Reversible Inhibition of the Calcium-pumping ATPase in Native Cardiac Sarcoplasmic Reticulum by a Calmodulin-binding Peptide

EVIDENCE FOR CALMODULIN-DEPENDENT REGULATION OF THE $V_{\text{max}}$ OF CALCIUM TRANSPORT

(Received for publication, August 2, 1999, and in revised form, October 28, 1999)

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Calmodulin (CaM) and Ca$^{2+}$/CaM-dependent protein kinase II (CaM kinase) are tightly associated with cardiac sarcoplasmic reticulum (SR) and are implicated in the regulation of transmembrane Ca$^{2+}$ cycling. In order to assess the importance of membrane-associated CaM in modulating the Ca$^{2+}$ pump (Ca$^{2+}$-ATPase) function of SR, the present study investigated the effects of a synthetic, high affinity CaM-binding peptide (CaM BP; amino acid sequence, LKWKLLKLLKLLKLG) on the ATP-energized Ca$^{2+}$ uptake, Ca$^{2+}$-stimulated ATP hydrolysis, and CaM kinase-mediated protein phosphorylation in rabbit cardiac SR vesicles. The results revealed a strong concentration-dependent inhibitory action of exogenous CaM (1–3 μM) on Ca$^{2+}$-ATPase activities of SR (50% inhibition at −2–3 μM CaM BP). The inhibition, which followed the association of CaM BP with its SR target(s), was of rapid onset (manifested within 30 s) and was accompanied by a decrease in $V_{\text{max}}$ of Ca$^{2+}$ uptake, unaltered $K_{\text{m}}$ for Ca$^{2+}$ activation of Ca$^{2+}$ transport, and a 10-fold decrease in the apparent affinity of the Ca$^{2+}$-ATPase for its substrate, ATP. Thus, the mechanism of inhibition involved alterations at the catalytic site but not the Ca$^{2+}$-binding sites of the Ca$^{2+}$-ATPase. Endogenous CaM kinase-mediated phosphorylation of Ca$^{2+}$-ATPase, phospholamban, and ryanodine receptor-Ca$^{2+}$ release channel was also strongly inhibited by CaM BP. The inhibitory action of CaM BP on SR Ca$^{2+}$ pump function and protein phosphorylation was fully reversed by exogenous CaM (1–3 μM). A peptide inhibitor of CaM kinase markedly attenuated the ability of CaM to reverse CaM BP-mediated inhibition of Ca$^{2+}$ transport. These findings suggest a critical role for membrane-bound CaM in controlling the velocity of Ca$^{2+}$ pumping in native cardiac SR. Consistent with its ability to inhibit SR Ca$^{2+}$ pump function, CaM BP (1–2.5 μM) caused marked depression of contractility and diastolic dysfunction in isolated perfused, spontaneously beating rabbit heart preparations. Full or partial recovery of contractile function occurred gradually following withdrawal of CaM BP from the perfusate, presumably due to slow dissociation of CaM BP from its target sites promoted by endogenous cytosolic CaM.

By regulating cytosolic Ca$^{2+}$ concentration, the sarcoplasmic reticulum (SR) plays a central role in the contraction-relaxation cycle of heart muscle. Upon excitation of the cardiomyocyte, Ca$^{2+}$ is released from the SR through Ca$^{2+}$-release channels (known as RYR-CRC) to initiate muscle contraction (1–5). Subsequent muscle relaxation occurs upon sequestration of Ca$^{2+}$ back into the SR lumen by a Ca$^{2+}$-pumping ATPase (Ca$^{2+}$-ATPase) present in the SR (1, 4, 6, 7). A well known mechanism for the regulation of the cardiac SR Ca$^{2+}$-ATPase involves phosphorylation of another intrinsic SR protein, phospholamban (8–11). In its unphosphorylated state, phospholamban is thought to interact with the Ca$^{2+}$-ATPase exerting an inhibitory effect; phosphorylation of phospholamban by CaM kinase disrupts this interaction resulting in stimulation of Ca$^{2+}$ pump activity (8–11). In cardiac SR, the RYR-CRC also undergoes phosphorylation by CaM kinase (12–14), and this may result in stimulation of Ca$^{2+}$ release from the SR (12, 15–17).

