Cardiac High Molecular Weight Calmodulin Binding Protein Contains Calpastatin Activity†

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ABSTRACT: A high molecular weight calmodulin binding protein (HMWCaMBP) was previously identified and purified from bovine heart cytosolic fraction [Sharma, R. K. (1990) *J. Biol. Chem. 265*, 1152−1157]. In this study, we report the biological function of this protein. HMWCaMBP was subjected to peptide mapping and three peptides were sequenced. Two of the three peptide sequences were shown to be highly homologous to the calpain inhibitor, calpastatin. However, the third peptide did not show homology to any known proteins. The Western blot analysis of HMWCaMBP and purified calpastatin from bovine cardiac muscle showed immunoreactivity with polyclonal antibody raised against HMWCaMBP. Furthermore, HMWCaMBP inhibited calpain II and calpain I activities in a dose dependent fashion. Our data based on sequence homology, amino acid analysis, antibody reactivity and calpain inhibition suggests that HMWCaMBP is homologous to calpastatin and may be a CaM-binding form of calpastatin.

Intracellular calcium [Ca^{2+}] plays an important role in regulating various cellular functions as a second messenger (Clapham, 1995). Stimulation of cell surface receptors induces the increase in intracellular Ca^{2+} concentrations (Abdel-latif, 1986). In many instances, Ca^{2+} achieves its intracellular effects by binding to receptor proteins such as calmodulin (CaM),[1] troponin C, S-100 proteins, and calcyclin to transmit the Ca^{2+} signal (Cheung, 1980; Klee, 1991). CaM is a ubiquitous cellular, major calcium binding protein and a critical regulator in transducing the Ca^{2+} signal in a number of systems. It has the ability to associate with and modulate different proteins in a Ca^{2+}-dependent and reversible manner (Klee, 1991). In the heart, intracellular Ca^{2+} plays a central role in mechanical, metabolic and regulatory processes (Abdel-latif, 1986). The specific binding of Ca^{2+}−CaM to target proteins (CaM binding proteins, CaMBPs) regulates a variety of cellular responses (Klee, 1991). The distribution and expression of these CaM regulated proteins appears to vary among tissues. A number of CaM-dependent enzymes and proteins exist as tissue specific isoforms. In this respect, a number of CaM-dependent enzymes and proteins were discovered and purified before their intrinsic biological function was known, e.g. a brain tissue abundant CaM-binding protein known as calcineurin was extensively characterized before it was found to be a CaM-stimulated phosphatase (Stewart et al., 1982). Similarly caldesmon, a smooth muscle abundant CaM-binding protein was initially discovered as a CaM-binding protein, and now it has been implicated in smooth muscle cell contraction (Sobue et al., 1981; Walsh, 1985). The expression and tissue specific function of CaM is dependent on the properties and distribution of these calmodulin binding proteins.

The cardiac high molecular weight calmodulin binding protein (HMWCaMBP) was purified to near homogeneity in our laboratory (Sharma, 1990). Western blot analysis further revealed that HMWCaMBP was also present in lung and brain at a lower level than found in heart (Sharma, 1991a). However, the biological function of this protein was not known. In the present study, we have demonstrated the function of cardiac HMWCaMBP. On the basis of sequence homology, amino acid analysis, antibody reactivity, and calpain inhibition our findings suggest that HMWCaMBP is homologous to calpastatin, an inhibitor of the Ca^{2+}-activated cysteine proteases, calpains.

EXPERIMENTAL PROCEDURES

Materials. Hammersten-grade casein was purchased from ICN Biomedical Inc., USA. Bovine brain CaM and all other chemicals were obtained as described previously (Sharma, 1990).

Methods

Purification of HMWCaMBP. HMWCaMBP was purified by DEAE-Sepharose CL-6B, CaM-affinity, and Sepharose-6B gel filtration column chromatographies as previously described (Sharma, 1990) and further purified by phenyl-Sepharose-4B column chromatography. The major step in the purification of HMWCaMBP was through CaM-affinity column chromatography. The dialyzed sample from the CaM Sepharose-4B column was applied to a Sepharose-6B column (1.5 × 95 cm) which was pre-equilibrated with buffer A (20 mM Tris-HCl buffer, pH 7.0, 1 mM imidazole, 1 mM magnesium acetate, 10 mM mercaptoethanol, 0.1 mM EGTA) containing 0.1 M NaCl and 10% (w/v) sucrose. The presence of HMWCaMBP in the eluted fractions was detected by SDS−PAGE and fractions containing HMW-CaMBP were pooled. Ammonium sulfate was added to the pooled fraction to a final saturation of 10% and was applied

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[1] Abbreviations: HMWCaMBP, high molecular weight calmodulin binding protein; CaM, calmodulin; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride.

