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ON THE MECHANISM OF BINDING OF CALPASTATIN, THE PROTEIN INHIBITOR OF CALPAINS, TO BIOLOGIC MEMBRANES

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Bovine myocardial calpastatin, the endogenous inhibitor of the calcium-dependent proteinases, calpains, could bind to sarcoplasmic reticulum preparations at neutral pH and low ionic strength. Even in the presence of 100 to 200 mM KCl, 4 to 5 μg of calpastatin was bound per mg of membrane. Although calpastatin is found associated with bovine myocardial sarcolemma, neither canine nor human erythrocyte calpastatins were present in isolated erythrocyte membrane preparations. The bovine myocardial calpastatin, but not human erythrocyte calpastatin, could associate with purified phospholipid vesicles at low ionic strength. Thus, phospholipids appear to be involved in the binding of calpastatin to membranes.

The calcium-dependent intracellular proteinases, calpains, are present in many different mammalian tissues (1-3). Although the exact physiologic function of these enzymes is not yet established, they may be important in the proteolytic processing of various proteins at cellular membranes in response to elevated calcium concentrations (4,5). A highly specific and potent inhibitory protein (calpastatin) is also ubiquitously distributed, and it is probably an important factor in the in vivo regulation of calcium-dependent proteolysis (1,2). Like the calpains, calpastatin appears to be predominantly cytosolic. However, in previous studies it has been proposed that some bovine myocardial calpastatin is associated in vivo with sarcolemma and sarcoplasmic reticulum (6-8). Turnover of this membrane-associated

The abbreviations used are: MOPS, 4-morpholinepropane sulfonic acid; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetra acetic acid; PI, phosphatidylinositol; PS, phosphatidylserine, R_S, Stokes radius; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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calpastatin may be important in regulating the activity of calpains at membranes, thus it is important to further investigate the nature of the interaction of calpastatins with biologic membranes. Herein, it is shown that association with phospholipids is important in binding of calpastatins to membranes.

MATERIALS AND METHODS

Materials. Gel electrophoresis supplies, nitrocellulose sheets, alkaline phosphatase-conjugated goat anti-mouse IgG, 5-bromo-4-chloro-3-indolyl phosphate, and p-nitro blue tetrazolium chloride were purchased from Bio-Rad. DEAE-Sepharose and Sepharose CL-2B, products of Pharmacia Fine Chemicals, were obtained from Sigma. Affi-Gel Blue was obtained from Bio-Rad. Soybean phosphatidylinositol and bovine brain phosphatidylserine were purchased in chloroform/methanol solution from Sigma. Vesicles were prepared by sonication of the dried lipids in the appropriate aqueous buffer.

Buffers. The experiments and purification procedures were performed in either buffer A: 50 mM imidazole-HCl, 1 mM dithiothreitol, pH 7.4 measured at room temperature; or buffer B: 20 mM MOPS , 1 mM dithiothreitol, pH 7.2 measured at room temperature.

Protein determination. Protein concentrations were determined by the method of Lowry, et al. (9) utilizing bovine serum albumin as the standard.

Calpastatin assay. Calpastatin was assayed by inhibition of purified bovine myocardial calpain 2 in the standard spectrophotometric caseinolytic assay (10). One unit of calpastatin produces 50% inhibition of 0.1 unit of calpain 2.

Membrane-bound calpastatin preparation. All steps were performed at 4°C to 7°C unless otherwise stated. One kilogram of bovine heart tissue was homogenized in 2.5 l of buffer A containing 5 mM EGTA and centrifuged at 12,000 x g for 10 min. The pellet was resuspended in 2.5 l of the same buffer and re-centrifuged. The washed pellet was resuspended in buffer A containing 1.0 M NaCl, which effectively solubilized the particulate calpastatin, and heated to 95°C for 10 min. The preparation was centrifuged at 12,000 x g for 10 min, and trichloroacetic acid was added to the supernatant to a final concentration of 15% w/v. The precipitate was collected by centrifugation, redissolved in a small volume of 0.5 M imidazole base, and dialyzed against buffer A. The sample was applied to a 100 ml column of DEAE-Sepharose, and the gel was washed with 400 ml of buffer A containing 0.09 M NaCl. Calpastatin was then eluted from the gel with buffer A containing 0.18 M NaCl. The DEAE-Sepharose eluate was diluted with one-half volume of water and applied to a 50 ml column of Affi-Gel Blue. This column was washed with 200 ml of buffer A containing 0.12 M NaCl, and the calpastatin was eluted in near-homogeneous form with buffer A containing 0.5 M NaCl (figure 1). About 1 mg of purified calpastatin was obtained per kg of bovine heart.