Recent studies from this laboratory (14, 18–22) and other laboratories (23–26) have demonstrated that in cardiac SR, a membrane-associated CaM kinase phosphorylates the Ca$^{2+}$-ATPase in addition to RYR-CRC and phospholamban. The phosphorylation occurred at a serine residue and was specific for the cardiac/slow-twitch muscle isoform (SERCA2a) of the Ca$^{2+}$-ATPase (18). Site-directed mutagenesis studies by Toyofuku et al. (23) resulted in the identification of Ser$^{38}$ as the site in SERCA2a that is phosphorylated by CaM kinase. Studies using native cardiac SR vesicles (14), purified SR Ca$^{2+}$-ATPase preparations (14, 18), and SERCA2a expressed in HEK-293 cells (23) suggested that Ser$^{38}$ phosphorylation of the Ca$^{2+}$-ATPase results in activation of the $V_{\text{max}}$ of Ca$^{2+}$ transport. Some studies have, however, questioned the physiological role of Ca$^{2+}$-ATPase phosphorylation. Thus, a study by Odermatt et al. (24) showed CaM kinase-mediated phosphorylation of the Ca$^{2+}$-ATPase in native rabbit cardiac SR as well as SERCA2a expressed in HEK-293 cells but failed to observe a significant stimulatory effect of phosphorylation on Ca$^{2+}$-ATPase function. Another study by Reddy et al. (27) reported failure to observe phosphorylation of the Ca$^{2+}$-ATPase in canine cardiac SR or purified Ca$^{2+}$-ATPase reconstituted in lipid vesicles. These studies have attributed the stimulatory effect of CaM kinase to the phosphorylation of phospholamban and a consequent increase in Ca$^{2+}$ affinity of the Ca$^{2+}$-ATPase. In native cardiac SR, analysis of the selective effects of Ca$^{2+}$-ATPase phosphorylation on Ca$^{2+}$-pumping activity of this enzyme is hampered by the concomitant phosphorylation of phospholamban and RYR-CRC by the membrane-bound CaM kinase. Recently, we achieved selective phosphorylation of the Ca$^{2+}$-ATPase by treating SR vesicles with a specific Ca$^{2+}$-ATPase inhibitor and using CaM kinase II, CaM BP, and CaM kinase II as substrates for CaM kinase.
ATPase by the SR-associated CaM kinase by utilizing a phospholamban monoclonal antibody, which inhibits phospholamban phosphorylation, and the RYR-CRC blocking drug, ruthenium red, which was found to inhibit RYR-CRC phosphorylation (22). Under these conditions, Ca\(^{2+}\)-ATPase phosphorylation by endogenous CaM kinase resulted in enhanced \(V_{\text{max}}\) of Ca\(^{2+}\) transport (22). During the course of these studies we have found that, in addition to the endogenous CaM kinase, SR vesicles isolated from cardiac muscle contains significant amount of calmodulin that is resistant to extraction with high salt (0.6 M KCl). The presence of calmodulin in isolated SR vesicles may mask the true potential of calmodulin-dependent regulation of SR function in \textit{in vitro} experiments. For example, since both calmodulin and CaM kinase are structured in the same way, these proteins could contribute to the overall activity of CaM kinase. These findings imply that a calmodulin-dependent process of Ca\(^{2+}\) transport would also result in concurrent activation of CaM kinase and other Ca\(^{2+}\)/calmodulin-dependent membrane events. This issue assumes a higher level of complexity given that CaM kinase, once activated, undergoes autophosphorylation and retains activity independently of Ca\(^{2+}\)/calmodulin (28). In the present study, we utilized a previously characterized amphiphilic, high affinity calmodulin-binding peptide (29) to unmask the potential influence of SR-associated calmodulin on cardiac SR Ca\(^{2+}\)-ATPase function. The results presented here demonstrate a strong inhibitory action of CaM BP on the Ca\(^{2+}\) ion-transporting as well as energy-transducing functions of the Ca\(^{2+}\)-ATPase. This inhibition stems from the association of CaM BP with SR membrane target(s) and is readily reversed by calmodulin. These findings imply that a calmodulin-dependent process controls the velocity of Ca\(^{2+}\) pumping in native cardiac SR.

**EXPERIMENTAL PROCEDURES**

**Materials**—
4CaCl\(_2\) was purchased from NEN Life Science Products, and \(\gamma\)-\[^{32}\text{P}\]\textit{ATP} was from Amersham Pharmacia Biotech. Reagents for electrophoresis were obtained from Bio-Rad. Monoclonal antibody against calmodulin was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). All other chemicals were from Sigma.

**Synthesis and Purification of Peptides**—A 17-amino acid high affinity calmodulin-binding peptide (designated CaM BP in this report), designed and characterized previously by DeGrado et al. (29), and three fragments of this peptide with overlapping residues were synthesized by the University of Victoria Protein Micro-chemistry Center using a model 430A Applied Biosystems peptide synthesizer. The C termini of the peptides were amidated. All peptides were purified by high performance liquid chromatography, analyzed by mass spectrometry, and sequenced on Applied Biosystems model 473A protein sequencer. The sequences included the following: CaM BP, LKWKLLKLKKLLKKLG; fragment A, LKWKLL; fragment B, LKLLKK; and fragment C, KLLKLG.

**Preparation of SR Vesicles**—SR membrane vesicles were prepared from heart ventricles and fast-twitch (adductor magnus) skeletal muscle of New Zealand White rabbits (body weight 2.5–3 kg) as described previously (30). Following isolation, the SR vesicles were suspended in 10 mM Tris maleate (pH 6.8) containing 100 mM KCl and stored at −80 °C after quick-freezing in liquid N\(_2\). Protein concentration was determined by the method of Lowry et al. (31) using BSA as standard.

**Ca\(^{2+}\) Transport and Ca\(^{2+}\)-ATPase Assays**—ATP-dependent, oxalate-facilitated Ca\(^{2+}\) uptake by SR was determined using a Millipore filtration technique as described previously (32). The standard incubation medium for Ca\(^{2+}\) uptake (total volume 250 \(\mu\)l) contained 50 mM HEPES (pH 7.2), 5 mM MgCl\(_2\), 5 mM Na\(_2\)ATP, 200 mM KCl, 0.1 mM EDTA, 5 mM potassium oxalate, 5 mM ATP, 0.1 mM \[^{45}\text{Ca}\]Cl\(_2\) (−8000 cpm/nmol, free Ca\(^{2+}\) 7.5 \(\mu\)M), 25 \(\mu\)M ruthenium red, and SR (6 \(\mu\)g of protein). In experiments where Ca\(^{2+}\) concentration dependence was studied, the EGTA concentration in the assay medium was held at 0.1 mM, and the amount of total \[^{45}\text{Ca}\]Cl\(_2\) added was varied in the range 1 to 200 \(\mu\)l to yield the desired free Ca\(^{2+}\). Modifications to the standard incubation medium are specified in the figure legends. Unless indicated otherwise, all assays were carried out at 37 °C; the Ca\(^{2+}\) transport reaction was initiated by the addition of SR vesicles after preincubiation of the rest of the assay components for 3 min. The initial free Ca\(^{2+}\) concentrations in the assay medium were determined using the computer program of Fabiato (33). The data on Ca\(^{2+}\) concentration dependence on Ca\(^{2+}\) uptake were analyzed by nonlinear regression curve fitting using SigmaPlot scientific graph program (Jandel Scientific) run on an IBM-PC computer. The data were fit to Equation 1,

