Cardiac Calmodulin-Stimulated Protein Phosphatase: Purification and Identification of Specific Sarcolemmal Substrates

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A calmodulin-stimulated protein phosphatase has been purified from bovine myocardium. The purification procedure involves sequential DEAE-Sephadion ion exchange chromatography, calmodulin-Sepharose affinity chromatography, and high performance liquid chromaturgy using a Spheroel DEAE SPW column. By SDS polyacrylamide gel electrophoresis, the purified cardiac phosphatase consists of two subunits of Mr 61,000 and 19,000, similar to the brain enzyme, calcineurin. Protein phosphatase activity of the cardiac enzyme is stimulated by Ca\(^{2+}\)-calmodulin and inhibited by the calmodulin antagonist drug, calmidazolium. Effects of a series of divalent cations on catalytic activity of the cardiac calmodulin-stimulated protein phosphatase are similar to those observed with calcineurin, when the two enzymes are assayed under identical conditions. Highly enriched preparations of bovine cardiac sarcolemma contain substrates of cAMP-dependent protein kinase of Mr 166 K, 133 K, 108 K, 79 K, 39 K, and 14 K, which are specifically dephosphorylated by the calmodulin-stimulated phosphatase with pseudo-first-order rate constants of 0.23, 0.46, 0.69, 0.35, 0.69, and 0.115 min\(^{-1}\), respectively. These substrates are not present in purified preparations of cardiac sarcoplasmic reticulum. These results support a role of the calmodulin-stimulated phosphatase in the Ca\(^{2+}\)-regulation of specific sarcolemmal processes by protein dephosphorylation. (Circulation Research 1987; 60:602-611)

Biochemical responses to regulatory stimuli are mediated by at least two major intracellular signals, cAMP and calcium. Available evidence clearly demonstrates that the cAMP and calcium dependent regulatory systems are extensively interrelated.1 This interrelation is particularly evident in cardiac tissue, where both sarcolemmal and sarcoplasmic reticulum Ca\(^{2+}\)-ATPases involved in control of cytosolic Ca\(^{2+}\) appear to be regulated by cAMP-dependent phosphorylation.2-5

In the case of cAMP, intracellular biochemical effects result from activation of the enzyme cAMP-dependent protein kinase, which in turn regulates a variety of cellular processes through the mechanism of protein phosphorylation. The calcium-dependent regulatory system is also known to involve a phosphorylation mechanism, and both Ca\(^{2+}\), calmodulin-regulated6-17 and Ca\(^{2+}\), phospholipid-regulated protein kinases18 have been identified. The study of specific substrates of these kinases has provided important insights into the biochemical mechanisms involved in physiological regulation. In contrast, equally important information concerning the phosphatases responsible for the dephosphorylation of these substrates remains more limited.

The recent discovery of a family of Ca\(^{2+}\)- and calmodulin-stimulated phosphatases establishes another important mechanism by which calcium exerts its intracellular effects.19-22 Of these phosphatases, calcineurin, a major calmodulin-binding protein present in brain,23-26 has been most extensively studied. This protein is a heterodimer consisting of two subunits, Mr 61,000 calmodulin-binding subunit termed calcineurin A, and Mr 19,000 Ca\(^{2+}\)-binding subunit, called calcineurin B.26 Available evidence indicates that the catalytic site resides on the large calmodulin-binding subunit of the enzyme.27

The physiological role of the Ca\(^{2+}\)-, calmodulin-stimulated phosphatase is unknown. Several lines of evidence suggest a role of the phosphatase in membrane regulation,28-32 and interestingly, the most important clue concerning the physiological significance of the calmodulin-stimulated phosphatase comes from recent studies in heart. These studies have suggested that this phosphatase is involved in regulation of sarclemmal Na\(^{+}\)-Ca\(^{2+}\) exchange.33 Available evidence indicates that calmodulin-stimulated phosphatases may be tissue specific,34 and information accumulated in several laboratories indicates that a calcineurin-like protein is present in heart.31,22,34,35

