{2+}. With calpain IM, 90% activation was attained at 8 μM Ca^{2+}, and a further increase in Ca^{2+} concentration caused a gradual increase in activity, which continued up to 0.8 mM Ca^{2+}. Such a gradual increase was not found for calpains ID and IID, as had previously been reported (Kitahara et al., 1984).

pH Dependence. The caseinolytic activities of calpains IM, ID, and IID were determined under standard assay conditions but at various pHs, as shown in Figure 6. For each enzyme, the highest activity attained was taken as 100%. Similar pH optima were found: pH 7.3 for calpain IM: pH 7.5 for calpain ID; and pH 7.7 for calpain IID. These values are in good agreement with those reported earlier for rat calpains (Yoshimura et al., 1983). However, the pH profile of calpain IM was significantly narrower than that of calpains ID or IID.

Heat Stability. Solutions of the purified calpains IM, ID, and IID were preincubated at given temperatures in the absence of Ca^{2+} for 10 min, and then the remaining activity was determined at pH 7.5 by the standard procedure. The preincubation was carried out at pH 7.3, 7.5, or 7.7. The results obtained are shown in Figure 7. As had already been reported (Yoshimura et al., 1983), calpain IID was found to be more labile than calpain ID. However, calpain IM was even more labile. At pH 7.5, 50% loss of activity occurred at 35

Table II: Amino Acid Compositions of Various Preparations of Pig Calpains[a]

	mol % (residues/100 residues)					
	PMN cell calpain IM					
amino acid	prepn 1	prepn 2	PMN cell calpain ID	PMN cell calpain IID	erythrocyte calpain I[b]	kidney calpain II[b]
aspartic acid	11.5	11.9	11.7	11.9	11.4	11.5
threonine	4.9	5.1	5.0	4.9	5.4	5.0
serine	6.2	5.9	5.8	5.8	7.3	6.5
glutamic acid	15.1	15.2	14.7	13.6	11.4	12.9
proline	3.9	3.8	3.1	3.3	3.9	3.0
glycine	8.7	8.3	9.2	10.5	11.0	10.3
alanine	7.7	7.7	7.3	7.3	6.6	6.8
valine	4.6	4.7	5.3	5.0	6.1	5.5
methionine	1.1	0.6	1.8	2.1	1.7	1.9
isoleucine	5.4	5.6	5.3	5.5	3.7	5.4
leucine	9.9	10.3	10.0	9.2	10.3	9.6
tyrosine	2.3	1.4	2.5	2.3	2.5	3.4
phenylalanine	5.1	5.7	4.6	4.9	5.9	5.5
lysine	6.2	6.0	6.1	5.7	5.1	5.7
histidine	1.6	1.6	2.3	2.4	1.4	1.6
arginine	5.8	6.2	5.3	5.6	6.3	5.6

[a] The contents of half-cystine and tryptophan were omitted for the calculation of mole percent. [b] The individual compositions of the respective heavy and light subunits of calpains I and II were taken from Yumoto et al. (1984), and they were combined with due correction to obtain mole percent data for the heterodimer as a whole.

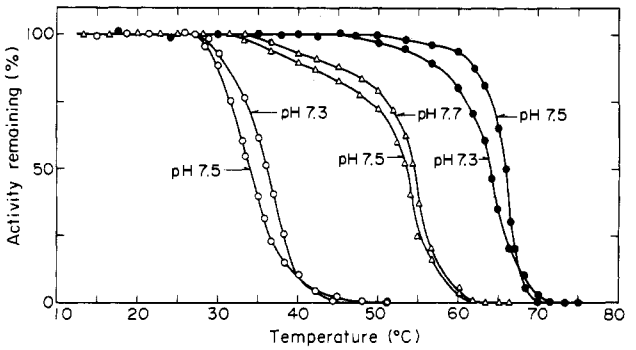

FIGURE 7: Effect of heat treatment on the activities of PMN calpains IM, ID, and IID. Four micrograms of calpain IM (○), ID (●), or IID (△) in 0.5 mL of buffer A, adjusted to pH 7.3, 7.5, or 7.7, was preincubated in the absence of calcium ions for 10 min at the indicated temperatures. The enzyme solution was immediately chilled in an ice bath. The remaining activity was then assayed under standard conditions.