\[
\nu = V_{\text{max}} \left[\text{Ca}^{2+}\right] / K_{\text{Ca}} + \left[\text{Ca}^{2+}\right] \tag{1}
\]

where \(\nu\) is the measured Ca\(^{2+}\) uptake activity at a given Ca\(^{2+}\) concentration; \(V_{\text{max}}\) is the maximum activity reached; \(K_{\text{Ca}}\) is the Ca\(^{2+}\) concentration giving half of \(V_{\text{max}}\), and \(n\) is the equivalent to the Hill coefficient.

**Western Immunoblotting**—Western blotting analysis of endogenous calmodulin in SR vesicles was performed using a monoclonal antibody specific for calmodulin (35). SR proteins were fractionated on SDS-polyacrylamide (15%, homogenous) mini-gels and then electrophoetolotted to nitrocellulose sheets. The sheets were incubated in 0.2% glutaraldehyde in PBS for 45 min at 24 °C and then rinsed in PBS. Nonspecific binding sites were blocked with 2% BSA, 0.1% gelatin in PBS for 60 min at 37 °C. Following three 15-min washes with 0.05% Tween 20 in PBS, the sheets were incubated with anti-calmodulin monoclonal antibody (0.5 \(\mu\)g/ml in PBS) for 60 min at 37 °C and then with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (dilution 1:1000). After five 10-min washes in PBS/Tween, the sheets were rinsed with deionized water, and the immunoreactive peptide band representing calmodulin was visualized following color development using a Bio-Rad assay kit.

**Phosphorylation Assay**—Endogenous CaM kinase-catalyzed SR protein phosphorylation was measured as described previously (18). The standard incubation medium (total volume 50 \(\mu\)l) for phosphorylation by endogenous CaM kinase contained 50 mM HEPES (pH 7.4), 10 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 0.1 mM EGTA, 1 \(\mu\)M calmodulin, 0.8 mM \(\gamma\)-\[^{32}\text{P}\]\textit{ATP} (specific activity, 300–400 cpm/pmol), and SR (30 \(\mu\)g of protein). The phosphorylation reaction was initiated by the addition of SR after preincubation of the rest of the assay components for 3 min at 37 °C. The reaction was terminated after 2 min by the addition of 15 \(\mu\)l of SDS sample buffer, and the samples were analyzed in 4–18% SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed. Quantification of phosphorylation was carried out by liquid scintillation counting after excision of the radioactive bands from the gels (18).

**Heart Perfusion and Measurement of Contractile Function**—Rabbits were anesthetized with sodium pentobarbital (35 mg/kg, intravenously), and the hearts were excised and immediately cannulated for retrograde aortic perfusion of the coronary arteries with a modified Langendorf solution consisting of 154 mM NaCl, 5 mM KCl, 2.2 mM CaCl\(_2\), 6 mM NaHCO\(_3\), and 5.5 mM dextrose. The perfusion buffer was equilibrated with 95% O\(_2\), 5% CO\(_2\), which maintained a pH of 7.4; the perfusion temperature was set at 37 ± 0.2 °C. The hearts were perfused at a constant flow rate of 25 ml/min using a peristaltic pump. After an initial 15–20 min of perfusion, when the spontaneous beating had stabilized, a latex balloon-tipped cannula filled with degassed H\(_2\)O was inserted into the lumen of the left ventricle for obtaining systolic left ventricular pressure development. The cannula was connected via a pressure transducer (COBE, Bramalea, Canada) to a Biopac System Digital Monitor (model MP100) and a personal computer that allowed on-line monitoring of left ventricular pressure and offline calculation of developed pressure, rate of pressure development (+dP/dt), and rate of relaxation (−dP/dt).

**Data Presentation**—Unless specified otherwise, the experimental values represent the average of at least three independent experiments using separate SR preparations performed in duplicate. The data are presented as mean ± S.E.

**RESULTS**

**Effects of Varying Concentrations of CaM BP and Its Fragments on ATP-dependent Ca\(^{2+}\) Uptake by Cardiac SR in the**
**Fig. 1. Effects of varying concentrations of CaM BP and its fragments on ATP-dependent Ca\(^{2+}\) uptake by cardiac SR in the absence and presence of calmodulin.** The Ca\(^{2+}\) uptake reaction was carried out for 2 min in the standard assay medium (see "Experimental Procedures") with 6 \(\mu\)g of SR protein in the assay (A and inset in B) or with varying amounts of SR protein in the assay (B). In the experiments shown in A, Ca\(^{2+}\) uptake was determined in the absence of CaM BP and in the presence of varying concentrations of CaM BP without calmodulin in the assay (O) and with 5 \(\mu\)M calmodulin in the assay (C). In the experiments shown in the inset in A, Ca\(^{2+}\) uptake was determined in the absence of CaM BP and in the presence of varying concentrations of CaM BP (O) or CaM BP fragments (\(\triangle\), CaM BP fragment A; \(\bullet\), CaM BP fragment B; \(\bullet\), CaM BP fragment C). In the experiments shown in B, Ca\(^{2+}\) uptake was determined in the absence of CaM BP and in the presence of varying concentrations of CaM BP with differing amounts of SR protein (O, 3 \(\mu\)g; \(\bigcirc\), 6 \(\mu\)g; \(\triangle\), 9 \(\mu\)g; \(\bullet\), 15 \(\mu\)g; \(\bigcirc\), 20 \(\mu\)g) in the assay. Each data point in A represents the mean ± S.E. of four experiments using separate SR preparations. Each data point in B and the inset in A represent the average value from duplicate determinations using a single SR preparation.