We presently describe the purification and initial characterization of a Ca\(^{2+}\)-, calmodulin-stimulated phosphatase from bovine heart. In addition, the calmodulin-stimulated phosphatase dephosphorylates specific sarcolemmal substrates of cAMP-dependent protein kinase in highly enriched cardiac sarcolemmal vesicles. These results are compatible with a role of the calmodulin-stimulated phosphatase in regulation of sarcolemmal processes.
Materials and Methods

Protein Purification

Calmodulin was purified from ram testes using ammonium sulfate precipitation (40–60% saturation) and sequential chromatography on phenyl-Sepharose and DEAE-Sepharose by modification of previously published methods.36,37 Catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart using the procedure of Bechtel et al.38 Calcineurin was purified from bovine brain by a scaled down version of the method of Klce et al.39

Purification of Calmodulin-Stimulated Protein Phosphatase From Bovine Heart

Preparation of a Soluble Extract. Bovine hearts, obtained from a local slaughterhouse, were used fresh or after storage at −70° C. All procedures were performed at 4° C. Bovine myocardium (600 g), trimmed to remove adherent fat, superficial blood vessels, and connective tissue, was homogenized in 3 volumes of Tris-HCl 0.1 M, pH 7.5, MgCl₂ 5 mM, EDTA 1 mM, EGTA 1 mM, and protease inhibitors phenyl methyl sulfonyl fluoride (PMSF) 75 μg/mL, leupeptin 1 μg/mL, trypsin inhibitor 10 μg/mL, and L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK) 10 μg/mL in a Waring blender two times for 30 seconds. The homogenate was sedimented at 10,000g for 20 minutes, and the soluble extract was decanted and filtered through glass wool.

DEAE-Sepharose Chromatography. The soluble extract was brought to 8,000 mL by addition of equilibration buffer containing 0.05 M ammonium sulfate and batch adsorbed to 200 mL DEAE-Sepharose previously equilibrated in the same buffer. The DEAE-Sepharose was isolated and washed with 3 vol of this buffer using a Buchner funnel, and a 5 x 10 cm column was poured. After loading, the DEAE-Sepharose column was washed with equilibration buffer containing 0.05 M ammonium sulfate to bring the A₂₈₀ of the eluate that activity inhibitable by 1 mM Tris/EGTA. In all membrane preparations of bovine cardiac sarcoplasmic reticulum, the highly enriched preparations of bovine cardiac sarcoplasmic reticulum were isolated by differential centrifugation, calcium oxalate loading, and discontinuous sucrose gradient sedimentation according to the procedure of Jones et al (for canine myocardium).40 The free sarcoplasmic reticulum fractions (SRₑ) employed in the present study had Ca²⁺-ATPase activity > 150 μmol Pi/hr/mg and (Na⁺,K⁺)-ATPase activity < 3 μmol Pi/hr/mg. Membrane protein determinations were performed by the method of Lowry et al.

ATPase Assays. (Na⁺,K⁺)-ATPase activity was measured at 37° C in HEPES 50 mM, pH 7.4, MgCl₂ 3 mM, EGTA 1 mM, NaCl 100 mM, and Tris-ATP 3 mM. Total (Na⁺,K⁺)-ATPase activity was determined in the membrane fractions after unmasking by treatment with sodium dodecyl sulfate (0.3 mg/mL). (Na⁺,K⁺)-ATPase activity was defined as that activity inhibited by 1 mM ouabain. Sarcoplasmic reticulum Ca²⁺-ATPase activity was determined in HEPES 50 mM, pH 7.4, MgCl₂ 3 mM, KCl 100 mM, CaCl₂ 50 μM, and Tris-ATP 3 mM. Ca²⁺-ATPase activity was that activity inhibitable by 1 mM Tris/EGTA. In all assays of Ca²⁺-ATPase, the divalent cation ionophore A23187 was included at 3 μM to eliminate Ca²⁺ accumulation within the vesicles. Inorganic phosphate released from ATP was determined colorimetrically.

