The phosphorylation of the cardiac muscle isofrom of the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase (SERCA2a) on serine 38 has been described as a regulatory event capable of very significant enhancement of enzyme activity (Hawkins, C., Xu, A., and Narayanan, N. (1994) J. Biol. Chem. 269, 31198–31206). Independent confirmation of these observations has not been forthcoming. This study has utilized a polyclonal antibody specific for the phosphorylated serine 38 epitope on the Ca\textsuperscript{2+} transporter. The detection sensitivity of assays was adequate to detect phosphorylation in isolated sarcoplasmic reticulum vesicles or suitably stimulated cardiomyocytes. A quantitative Western blot approach failed to detect serine 38-phosphorylated Ca\textsuperscript{2+}-ATPase in either kinase-treated sarcoplasmic reticulum vesicles or suitably stimulated cardiomyocytes. Calibration standards confirmed that the detection sensitivity of assays was adequate to detect Ser-38 phosphorylation if it occurred on at least 1% of Ca\textsuperscript{2+}-ATPase molecules in SR vesicle experiments or on at least 0.1% of Ca\textsuperscript{2+}-ATPase molecules in cardiac myocytes. The failure to detect a phosphorylated form of the Ca\textsuperscript{2+}-ATPase in either preparation (isolated myocyte, purified sarcoplasmic reticulum vesicles) suggests that Ser-38 phosphorylation of the Ca\textsuperscript{2+}-ATPase is not a significant regulatory feature of cardiac Ca\textsuperscript{2+} homeostasis.

Regulation of Ca\textsuperscript{2+} sequestration by cardiac sarcoplasmic reticulum has been identified as a key control point in cardiac muscle contraction (1, 2). Stimulation of the rate of Ca\textsuperscript{2+} uptake occurs during exercise, or following β-adrenergic stimulation (3) and is associated with an enhanced force of contraction and an increased rate of relaxation. This accounts for much of the positive inotropic and positive lusitropic effects of these interventions. In contrast, Ca\textsuperscript{2+} sequestration by cardiac SR is abnormally slow in the muscle of individuals with heart failure (4–6). In animal studies, normalization of the rate of Ca\textsuperscript{2+} sequestration has been shown to prevent progression of heart failure (7) and thus the molecular mechanism of control of Ca\textsuperscript{2+} transport into the sarcoplasmic reticulum has become a focus for research into purposeful therapies to combat human heart failure (7, 8).

Ca\textsuperscript{2+} transport into cardiac SR is an enzymatic process performed by the Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase (9) (or SERCA2a, Ref. 10), a member of the P-type ATPase family (11). The transport process involves the movement of two Ca\textsuperscript{2+} from the cytoplasm into the lumen of the SR following the hydrolysis of a single molecule of ATP (12), although significantly lower coupling efficiencies have been measured empirically (13). The reaction cycle is relatively slow and thus a large number of SERCA molecules are expressed in cardiac muscle (up to 45% of total SR protein content, 14) to achieve the rates of Ca\textsuperscript{2+} sequestration required by the kinetics of contraction and relaxation.

Regulation of Ca\textsuperscript{2+} transport into the SR occurs on an acute time scale (seconds to minutes) through the transient modification of the proteins involved. The most important of these is phospholamban, which is a naturally occurring inhibitor of the Ca\textsuperscript{2+}-ATPase. In its dephosphorylated state, phospholamban is able to interact with SERCA and reduce Ca\textsuperscript{2+}-pump activity through a reduction in the apparent Ca\textsuperscript{2+} affinity of the pump (15–18).

Phosphorylation of phospholamban on one of a number of sites (Ser-16, cAMP-dependent protein kinase; Thr-17, CaMKII; Ref. 19) abrogates the inhibitory influence of phospholamban (20), to reveal enhanced Ca\textsuperscript{2+} transport activity at all physiological Ca\textsuperscript{2+} concentrations. The kinetic basis of inhibition and subsequent activation of Ca\textsuperscript{2+} transport is complex; involving contributions from the acceleration of particular reaction steps in the catalytic cycle of SERCA (21) and an increase in the coupling efficiency between ATP hydrolysis and Ca\textsuperscript{2+} movement (13).