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to a phenyl-Sepharose-4B column (1.5 × 10 cm) which was pre-equilibrated with buffer B (buffer A containing 10% ammonium sulfate, w/v). The column was washed with buffer B and the bound protein was eluted with buffer A. The highest protein concentration fractions were pooled, dialyzed against buffer A, and used for further studies.

Amino Acid Composition and Sequence Analysis. The purified HMWCaMBP was subjected to the SDS−PAGE (7.5%) and was transferred onto Immobilon^{PSQ}-PVDF sequence membrane (Millipore Inc., U.S.A.) at 100 V for 2 h in 25 mM Tris buffer, pH 7.5, containing 10% methanol. After transfer the membrane was stained with 0.1% Ponceau S in 40% methanol until the 140 kDa HMWCaMBP band was visible. The band was cut out, destained with 50% methanol, washed 3−4 times with double-distilled water, and used for amino acid and partial peptide sequence analysis (Chait & Kent, 1992). Because the N-terminal region of the HMWCaMBP was blocked, the HMWCaMBP was digested with lysyl endopeptidase. The proteolytic peptides were purified by reverse phase high performance liquid chromatography and characterized by automated sequencing on an Applied Biosystems 477A Protein Sequencer (Harvard Microchem Lab., U.S.A.). The partial peptide sequences were compared with known proteins in the database using a BLAST (basic local alignment search tool) search (Altshul et al., 1990).

Immunoblotting Method. Polyclonal antibodies against HMWCaMBP were raised in New Zealand white rabbits as described previously (Sharma, 1991a). Immunoblotting was carried out essentially according to the method of Towbin et al. (1979).

Purification of Calcium Dependent Protease Calpain II and Calpain I. Calcium-activated neutral protease, calpain II from bovine cardiac muscle was partially purified as described (Hara et al., 1983) by DEAE-Sepharose CL-6B and Sepharose-6B gel filtration column chromatographies. The eluted fractions from Sepharose-6B column were examined for calpain activity at micromolar and millimolar concentrations of Ca^{2+}. The fractions containing protease activity at millimolar Ca^{2+} concentration were pooled and used for inhibition study.

Calpain I was purified from human erythrocytes to greater than 90% purity utilizing DEAE-Sepharose, Bio-Gel, and phenyl-Sepharose column chromatographies as previously described (Mellgren, 1991).

Assay of Calpain II and I Activity

Calpain II. Calpain II activity was determined by using Hammersten-grade casein as a substrate (Yoshimura et al., 1983). The assay mixture contained 20 mM imidiazole-HCl buffer containing 5 mM cysteine, pH 7.5, 5 mg of casein, and either 5 mM Ca^{2+} or 5 mM EGTA and calpain II in a total volume of 1 mL. The reaction mixture was incubated at 30 °C for 30 min, and the reaction was stopped by the addition of an equal volume of 5% trichloroacetic acid (TCA). After 10 min on ice, the samples were centrifuged at 1000 rpm for 10 min, and the supernatant containing TCA soluble products was used for measurement by the dye binding method (Bradford, 1976). One unit of calpain activity was defined as the amount of enzyme which caused an increase of one absorbance unit at 595 nm per 30 min at 30 °C.

Table 1: Amino Acid Composition of Bovine Cardiac HMWCaMBP and Calpastatin[a]

	HMWCaMBP	calpastatin[b]
aspartic acid	11.2	10.1
glutamic acid	13.1	11.4
serine	11.1	8.0
glycine	8.5	6.3
histidine	1.0	1.7
arginine	2.1	1.7
threonine	6.1	6.6
alanine	9.8	11.9
proline	9.4	14.0
tyrosine	1.2	1.3
valine	4.3	5.4
methionine	0.7	1.3
isoleucine	1.8	2.1
leucine	6.0	8.4
phenylalanine	1.1	1.0
lysine	12.6	8.0

[a] The results are expressed as percentage of total residues (100). [b] Data from Mellgren et al. (1985).

