Identification of Ser\textsuperscript{38} as the Site in Cardiac Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase That Is Phosphorylated by Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase*

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In previous studies (Xu, A., Hawkins, C., and Narayanann, N. (1993) J. Biol. Chem. 268, 8394–8397), the Ca\textsuperscript{2+}-ATPase of cardiac muscle sarcoplasmic reticulum (SERCA2) was shown to be phosphorylated by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM kinase) on a serine residue, likely to be either Ser\textsuperscript{38}, Ser\textsuperscript{167}, or Ser\textsuperscript{531}. SERCA2 and SERCA2 mutants S38A, S167A, and S531A were expressed in HEK-293 cells and tested for phosphorylation with CaM kinase. Mutant S38A was not phosphorylated, while mutants S167A and S531A were phosphorylated, suggesting that Ser\textsuperscript{38} is the site of CaM kinase phosphorylation in SERCA2. This conclusion was supported by the observation that phosphorylation of SERCA2 and mutants S167A and S531A by CaM kinase increased the $V_{\text{max}}$ for Ca\textsuperscript{2+} transport, while the $V_{\text{max}}$ for Ca\textsuperscript{2+} transport by mutant S38A was unaffected by exposure to a phosphorylation reaction mix. SERCA1, containing a potential CaM kinase phosphorylation site at Ser\textsuperscript{187} and two SERCA1 mutants, K35R plus H38S and T532S, in which potential CaM kinase sites were created, were not phosphorylated by CaM kinase, and $V_{\text{max}}$ for Ca\textsuperscript{2+} transport was unaffected by exposure to a phosphorylation reaction mix. Thus phosphorylation of Ser\textsuperscript{38} in SERCA2 results in an enhancement of Ca\textsuperscript{2+}-dependent transport, providing a potential regulatory mechanism for Ca\textsuperscript{2+} removal from cardiac and other tissues in which SERCA2 is expressed.

The Ca\textsuperscript{2+}-ATPase of sarcoplasmic reticulum forms part of a system of pumps and channels that regulates intracellular Ca\textsuperscript{2+} concentrations. At least three Ca\textsuperscript{2+}-ATPase isoforms are expressed in tissue-specific and developmentally regulated patterns (Brandl et al., 1987; Lytton et al., 1989; Burk et al., 1989). The fast-twitch muscle isoform (SERCA1) is expressed at high levels in striated skeletal muscle. The cardiac/slow-twitch muscle isoform (SERCA2) is expressed at high levels in cardiac cells and, in an alternatively spliced form, in nonmuscle cells. SERCA5 is also widely expressed. These Ca\textsuperscript{2+}-ATPases are 110-kDa proteins composed of a globular cytoplasmic domain and a transmembrane domain connected through a stalk region (Toyofukū et al., 1993). The transmembrane domain is predicted to be made up of 10 transmembrane helices, the first 5 of which are extended on the cytoplasmic surface to form helical stalk sequences 1–5 (MacLennan et al., 1985). A β-strand domain is situated between stalk sequences 2 and 3, and a larger domain, containing phosphorylation and nucleotide-binding/hinge subdomains, is situated between stalk sequences 4 and 5.

Ca\textsuperscript{2+}-ATPase pumps 2 mol of Ca\textsuperscript{2+} from the cytoplasm to the lumen at the expense of 1 mol of ATP. The activity of these pumps is regulated largely by intracellular Ca\textsuperscript{2+} concentrations, but in heart, SERCA2 interacts with and is inhibited by the dephospho form of phospholamban, a low molecular weight, membrane-bound regulatory protein (Tada et al., 1988). As part of the inotropic response, phospholamban can be phosphorylated by cAMP-dependent protein kinase and CaM kinase, reducing its ability to inhibit the Ca\textsuperscript{2+}-ATPase. Recently, Xu et al. (1993) demonstrated that CaM kinase also phosphorylates a serine residue in SERCA2, resulting in an enhancement of Ca\textsuperscript{2+}-dependent ATP hydrolysis by the enzyme. On the basis of the minimal consensus sequence (R-X-X-S/T) for CaM kinase phosphorylation (Pearson et al., 1985), Xu et al. (1993) proposed that the phosphorylated residue might be Ser\textsuperscript{38}, Ser\textsuperscript{167}, or Ser\textsuperscript{531}. In the present study, we used expression in a heterologous system to show that phosphorylation alters the Ca\textsuperscript{2+} transport function of SERCA2, and we used site-directed mutagenesis to determine that Ser\textsuperscript{38} is the site in SERCA2 phosphorylated by CaM kinase.