Other members of the P-type ATPase family are regulated by direct phosphorylation (e.g. plasma membrane Ca\textsuperscript{2+}-ATPase, 22; Na\textsuperscript{+},K\textsuperscript{+}-ATPase, Ref. 23). A comparable form of regulation has been described for SERCA2 by a number of groups (24–29), namely the phosphorylation of Ser-38 by calmodulin-dependent kinase II. Xu et al. (24) observed SERCA phosphorylation by CaMKII following the immunoprecipitation of SERCA from a solubilized, kinase-treated SR preparation. In addition, Xu et al. (24) demonstrated the phosphorylation of purified SERCA2a by CaMKII, which was accompanied by a 1-fold stimulation of Ca\textsuperscript{2+}-ATPase activity at all [Ca\textsuperscript{2+}]. Subsequent studies identified Ser-38 as the principal site of phosphorylation (25, 30), and still further studies noted substantial enhancement of Ca\textsuperscript{2+}-ATPase activity (1–1.5-fold increase in activity) despite the low stoichiometry of SERCA phosphorylation (12–13% of total SERCA; Ref. 26). Simple extrapolation of these latter data sets might suggest that phosphorylation of SERCA has the capacity to stimulate Ca\textsuperscript{2+}-pump activity ~10-fold, making this form of regulation more significant than that achieved by phospholamban.

The control of Ca\textsuperscript{2+}-pump function by direct phosphorylation is not observed by all investigators. Odermatt et al. (30) de-
Ser-38 Phosphorylation of SERCA

A number of physiological and pathological observations might be explained in terms of altered SERCA activity upon phosphorylation of Ser-38. The lack of independent confirmation of the incidence or importance of Ser-38 phosphorylation of SERCA has prevented confident implication of this event as a molecular explanation of particular physiological observations (6, 31). In the present study, we have produced and characterized an antibody specific for the Ser-38 phosphorylated form of SERCA2a in an attempt to evaluate whether Ser-38 phosphorylation occurs. This antibody is able to detect Ser-38 phosphorylation at levels of 0.03 pmol and above; however, it failed to detect SERCA in any experiment performed (included those involving kinase-treated SR containing up to 60 pmol of SERCA2a, and those involving myocytes exposed to CaMKII-activating interventions). As such we conclude that Ser-38 phosphorylation of SERCA does not occur to a significant extent in the situations examined.

EXPERIMENTAL PROCEDURES

Materials—Calmodulin was purified from bovine testes by the method of Gopalakrishna and Anderson (32). CaMKII was provided by Professor Howard Schulman (Stanford, CA). SERCA2a peptide (YLE-PAILE), and phosphorylated and dephosphorylated SERCA2a peptides (KKLKERWS/PQINEL41 and KKLKERWS/ NEL41) were purchased from NeoSystem, France. PKI peptide (TTYADFAISGRTGRNRHAD-amide) was obtained from Alta Bioscience, Birmingham, UK. Sulfo-cinimydiyl 4-(N-maleimidonethyl)-cyclohexane-1-carboxylate (sulfo- SMCC) cross-linker was from Pierce. Calyculin A was obtained from Alexis Corporation; IGEPAL CA-630, BSA and protein A-Sepharose 4B from Sigma; Triton and SDS from BHD; and 32P-ATP (specific activity of 400 Ci/mmol) was from ICN. Sure 2 supercompetent cells were obtained from Stratagene and BLR competent cells from Novagen. Preparation of Phosphospecific Antibodies—Ca2+/ATPase peptide phosphorylated on Ser-38 (KKLKERWS/PQINEL)41) was prepared by the CaMKII phosphorylation of peptide KKLKERWS/NEL41. The phosphopeptide was purified to homogeneity by reverse phase high performance liquid chromatography. Peptide was conjugated to keyhole limpet hemocyanin (KLH) using carbodiimide cross linkage (33) and dialyzed extensively against buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl). Adult New Zealand White rabbits were immunized with ~100 µg KLH and attached peptide at 6-week intervals and immune serum collected 11 days after immunizations. Serum was produced and stored at ~70 °C. A polyclonal antiserum is described herein: SERCA PS-38 (1:5000) to (I27)5 concatamer. Protein concentration was determined by a BCA assay (35).

Phosphorylation of SR Proteins by CaMKII—Rabbit and canine cardiac sarcoplasmic reticulum vesicles were prepared as described previously (36). Phosphorylation reactions were conducted at 37 °C in 100 µl of buffer (adapted for Hawkins et al., Ref. 26), containing 1 mg/ml SR vesicles and 50 mM Hepes-NaOH (pH 9.0), 5 mM MgSO4, 2 mM calyculin A, and 1 µM PKI. For phosphorylation by CaMKII (3 µg/ml or 6 µg/ml), the buffer contained 0.1 mM CaCl2 and 37.5 µg/ml of calmodulin. For the control samples, Ca2+ (1 mM EGTA present), calmodulin and CaMKII were omitted from the assay medium. The phosphorylation reactions were initiated by the addition of ATP to a final concentration of 1 mM [γ-32P]ATP (specific activity of 400 Ci/mmol) was used. After 2 min of incubation, the reactions were stopped by adding 25 µl of concentrated Laemmli sample buffer (5× strength) resulting in a final solution composition of 62.5 mM Tris-HCl (pH 6.8), 4% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% bromophenol blue (37). Samples were subjected to SDS-PAGE/Western blotting as described previously (38).