Calpain I. The activity of calpain I was assayed in the presence of 0.1 mM Ca^{2+} by its ability to generate TCA soluble fragments from ^{14}C-methylated casein as previously described (Mellgren et al., 1982).

Inhibition of Calpain II and I Activity by HMWCaMBP and Calpastatin. The HMWCaMBP was assayed for its inhibitory activity against calpain II. In addition, the inhibitory activity of purified bovine calpastatin (Mellgren et al., 1988) and HMWCaMBP was determined against calpain I. The procedure involved measuring the extent of inhibition by HMWCaMBP and calpastatin of a standard calpain reaction as described above.

Immunohistochemical Studies. The study material consisted of autopsy specimens of human myocardium. Approximately 5-μm thick sections were cut and subjected to the avidin−biotin complex method as previously described (Hsu et al., 1981) with the HMWCaMBP antibody being used as the primary antibody at a concentration of 14 μg/mL. The negative controls included the substitution of a preimmune serum. The immunogold study was carried out by utilizing the colloidal gold method as described (Hisano et al., 1993). Ultra thin sections were stained with uranyl acetate and examined under the electron microscope for deposition of gold particles.

Other Methods. SDS−PAGE was carried out according to the method of Laemmli (1970). Coomassie brilliant blue was used to visualize the protein bands on the gel. Protein assay was carried out as described (Bradford, 1976) using bovine serum albumin as a standard.

RESULTS

The amino acid composition of cardiac HMWCaMBP is shown in Table 1. The purified protein contained a high content of aspartic acid, glutamic acid, proline, and serine and a low content of histidine, tyrosine, and phenylalanine. Bovine cardiac HMWCaMBP was subjected to peptide mapping and three peptides (H_1, H_2, and H_3) were purified and sequenced. The homology of HMWCaMBP peptides to calpastatin from various species is given in Table 2. Peptide H_1 matched to *Bos taurus* at two different sites with sequence homology of 100 and 94%, respectively. Comparison with porcine, *Oryctolagus cuniculus*, and *Homosapiens* sequence showed 94 and 88% homology in domain

Table 2: Summary of the Homology of Partial Peptide Sequence of HMWCaMBP to Calpastatin[a]

species	sequence: peptide H_1	sequence: peptide H_2
HMWCaMBP	ELDDALDQLSDSLGQRQP	DNTTYXGPEVSXGMFXXTIE
Bos taurus[b]	25 ELDDALDQLSDSLGQRQP 42 [100%] 554 ELDDALDQLSDTLGQRQP 571 [94%]	153 DNTTYTGPEVSDPMSSTYIE 172 [65%]
porcine[c]	566 KLDDALDQLSDSLGQRQP 583 [94%]	174 DNTTYTGPEVLDPMSSTYIE 193 [60%]
Oryctolagus cuniculus[d]	570 ELDDALDKLSDSLGQRQP 587 [94%]	173 DSTAYTGPEISDPMSSTYIE 192 [50%]
Homosapiens[e]	531 DLDDALDKLSDSLGQRQP 548 [88%]	151 ENTTYTGPEVSDPMSSTYIE 170 [60%]

[a] The amino acids marked with asterisks are different from those of HMWCaMBP. The third HMWCaMBP peptide (H_3) DAMTAGALEALSESL--SEGIEHPG showed no homology. The data presented in parentheses indicates percent homology to HMWCaMBP. [b] Killefer and Koohmaraie, 1994. [c] Takano et al., 1988. [d] Emori et al., 1987. [e] Asada et al., 1989.

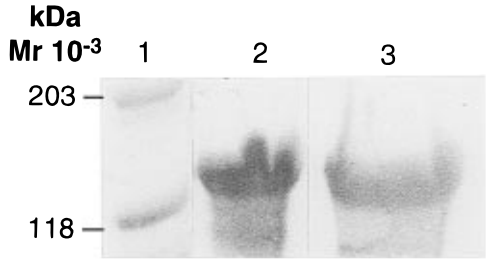

FIGURE 1: Western blot analysis of HMWCaMBP and calpastatin from bovine cardiac muscle. Purified HMWCaMBP (10 μg) and calpastatin (10 μg) from bovine cardiac muscle was subjected to 7.5% SDS−PAGE, transferred on to nitrocellulose membrane and probed with anti-HMWCaMBP antibody (10 μg/mL) as described under Experimental Procedures. Lane 1, prestained molecular weight markers (Bio-Rad), myosin (203 kDa) and β-galactosidase (118 kDa); lane 2, HMWCaMBP; and lane 3, bovine calpastatin.