SDS polyacrylamide gel electrophoresis was performed in slabs consisting of a 7–15% linear gradient of acrylamide by modification of the method of Laemmli.41 Molecular weight markers were myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (68,000), catalase (58,500), fumarase (48,000), actin (42,000), lactate dehydrogenase (35,000), and β-lactoglobulin (17,500).

[125I] Calmodulin Gel Overlay. Fractions for assay were subjected to slab SDS gel electrophoresis and gels processed for [125I] calmodulin gel overlay as previously described.39 Labelled-bands localized by autoradiography were excised from gels and radioactivity determined by gamma counting. Gels were internally calibrated with known amounts of purified Tris-acetate 0.02 M, pH 7.5, and magnesium acetate 1 mM, washed for 5 minutes in this buffer, and eluted with a 20 minute linear 0–0.5 M sodium acetate gradient in this buffer at constant flow rate of 0.8 mL/min. The calmodulin-stimulated phosphatase eluted as a single symmetrical peak at 11–14 mS, well-resolved from other proteins (Figure 3).
brain calcineurin run in adjacent lanes as previously described. 39

**Phosphatase Assay.** Phosphatase activity was assayed at 37°C in 50 μL 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, 6 mM MgCl₂, 0.1 mg/mL BSA, 0.5 mM DTT, 1 μM [³²P] histone VS, and indicated concentrations of calmodulin, divalent cations, or calmodulin antagonists. Inorganic ³²P released by the phosphatase was extracted by the method of Martin and Doty 40 and quantified by liquid scintillation counting.

**Membrane Phosphorylation.** Membrane vesicle fractions (30–40 μg protein) were incubated for 5 minutes at 37°C in 40 μL 0.04 M Tris-HCl, pH 7.5, 3 mM MgCl₂, 0.1 M NaCl, 10–100 μM [³²P]ATP and 0.2 μg catalytic subunit of cAMP-dependent protein kinase. Reactions were stopped by addition of 20 μL SDS gel dissociation medium (0.14 M Tris, 22.4% glycerol, 6% sodium dodecyl sulfate, 10% 2-mercaptoethanol and trace bromphenol blue). Samples were heated at 90°C for 90 seconds prior to SDS-polyacrylamide gel electrophoresis.

**Membrane Dephosphorylation.** Sarcolemmal vesicles were incubated at 37°C for 15 minutes in Tris-HCl 0.04 M, pH 7.5, MgCl₂ 3 mM, NaCl 0.1 M, and [³²P]ATP 50 μM in the presence of 0.6 μg catalytic subunit of cAMP-dependent protein kinase. Membranes were reisolated and washed to remove remaining [³²P]ATP by sedimentation for 5 minutes in a Beckman Airfuge at full speed. The dephosphorylation reaction was initiated by addition of 50 μL [³²P] phosphorylated vesicles to dephosphorylation buffer containing Tris-HCl 0.04 M, pH 7.5, MgCl₂ 3 mM, NaCl 0.1 M, MnCl₂ 1 mM, DTT 0.2 mM, CaCl₂ 1 mM, or EGTA 2 mM at 37°C in the presence or absence of 1 μM calmodulin and 2–15 μg calmodulin-stimulated phosphatase. At the indicated times, 25 μL aliquots of the reaction mixtures were withdrawn, suspended in 20 μL SDS gel dissociation medium, and immediately heated to 90°C for 90 seconds. The time course of dephosphorylation of specific sarcolemmal substrates was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Radioactive bands were excised from the dried gels and radioactivity determined by liquid scintillation counting.