Human erythrocyte calpastatin preparation. Human blood was obtained from the Medical College of Ohio Blood Bank. The erythrocytes from 150 ml of blood were collected by centrifugation at 1000 x g and washed 3 times with 50 mM imidazole-HCl, 150 mM NaCl, pH 7.4. The erythrocytes were then lysed by resuspending in 5 mM EDTA, 7 mM β-mercaptoethanol, pH 7.4. The lysed cells were centrifuged for 20 min at 12,000 x g, and the hemolyzate was applied to a 100 ml column of DEAE-Sepharose and chromatographed as described above for purification of the bovine membrane-bound calpastatin. The DEAE-eluate was heated to 60°C for 10 min, and the precipitated protein removed by centrifugation. The supernatant was subjected to Affi-Gel Blue chromatography as described above for the bovine calpastatin. However, the erythrocyte calpastatin did not bind to this gel, and all of the applied activity was

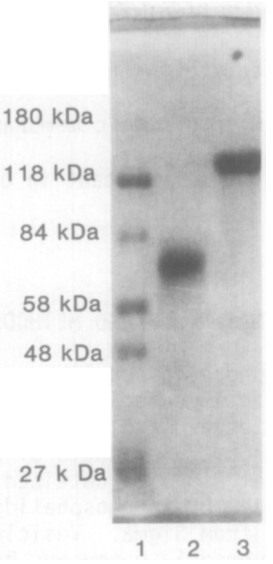


Figure 1. SDS-PAGE of purified human erythrocyte and bovine myocardial calpastatins. Two microgram samples of the purified human erythrocyte (lane 2) or bovine myocardial (lane 3) calpastatins were electrophoresed in an 8% polyacrylamide slab gel, and stained with coomassie blue dye. In lane 1, a mixture of protein standards was applied: α_2-macroglobulin (180 kDa), β-galactosidase (118 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48 kDa), and triose phosphate isomerase (27 kDa).

recovered in the break-through fraction. Highly purified erythrocyte calpastatin was prepared by heating the Affi-Gel Blue break-through fraction to 100°C for 20 min and centrifuging to remove virtually all of the contaminating protein, which was denatured by the heat treatment. The purified human calpastatin displayed the lower molecular weight typically associated with erythrocyte calpastatins (11) (figure 1). The final specific activity was 5,500 units/mg of protein, which is comparable to the specific activity of purified bovine myocardial calpastatin (12), and 1.16 mg of calpastatin was recovered.

Membrane preparations. Erythrocyte ghosts were prepared by resuspending the pellets from the lysis step of the erythrocyte calpastatin preparation in buffer A, and re-centrifuging at 20,000 x g for 20 min. This wash step was repeated 3 times. SR was purified from bovine myocardium as previously described (13), and further washed in buffer A containing 1.0 M NaCl to remove most of the endogenous calpastatin (7). The NaCl-washed SR was stored in buffer B.

Electrophoresis and protein electroblotting. Slab gel SDS-PAGE was performed using the Laemmli buffer system (14). Proteins were stained with coomassie brilliant blue dye. Electroblotting and immunostaining of the nitrocellulose blots were performed as previously described (13). Alkaline phosphatase-conjugated second antibody was utilized, and an indolyl phosphate, tetrazolium blue staining procedure was employed (15). The first antibody was either of two monoclonal antibodies specific for calpastatins, PI-2 or PI-4 (16).