**EXPERIMENTAL PROCEDURES**

Oligonucleotide-directed Mutagenesis and cDNA Expression in HEK-293 Cells—Mutagenesis of SERCA1 and SERCA2 cDNA was performed by methods described previously (Kunkel, 1985; Maruyama and MacLennan, 1988; Clarke et al., 1988b; Toyofuku et al., 1993). Mutant cDNAs were cloned into the pMT2 expression vector (Kaufman et al., 1989) and transfected into HEK-293 cells using calcium phosphate precipitation (Toyofuku et al., 1994). Microsomes prepared from transfected cells were suspended in solution A (0.25 M sucrose, 0.15 M KCl, 3% P-mercaptoethanol, 20 mM CaCl\textsubscript{2}, and 10 mM Tris-HCl, pH 7.4) at 4°C for 5 min. Aliquots were removed for determination of protein concentration and immunoblotting with mouse monoclonal anti-SERCAl antibody, A52, or mouse monoclonal anti-SERCA2 antibody, 11D8F6 (a generous gift from Dr. K. P. Campbell). The immunoreactive protein bands were visualized by the alkaline phosphatase reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates, as described previously (Toyofuku et al., 1993).

Protein Phosphorylation and Immunoprecipitation of Ca\textsuperscript{2+}-ATPase—Aliquots of 100 μg of microsomes expressing Ca\textsuperscript{2+}-ATPase were phosphorylated by 0.25 μM exogenous CaM kinase II in 100 μl of solution containing 50 mM Hepes, pH 7.4, 10 mM MgCl\textsubscript{2}, 2 μM CaCl\textsubscript{2}, and 0.4 μM γ-32P-ATP (specific activity, 300–400 cpdpmol) at 37°C for 5 min. The CaM kinase used was the α-subunit of mouse CaM kinase, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase; MOPS, 3-(N-morpholino)propanesulfonic acid.

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The abbreviations used are: CaM kinase, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase; MOPS, 3-(N-morpholino)propanesulfonic acid.
Expression and Function of Normal and Mutant Ca\textsuperscript{2+}-ATPases in HEK-293 Cells—The demonstration by Xu et al. (1993) that CaM kinase phosphorylation of SERCA2 leads to an increase in $V_{\text{max}}$ for ATP hydrolysis led us to express SERCA2 in a heterologous system, in the absence of phospholamban, and to test whether CaM kinase phosphorylation would affect $V_{\text{max}}$ or Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport. We also carried out a series of mutations in SERCA2 that were designed to identify the site of phosphorylation. Since only 3 serines in SERCA2, Ser\textsuperscript{284}, Ser\textsuperscript{167}, and Ser\textsuperscript{531}, are located in CaM kinase II consensus phosphorylation sites, each was mutated to Ala to make the three SERCA2 mutants S2(S38A), S2(S167A), and S2(S531A). In a third series of experiments, we created consensus CaM kinase II phosphorylation sites in SERCA1 by mutating the corresponding residues in SERCA1 to those found in SERCA2. This involved the double mutation of Lys:' to Arg plus His38 to Leu in SERCA1 and the single mutation of Ser167 to Ala in SERCA1. In the second series of experiments, we created consensus CaM kinase II phosphorylation sites in SERCA1 by mutating the corresponding residues in SERCA1 to those found in SERCA2. This involved the double mutation of Lys\textsuperscript{35} to Arg plus His\textsuperscript{38} to Ser in the mutant S1(K35R-H38S). Ser\textsuperscript{167} was located in a CaM kinase II consensus site and did not require mutation. Thr\textsuperscript{532}, however, was mutated to Ser in the second SERCA1 mutant S1(T532S).