**Results**

**Purification of Calmodulin-Stimulated Phosphatase From Bovine Myocardium**

Several lines of evidence indicated the presence of a calcineurin-like protein in heart. A calcineurin-like protein can be detected in myocardial homogenates by [¹²⁵I] calmodulin gel overlay and immunoblotting techniques. 26 Given this evidence of structural similarity, purification of a calmodulin-stimulated phosphatase from heart was undertaken by modification of methods that have proven successful for purification of the calmodulin-stimulated phosphatase, calcineurin, from bovine brain. This approach allowed comparison of the behavior of the cardiac protein with that of the brain enzyme throughout the course of purification. The heart enzyme, like calcineurin, was highly susceptible to proteolysis during the course of purification. Therefore, EGTA and protease inhibitors leupeptin, PMSF, TPCK, and trypsin inhibitor were included beginning with the homogenization step to retard proteolysis. Furthermore, following five-fold dilution, the soluble extract could be directly adsorbed to DEAE-Sephacel batchwise, avoiding concentration of contaminating proteases and eliminating dialysis time. At the early stages of purification, a calcineurin-like protein could not be clearly identified by simple inspection of a Coomassie blue stained SDS-gel. However, a 61,000–62,000 Mr calmodulin-binding polypeptide similar to the calcineurin A subunit could be identified and quantified by [¹²⁵I] calmodulin gel overlay (Figure 1). On elution of the DEAE-Sephacel column, the cardiac calmodulin-stimulated phosphatase, identified by [¹²⁵I] calmodulin gel overlay, eluted early at a conductivity of 8–14 mS similar to the brain enzyme. Assays of column fractions using [³²P] phosphorylated histone VS as a substrate revealed a Ca²⁺, calmodulin-stimulated, and calmidazolium-inhibited phosphatase activity, which eluted at 8–15 mS. However, at this stage, the Ca²⁺, calmodulin regulated component constituted less than 25% of total histone phosphatase activity eluting in this range of conductivity, and quantitative estimates of calmodulin-stimulated phosphatase activity were somewhat variable at this early step of purification (data not shown). Adsorption and elution of the DEAE-Sephacel column in the presence of EGTA allowed essentially complete resolution of the calmodulin-stimulated phosphatase from calmodulin, which eluted in fractions 60–80 (Figure 1). Thus, the DEAE column fractions containing the calmodulin-stimulated phosphatase could be pooled, brought to 0.5 mM CaCl₂, and directly loaded on calmodulin-Sepharose.

Electrophoretic analysis of the calmodulin-binding proteins eluting from the calmodulin-Sepharose column in EGTA clearly revealed two major polypeptides of mobility similar to the subunits of brain calcineurin (Figure 2). The Mr 61,000–62,000 polypeptide, which bound calmodulin on [¹²⁵I] calmodulin gel overlay, is similar to brain calcineurin A, but the slightly lower electrophoretic mobility of this polypeptide compared to brain calcineurin was apparent at this stage of purification. While these results suggest the possibility of structural differences in the large catalytic subunits, they may also reflect differences in the extent of limited proteolysis encountered during the course of purification. A small polypeptide had identical electrophoretic mobility to the Mr 19,000 Ca²⁺ binding subunit of brain calcineurin.* Like calcineurin B, this polypeptide from heart demonstrated an identical Ca²⁺-dependent in increase in electrophoretic mobility, a finding typical of small homologous intracel-
lular Ca\(^{2+}\)-binding proteins, including calmodulin and troponin C. At this step of purification, the only remaining significant contaminants detectable on the Coomassie blue stained gel were calmodulin-binding proteins of Mr 220 K, 170 K and 70 K (minor bands of approximate Mr 120 K and 140 K were also faintly visualized) (Figure 2). The remainder of the bands seen at this stage represent a series of proteolytic fragments of Mr 44,000–60,000 derived from the large subunit of the calmodulin-stimulated phosphatase. A similar series of fragments are seen in the purified calcineurin, which also undergoes similar limited proteolysis (Figure 2).