RESULTS

Binding of bovine calpastatin to salt-washed SR vesicles. Bovine calpastatin, purified from a myocardial 12,000 x g fraction as described in

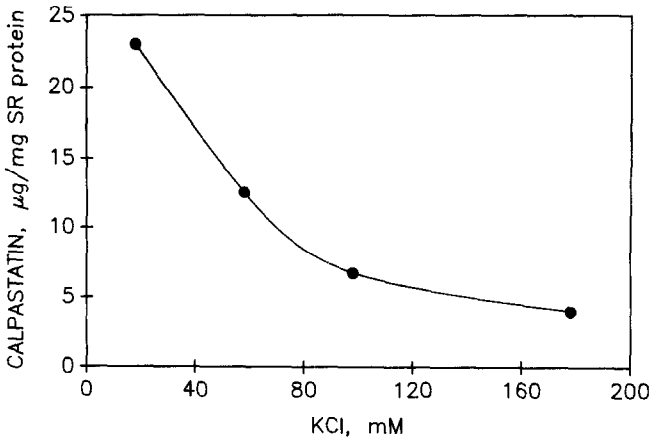
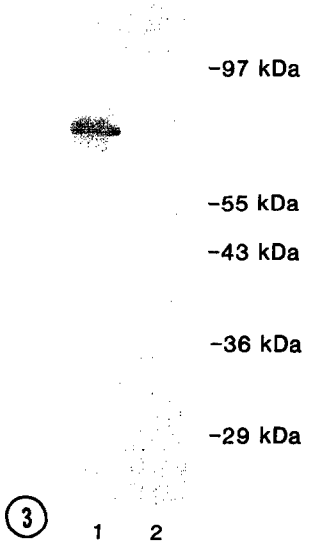


Figure 2. Binding of purified bovine myocardial calpastatin to SR. Calpastatin was incubated at a concentration of 20 μg/ml with SR (200 μg/ml) in 100 μl of buffer B containing varying amounts of KCl. After 30 min at 4°C, the samples were centrifuged at 12,000 x g for 15 min. The pellets were resuspended and 6.7 μg samples were subjected to SDS-PAGE and protein immunoblotting using PI-2 as the first antibody. The immunoblot was scanned using a laser densitometer, and the amount of calpastatin bound was estimated relative to the staining intensity of a known amount of purified calpastatin in the same blot.

the Methods section, bound to salt-washed SR (figure 2). The binding was pH-dependent: approximately equal amounts of calpastatin were bound to the salt-washed SR at pH 6.5 or 7.2, but there was less binding at pH 8.0 (data not shown). The binding was also greatly influenced by ionic strength (figure 2). Under conditions approximating physiologic ionic strength, there was only about 20% as much calpastatin bound to the salt-washed SR as there was at low ionic strength. Even under these conditions about 4 or 5 μg of calpastatin was bound per mg of SR protein (figure 2). This is comparable to or slightly higher than the amount of endogenous calpastatin in purified SR vesicles (7).

Absence of endogenous bound calpastatin on erythrocyte ghosts. Canine erythrocyte ghosts (plasma membrane) were prepared as described in the Methods section, and subjected to protein electroblotting and immunostaining (figure 3). Although there was readily detectable calpastatin immunoreactivity in the hemolyzate (lane 1), there appeared to be no calpastatin in the ghosts (lane 2). Direct assay did not reveal detectable calpastatin activity in the canine erythrocyte ghosts, nor in human erythrocyte ghosts. These results are in marked contrast with our studies of myocardial sarcolemma and SR, in which



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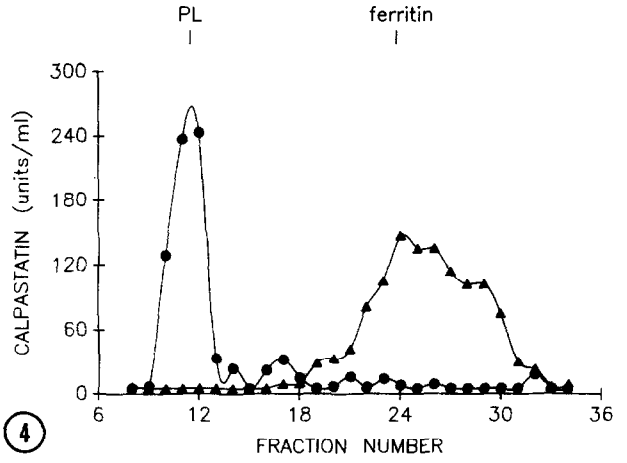

