A novel protein which represents the most abundant calmodulin-binding protein in bovine heart cytosolic fraction was purified to apparent homogeneity. The purification procedure involved DEAE-Sepharose CL-6B (to remove calmodulin), calmodulin-Sepharose 4B affinity, and Sephacryl S-400 column chromatographies. This purified calmodulin-binding protein is a highly symmetric protein with a sedimentation coefficient of approximately 5.0 S and a Stokes radius of about 83.0 A. The molecular weight of the calmodulin-binding protein was determined to be 175,000 from the sedimentation constant and Stokes radius of the protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the protein showed a single protein band with an apparent molecular weight of 140,000. The result suggests that the protein is monomeric. Although this molecular weight is similar to that of caldesmon, a known ubiquitous calmodulin-binding protein, the protein did not react with caldesmon-specific antibodies, nor did it display a proteolytic fragmentation pattern similar to that of the former. In addition, caldesmon was found almost exclusively in the particulate fraction in low ionic strength cardiac muscle extract, whereas this protein is purified from the soluble fraction.

In this present study, the description of a novel calmodulin-binding protein is reported. The protein, which appears to be especially abundant in heart muscle extract, has been purified to homogeneity and characterized in terms of molecular properties.

**EXPERIMENTAL PROCEDURES**

*Materials—*Bovine brain calmodulin was prepared by using a phenyl-Sepharose column as described by Gropalakrishna and Anderson (10) and further purified by passage through a Sepharose 4B 200 gel filtration column which was pre-equilibrated with buffer A (20 mM Tris-HCl, 1 mM magnesium acetate, 1 mM imidazole, pH 7.0, 10 mM 2-mercaptoethanol) containing 0.01 mM Ca" and 0.1 M NaCl. The calmodulin-Sepharose 4B gel was prepared as described by Sharma et al. (11). The chicken gizzard smooth muscle caldesmon (12, 13) and polyclonal antibodies against chicken gizzard caldesmon (14) were a generous gift from Dr. M. P. Walsh (University of Calgary). Caldesmon from bovine heart was prepared as described by Ngai and Walsh (12).

*Preparation of Total Calmodulin-binding Proteins—*All steps were carried out at 4 °C. Fresh bovine tissues (heart, spleen, lung, skeletal muscle, uterus, and brain) were obtained from a local slaughterhouse and transferred to the laboratory in packed ice. All tissues were stored at -30 °C until use.

*Extraction—*After being partially thawed at room temperature, 100 g of bovine tissues, except uterus and brain, were ground in a meat grinder and were then homogenized in a Waring blender for 3 min, in 200 ml of ice-cold 20 mM Tris-HCl buffer, pH 7.6, containing 2 mM EDTA, 100 mg of phenylmethylsulfonyl fluoride/liter, 100 mg of soybean trypsin inhibitor/liter, and 200 mg of benzamidine/liter. These protease inhibitors were included in the buffers up to the calmodulin-Sepharose affinity chromatography step. The homogenate was centrifuged at 10,000 x g for 20 min, and the supernatant was filtered through glass wool. 2-Mercaptoethanol and EGTA were added to the supernatant to make final concentrations of 10 mM and 0.1 M NaCl, respectively.

*DEAE-Sepharose CL-6B Column Chromatography—*The supernatant from the extraction step was applied to a DEAE-Sepharose CL-6B (9.5 x 80 cm) column pre-equilibrated with buffer A containing 0.1 mM EGTA. The column was subsequently washed with 2/5 bed volumes of buffer A containing 0.1 mM EGTA, 0.38 M NaCl, and proteins were eluted with the same buffer containing 0.2 M NaCl.

*Calmodulin-Sepharose 4B Affinity Chromatography—*The pooled fractions from the DEAE-Sepharose CL-6B column were applied to a calmodulin-Sepharose 4B affinity column (1.5 x 10 cm), which had been previously equilibrated with buffer A containing 0.05 mM Ca" and 0.2 M NaCl until no protein could be detected in the eluate (about 4 bed volumes). The protein was then eluted with buffer A containing 1.0 mM EGTA and 0.2 M NaCl. Fractions containing high protein concentrations were pooled.

*Gel Electrophoresis—*Polyacrylamide gel electrophoresis in the
presence of SDS was carried out according to the procedure of Laemmli (15) employing 12% gel. Coomassie Blue was used to visualize the protein bands on the gel.