The expression of wild-type and mutant cDNAs in HEK-293 cells is shown in Fig. 1A. Expression was monitored by immunoblotting, and activity was measured as a function of Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport. Immunoblots with antibody IID8F6 illustrate the expression of SERCA2, and its mutants and immunoblots with antibody A52 illustrate the expression of SERCA1 and its mutants. Each mutant protein was expressed at a high level and migrated with the same mobility as brain CaM kinase II, expressed in baculovirus and purified as described by Bricey et al. (1990). As a control, microsomes were incubated in a medium lacking CaCl\textsubscript{2}, calmodulin, and exogenous CaM kinase II. The phosphorylated and control microsomes were washed with 1 ml of a solution containing 10 mM Tris-HCl, pH 7.2, 2% β-mercaptoethanol, and 25 mM NaF. The washed microsomes were solubilized in a solution containing 50 mM Hapes, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5% SDS, 1 mM EGTA, 1.5 mM MgCl\textsubscript{2}, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM phenylmethylsulfonyl fluoride, 10 pg/ml aprotinin, and 10 pg/ml leupeptin and then incubated with 50 μl of Protein A-Sepharose at 4 °C for 1 h with rocking. The supernatants were then incubated with 30 μl of Protein A-Sepharose precoated with rabbit anti-mouse IgG Fc fragment-specific antibody and either mouse monoclonal anti-SERCAl antibody, A52, or mouse monoclonal anti-SERCA2 antibody, IID8F6, at 4 °C for 2 h with rocking. The beads were washed 3 times with the solubilizing buffer, dissolved in SDS-loading buffer, and subjected to SDS-polyacrylamide gel electrophoresis in 10% polyacrylamide, followed by autoradiography.

**Ca\textsuperscript{2+} Transport Assay**—Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport activity was assayed as described by Toyofuku et al. (1992) in 150 μl of a solution containing 20 mM MOPS, pH 6.8, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 5 mM ATP, 0.5 mM EGTA, 5 mM potassium oxalate, and various molar concentrations of Ca\textsuperscript{2+} containing 45Ca at a specific activity of 10⁶ cpm/μmol. Free Ca\textsuperscript{2+} concentrations were calculated using the computer program of Fabiato and Fabiato (1979). Microsomes containing the expressed Ca\textsuperscript{2+}-ATPases were phosphorylated by exogenous CaM kinase II in the medium described above, except that 45Ca was deleted and 5 mM ATP was replaced with 0.4 mM ATP containing [γ-32P]ATP. The phosphorylated and control microsomes were washed 3 times with 1 ml of solution A and resuspended in 150 μl of solution A. Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport activity was measured using 10 μl of these phosphorylated microsomes. Data were analyzed by nonlinear regression using the Sigma Plot scientific graph system obtained from Jandel Science.
Phosphorylation of Ca\textsuperscript{2+}-ATPase

Fig. 2. Phosphorylation and immunoprecipitation of wild-type and mutant SERCA2 and SERCA1 proteins. Microsomes from HEK-293 cells transfected with the SERCA constructs indicated were phosphorylated with CaM kinase and immunoprecipitated with anti-SERCAl antibody (lanes 1-8) or anti-SERCA1 antibody (lanes 9-14). The approximate mass of the marker proteins is indicated on the left. The upper panel shows the Coomassie Blue-stained gel, and the lower panel shows the autoradiogram derived from it.

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KDa 106 117 125 130 135 140

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The Ca\textsuperscript{2+} transport function of each of the expressed proteins is illustrated in Fig. 1, B and C. The Ca\textsuperscript{2+} transport activity of microsomes from transfected cells was at least 6 times higher than that of control microsomes from untransfected cells. The Ca\textsuperscript{2+} concentrations required to attain the half-maximum Ca\textsuperscript{2+} uptake rate (K_{Ca}), the Hill coefficient (n_H), and maximum Ca\textsuperscript{2+} uptake rate (V_{max}) were calculated from these Ca\textsuperscript{2+} uptake curves. The K_{Ca} for Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport for expressed Ca\textsuperscript{2+}-ATPases ranged from pC_{a} 6.70 ± 0.04 to pC_{a} 6.76 ± 0.03 (not significantly different at p = 0.01). The n_H ranged from 1.7 ± 0.3 to 1.8 ± 0.3 (not significantly different at p = 0.01). The V_{max} values for the various proteins prior to their exposure to phosphorylation reaction mix, expressed as nmol/min/mg of microsomal protein, were 238 ± 35 for SERCA2, 117 ± 23 for S2(S38A), 142 ± 30 for S2(S167A), 98 ± 19 for S2(S531A), 290 ± 32 for SERCA1, 173 ± 22 for S1(K35R-H38S), 235 ± 28 for S1(T532S), and 18 ± 2 for untransfected control cells.