It should be noted that brain calcineurin, like the heart enzyme, is not completely purified at the calmodulin-Sepharose step, and further purification is accomplished using gel filtration on Sephadex G-200. However, some of the remaining contaminating calmodulin-binding proteins observed at this stage of purification are different from those encountered with the heart enzyme. The cardiac enzyme purification was further complicated by the fact that the amount of enzyme in heart is substantially lower than that in brain. Therefore, a new method for further purification of the heart enzyme was required.

Complete purification of the cardiac enzyme was accomplished using HPLC techniques. The two subunits of the cardiac calmodulin-stimulated phosphatase were resolved from these other proteins by high performance liquid chromatography using a Spherogel TSK DEAE 5PW column. Use of this polymer based support allows elution of the cardiac enzyme at neutral pH. The two subunits of the cardiac phosphatase (Mr 61,000 and 19,000) elute as a single symmetrical peak at 11–14 mS (22 minutes), well resolved from other protein contaminants (Figure 3). The retention time of the heart enzyme was compared to that of purified brain calcineurin and was found to be indistinguishable. The arrow in Figure 3 indicates the elution position of purified brain calcineurin. Electrophoretic analysis of the purified cardiac calmodulin-stimulated phosphatase reveals two subunits of Mr 61,000 and 19,000 (Figure 4). The small subunit of the heart enzyme exhibits a Ca\(^{2+}\)-dependent increase in mobility identical to calcineurin B (Figures 3 and 4).

**Enzyme Properties and Regulation**

The activity of the cardiac enzyme assayed using [\(^{32}\)P]-phosphorylated histone VS as a substrate was stimulated by Ca\(^{2+}\) and calmodulin, and inhibited by the calmodulin antagonist, calmidazolium (R24571) (Figure 5). The activity and pattern of regulation of the cardiac phosphatase was compared to the brain enzyme and found to be quite similar when assayed under identical conditions. It should be noted that the concentration of histone VS in these assays (1 \(\mu\)M) is below \(K_m\) for calcineurin, and measured activities do not represent \(V_{max}\). Nonetheless, under the present con-
Conditions of assay, the level of phosphatase activity of the cardiac enzyme is quite similar to that exhibited by the brain enzyme calcineurin (Figure 5). Typical of several phosphatases, calmodulin-stimulated enzymes from both heart and brain demonstrated higher activity when 1 mM Mn$^{2+}$ was present in the assay. The effects of a series of divalent cations on phosphatase activity were also tested using histone VS as a substrate. The results of these experiments comparing the cardiac and brain enzymes are shown in Figure 5. The inhibitory or stimulatory effects on activity of both the cardiac and brain phosphatases by the different cations was the same under the present assay conditions. Addition of 1 mM Mn$^{2+}$ in the presence of 1 mM Ca$^{2+}$ and calmodulin further stimulated activity above that observed in the presence of Ca$^{2+}$ and calmodulin alone. In contrast, with 0.5 mM dithiothreitol present in the assay mixture, addition of 1 mM Ni$^{2+}$ or 1 mM Co$^{2+}$ resulted in lower activity than that observed with Ca$^{2+}$ and calmodulin alone.

Identification of Cardiac Sarcolemmal Substrates of Calmodulin-Stimulated Phosphatase

In evaluating the role of calmodulin-stimulated phosphatases in muscle, attention has previously focused on the potential role of the phosphatases in linking control of glycogen metabolism to contraction, and the specificity of dephosphorylation of a number of soluble substrates has been examined. Many of the soluble phosphoproteins known to be dephosphorylated by the phosphatase at a significant rate are substrates of cAMP-dependent protein kinase. In addition, several lines of evidence suggest that the calmodulin-stimulated phosphatase has a physiologically significant role in regulating membrane function. In cardiac sarcolemma, an intrinsic cAMP-dependent protein kinase is known to be present, and cAMP-dependent phosphorylation has been implicated in regulation of several sarcolemmal transport processes. In view of this evidence, efforts to identify specific substrates for the calmodulin-stimulated phosphatase were focused on evaluation of sarcolemmal substrates phosphorylated by cAMP-dependent protein kinase.