**Absence and Presence of Calmodulin—** The ATP-dependent, oxalate-facilitated Ca\(^{2+}\) uptake by SR vesicles is a useful, commonly used parameter to measure the Ca\(^{2+}\)-pump (Ca\(^{2+}\)-ATPase) function of SR in vitro (6). The results presented in Fig. 1 demonstrate the effects of varying concentrations of CaM BP and its fragments on ATP-dependent Ca\(^{2+}\) uptake by cardiac SR vesicles measured in the absence and presence of calmodulin in the assay medium. When assays were performed in the absence of calmodulin, CaM BP caused strong, concentration-dependent inhibition of Ca\(^{2+}\) uptake by SR with virtually complete inhibition occurring at <5 \(\mu\)M CaM BP (Fig. 1A). Fragmented molecules of CaM BP (CaM BP fragments A–C) failed to inhibit Ca\(^{2+}\) uptake by SR (Fig. 1A, inset). Addition of low micromolar concentrations of calmodulin to the assay medium prevented the inhibitory action of CaM BP on Ca\(^{2+}\) uptake by SR, in a concentration-dependent manner, and caused appreciable stimulation (40–55%) of Ca\(^{2+}\) uptake (Fig. 1, A and B, inset). The results presented in Fig. 1A and the inset in Fig. 1B were obtained under the standard Ca\(^{2+}\) uptake assay conditions with 6 \(\mu\)g of SR protein in the assay medium (see under "Experimental Procedures"). In additional experiments, the effect of CaM BP on Ca\(^{2+}\) uptake by SR was determined with varying amounts of SR in the assay medium. The results presented in Fig. 1B show that the concentration dependence curve for CaM BP inhibition of Ca\(^{2+}\) uptake is progressively shifted to the right with increasing concentration of SR in the assay. These findings suggest that the inhibitory action of CaM BP stems from its apparently stoichiometric interaction with one or more targets in the SR, and such interaction is prevented by calmodulin.

**Effect of CaM BP on Cardiac SR Ca\(^{2+}\)-ATPase Activity—** Since CaM BP inhibited ATP-dependent Ca\(^{2+}\) uptake by cardiac SR, the effect of CaM BP on Ca\(^{2+}\)-ATPase activity (ATP hydrolysis) was investigated. The results presented in Fig. 2 show that, under the assay conditions identical to that used for Ca\(^{2+}\) uptake, CaM BP caused concentration-dependent inhibition of Ca\(^{2+}\)-stimulated ATPase activity. The inhibition of Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\) uptake by CaM BP occurred at similar concentration range with only a minor difference in \(K_i\) values for Ca\(^{2+}\) uptake (50% inhibition at ~2 \(\mu\)M CaM BP) and Ca\(^{2+}\)-ATPase activity (50% inhibition at ~2.8 \(\mu\)M CaM BP) (Fig. 2, inset). Thus the observed reduction in Ca\(^{2+}\) uptake is mainly a consequence of a primary inhibition of ATPase activity by CaM BP. Addition of calmodulin (3 \(\mu\)M) to the assay medium reversed the inhibitory effect of CaM BP on Ca\(^{2+}\)-ATPase activity (Fig. 2).

**Effect of CaM BP on the Time Course of Ca\(^{2+}\) Uptake by Cardiac SR—** Fig. 3A shows the time course of ATP-dependent Ca\(^{2+}\) uptake by cardiac SR measured in the absence of CaM BP and in the presence of two selected concentrations of CaM BP (2 and 4 \(\mu\)M) with or without calmodulin. The rates of Ca\(^{2+}\) uptake by SR is strongly inhibited by CaM BP; the inhibition was of rapid onset (manifested within 30 s) and the degree of inhibition increased with increasing concentration of CaM BP. Addition of calmodulin (3 \(\mu\)M) to the assay medium reversed the inhibitory action of CaM BP.

In the experiments described thus far, the effect of CaM BP was assessed by adding this peptide directly to the Ca\(^{2+}\) uptake assay medium. In order to determine whether the inhibitory action of CaM BP results from its association with SR membrane target(s), in subsequent experiments, the time course of Ca\(^{2+}\) uptake was measured using CaM BP-pretreated and control SR vesicles obtained as follows. Cardiac SR vesicles were incubated with 5 \(\mu\)M CaM BP in the absence of calmodulin and in the presence of 3 \(\mu\)M calmodulin for 10 min at 24 °C. Subsequently, the SR vesicles were recovered by centrifuga-
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![Graph showing concentration-dependent inhibitory action of CaM BP on Ca\textsuperscript{2+}-ATPase activity of cardiac SR and correlation between inhibition of Ca\textsuperscript{2+} uptake and ATP hydrolysis.](image)

**Fig. 2.** Concentration-dependent inhibitory action of CaM BP on Ca\textsuperscript{2+}-ATPase activity of cardiac SR and correlation between inhibition of Ca\textsuperscript{2+} uptake and ATP hydrolysis. The Ca\textsuperscript{2+}-ATPase and Ca\textsuperscript{2+} uptake reactions were carried out for 2 min in the standard assay medium (see “Experimental Procedures”). The main figure shows the effects of varying concentrations of CaM BP on Ca\textsuperscript{2+}-ATPase activity measured in the absence of calmodulin (○) and in the presence of 3 μM calmodulin (□) in the assay medium; each data point represents mean ± S.E. of four experiments using separate SR preparations. In the experiments shown in the inset, the effects of varying concentrations of CaM BP on Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+}-ATPase activities were determined using the same SR preparation; the results are presented as percent inhibition of Ca\textsuperscript{2+} uptake or Ca\textsuperscript{2+}-ATPase activity as a function of CaM BP concentration in the assay medium.