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Figure 3. Protein immunoblotting of canine erythrocyte ghosts and hemolyzate. A sample of canine erythrocyte hemolyzate was heated at 70°C for 10 min, and a 150 μg sample of the supernatant was applied to a 10% polyacrylamide gel, electrophoresed, and immunoblotted using PI-4 as the first antibody (lane 1). In lane 2, a 200 μg sample of canine erythrocyte ghosts, prepared as described in the Methods section, was applied. The pre-stained molecular weight standards used were: phosphorylase b (97 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa) and carbonic anhydrase (29 kDa).

Figure 4. Gel filtration analysis of calpastatin binding to pure phospholipids. A 40 μg sample of purified bovine myocardial calpastatin was incubated with a 160 μg sample of PI:PS, 1:1, vesicles in 200 μl of buffer B for 10 min at room temperature. The sample was then applied to a 0.7 cm x 8.4 cm column of Sepharose CL-2B equilibrated in buffer B. Fractions of 100 μl were collected and assayed for calpastatin activity (circles). In another experiment, a 32 μg sample of purified human erythrocyte calpastatin was substituted for the bovine calpastatin (triangles). The column was calibrated with phospholipid alone (PL), and with ferritin (R_S = 5.9 nm).

membrane-bound calpastatin was easily detected by assay or immunostaining (7,8).

Binding of calpastatin to pure phospholipids. To further investigate the mechanism of calpastatin binding to biologic membranes, bovine myocardial calpastatin was incubated with mixed PI/PS vesicles, and applied to a Sepharose CL-2B column. The bovine myocardial calpastatin eluted with the phospholipids in the void volume (figure 4). Binding could be prevented by including 0.22 M NaCl in the sample incubation mixture and column buffer (data not shown). Calpastatin purified from the cytosolic fraction of bovine heart homogenates (12) also bound to the purified phospholipids (data not shown).

However, purified human erythrocyte calpastatin did not bind to the vesicles under low salt conditions (figure 4).

DISCUSSION

In this study it has been demonstrated that soluble myocardial calpastatin can bind to biologic membranes in low ionic strength buffers. The effects of pH and ionic strength on calpastatin binding to SR suggest that ionic interactions of titratable groups on the protein and membranes are important in this process. This is consistent with the present knowledge of calpastatin structure: it is a hydrophilic protein containing many basic and acidic amino acid residues (6,17).

The experiments with pure phospholipids indicate that one mechanism of binding of bovine myocardial calpastatin to biologic membranes is through association with membrane phospholipids. Erythrocyte calpastatin, which does not appear to bind to the erythrocyte plasma membrane (figure 3), does not bind to pure phospholipids either (figure 4). This latter observation represents, to my knowledge, the first demonstration of a functional difference between the "small" calpastatins (apparent Mr on SDS-PAGE = 60,000 to 70,000) and the "large" calpastatins (apparent Mr = 110,000 to 130,000). The large and small calpastatins appear to share the same primary sequence, except that the small form is missing the amino terminal sequence including one of the four calpain binding sites (18). Therefore, it is likely that binding of calpastatin to membranes requires the presence of an amino terminal region. This finding lends support to the hypothesis that limited proteolysis of bovine or canine myocardial calpastatin results in its release from biologic membranes, leading to potential turnover of membrane proteins upon subsequent stimulation of the proteinases (5,6,8). A selective fragmentation near the amino terminus would result in the release of most of the calpastatin molecule. Indeed, the major calpastatin fragment released from a canine myocardial 1000 x g pellet by incubation with calcium ion had about the same apparent mass on SDS-PAGE as the erythrocyte calpastatins (6).

In further studies, it will be important to investigate the influence of

binding, or lack of binding, to membrane lipids on the ability of the calpastatins to inhibit membrane-associated calpains.

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