Phosphorylation of Mutant Ca\textsuperscript{2+}-ATPases by CaM Kinase—Microsomes expressing wild-type and mutant Ca\textsuperscript{2+}-ATPases were phosphorylated by exogenous CaM kinase, solubilized, and immunoprecipitated with an isoform-specific antibody against either SERCA1 or SERCA2 (Fig. 2). For each of the wild-type and mutant expressed proteins, a major protein band, which corresponded to the Ca\textsuperscript{2+}-ATPase, was visualized on Coomassie Blue-stained SDS-polyacrylamide gels (Fig. 2, upper panel). When the phosphorylation reaction was carried out in the presence of Ca\textsuperscript{2+}, CaM, and CaM kinase II, a major \textsuperscript{32}P-labeled protein, which was superimposable with the immunoprecipitable SERCA2 protein, was visualized on autoradiography (Fig. 2, lower panel). The amount of \textsuperscript{32}P incorporated into SERCA2 or into SERCA2 mutants S2(S167A) and S2(S531A) was very low in control reaction but was enhanced severalfold over control by the addition of Ca\textsuperscript{2+}, CaM, and CaM kinase II. When these components were added to the mutant S2(S38A), we observed only a very low level of phosphorylation. This level of phosphorylation, amounting to a few percent of the level of wild-type phosphorylation under the same conditions, corresponds to the level of threonine phosphorylation of the protein, about 5% of serine phosphorylation, that was observed by Xu et al. (1993) and studied in more detail by Hawkins et al. \textsuperscript{3,4} No enhancement over control levels of phosphorylation was observed when Ca\textsuperscript{2+}, CaM, and CaM kinase were added to phosphorylation reaction mixes for SERCA1 or SERCA1 mutants S1(K35R-H38S) or S1(T532S).

Effects of Phosphorylation on Ca\textsuperscript{2+} Transport Rate—In previous studies, phosphorylation of SERCA2 by CaM kinase resulted in a 2-fold enhancement of the V_{max} of Ca\textsuperscript{2+}-dependent ATP hydrolysis (Xu et al., 1993). When we measured the effect of CaM kinase-dependent phosphorylation on the Ca\textsuperscript{2+} transport function of wild-type and mutant forms of SERCA2 and SERCA1, we observed an increase in V_{max} for SERCA2 and for mutants S2(S167A) and S2(S531A) (Fig. 3). We did not observe any effect on either V_{max} or K_{Ca} for Ca\textsuperscript{2+} transport for SERCA2 mutant S2(S38A) or for SERCA1 or SERCA1 mutants S1(K35R-H38S) or S1(T532S) following exposure of these proteins to phosphorylation reaction mixtures.

DISCUSSION

We observed that the V_{max} for Ca\textsuperscript{2+} uptake for expressed SERCA2 was increased 1.7-fold by exposure to CaM kinase II under conditions where the V_{max} for SERCA1 was unchanged (Fig. 3). These results clearly demonstrate that the effect of CaM kinase phosphorylation on V_{max} of ATP hydrolysis by SERCA2, previously reported by Xu et al. (1993), is also reflected in an increase in V_{max} of Ca\textsuperscript{2+} transport. Similar studies of mutant enzymes revealed that V_{max} values for S2(S167A) and S2(S531A) were each stimulated about 1.4-fold by exposure to CaM kinase. By contrast, exposure of SERCA2 mutant S38A or SERCA1 mutants K35R-H38S or T532S to CaM kinase had no effect on V_{max}.

These functional studies were correlated with studies of CaM kinase-dependent phosphorylation. SERCA2 and SERCA2 mutants S167A and S531A were all phosphorylated by CaM kinase II (Fig. 2). Mutant S38A was phosphorylated only slightly above background, at a level that could be attributed to threonine phosphorylation of the protein. SERCA1 and SERCA1 mutants were not phosphorylated by CaM kinase. Thus those proteins in which CaM kinase induced a stimulation of V_{max} were phosphorylated, while those in which CaM kinase was ineffective were not phosphorylated.