![Graph showing effect of CaM BP on the time course of Ca\textsuperscript{2+} uptake by cardiac SR.](image)

**Fig. 3.** Effect of CaM BP on the time course of Ca\textsuperscript{2+} uptake by cardiac SR. The Ca\textsuperscript{2+} uptake reaction was carried out in the standard assay medium (see “Experimental Procedures”) for various time intervals as indicated. In the experiments shown in A, the time course of Ca\textsuperscript{2+} uptake was measured in the absence of CaM BP (○), in the presence of CaM BP without calmodulin (○), 2 μM CaM BP □, 4 μM CaM BP ▲, and in the presence of CaM BP plus calmodulin (△, 4 μM CaM BP plus 3 μM calmodulin). In the experiments shown in B, the time course of Ca\textsuperscript{2+} uptake was measured using control SR (○), and CaM BP-pretreated SR without calmodulin (□) or with 3 μM calmodulin (■) in the Ca\textsuperscript{2+} uptake assay medium. In the experiments shown in C, the time course of Ca\textsuperscript{2+} uptake was measured using control SR (○) and SR pretreated with CaM BP alone (□) or CaM BP plus calmodulin (△). CaM BP-pretreated SR was obtained by incubating SR vesicles (250 μg of protein) in buffer A (total volume 600 μl) containing 50 mM HEPES (pH 7.2), 5 mM MgCl\textsubscript{2}, 5 mM Na\textsubscript{2}SO\textsubscript{4}, 120 mM KCl, 0.1 mM EGTA, 0.1 mM CaCl\textsubscript{2}, 5 mM ATP, and 5 μM CaM BP, in the absence or in the presence of 3 μM calmodulin, for 10 min at 24 °C. The SR vesicles were then recovered by centrifugation, washed twice with buffer B (10 mM Tris maleate, 100 mM KCl (pH 6.8)), resuspended in the same buffer, and used for Ca\textsuperscript{2+} uptake assays. SR vesicles subjected to the same experimental protocol but without CaM BP in buffer A served as the control for these experiments. Each data point in A and B represents mean ± S.E. of three experiments using separate SR preparations. Each data point in C represents the average of duplicate determinations using a single SR preparation.
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effects by interfering with calmodulin-dependent processes that are normally involved in the control of Ca\(^{2+}\) sequestering and Ca\(^{2+}\) release functions of the SR.

**Effect of Calmodulin BP on Ca\(^{2+}\) Uptake by Cardiac SR at Varying Concentrations of Ca\(^{2+}\) and ATP**—The results presented in Fig. 5 show the effect of two selected concentrations of CaM BP (1.5 and 3 \(\mu M\)) on Ca\(^{2+}\) uptake by cardiac SR at a wide range of Ca\(^{2+}\) concentrations (9 nM to 67 \(\mu M\)). CaM BP inhibited Ca\(^{2+}\) uptake at all Ca\(^{2+}\) concentrations tested. At the submaximally effective concentrations of CaM BP used, the inhibitory effect could not be overcome with increasing Ca\(^{2+}\) concentration. On the other hand, addition of calmodulin (3 \(\mu M\)) to the assay medium fully reversed the inhibitory effect of CaM BP. The kinetic parameters derived from the data shown in Fig. 5 are summarized in Table I. It can be seen that the inhibitory action of CaM BP is associated with a decrement in \(V_{\text{max}}\) without appreciable changes in the apparent affinity of the Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) or the Hill coefficient \((n_\text{H})\) for Ca\(^{2+}\).

As shown in Fig. 6, CaM BP (1.5 and 3 \(\mu M\)) inhibited Ca\(^{2+}\) uptake by cardiac SR at various ATP concentrations, and calmodulin (3 \(\mu M\)) reversed the inhibitory effect. Kinetic parameters derived from the data showed that CaM BP inhibition is associated with decrements in \(V_{\text{max}}\) (\(V_{\text{max}}\) (nmol Ca\(^{2+}\)/mg protein/min): control, 500 ± 83; +1.5 \(\mu M\) CaM BP, 387 ± 56; +3 \(\mu M\) CaM BP, 273 ± 30; +3 \(\mu M\) CaM BP and 3 \(\mu M\) calmodulin, 572 ± 72) and the apparent affinity of the Ca\(^{2+}\)-ATPase for ATP (\(K_{\text{app}}\) for ATP (mM): control, 0.285 ± 0.02; +1.5 \(\mu M\) CaM BP, 0.557 ± 0.09; +3 \(\mu M\) CaM BP, 2.87 ± 0.23; +3 \(\mu M\) CaM BP and 3 \(\mu M\) calmodulin, 0.274 ± 0.03).