IV region to calpastatin, respectively. Peptide H_2 also showed homology to *Bos taurus* (65%), porcine (60%), *Oryctolagus cuniculus* (50%), and *Homosapiens* (60%). However, the third peptide (H_3) did not show any similarity to known protein sequences. Polyclonal antibody raised against HMWCaMBP cross-reacted with calpastatin from bovine cardiac muscle (Figure 1), suggesting that HMW-CaMBP and calpastatin have common antigenic epitopes.

To further substantiate that HMWCaMBP contains calpastatin activity, the inhibitory effect of HMWCaMBP on the activity of calpain II and I was examined. Partially purified calpain II showed half-maximal activation at approximately 0.7 mM concentration of Ca^{2+} (data not shown). HMW-CaMBP inhibited the calpain II activity in a concentration dependent manner in the presence of Ca^{2+} with maximal inhibition at the concentration of 1.5 μg/mL and the half-maximal inhibition was at 0.3 μg/mL (Figure 2). HMW-CaMBP was also found to inhibit purified human erythrocyte calpain I (Figure 3). In this experiment, 8 nM calpain was utilized in the assay, and known concentrations of either purified bovine calpastatin, or HMWCaMBP were added. HMWCaMBP produced an essentially a linear inhibition vs concentration relationship until at least 70% inhibition of calpain (Figure 3). This is a typical of calpastatins, which are rapid, tightly binding inhibitors of calpains (Maki et al., 1990). In addition, HMWCaMBP appeared to possess four

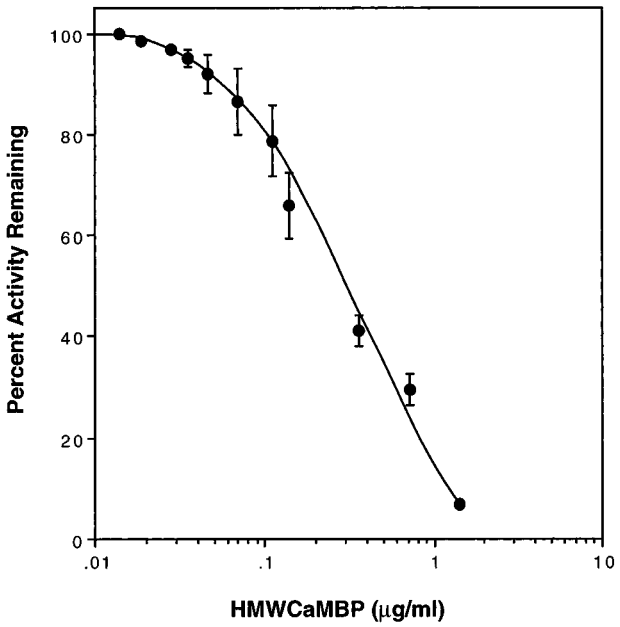

FIGURE 2: Dose-dependent inhibition of calpain II activity by HMWCaMBP. Partially purified calpain II (35 μg/assay) was incubated in the presence of various concentrations of HMW-CaMBP as described under Experimental Procedures. Error bars indicate standard deviation of triplicate assays.

inhibitory domains per 80 kDa mass unit, as predicted for a calpastatin (Takano et al., 1988).

The immunohistochemical study of human cardiac muscle showed strong staining of myocardial cells with HMW-CaMBP antibody. Ultrastructural and immunolocalization of HMWCaMBP utilizing the immunogold labeling technique demonstrated cytoplasmic and myofilament distribution of the HMWCaMBP (Figure 4).