Isolated Cardiac Myocytes—Isolated rat ventricular myocytes were prepared from male Wistar rats according to the method of Frampton et al. (39). After isolation, cells were resuspended in 1 ml of a HEPES-based solution of the following composition (mM): NaCl 130; KCl 5.4; CaCl2 1; MgCl2 1.4; NaH2PO4 0.4; HEPES 5; glucose 10; creatine 10, 1M of the phosphatase inhibitor calyculin A or to increased stimulated frequency (2.5 Hz). After 5 min, concentrated Laemmli sample buffer (5× strength), containing 50 mM NaF and 5 mM sodium pyrophosphate to prevent dephosphorylation of SR proteins during sample preparation (40), was added to each cell suspension. Samples were subjected to SDS-PAGE.

Immunoblot Analysis—Myocardial proteins were separated by SDS-PAGE using 10 and 15% polyacrylamide gels as described by Laemmli (37). Following separation, proteins were transferred to PVDF membranes (Pall BioSupport, Portsmouth, UK) by semi-dry blotting, and non-specific binding sites were blocked for 2–4 h at room temperature using 5% dried milk and Tris-buffered saline (pH 7.4), 0.1% Tween 20. Membranes were probed overnight at 4 °C with primary antibodies: PT-17 (1:5000) for the Thr-17 phosphorylated form of phospholamban (41); α-CLEP (1:5000) for SERCA2a (16); and SERCA PS-38 (1:5000) antisemier for the Ser-38-phosphorylated form of Ca2+/ATPase. A secondary horseradish peroxidase-labeled antibody raised in rabbit (goat anti-rabbit IgG (H+F); Jackson Immunomethods) was used for detection of the 2.5× enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maxi mum Sensitivity Substrate, Pierce) to visualize the primary antibodies. Data were captured using a Fuji LAS-1000 Imaging System CCD Camera (AIDA software for analysis).

Preparation of Stripping Membranes—Following immunoblot analysis, membranes were incubated at 55 °C in stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 0.7% β-mercaptoethanol for 30 min. Membranes were then washed in Tris-buffered saline (pH 7.4), 0.1% Tween 20 and 5% dried milk for 2–4 h at room temperature, and probed overnight at 4 °C with another primary antibody. The amount of 32P incorporation into the cardiac SR proteins was determined by phosphorimaging. Phosphorimaging plates were exposed to PVDF membranes for 48 or 72 h, and densitometric analysis of the data was conducted using Fuji BAS-1000 scanner (AIDA software for analysis). Phosphoproteins were also detected no direct stimulation of Ca2+/pump function following CaMKII treatment, although phospholamban-mediated control was clearly evident. Furthermore this study noted a sizeable inhibition of basal Ca2+/pump activity upon brief exposure to Ca2+/free conditions (30). These Ca2+/free conditions were similar to the “control” conditions of Xu et al. (24), making it unclear whether Xu et al. (24) had described stimulation of pump activity in response to CaMKII phosphorylation or anti-fungal inhibition of pump activity under (Ca2+/free) control conditions. In separate studies Ready et al. (14) explored the phosphorylation of SERCA using highly purified cardiac SR and highly purified SERCA2a. CaMKII was unable to phosphorylate SERCA in either of these preparations, however the phosphorylation of a junctional SR protein of 100 kDa was noted. This phosphoprotein co-migrated with SERCA in one-dimensional electrophoretic separations but did not share its distribution throughout the SR network. As such the identity of the phosphoprotein was suggested to be a protein unrelated to SERCA.

A secondary horseradish peroxidase-labeled antibody raised in rabbit (goat anti-rabbit IgG (H+F); Jackson Immunomethods) was used for detection of the 2.5× enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maxi mum Sensitivity Substrate, Pierce) to visualize the primary antibodies. Data were captured using a Fuji LAS-1000 Imaging System CCD Camera (AIDA software for analysis).
excised from PVDF membranes and quantified by scintillation counting.

**ELISA**—Competitive ELISA experiments were performed by standard protocols (42) using o-phenylene diamine as the substrate for peroxidase-conjugated second antibodies (Jackson Immunochemicals). ELISA plates were coated with phosphorylated Ser-38 peptide at 1 μg/well, and phosphorylated, and de-phosphorylated Ser-38 peptides were used as competing agents as described in the relevant figure.