It is difficult to quantify these results because each of the mutants affected the V_{max} of the unphosphorylated protein (Fig. 1). V_{max} values were reduced to less than 60% of wild type for S2(S167A), to less than 50% for S2(S38A), and to less than 40% for S2(S531A). Similarly, V_{max} values were reduced to about 80% of wild type for S1(T532S) and to about 60% for S1(K35R-

\textsuperscript{3} C. Hawkins, A. Xu, and N. Narayanun, unpublished data.

\textsuperscript{4} A. Xu, C. Hawkins, and A. Chen.
Phosphorylation of Ca\textsuperscript{2+}-ATPase

FIG. 3. Effects of CaM kinase phosphorylation on the \(V_{\text{max}}\) of wild-type and mutant SERCA2 and SERCA1 molecules. Ca\textsuperscript{2+} uptake assays were conducted with microsomes that were pretreated with (open circles) or without CaM kinase (closed circles) as described under "Experimental Procedures." Control and phosphorylation reactions were conducted with aliquots from the same experimental sample. In each experiment, the uptake rate (nmol/min/mg of microsomal protein) of phosphorylated microsomes was normalized to the activity of control microsomes and is shown as a percentage of the control Ca\textsuperscript{2+} uptake rate. Points and bars represent the mean \(\pm\) S.D. obtained from three independent experiments. The curve represents the best fit of the data to a cooperative model of enzyme activation.

Moreover, the levels of CaM kinase-dependent phosphorylation of S2(S167A) and S2(S531A) were diminished when compared with wild type (Fig. 2). Correlations can be made, however. The mutation of Ser\textsuperscript{38} led to loss of stimulation of \(V_{\text{max}}\) by CaM kinase and to a loss of protein phosphorylation. By contrast, proteins mutated at Ser\textsuperscript{537} or Ser\textsuperscript{531} retained a 1.4-fold increase in \(V_{\text{max}}\) and CaM kinase-dependent phosphorylation. Thus there is a strong correlation between loss of Ser\textsuperscript{38}, loss of CaM kinase-dependent phosphorylation, and loss of stimulation of \(V_{\text{max}}\) for Ca\textsuperscript{2+} transport. However, since we cannot quantify the results more precisely, we cannot rule out a minor contribution of Calmodulin kinase-dependent phosphorylation of Ser\textsuperscript{167} or Ser\textsuperscript{531} to the stimulation of \(V_{\text{max}}\).

Although SERCA2 is phosphorylated at Ser\textsuperscript{38}, the introduction of a potential CaM kinase phosphorylation site into SERCA1 by mutation of Lys\textsuperscript{58} to Arg and His\textsuperscript{38} to Ser, to duplicate the phosphorylation site in SERCA2, failed to create a site for CaM kinase-dependent phosphorylation of SERCA1. It is possible that the CaM kinase phosphorylation site has a different three-dimensional structure in SERCA2 than in SERCA1 so that the site in SERCA1 is inaccessible to CaM kinase. For both SERCA2 and SERCA1 molecules, the sequence comprising the potential CaM kinase phosphorylation site at Ser\textsuperscript{38} is predicted to form part of a loop structure between a predicted 11-residue cytoplasmic \(\alpha\)-helix near the NH\textsubscript{2} terminus of the molecule and a long \(\alpha\)-helix, which makes up predicted stalk sector 1 and transmembrane sector 1 (S\textsubscript{T}/M\textsubscript{1}) \(\alpha\)-helices. The sequence of the \(\alpha\)-helix found upstream of the phosphorylation site and the loop containing the site is not well conserved, since 9 residues are substituted in the 14 residues between Ser\textsuperscript{38} and Ser\textsuperscript{38} (Brandi \textit{et al.}, 1987). Six substitutions are highly conservative, but three are not, perhaps providing a structural basis...
for the differential phosphorylation of SERCA2 and the SERCA1 mutant, S1(K35R-H38S).