°C for calpain IM, at 54 °C for calpain IID, and at 67 °C for calpain ID. That calpain IM showed the lowest heat stability seems to be compatible with the fact that its activity was found to be the most rapidly lost on either side of the optimal pH when the three calpain species were compared (Figure 6).

Amino Acid Composition. Table II summarizes the results of amino acid analysis of two different preparations of PMN calpain IM and one preparation each of calpains ID and IID. Since only one hydrolysis time (24 h) was used and the values for half-cystine and tryptophan were omitted from the mole percent calculation, the values shown in the table should be regarded as rough ones for comparison among the three calpain species isolated. The table also includes the reported compositions of calpains I and II, both intact heterodimers, from pig tissues other than PMN cells. It can be seen that PMN calpains ID and IID correspond well to authentic pig calpains I and II, respectively. PMN calpain IM shows unique features; it contains significantly more glutamic acid and less glycine and methionine compared with PMN calpain ID or pig calpain I. These differences are consistent with the present finding that calpain ID is devoid of the light subunit, because the light subunit contains more glycine and methionine and less glutamic acid, as mole percent, than the calpain I heavy subunit does (Yumoto et al., 1984).

Discussion

In the initial phase of the present study, we concentrated on determining how to prevent possible degradation of calpain molecules during the course of their isolation from PMN cells. Special care was taken to avoid the involvement of lysosomal cathepsins, since PMN cells are known to contain well-developed lysosomes. The disruption of cells by means of nitrogen cavitation has been recommended as the method of choice when the destruction of intracellular organelles has to be minimized (Klempner et al., 1980). The optimal conditions for nitrogen cavitation, under which human PMN cells can be disrupted with minimal destruction of the lysosomes, had been previously established (Klempner et al., 1980), but we had to determine whether or not such conditions were applicable to pig PMN cells. Thus, we found that application of a pressure of 335 psi for 23 min at 4 °C gave the most satisfactory results: when carefully separated, fresh pig PMN cells were used, the release of lysosomal acid phosphatase into the lysate was less than 4.0 King-Armstrong units/L. We then proceeded to purify calpains from the lysate obtained on nitrogen cavitation at 335 psi, expecting to obtain calpain molecules as intact as possible. We could obtain two calpain species from the DEAE-cellulose column eluate, one eluting at 0.1 M NaCl and one at 0.25 M NaCl (Table I, upper half), which we first thought corresponded to calpains I and II, respectively (Murachi, 1983a). However, when these two species were subjected to electrophoretic analysis, we found that the "calpain I" species comprised only a 70-kDa protein which immunologically cross-reacted with only the anti-calpain I heavy subunit antibody, i.e., with neither the anti-calpain II heavy subunit antibody nor the anti-calpain light subunit antibody (Figure 4). The "calpain II" species isolated behaved normally, showing 80- and 29-kDa protein bands, each of which was immunologically identified properly (Figure 4). The occurrence of a "70-kDa calpain I" in the original nitrogen cavitation lysate was confirmed (Figures 1–3), which suggested two possibilities, either (1) PMN cells in vivo contained 70-kDa calpain I, instead of the usually encountered 83 plus 29 kDa molecular species, or (2) the 70-kDa species was artificially produced during the cell disruption procedure.