DISCUSSION

To establish the function of HMWCaMBP that had been purified from bovine cardiac muscle (Sharma, 1990), the protein was digested with lysyl endopeptidase and three peptides were purified. The partial peptide sequence analysis obtained from peptide H_1 and H_2 revealed that HMWCaMBP shares 88−100% and 50−65% homology to calpastatin, respectively. Partial peptide sequences of HMWCaMBP

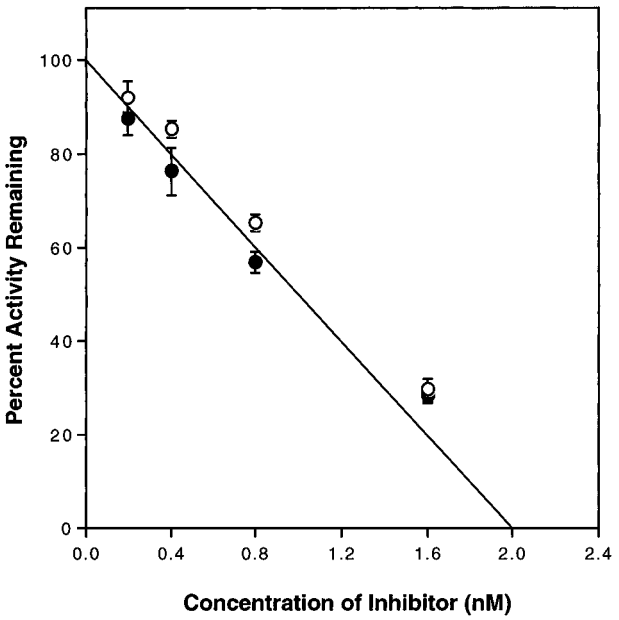

FIGURE 3: Inhibition of human calpain I by either bovine cardiac HMWCaMBP or calpastatin. Purified calpain I at a concentration of 8 nM was assayed for caseinolytic activity either in the presence of various concentrations of HMWCaMBP (●) or calpastatin (○). The line represents the expected inhibition produced by a stoichiometric inhibitor protein of 80 kDa mass possesing four independent calpain inhibitory domains (Maki et al., 1990). Error bars indicate standard deviation of triplicate assays.

showed 50−100% homology to the primary translational products of calpastatin from porcine (Takano et al., 1988), *Oryctolagus cuniculus* (Emori et al., 1987), *Bos taurus* (Killefer & Koohmaraie, 1994), and *Homosapiens* (Asada et al., 1989). The minor substitution of amino acid residues between HMWCaMBP and calpastatin from various sources could be due to divergence among animal species. Although peptide H_2 showed 50−65% homology to calpastatin, peptide H_3 did not show any sequence homology to the known proteins. These results suggest that HMWCaMBP could be a distinct protein which shares homology with calpastatin. Calpastatin is an endogenous inhibitor protein acting specifically on calpains (Saido et al., 1994). Both calpastatin and calpains are ubiquitously expressed in different tissues and are thought to play an important role in Ca^{2+}-dependent regulatory systems (Croall & Demartino, 1991; Saido et al., 1994). Calpains require calcium and reduced environment for their activity and play an important role in various physiological and pathological functions (Croall & Demartino, 1991). Calpains I and II require different concentration of Ca^{2+} for their activity. Calpain II requires millimolar concentration of Ca^{2+} (m calpain), whereas calpain I requires micromolar concentration of Ca^{2+} (μ calpain) for half maximal activation (Croall & Demartino, 1991). Recently, it has been reported that μ calpain activates m calpain and the concentration required for its half maximal activation is reduced to micromolar levels (Tompa et al., 1996). Calpains cause limited proteolysis of substrates resulting in modification of activity rather than inactivation or complete degradation of substrate. For example, protein kinase C after cleavage by calpain becomes fully active but no longer requires Ca^{2+} and phospholipid for activation (Kishimoto et al., 1989). Similarly calcineurin after cleavage by calpain does not require Ca^{2+} and CaM for activation (Tallant et al., 1988). Several structural proteins such as neurofilament, spectrin, microtubule associated protein, Tau and calspectin act as substrates of calpains, which suggests that calpains play a role in maintaining cellular structural integrity (Deshpande et al., 1995; Saito et al., 1993; Yoshida et al., 1995). It has also been reported that transcriptional factors c-Fos, c-Jun, and NF-κB are proteolyzed by calpains (Hirai et al., 1991; Liu et al., 1996). Thus calpastatin, an inhibitor of calpains may play an important role in various cellular functions.