Although there has been no evidence up until now that the sequence upstream of stalk sector S4 was significant in regulating the function of the Ca\(^{2+}\)-pump, it is now apparent that it does. There are two possible ways in which phosphorylation of Ser\(^{28}\) may regulate the function of the Ca\(^{2+}\)-ATPase. The site of CaM kinase phosphorylation in the molecule lies within approximately the same stratum (a few nanometers from the membrane cytoplasmic surface) as the site of interaction between the cytoplasmic domain of phospholamban (Toyofuku et al., 1994) and residues 336–412 in the cytoplasmic domain of SERCA2 (Toyofuku et al., 1993). This interaction regulates the apparent Ca\(^{2+}\) affinity of SERCA2. By analogy, there may be an intramolecular interaction between the NH\(_2\) terminus of SERCA2 and an internal site that regulates \(V_{\text{max}}\) max. Such a site would be different from the site that binds phospholamban, since the phospholamban-binding site affects \(K_{\text{m}}\), not \(V_{\text{max}}\) max.

A second possibility is that the phosphorylation site regulates the Ca\(^{2+}\)-ATPase through long range interactions. In earlier studies, two active site domains were located in the Ca\(^{2+}\)-ATPase. These are comprised of the nucleotide-binding and phosphorylation doms lying between stalk sequences S4 and S5 in the cytoplasmic domain (MacLennan et al., 1985; Clarke et al., 1990) and the sites of Ca\(^{2+}\) binding and translocation comprised of transmembrane sequences M\(_4\), M\(_5\), M\(_6\), and M\(_8\) (Clarke et al., 1989a). Long range interactions that occur between these sites determine the ability of Ca\(^{2+}\) bound in the transmembrane domain to control phosphorylation of the phosphorylation domain by ATP. They also control the ability of the phosphorylated intermediate in the cytoplasmic domain to bring about Ca\(^{2+}\) translocation in the transmembrane domain. Apparent Ca\(^{2+}\) binding affinity in the transmembrane domain can also be modified through long range interactions originating in the nucleotide-binding domain (Toyofuku et al., 1992). These long range interactions must all be mediated through stalk sectors S3 and S5 (MacLennan, 1990; MacLennan et al., 1992). It is, therefore, possible that long range interactions, regulated by the phosphorylation of a residue near the top of S3, can alter the function of SERCA2 so that \(V_{\text{max}}\) max is increased. This may occur through long range interactions among S\(_4\)/M\(_4\) and other stalk and transmembrane sequences involved in Ca\(^{2+}\) translocation. Future studies will be aimed at differentiating between these and other possible regulatory mechanisms.

The serine phosphorylation of cardiac sarcoplasmic reticulum proteins offers an important mechanism for the regulation of cardiac contractility. The cardiac regulatory protein, phospholamban, in its dephosphorylated form inhibits the Ca\(^{2+}\)-ATPase. When phosphorylated by CaM kinase or cAMP-dependent protein kinase, however, phosphorylated phospholamban dissociates from the cardiac Ca\(^{2+}\)-ATPase, resulting in a decrease in \(K_{\text{m}}\) for Ca\(^{2+}\) activation of the Ca\(^{2+}\) transport rate but with minimal change in \(V_{\text{max}}\) max (Morris et al., 1991; Briggs et al., 1992; Toyofuku et al., 1993). By contrast, the phosphorylation of cardiac Ca\(^{2+}\)-ATPase by CaM kinase enhances the \(V_{\text{max}}\) of Ca\(^{2+}\)-

dependent ATP hydrolysis (Xu et al., 1989) and of Ca\(^{2+}\) transport (this study) without changing \(K_{\text{m}}\). Therefore, the positive \(K_{\text{m}}\) effects brought about by phosphorylation of phospholamban and the positive \(V_{\text{max}}\) effects brought about by CaM kinase phosphorylation of the cardiac Ca\(^{2+}\)-ATPase may be synergistic in stimulation of cardiac function. Such a mechanism would be of particular physiological significance in the heart where myofilament activation and contraction result from binding of Ca\(^{2+}\) to the single Ca\(^{2+}\)-specific site on troponin C (Thompson et al., 1990). This site has much lower affinity for Ca\(^{2+}\) than the Ca\(^{2+}\)-binding sites in the Ca\(^{2+}\)-ATPase (Holroyde et al., 1989; Thompson et al., 1990). Therefore, an increase in Ca\(^{2+}\) binding affinity of the Ca\(^{2+}\)-ATPase may not be sufficient to produce maximal stimulation of Ca\(^{2+}\) pumping unless accompanied by an increase in \(V_{\text{max}}\).

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