Highly purified bovine cardiac sarcolemmal (SL) and sarcoplasmic reticulum (SR) vesicles contain several intrinsic substrates of cAMP dependent protein kinase as shown in the autoradiogram in Figure 6 (time 0, lanes 1 and 7). Several of the substrates phosphorylated in the sarcolemmal fraction are not present in purified preparations of SR. The most prominent substrates of cAMP dependent protein kinase which appear unique to sarcolemma exhibit apparent Mr 166 K, 133 K, 108 K, 79 K, 48 K, 39 K, and 14 K. This membrane localization supports the idea that these substrates participate in specific biochemical processes that are unique to sarcolemma. The most prominent substrate in SR is phospholamban, which migrates at approximately 10 K after heating in SDS gel dissociation medium as previously described.

Using highly purified sarcolemmal vesicles prepared from SL preparations, the calmodulin-stimulated phosphatase was purified through a series of high performance liquid chromatography columns. The elution profile of the calmodulin-Sepharose peak from bovine heart. The cardiac phosphatase elutes as a single symmetrical peak at 22 minutes. Purified brain calcineurin exhibits an identical retention time. The arrow indicates the elution position of brain calcineurin.

**FIGURE 3.** Spherogel TSK DEAE 5PW high performance liquid chromatography. The elution profile of the calmodulin-Sepharose peak from bovine heart. The cardiac phosphatase elutes as a single symmetrical peak at 22 minutes. Purified brain calcineurin exhibits an identical retention time. The arrow indicates the elution position of brain calcineurin.

**FIGURE 4.** SDS-polyacrylamide gel electrophoresis of the purified calmodulin-stimulated phosphatase from bovine heart. Samples of the phosphatase were brought to 2 mM Ca$^{2+}$ (lane 1) or 2 mM EGTA (lane 2) prior to electrophoresis to demonstrate the Ca$^{2+}$-dependent shift in mobility of the small subunit.
viously phosphorylated with exogenous catalytic subunit of cAMP-dependent protein kinase in the presence of [\(^{32}\)P]ATP, the specificity for dephosphorylation of sarcolemmal substrates by the calmodulin-stimulated phosphatase was examined. The time course of dephosphorylation was followed in the presence or absence of added calmodulin-stimulated phosphatase. The calmodulin-stimulated phosphatase dephosphorylated several sarcolemmal substrates of cAMP-dependent protein kinase present in the sarcolemmal fraction, as shown in the autoradiogram in Figure 6.

Figure 6. Time course of dephosphorylation of sarcolemmal substrates of cAMP-dependent protein kinase by the calmodulin-stimulated phosphatase (CSP). Sarcolemmal vesicles were phosphorylated with [\(^{32}\)P]ATP using 0.6 µg of purified catalytic subunit of cAMP-dependent protein kinase. The dephosphorylation was conducted in the presence (left panel) or absence (right panel) of CSP (15 µg) as described in the experimental procedures.

Discussion
Most of the studies of purified phosphatases indicate activity toward multiple phosphoproteins. This lack of specificity in vitro has hampered the understanding of
The calmodulin-stimulated phosphatase dephosphorylates specific substrates of cAMP-dependent protein kinase present in highly enriched bovine cardiac sarcolemmal vesicles. The fraction of \( ^{32}P \)-remaining is plotted as a function of time. Membrane vesicles, phosphorylated with cAMP-dependent protein kinase, were incubated in the absence (○) to detect endogenous phosphatase activity or presence of CSP (●), as described in Figure 6. The Ca\(^{2+}\)-calmodulin stimulation of dephosphorylation by the phosphatase was demonstrated by conducting parallel incubations in the presence of 2 mM EGTA (△) or 1 mM Ca\(^{2+}\) and 1 μM calmodulin (■).