Peptide Mapping—Peptide mapping by limited proteolysis was performed according to the method described by Cleveland et al. (16). The bands corresponding to caldesmon from chicken gizzard and bovine heart and the high molecular weight calmodulin-binding protein (10 µg) were cut from SDS-polyacrylamide gel. The gel slices were equilibrated with buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, and 1 mM Na,EDTA for 2 h. The gel slices were then placed in the sample wells of a second SDS gel (15%) and overlaid with either 0.02 or 0.1 µg of Staphylococcus aureus V8 protease prepared in the equilibration buffer containing 0.001% bromophenol blue. The samples were digested at room temperature for 60 min in the stacking gel during electrophoresis.

Immunoblotting Method—Immunoblotting procedure was essentially carried out according to the method of Towbin et al. (17). Nitrocellulose membranes were soaked in distilled, deionized water for 30 min and in TransBlot buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol, 0.1% SDS) for 30 min. After electrophoresis, gels for transblotting were soaked in ≈200 ml of TransBlot buffer with gentle agitation for 1 h. Transblotting was carried out at 20 V (constant voltage) and 4°C for 18 h. Following transfer (in duplicate), one membrane of nitrocellulose was stained with 0.7% Amido Black for approximately 5 s (total protein stain). A second incubation was carried for 60 min at 40°C with Tris saline (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.02% sodium azide) containing 3% bovine serum albumin. The blot was washed in Tris saline, and anti-caldesmon antiserum was applied (50 µg/ml overnight at room temperature). The primary antibody was washed away, and peroxidase-conjugated anti-rabbit was applied (1:1000 dilution) for 2 h. Again, the blot was washed, and the immunoreactive proteins were visualized by exposure to 60 mg of 4-chloro-1-naphthol in 20 ml of methanol with 100 ml of Tris saline containing 90 µl of 30% H2O2.

Amino Acid Composition Analysis—Sample containing 100 µg of purified protein (as shown in Fig. 6) was hydrolyzed with 6 N HCl containing 0.1% (w/v) phenol at 110°C in vacuo for 24 h. Analyses were carried out in a Beckman model 6300 amino acid analyzer equipped with a Spectra-Physics SP 4270 2-channel integrator.

Assay for Inhibition of Calmodulin-dependent Phosphodiesterase by Calmodulin-binding Protein—The assay was based on the ability of calmodulin-binding protein to compete with phosphodiesterase for the binding of calmodulin (18). This procedure involved measuring the extent of inhibition by calmodulin-binding protein of a standard phosphodiesterase reaction containing 500 ng/ml calmodulin and 0.012-0.016 unit/ml of the phosphodiesterase when it was fully activated.

RESULTS

During purification of bovine heart calmodulin-dependent phosphodiesterase from calmodulin-Sepharose 4B affinity column chromatography, it was found that EGTA-eluted samples contained several calmodulin-binding proteins including a high molecular weight calmodulin-binding protein which was the predominant protein and has a molecular weight of 140,000 (Fig. 1, lane 1) on SDS-gel electrophoresis. The total calmodulin-binding proteins were prepared as described under “Experimental Procedures” from several bovine tissues including spleen, lung, skeletal muscle, uterus, and brain to determine whether this high molecular weight calmodulin-binding protein is heart-specific. A protein of similar apparent molecular weight was not observed in any of the tissues examined, suggesting that this protein may be a heart-specific calmodulin-binding protein.

The identity and function of this protein is not clear, although its electrophoretic properties resemble those of caldesmon (6), a major calmodulin-binding protein originally found in chicken gizzard (6), and also present in heart (19). It has a molecular weight varying from 135,000 to 150,000 (6, 12, 13, 19-21). The possibility that this high molecular weight calmodulin-binding protein is caldesmon was investigated. The immunoblotting experiments were performed to establish the relationship between these proteins, using an anti-caldesmon polyclonal antibody, raised against chicken gizzard caldesmon. Immunoblot analysis of high molecular weight calmodulin-binding protein and caldesmon. Immunoblot analysis of high molecular weight calmodulin-binding protein using polyclonal antibodies against chicken gizzard caldesmon. Purified caldesmon (5 µg) and cardiac calmodulin-binding proteins (15 µg) were subjected to SDS-PAGE and either stained with Coomassie Blue (A) or immobiloblotted (B) using rabbit anti-chicken gizzard caldesmon (14). Lanes 1 and 3, purified chicken gizzard caldesmon; lanes 2 and 4, bovine heart total calmodulin-binding protein.

Fig. 1. The distribution of total calmodulin-binding proteins in diverse bovine tissues. Total calmodulin-binding protein was prepared as described under “Experimental Procedures.” Aliquots (15 µg) of each calmodulin-binding protein were subjected to SDS-PAGE. Lanes: 1, heart; 2, spleen; 3, lung; 4, skeletal muscle; 5, uterus, and 6, brain.