Reversible Association of Calmodulin BP with Cardiac SR and the Presence of Endogenous Calmodulin in Cardiac SR—As described earlier, pretreatment of cardiac SR with CaM BP (in the absence but not in the presence of calmodulin) resulted in diminished rates of Ca\(^{2+}\) uptake suggesting that the inhibitory action of CaM BP is dependent on its association with the SR membrane (cf. Fig. 3, B and C). In order to visualize the physical association of CaM BP with SR, experiments were performed in which cardiac SR vesicles were pretreated with CaM BP in the absence and presence of calmodulin and then the SR proteins were fractionated by electrophoresis on SDS-polyacrylamide (4–18% linear gradient) gels. In these experiments, the electrophoresis was terminated when the dye front had reached about 1 cm above the bottom of the gel so that the low molecular weight peptide (CaM BP molecular mass 2062 daltons) could be retained on the gel matrix. The protein profiles in Coomassie Blue-stained gels from these experiments showed association of CaM BP with the SR membrane (Fig. 7A). These findings clearly demonstrate that CaM BP associates with the SR, and calmodulin prevents and reverses this association.

Western immunoblotting analysis using a monoclonal antibody specific for calmodulin (35) showed considerable amount of endogenous calmodulin in the isolated cardiac SR vesicles (Fig. 7B). Since the procedure used for the isolation of SR vesicles involved extraction of the membranes with high salt (0.6 M KCl, cf. Ref. 30), it appears that the endogenous calmod-

**TABLE I**

| Parameter                        | Control          | 1.5 \(\mu M\) CaM BP | 3 \(\mu M\) CaM BP | 3 \(\mu M\) CaM BP + 3 \(\mu M\) calmodulin |
|----------------------------------|------------------|------------------------|---------------------|------------------------------------------|
| \(V_{\text{max}}\) (nmol Ca\(^{2+}\)/mg protein/min) | 489 ± 85         | 362 ± 68               | 255 ± 46            | 616 ± 76                                 |
| \(K_{\text{app}}\) for Ca\(^{2+}\) (\(\mu M\)) | 0.62 ± 0.07      | 0.55 ± 0.08            | 0.54 ± 0.04         | 0.91 ± 0.10                              |
| Hill coefficient \((n_\text{H})\) | 1.40 ± 0.04      | 1.47 ± 0.13            | 1.35 ± 0.05         | 1.20 ± 0.02                              |
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Reversible Inhibition of Endogenous CaM Kinase-mediated Cardiac SR Protein Phosphorylation by CaM BP—Activation of SR-associated 9CaM kinase by calmodulin and consequent phosphorylation of phospholamban, Ca2+-ATPase, and RYR-CRC are thought to regulate both the Ca2+ uptake and release functions of the SR (8–17, 22). In view of this, experiments were performed to determine the effects of CaM BP on endogenous CaM kinase-mediated SR protein phosphorylation. The results presented in Fig. 8 demonstrate that CaM BP causes concentration-dependent inhibition of phosphorylation of phospholamban, Ca2+-ATPase, and RYR-CRC; this inhibition is reversed by increasing the concentration of calmodulin in the phosphorylation assay medium. Thus, CaM kinase, a major calmodulin target in the SR, is inhibited by CaM BP.2 The inhibitory effects of CaM BP on SR protein phosphorylation and SR Ca2+ pump function are manifested at the same concentration range of CaM BP (e.g., see Fig. 1 and Fig. 8).

Relationship between the Inhibitory Effects of CaM BP on Cardiac SR CaM Kinase and SR Ca2+ Uptake—In additional experiments, we utilized a synthetic CaM kinase inhibitor peptide (corresponding to amino acid residues 290–309 of CaM kinase II, cf. Ref. 28) to investigate the potential relationship between the inhibitory effects of CaM BP on cardiac SR CaM kinase and SR Ca2+ uptake. In these experiments, the effects of varying concentrations of CaM kinase inhibitor peptide on Ca2+ uptake by SR was determined in the absence and presence of CaM BP and/or calmodulin in the assay medium. The results are summarized in Fig. 9. CaM kinase inhibitor peptide abolished the stimulatory effect of calmodulin on Ca2+ uptake by SR but did not affect the basal Ca2+ uptake measured in the absence of calmodulin. Interestingly, the ability of calmodulin to reverse the inhibitory effect of CaM BP on Ca2+ uptake by SR was markedly attenuated by the CaM kinase inhibitor peptide. These findings indicate that reversal of the inhibitory effect of CaM BP by calmodulin is dependent, at least in part, on CaM kinase activation.

Effects of CaM BP on Ca2+-ATPase and Ca2+ Uptake Activities of Fast-twist Skeletal Muscle SR—It has been demonstrated previously that the Ca2+-ATPase in cardiac SR, but not fast-twist skeletal muscle SR, undergoes phosphorylation by endogenous and exogenous CaM kinase (18). Therefore, it was of interest to examine whether CaM BP influenced the Ca2+-ATPase activity and Ca2+ transport function of fast skeletal muscle SR. As shown in Fig. 10A, under assay conditions identical to those used in the experiments using cardiac SR (cf. Fig. 1A and Fig. 2), CaM BP (0.5–5 m) did not inhibit the Ca2+-ATPase activity of fast skeletal muscle SR; instead a stimulatory effect was observed. On the other hand, the ATP-dependent Ca2+ uptake activity of fast skeletal muscle SR was inhibited by CaM BP at the same concentration range in which no inhibitory effect on Ca2+-ATPase activity could be observed (Fig. 10B). Thus, the observed inhibition of Ca2+ uptake re-

![Fig. 6. Effect of CaM BP on ATP-dependent Ca2+ uptake by cardiac SR at varying ATP concentrations.](image-url)
reflects CaM BP-induced activation of Ca\textsuperscript{2+} release from the SR. This is in direct contrast to the concurrent inhibition of Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+}-ATPase activity by CaM BP observed in cardiac SR (cf. Fig. 1 and Fig. 2). Inclusion of calmodulin (3 \textmu M) in the assay medium prevented the effects of CaM BP on Ca\textsuperscript{2+}-ATPase and Ca\textsuperscript{2+} uptake activities of fast skeletal muscle SR (Fig. 10, A and B).