When pig PMN cells were disrupted by conventional homogenization and the supernatant obtained was subjected to electrophoretic as well as chromatographic analysis, it was

found that the cytosol contained both the 83-kDa calpain I heavy subunit and the 80-kDa calpain II heavy subunit, in addition to the common 29-kDa light subunit (Figures 1–3). Starting from the same cytosol preparation, both heterodimeric calpains I and II, of ordinary molecular sizes, could actually be isolated with reasonably high specific activities and in good yields (Table I, lower half, and Figure 4). The Ca^{2+} requirements for activation of these calpains I and II (Figure 5) were in perfect agreement with those reported for authentic calpain I from pig erythrocytes and calpain II from pig kidney (Kitahara et al., 1984). These results clearly indicate that the intact form of calpain I in PMN cells should have a subunit composition of 83 plus 29 kDa, as a heterodimer of the same composition has been found in many other tissues and cells of pigs (Kitahara et al., 1984; Yoshimura et al., 1984; Hatanaka et al., 1984; Ando et al., 1988). We thus concluded that the 70-kDa monomeric calpain I, calpain IM, found and isolated on nitrogen cavitation at 335 psi, is an artifact formed from heterodimeric calpain I, calpain ID, during the course of the cell disruption. The low yield of calpain IM compared with those of calpains ID and IID (Table I) seems to be consistent with the artifact nature of IM. Calpain II, on the other hand, was found to always exist as its heterodimeric form, IID, whose subunit composition, 80 plus 29 kDa, is identical with that of calpain II in other pig tissues (Kitahara et al., 1984; Yoshimura et al., 1984; Ando et al., 1988). The known existence of only two different heavy subunit genes, one for calpain I and the other for calpain II, in a few mammals (Suzuki, 1987) is also compatible with the present finding for PMN cells that 70-kDa calpain IM is an artifact, and so the expression of an additional kind of gene in these particular cells can be ruled out.

Pontremoli and Melloni (1986) claimed that human PMN cells contain only the "mM form" of calpain, which is a monomer of 85 kDa. Despite the difference in animal species used by these authors and us, our results, described above, strongly indicate that their 85-kDa monomeric calpain must be an artifact, as our 70-kDa species is. These authors (Pontremoli et al., 1985) lysed PMN cells through three cycles of freezing and thawing, which could well have caused the breakdown of the naturally occurring, heterodimeric calpain molecule into a monomeric form, as the nitrogen cavitation method did in our case. However, human monomeric 85-kDa calpain was reported to require 0.05–0.06 mM Ca^{2+} for half-maximal activation, which greatly differs from our finding that pig calpain IM is 50% activated at approximately 1 μM Ca^{2+} (Figure 5). It is most likely that the freezing and thawing treatment of the human PMN cells caused preferential cleavage of calpain II heterodimers to yield 85-kDa monomers, while nitrogen cavitation at 335 psi caused predominant breakdown of calpain ID into calpain IM, reflecting the differences in the cells and/or in the methods employed.

The question then arises as to the mechanism by which calpain ID is broken down into calpain IM. The breakdown should be the result of a partial loss of the heavy subunit and the complete loss of the light subunit during the course of the cell disruption. Three possibilities for the underlying mechanism may be considered: (1) physical destruction; (2) autolysis; and (3) nonautolytic proteolysis. The physical destruction of the subunit proteins due to an abrubt change in pressure with the nitrogen cavitation procedure is unlikely, because the application of 475 psi, which is much higher than 335 psi, left the 83-kDa calpain I protein partially intact. Thus, after exposure to 475-psi pressure, the lysate gave both 70- and 83-kDa bands (Figure 1). The autolysis of calpains is a well-known phenomenon, which usually causes limited cleavage of N-terminal portions of both the heavy and light subunits, which in turn significantly lowers the Ca^{2+} requirement of the enzyme (Suzuki, 1987). Calpain IM showed a decreased Ca^{2+} requirement compared with that of the parent calpain ID (Figure 5), but it differs from the reported autolysis products in having completely lost the light subunit protein (Figures 2 and 3). Also, while autolysis can only occur when Ca^{2+} is added at significant concentrations to the medium, PMN cells in the present study were always kept in a medium containing 2.5 mM EGTA, so that the risk of autolysis should have been minimal, if not nil.