Fig. 2. Immunological dissimilarity of high molecular weight calmodulin-binding protein and caldesmon. Immunoblot analysis of high molecular weight calmodulin-binding protein using polyclonal antibodies against chicken gizzard caldesmon. Purified caldesmon (5 µg) and cardiac calmodulin-binding proteins (15 µg) were subjected to SDS-PAGE and either stained with Coomassie Blue (A) or immobiloblotted (B) using rabbit anti-chicken gizzard caldesmon (14). Lanes 1 and 3, purified chicken gizzard caldesmon; lanes 2 and 4, bovine heart total calmodulin-binding protein.
Calmodulin-binding Protein from Heart

that the high molecular weight calmodulin-binding protein is a novel calmodulin-binding protein which is present in cytosolic fraction, whereas bovine heart caldesmon was present in the particulate fraction (14).

To further substantiate that the high molecular weight calmodulin-binding protein was not caldesmon, caldesmon from chicken gizzard and caldesmon and high molecular weight calmodulin-binding protein from bovine heart were subjected to peptide mapping by partial proteolysis (16). The proteolytic cleavage pattern of chicken gizzard and bovine heart caldesmon were indistinguishable (Fig. 4, lanes 1, 2, 4, and 5), whereas the high molecular weight calmodulin-binding protein exhibited a different proteolytic profile (Fig. 4, lanes 3 and 6). The result suggests that peptide maps of high molecular weight calmodulin-binding protein from bovine heart is distinct, thus ruling out the possibility that the high molecular weight calmodulin-binding protein is caldesmon.

Comparison of Amino Acid Composition of High Molecular Weight Calmodulin-binding Protein with Caldesmon—The complete amino acid composition analysis of bovine heart high molecular weight calmodulin-binding protein is given in Table I, which also includes the composition of the bovine heart caldesmon and chicken gizzard caldesmon for comparison. Although these proteins bind calmodulin in a Ca**+-dependent manner and have similar molecular weight, notable differences do exist in amino acid composition between high molecular weight calmodulin-binding protein and caldesmon, e.g. bovine heart high molecular weight calmodulin-binding protein has substantially larger amounts of aspartic, proline, and leucine and lower amounts of arginine, glutamic acid, and phenylalanine. The differences between the amino acid compositions of high molecular weight calmodulin-binding protein and caldesmon indicate significant differences in amino acid sequence.

Purification of Calmodulin-binding Protein from Bovine Heart—Endogenous calmodulin was removed from a crude extract of bovine heart (1 kg) by using DEAE-Sepharose CL-6B column chromatography as described under “Experimental Procedures.” The 0.2 M NaCl eluent from DEAE-Sepharose CL-6B column was applied on calmodulin-Sepharose 4B affinity column as described under “Experimental Procedures.” After removal of nonspecific proteins by washing the column with buffer A containing 0.1 mM and 0.2 mM Ca**+, the column was further washed with 1 bed volume of the same buffer without NaCl. A protein peak was eluted with buffer A containing 1 mM EGTA (Fig. 5, peak A) which was associated mainly with calmodulin-dependent phosphodiesterase, calmodulin-dependent protein kinase activity, and some of the other calmodulin-binding proteins. After elution with buffer A containing 1 mM EGTA, the protein concentration in the column returned to the basal level, and 0.2 M NaCl was added to the elution buffer, resulting in the appearance of a second peak of proteins in the column eluent (Fig. 5, peak B). As can be seen in Fig. 5, peak A contains 30-35% of total calmodulin-binding proteins, whereas peak B represents 67-70% of total calmodulin-binding proteins. Analysis of the SDS-PAGE patterns (Fig. 5, inset) and enzyme activities of the two protein fractions of high molecular weight calmodulin-binding protein and comparison with bovine heart and chicken gizzard caldesmon indicated significant differences.

Table I

<table>
<thead>
<tr>
<th>Amino acid composition of bovine heart high molecular weight calmodulin-binding protein and comparison with bovine heart and chicken gizzard caldesmon</th>
<th>Bovine heart high molecular weight calmodulin-binding protein</th>
<th>Bovine heart caldesmon*</th>
<th>Chicken gizzard caldesmon*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>10.6</td>
<td>7.3</td>
<td>7.3</td>
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<tr>
<td>Histidine</td>
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<td>7.2</td>
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<td>Threonine</td>
<td>6.4</td>
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</tr>
<tr>
<td>Serine</td>
<td>9.5</td>
<td>6.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.5</td>
<td>22.3</td>
<td>25.4</td>
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<td>Proline</td>
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<td>3.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.3</td>
<td>7.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.6</td>
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<td>11.0</td>
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<td>Valine</td>
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<td>Methionine</td>
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<tr>
<td>Tryptophan</td>
<td>NT*</td>
<td>+*</td>
<td>0.7</td>
</tr>
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</table>

* From Clark et al. (19).  
* NT, not tested.  
+ Indicates tryptophan was detected but could not be quantified accurately.
peaks indicated that peak A contained calmodulin-dependent phosphodiesterase and calmodulin-dependent protein kinase activities, whereas peak B was enriched with the high molecular weight calmodulin-binding protein. Peak B was pooled and concentrated by ultrafiltration through an Amicon PM-10 membrane to 5.0 mg/ml protein.