**Effect of CaM BP on Cardiac Contractile Function**—In view of the strong inhibitory action of CaM BP on Ca\textsuperscript{2+} uptake by cardiac SR observed in vitro, it was of considerable interest to examine the effect of this peptide on cardiac contractile function. The hydrophobic nature of CaM BP (29) facilitated such investigation using isolated, perfused heart preparations. In isolated, spontaneously beating rabbit heart preparations perfused at a constant flow rate, CaM BP (1 and 2.5 \textmu M) produced marked concentration-dependent depression of contractile function as evidenced by decrements in developed left ventricular pressure, rates of pressure development and relaxation, as well as pronounced elevation of end diastolic pressure (Fig. 12).

**Fig. 8.** Reversible inhibition of endogenous CaM kinase-mediated cardiac SR protein phosphorylation by CaM BP. The phosphorylation reaction was carried out for 2 min in the standard assay medium (see "Experimental Procedures"). The concentrations of CaM and CaM BP were varied as indicated. The top left panel shows Coomassie Blue-stained SDS-polyacrylamide gel depicting SR protein profile; the top right panel shows an autoradiogram of the same gel depicting protein phosphorylation. The phosphorylated peptide bands representing RYR-CRC, Ca\textsuperscript{2+}-ATPase, and phospholamban (PLN; H, high molecular weight form; L, low molecular weight form) were excised from the gel, and \textsuperscript{32}P incorporation was quantified by liquid scintillation counting, and the results are presented in the bottom panels.

**Fig. 9.** Attenuation of calmodulin-mediated reversal of the inhibitory action of CaM BP on cardiac SR Ca\textsuperscript{2+} uptake by a CaM kinase II inhibitor peptide. The Ca\textsuperscript{2+} uptake reaction was carried out for 2 min under standard conditions (see "Experimental Procedures") without and with varying concentrations of CaM kinase II inhibitor peptide in the assay medium as indicated. The assays were performed in the absence of CaM BP and calmodulin ( ), in the presence of 3 \textmu M CaM BP ( ), 3 \textmu M calmodulin ( ), and 3 \textmu M each of CaM BP and calmodulin ( ). Results from a typical experiment are shown. Similar results were obtained in two additional experiments using separate SR preparations.
CaM BP, but an enhancement in heart rate was not altered significantly during perfusion with CaM BP. The observed depression of contractile function induced by a low concentration of CaM BP (1 μM) was fully reversible upon reperfusion with normal buffer over a period of 20–30 min. However, only partial recovery of contractile function was observed upon reperfusion following infusion of a higher concentration (2.5 μM) of CaM BP. In these spontaneously beating preparations, the heart rate (beats/min) was not altered significantly during perfusion with CaM BP, but an enhancement in heart rate was observed during reperfusion with normal buffer subsequent to infusion of 2.5 μM (but not 1 μM) CaM BP (Fig. 12 and Table II).

**DISCUSSION**

In this study, we have made the following novel, key observations: (i) At low micromolar concentrations, CaM BP strongly inhibits active Ca²⁺ sequestration by isolated cardiac SR vesicles. (ii) The inhibition of Ca²⁺ transport is mainly the consequence of a primary inhibition of the SR Ca²⁺-ATPase. (iii) Cardiac SR vesicles contain firmly bound endogenous calmodulin, and exogenously added calmodulin readily reverses the inhibitory action of CaM BP on the energy transduction and Ca²⁺ ion transport functions of the SR Ca²⁺-ATPase. Taken together, these findings suggest a crucial role for SR-associated calmodulin in the regulation of cardiac SR Ca²⁺ pump function. As discussed below, analysis of the characteristics of Ca²⁺-ATPase inhibition by CaM BP, and the reversal of inhibition by calmodulin, has provided insights into the mechanisms of action of CaM BP.

CaM BP inhibits the SR Ca²⁺-ATPase rapidly (Fig. 3A), and the inhibition results from the association of CaM BP with SR membrane target(s) (Fig. 3, B and C and Fig. 7). The association of CaM BP with its SR target(s) appears to be stoichiometric (Fig. 1B) although actual stoichiometry could not be determined as CaM BP binding to SR was not quantified in this study. CaM BP is an amphiphilic peptide with high affinity for calmodulin (dissociation constant for binding 0.2 nM, cf. Ref. 29), and several lines of evidence presented here suggest that SR-associated calmodulin is a prime target of CaM BP. Thus, we have found that (i) presence of exogenous calmodulin in the incubation medium prevents association of CaM BP with SR (Fig. 7A) and blocks the inhibition of Ca²⁺-ATPase by CaM BP; (ii) exogenous calmodulin is effective in displacing CaM BP previously bound to SR (Fig. 7A) and in reversing pre-existing CaM BP-induced inhibition of Ca²⁺-ATPase (Fig. 3B); and (iii) the intact CaM BP molecule capable of high affinity calmodulin binding, but not its truncated fragments that lack the ability to bind calmodulin, inhibits the SR Ca²⁺-ATPase (Fig. 1A). From
should be noted that unlike CaM BP, the CaM kinase inhibitor treated, at least in part, through inhibition of SR CaM kinase. It cannot access a functionally important pool of calmodulin structured with high affinity for calmodulin, is apparently able to autophosphorylation (36). A hydrophobic molecule like CaM kinase molecule through a process of sequential intersubunit scavenging calmodulin added to the assay medium (28). However, this peptide is membrane-impermeant (28) and, hence, from its ability to interact with endogenous calmodulin in the SR and consequent perturbations in calmodulin-dependent membrane events.