The comparison of amino acid composition between HMWCaMBP and calpastatin from various sources showed considerable similarity (Mellgren & Carr, 1983; Mohan & Nixon, 1995). The amino acid composition revealed that HMWCaMBP has large amounts of aspartic acid and glutamic acid and lower amounts of histidine, tyrosine and phenylalanine. A similar profile was observed for calpastatin purified from bovine myocardium by a standard method (Table 1, Mellgren et al., 1985). Under non-denaturing conditions the HMWCaMBP exhibits an apparent molecular mass of 175 kDa, whereas under denaturing conditions the protein shows an apparent molecular mass of 140 kDa. The amino acid composition of HMWCaMBP may influence the electrophoretic mobility because of high amounts of negatively charged amino acids (Table 1). HMWCaMBP contains large amounts of proline and it has been shown that proline alters the binding of SDS to protein (Kaufmann et al., 1984). Some nuclear proteins such as c-myc and c-fos also have clusters of proline residues in their sequences and have apparent molecular weights higher than those predicted from their cDNA sequences (Persson et al., 1984; Van Beveran et al., 1983). Most importantly, calpastatin have been shown to display anamolously high molecular weight on SDS−PAGE (Takano et al., 1988). Porcine heart calpastatin has been shown to be 107 kDa protein on SDS−PAGE (Takano et al., 1988). Mellgren et al. (1983) reported the bovine heart calpastatin as a 145 kDa protein; however, Otsuka & Goll (1987) reported it to be a 115 kDa protein. The difference in size in calpastatin from different species and tissues could be due to either a post transcriptional or post translational processing. The HMWCaMBP showed molecular weight of 140 kDa and Stoke radius of 83 Å which was different than previously reported calpastatins (Mellgren et al., 1983; Otsuka & Goll, 1987; Takano et al., 1988), suggesting that HMWCaMBP could be a novel structural form of calpastatin.

Immunoblotting analysis of HMWCaMBP and bovine calpastatin indicated that HMWCaMBP antibody cross-reacts with calpastatin suggesting that both proteins have common antigenic epitopes. To test the possibility that HMWCaMBP contains calpastatin activity, HMWCaMBP was incubated either with calpain II or calpain I. Both forms of calpain were inhibited by HMWCaMBP in a concentration dependent manner (Figures 2 and 3). Importantly, HMWCaMBP inhibited calpain I at sub-stoichiometric concentrations, and appeared to possess four independent inhibitory domains per 80 kDa mass unit. This rules out the co-purification of small amounts of contaminating calpastatin in the preparation, and indicates that HMWCaMBP has the same number of calpain inhibitory domains as previously characterized calpastatins (Maki et al., 1990; Mellgren et al., 1983; Takano et al., 1988).

Immunohistochemical localization of HMWCaMBP from autopsy specimens of human heart revealed a strong staining of myocardium muscle fibre with polyclonal antibody raised