**Sepharose 6B Column Chromatography**—A column of Sepharose 6B (1.5 × 95 cm) was prepared and equilibrated with buffer A containing 0.1 mM EGTA, 0.1 M NaCl, and 10% sucrose. Peak B from the last step was applied to the Sepharose 6B column. Proteins were eluted with equilibration buffer, and selected column fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The fractions (105–118) which contained high molecular weight protein were pooled as shown in Fig. 6 and concentrated by ultrafiltration. The concentrated samples were stored at −70 °C in 1-ml aliquots.

The purity of the pooled sample was examined by SDS-gel electrophoresis (Fig. 6, inset). The sample showed a predominant polypeptide of Mr = 140,000 and minor polypeptides. These polypeptides are likely contaminants since the amount varied from preparation to preparation relative to the Mr = 140,000 polypeptide.

**Molecular Weight and Subunit Structure of Bovine Heart Calmodulin-binding Protein**—Gel filtration and sucrose gradient sedimentation analysis were used to examine the physical characteristics of the calmodulin-binding protein. On Sepharose 6B chromatography, the calmodulin-binding protein eluted with a symmetrical profile of estimated Stokes radius of about 83.0 Å (Fig. 6). Sedimentation in sucrose gradients together with standard proteins indicated that bovine heart calmodulin-binding protein had a sedimentation coefficient of about 5 (data not shown). The molecular weight of the protein was then calculated to be 175,000, from the sedimentation constant and Stokes radius according to the method of Siegel and Monty (22). The result suggests that the protein is a highly asymmetric molecule, since its apparent molecular weight by gel filtration greatly exceeds the calculated molecular weight.

**Inhibition of Phosphodiesterase Activity by the Calmodulin-binding Protein**—The ability of the high molecular weight protein to bind calmodulin was confirmed by its ability to inhibit phosphodiesterase activity. Calmodulin-dependent phosphodiesterase activity was markedly inhibited by this calmodulin-binding protein in a dose-dependent fashion. The amount of protein required for 50% inhibition was 5 μg/ml under the standard assay condition as described under “Experimental Procedures” (Fig. 7). In contrast, caldesmon was shown not to inhibit the phosphodiesterase reaction (Fig. 7). This is consistent with the previous report of Ngai and Walsh (12) that caldesmon does not inhibit two calmodulin-dependent enzymes, i.e. myosin light chain kinase and phosphodiesterase.

**Reversibility of Phosphodiesterase Inhibition by Calmodulin-binding Protein**—The inhibition of phosphodiesterase by high molecular weight calmodulin-binding protein (shown in Fig. 7) is likely to have been the result of competition between...
The biological activity of the purified calmodulin-binding protein was examined by testing such a possibility. The high molecular weight calmodulin-binding protein (G) and caldesmon (W) as described previously by Sharma et al. (18). The enzyme activity was represented as a percentage of the activity of the calmodulin-dependent phosphodiesterase activity. The inhibition of phosphodiesterase activity, again in contrast to caldesmon which was not shown to inhibit the phosphodiesterase reaction (Fig. 7). Furthermore, while caldesmon appears to exist exclusively in the particulate fraction of a lower ionic strength cardiac extract, the high molecular weight calmodulin-binding protein appeared to be mostly, if not exclusively, present in the soluble fraction.

The physiological functions of calmodulin in mammalian heart, as judged by the various calmodulin-dependent heart proteins, are multifold. It participates in the control of cAMP and cGMP hydrolysis (24), phosphorylation, and dephosphorylation of a number of proteins involved in Ca2+ homeostasis (e.g. phospholamban (25) and Ca2+ channel proteins (26)) and in energy metabolism (27, 28). The observation that the high molecular weight calmodulin-binding protein exists as the predominant protein in the calmodulin-binding protein function of heart suggests strongly that one of the important functions of calmodulin in heart is related to the regulation of this protein. The observation that this protein appears specifically enriched in heart suggests that it has an intrinsic activity uniquely related to the function or operation of the mammalian heart. The identification and successful purification of this protein have made it possible for us to focus on examining such a possibility.

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Purification and characterization of novel calmodulin-binding protein from cardiac muscle.

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