The present results indicate that a calmodulin-stimulated protein phosphatase can be purified from bovine heart. Previously, members of this family of Ca\(^{2+}\), calmodulin-stimulated phosphatases have been successfully purified from brain and skeletal muscle, where the respective enzymes exist in substantially greater amounts than the cardiac enzyme. To accomplish the purification from heart, HPLC based ion exchange was used as the final step in the purification, taking advantage of the high resolution and excellent recovery afforded by this method to complete the purification of the phosphatase. This HPLC procedure may be generally useful in the purification of calmodulin-stimulated phosphatases from other tissues, in which the amount of the enzyme is limited.

Although the calmodulin-stimulated phosphatase can be detected in a crude myocardial homogenate by \(^{125}\)I calmodulin gel overlay, levels of \(^{125}\)I calmodulin labeling of the 61,000 Mr subunit were below the linear range of this method under the conditions employed. However, calculations based on gel overlay analysis of fractions following the initial chromatographic step, indicate that the phosphatase is present at a level of at least 2.5 mg/kg heart. Because of incomplete recoveries associated with tissue extraction as well as adsorption and elution from DEAE Sephacel, this is a minimum value.

The purified cardiac calmodulin-stimulated phosphatase is a heterodimer consisting of Mr 61,000 calmodulin-binding subunit and a small subunit that exhibits a Ca\(^{2+}\)-dependent change in electrophoretic mobility identical to calcineurin. Previous studies of brain calcineurin have revealed that the large subunit of the enzyme, calcineurin A, contains not only the catalytic domain, but also distinct binding sites for calmodulin and the Mr 19,000 Ca\(^{2+}\)-binding subunit, calcineurin B. The calmodulin-binding domain is quite sensitive to proteolysis, and tryptic digestion of calcineurin generates an Mr 44,000 fragment of calcineurin A, which has lost the ability to bind calmodulin. This fragment retains the ability to bind calcineurin B, and the complex retains catalytic activity, suggesting that the Mr 44,000 fragment contains the catalytic site as well as the binding site for calcineurin B. A similar pattern of limited proteolysis is observed during purification, and small amounts of proteolytic fragments can be detected by SDS-polyacrylamide gel electrophoresis of both the cardiac and brain enzymes (Figure 2). Electrophoretic analysis of several preparations of the cardiac enzyme demonstrates the presence of a small subunit indistinguishable from brain calcineurin B. Although the large subunit typically exhibited a Mr of 61,000 (as in Figure 4), similar to calcineurin A, slight variations in the mobility of the large subunit were observed from preparation to preparation. Thus, although it is tempting to suggest tissue specific differences between the heart and brain enzymes, differing amounts of limited proteolysis during the course of purification could also account for small differences in mobility of the large subunit of the cardiac enzyme observed on electrophoresis of some preparations.

Results of the present study support the role of Ca\(^{2+}\)-calmodulin in regulating the cardiac phosphatase. The phosphatase activity of the cardiac enzyme has been demonstrated using \(^{32}\)P phosphorylated histone VS as a substrate. The specific activity and level of calmodulin-stimulated dephosphorylation of \(^{32}\)P-histones by the cardiac enzyme appears to be similar to purified brain calcineurin assayed under identical conditions. Similarly, effects of divalent cations on phosphatase
activity appear similar for the cardiac and brain enzymes. Conflicting results have been reported regarding the effect of specific divalent cations on the activity of the Ca$^{2+}$-calmodulin–stimulated phosphatases. Several investigators have reported that Ni$^{2+}$ activates calcineurin either in the presence or absence of calmodulin. Under the present experimental conditions, the cardiac enzyme was inhibited by Ni$^{2+}$ and Co$^{2+}$. Since cation effects on phosphatase activity appear to depend on pH, substrate, the presence of dithiothreitol, and possibly even the method of phosphatase preparation, we performed comparisons of enzyme activity for the cardiac and brain phosphatases under identical conditions. When assayed using histone VS at pH 7.5, in the presence of 0.5 mM dithiothreitol, Ni$^{2+}$ was noted also to inhibit enzyme activity of calcineurin. These results emphasize the need for direct comparisons of these enzymes when attempting to draw conclusions regarding their comparative regulation.