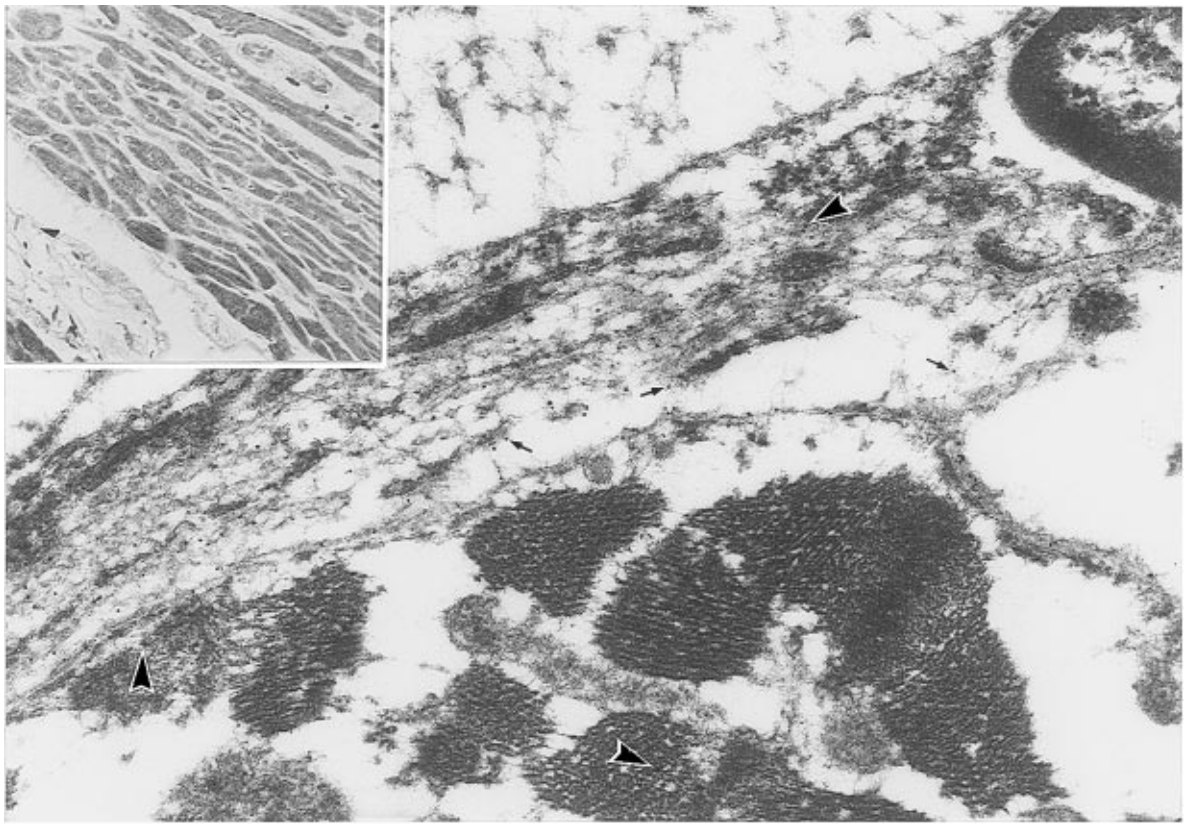

FIGURE 4: Ultrastructural localization of HMWCaMBP in human cardiac myocyte by using immunogold labeling technique demonstrates cytoplasmic (arrow) and myofilaments (arrowhead) immunoreactivity. Inset, micrograph of human cardiac muscle with strong immunostaining for polyclonal HMWCaMBP (EM magnification × 50 000).

against HMWCaMBP. Immunoreactivity was observed throughout the cytoplasm of the myocardial cells. Ultrastructural localization of HMWCaMBP demonstrated cytoplasmic and myofilament distribution suggesting an important role of this protein in cardiac muscle. The distribution of HMWCaMBP is reminiscent of the previously published immunoelectron microscopic localization of bovine myocardial calpastatin (Mellgren et al., 1989).

The majority of the CaM-regulated proteins undergo Ca^{2+}-dependent and reversible association with CaM. It was interesting to note that HMWCaMBP has a high affinity for CaM and strongly binds to CaM-Sepharose 4B column (Sharma, 1990). To further substantiate that the HMW-CaMBP is CaM-binding protein, CaM-dependent cyclic nucleotide phosphodiesterase (CaMPDE) activity was carried out in the presence of HMWCaMBP in a dose dependent manner. CaMPDE activity was markedly inhibited by the HMWCaMBP, and this inhibition was reversed by a high concentration of CaM, suggesting that binding is specific and reflects a simple competition for available CaM (Sharma, 1990). The observation that HMWCaMBP exists predominantly in heart and binds to CaM suggests that one potential function of CaM in heart is the regulation of calpain activity. This might be accomplished by competition between the protease and CaM for binding to HMWCaMBP under different intracellular conditions. Although it has been reported previously that calpastatin is not a CaM binding protein (Takano, 1995), HMWCaMBP binds CaM and exhibits calpastatin activity. It appears from our study that HMWCaMBP is a calpastatin based on the sequence homology, antibody reactivity, and calpain II and I inhibition. However, on the other hand, one of the peptide sequences (H_3) did not show any homology to known calpastatins and other properties like molecular mass, Stokes radius and CaM affinity are different from known calpastatins (Mellgren & Carr, 1983; Otsuka & Goll, 1987; Takano et al., 1988). These observations further suggest that HMWCaMBP could be a new protein or an isoform of calpastatin which contains CaM binding site(s). In recent years, it has become clear that various CaM-dependent enzymes exist as distinct isozymes and their functions are tissue specific (Cohen, 1988; Sharma & Hickie, 1996; Stull, 1988). The CaM-dependent isozymes have been demonstrated for various signal transduction systems including CaM-dependent cyclic nucleotide phosphodiesterase (Sharma et al., 1984, 1997; Sharma, 1991b; Sharma & Kalra, 1994), myosin light chain kinase (Stull, 1988), CaM-dependent protein kinase (Cohen, 1988), CaM-stimulated protein phosphatase (Yokoyama & Wang, 1991), and protein kinase C (Nishizuka, 1988). The regulatory and physiological significance of these isozymes is only beginning to be appreciated.

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