In the above evidence, it appears that the effects of CaM BP arise from its contractile function. Contractile function was assessed in isolated perfused, spontaneously beating rabbit heart as described under "Experimental Procedures." Segments of 15 consecutive contractions such as those depicted in Fig. 12 were analyzed to obtain the average value shown for each parameter. Similar findings were obtained in three additional isolated heart preparations. LVP, left ventricular pressure; +dP/dt, maximum rate of pressure development; −dP/dt, maximum rate of relaxation.

| Parameter | Control buffer | 1 μM CaM BP | Control buffer after 1 μM CaM BP | 2.5 μM CaM BP | Control buffer after 2.5 μM CaM BP |
|-----------|----------------|-------------|-------------------------------|---------------|---------------------------------|
| LVP, peak systolic (mm Hg) | 102 | 82 | 138 | 112 | 136 |
| LVP, end diastolic (mm Hg) | 25 | 29 | 31 | 81 | 76 |
| +dP/dt (mm Hg/s) | 476 | 456 | 612 | 382 | 470 |
| −dP/dt (mm Hg/s) | 333 | 271 | 486 | 360 | 392 |
| Heart rate (beats/min) | 144 | 141 | 159 | 139 | 222 |

The effects of CaM BP on contractile function parameters are summarized in Table II. Results similar to those shown here were obtained in three additional isolated perfused heart preparations studied. LVP, left ventricular pressure.

| TABLE II Effects of CaM BP on cardiac contractile function |

Analysis of the influence of CaM BP on the kinetic parameters of Ca2+-transport has provided further insights into the mechanism underlying Ca2+-ATPase inhibition. The inhibitory effect of CaM BP was associated with a decrease in the Vmax of Ca2+-transport without appreciable changes in the K0.5 for Ca2+ activation of Ca2+-transport or the Hill coefficients for Ca2+ (Table I). These findings suggest that CaM BP-mediated structural perturbations in the Ca2+-ATPase does not alter the functional properties of the Ca2+-binding sites located in the transmembrane region of the ATPase (6, 7). On the other hand, analysis of the effect of CaM BP on ATP concentration dependence revealed that 50% decrease in Vmax of Ca2+-transport was accompanied by a 10-fold decrease in the apparent affinity of...
the ATPase for its substrate, ATP (Fig. 6). Since the inhibitory effect of submaximally effective concentration of CaM BP could not be overcome by increasing the concentration of ATP, CaM BP inhibition is non-competitive with respect to ATP. These findings imply that CaM BP-mediated inhibition involved a major alteration at the catalytic site located in the extramembranous region of the Ca\textsuperscript{2+}-ATPase (6, 7).

Our observations on the effects of CaM BP on SR Ca\textsuperscript{2+}-ATPase and its mechanism of action are unique when compared with those reported for other basic calmodulin-binding peptides such as melittin (derived from bee venom) and C28R2 (derived from the autoinhibitory domain of plasma membrane Ca\textsuperscript{2+}-ATPase). Melittin has been shown to inhibit skeletal muscle SR Ca\textsuperscript{2+}-ATPase (43–46), and C28R2 has been shown to inhibit both cardiac and skeletal muscle SR Ca\textsuperscript{2+}-ATPase (47). It has been suggested that these peptides inhibit enzyme activity by electrostatically cross-linking Ca\textsuperscript{2+}-ATPase into large inactive aggregates (43, 44, 47) or by binding to hydrophilic cytoplasmic domain of the Ca\textsuperscript{2+}-ATPase without causing enzyme aggregation (45, 46). Unlike melittin and C28R2, CaM BP does not inhibit skeletal muscle SR Ca\textsuperscript{2+}-ATPase. Furthermore, the inhibitory action of melittin on skeletal muscle SR Ca\textsuperscript{2+}-ATPase is accompanied by a decrease in the enzyme’s affinity for Ca\textsuperscript{2+} and unaltered affinity for ATP (46). In contrast, CaM BP inhibition of cardiac SR Ca\textsuperscript{2+}-ATPase is associated with a pronounced decrease in the affinity of enzyme for ATP and unaltered affinity for Ca\textsuperscript{2+}.

The characteristics of Ca\textsuperscript{2+}-ATPase inhibition by CaM BP also differ from those of other inhibitors of SR Ca\textsuperscript{2+}-ATPase such as thapsigargin, cyclopiazonic acid, and clotrimazole. Unlike melittin and C28R2, CaM BP inhibits SR \textsuperscript{2+}-ATPase without causing a decrease in the affinity of enzyme for Ca\textsuperscript{2+} and unaltered affinity for ATP (46). In contrast, CaM BP inhibition of cardiac SR Ca\textsuperscript{2+}-ATPase is associated with a pronounced decrease in the affinity of enzyme for ATP and unaltered affinity for Ca\textsuperscript{2+}.

Acknowledgments—We are grateful to Dr. J. A. Hudspeth, 1997 Stevenson Lecturer from The Rockefeller University, for helpful discussions regarding the use of CaM BP as a CaM kinase II inhibitor. We thank Lily Jiang for secretarial assistance.

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