The use of cardiac muscle for these studies has proven to be a very useful approach. Previous studies have used the substrate specificity of protein phosphatases in vitro in attempt to provide clues regarding the role of phosphatases in physiological regulation. In the case of the calmodulin-stimulated phosphatases, a somewhat restricted substrate specificity has been demonstrated using a series of soluble proteins as substrates. However, attempts to identify distinct amino acid sequences common to phosphoprotein substrates of this or other phosphatases have not provided a conclusive basis for the observed specificity. As a result, the potential importance of subcellular compartmentalization in defining the physiological specificity of the phosphatases has been suggested.

Several lines of evidence suggest involvement of calmodulin-stimulated phosphatases in membrane interaction and membrane regulation. However, attention to the activity of the calmodulin–stimulated phosphatase in dephosphorylation of membrane phosphoproteins has not been previously examined. Extensive studies detailing the purification and characterization of cardiac sarcolemmal and SR membranes has enabled us to focus the present studies on specific membrane phosphoproteins. In view of the role in calcium transport, cardiac sarcolemmal membranes were chosen for the initial experiments to ascertain whether specific substrates for the cardiac Ca$^{2+}$-calmodulin-stimulated phosphatase exist. Further specificity was obtained by limiting these studies to sarcolemmal substrates phosphorylated only by cAMP-dependent protein kinase. Attempts to identify specific phosphoproteins in crude homogenates are often limited or unsuccessful due to the multiple kinases and extensive numbers of phosphoproteins found on SDS polyacrylamide gels of these homogenates. However, by using the present approach, specific substrates of cAMP-dependent protein kinase that are dephosphorylated by the calmodulin-stimulated phosphatase have been identified in highly enriched preparations of bovine cardiac sarcolemma. It is significant that the rate constants of dephosphorylation of the sarcolemmal phosphoproteins by the calmodulin-stimulated phosphatase vary widely. This evidence of specificity is observed despite the fact that all phosphoproteins are present in the sarcolemmal fraction, and all have been phosphorylated by cAMP-dependent protein kinase. Thus, it is clear that the substrate specificity of the calmodulin-stimulated phosphatase involves factors other than subcellular compartmentalization and amino acid sequences around the phosphorylated site.

Attention can now be focused on specific substrates. Figure 6 is an autoradiogram, and the intensity of the individual bands does not indicate the actual quantity of these proteins in the membrane fractions. The actual identification of these substrates remains to be determined. At the present time, the definite identification of one of the proteins as a putative Na$^{+}$-Ca$^{2+}$ exchanger is not possible. However, based on our knowledge of the proposed exchanger, several of the observed substrates are potential candidates. Available evidence suggests that reconstitution of Na$^{+}$-Ca$^{2+}$ exchange activity in heart correlates with either a 82,000 Mr$^{24}$ or 33,000 Mr$^{28}$ membrane protein. We have found specific substrates of the Ca$^{2+}$-calmodulin–stimulated phosphatase in sarcolemmal membranes, which may correspond to either of these species. Additional data regarding the structure of the Na$^{+}$-Ca$^{2+}$ exchanger will be required for improved understanding of this highly significant mechanism of regulation. However, the present results have identified a number of potentially important substrates of the calmodulin-stimulated phosphatase in sarcolemma. Identification of these substrates is needed to fully elucidate the biochemical mechanisms involved in Ca$^{2+}$-regulation of cardiac sarcolemmal function.

**Addendum.** Since this manuscript was submitted, a report of the large-scale purification of calmodulin-stimulated phosphatase from heart has appeared.

**References**


**KEY WORDS** • phosphatase • calmodulin • calcineurin • calcium • heart • sarcolemma
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