The possibility thus remaining is nonautolytic proteolysis. The involvement of lysosomal proteases can be disregarded, because the production of IM occurred with the nitrogen cavitation method, which guarantees the integrity of lysosomes, whereas ID did not undergo proteolysis even when it was made readily accessible to lysosomal proteases during the course of homogenization (Table I and Figures 1–3). It is therefore most likely that nitrogen cavitation at 335 psi rendered some extra lysosomal protease or proteases, other than calpain itself, attacking calpain ID to break it down to calpain IM. Candidates for such proteases are several known enzymes such as high molecular weight protease (or ingensin) (Tanaka et al., 1986; Ishiura et al., 1985; Dahlmann et al., 1985), chymase and tryptase (Kido et al., 1985), and certain yet unidentified proteases, bound as well as unbound to the cell membrane. The results of the present study indicate that the known uniformity of intact calpains I and II in various pig tissues can be extended to PMN cells, settling the hitherto somewhat controversial issue as to whether or not PMN cells are exceptional with regard to the calpain molecular species they contain.

The 70-kDa molecular species, calpain IM, is an artifact derived from calpain ID, and it may not have any physiological significance. However, the findings that calpain IM acquires such unique properties as that it requires a lower Ca^{2+} concentration for activation compared with calpain ID (Figure 5) and that it exhibits lower pH stability (Figure 6) and very much lower heat stability (Figure 7) may indicate that this artifact molecule deserves further investigation as to the mechanism of action of calpain, particularly, as to the function of the light subunit. It would be most interesting to determine whether or not calpain IM, which is devoid of the light subunit, has the ability to interact with the cell membrane (Melloni et al., 1985) and with phospholipids (Imajoh et al., 1986).

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Registry No. Ca, 7440-70-2; calpain, 78990-62-2.

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Racemization of Alanine by the Alanine Racemases from *Salmonella typhimurium* and *Bacillus stearothermophilus*: Energetic Reaction Profiles†

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ABSTRACT: Alanine racemases are bacterial pyridoxal 5′-phosphate (PLP) dependent enzymes providing D-alanine as an essential building block for biosynthesis of the peptidoglycan layer of the cell wall. Two isozymic alanine racemases, encoded by the *dadB* gene and the *alr* gene, from the Gram-negative mesophilic *Salmonella typhimurium* and one from the Gram-positive thermophilic *Bacillus stearothermophilus* have been examined for the racemization mechanism. Substrate deuterium isotope effects and solvent deuterium isotope effects have been measured in both L → D and D → L directions for all three enzymes to assess the degree to which abstraction of the α-proton or protonation of substrate PLP carbanion is limiting in catalysis. Additionally, experiments measuring internal return of α-^{3}H from substrate to product and solvent exchange/substrate conversion experiments in ^3H_2O have been used with each enzyme to examine the partitioning of substrate PLP carbanion intermediates and to obtain the relative heights of kinetically significant energy barriers in alanine racemase catalysis.

The alanine racemases are a group of pyridoxal 5′-phosphate (PLP)[1] containing bacterial enzymes that catalyze the racemization of D- and L-alanine. These enzymes are essential in bacteria, as D-alanine is an important component in the biosynthesis of the cell wall and can be obtained only by isomerization of L-alanine. Work in these laboratories has centered on the racemases from the Gram-negative bacteria *Salmonella typhimurium* (Badet et al., 1984; Esaki & Walsh, 1986) and *Pseudomonas striata* (Roise et al., 1984) and the Gram-positive thermophile *Bacillus stearothermophilus* (Badet et

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; DAAO, D-amino acid oxidase; LDH, lactate dehydrogenase; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; LADH, L-alanine dehydrogenase; CD, circular dichroism; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD.

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