**Statistics**—All values are expressed as mean ± S.D. for n experiments, and statistical significance was evaluated using the Student’s t test for unpaired data.

**RESULTS**

The phosphorylation of SERCA2 on Ser-38 has been described as a regulatory feature capable of very significant activation of Ca^{2+}-ATPase activity (24, 26), although its occurrence and implication have been disputed (14, 30). This site, although unique to SERCA2, is contained within a segment of the protein which is highly conserved between SERCA1 and SERCA2 (Fig. 1A), particularly from residue 39 onwards (43). As such, the two proteins are likely to display comparable structures in this region. This segment of SERCA1, for which two high resolution structures exist (11, 44), is a surface exposed, highly mobile segment of the protein (Fig. 1B, highlighted in black). This segment remains solvent exposed in both conformational extremes of the enzyme (E1, E2; Fig. 1B). Assuming that the structure of this segment of SERCA2 is comparable, Ser-38 would be surface exposed and mobile. This would make it accessible to kinases and phosphatases in the major conformational states of the enzyme, and would also make it accessible to an antibody specific for this site. We have produced a phosphorylation site-specific antibody to this feature (highlighted in black in the equivalent structure of SERCA1, Fig. 1) in an effort to define the incidence and role of Ser-38 phosphorylation in cardiac muscle. A polyclonal antibody was produced to the sequence 31KLKERWGS(PO4)NEL41, phosphorylated at Ser-38 as described under “Experimental Procedures.” Fig. 2 shows that this polyclonal antiserum, SERCA PS-38, was specific for the phosphorylated peptide, because the phosphopeptide was a potent inhibitor of antibody binding to antigen (IC_{50} 18 nM), whereas the equivalent dephosphorylated peptide was unable to interfere with antibody: antigen recognition.

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**Fig. 1.** Residue 38 is located in an accessible, highly mobile domain on the surface of SERCA. **A**, the primary sequence of SERCA2 and SERCA1 in the region of the proposed phosphorylation site (Ser-38, SERCA2) is compared (taken from Brandl et al., Ref. 43). Shading denotes immunogen sequence. **B**, x-ray crystal structure of SERCA1 in E1 conformation (44) and E2 conformation (11). Segment analogous to the (SERCA2) phosphoantibody epitope is highlighted in black, residue 38 side chain is displayed.
Ser-38 Phosphorylation of SERCA2

Fig. 2. Antibody discriminates between peptide and phosphopeptide. ELISA plates were coated with phosphorylated SERCA2a peptide ([31]KLKERWS([PO4]NEL)[31]; 1 μg/well) and probed with SERCA PS-38 (1:270) in the presence of phosphorylated peptide (SERCA P; ●) or dephosphorylated peptide ([31]KLKERWSNEL[31]) (SERCA deP; □) at a concentration range of 0.08 nM to 5 μM. Antibody binding to antigen immobilized on the ELISA plate was detected using peroxidase-conjugated secondary antibody as described under “Experimental Procedures.” Data represent mean ± S.D. (n = 2). In some cases S.D. is smaller than symbol size.

SERCA PS-38 Recognition of a Calibration Standard—Having confirmed that polyclonal antibody SERCA PS-38 was specific for the phosphorylated Ser-38 epitope (Fig. 2), we examined the phosphorylation status of this residue in SERCA following exposure of cardiac SR vesicles to CaMKII. SERCA was not detected in these Western blot experiments (see Fig. 5 for an example). It was important to establish the basis of this negative result, to ensure that it was providing information about the incidence of Ser-38 phosphorylation, rather than recording a technical failing of the antibody or experiment. To this end, we constructed a calibration standard comprising the phosphopeptide epitope attached to an irrelevant scaffolding protein. The scaffolding protein was chosen as it contained a phosphopeptide epitope attached to an irrelevant scaffolding protein. The scaffolding protein was chosen as it contained a phosphopeptide epitope attached to an irrelevant scaffolding protein. A contaminant of 51,230 Da, seen on the mass spectrum and therefore is present in low amounts in the calibration standard at loadings of 0.05 pmol and above; however, it failed to recognize SERCA protein, or any other protein, in the samples from dog (Fig. 4A). Immunorecognition of a protein of ~100 kDa, consistent in size to SERCA, was observed with rabbit SR samples (Fig. 4A). This interaction, however, was nonspecific, as it was not affected by inclusion of competing phosphopeptide epitope in the blot (Fig. 4B), and was observed without exposing the blot to primary antiserum (SERCA PS-38, Fig. 4C). Thus the immunoreactivity must be explained by recognition of an unidentified rabbit protein of ~100 kDa by the goat-anti-rabbit-peroxidase reagent.

Rabbit immunoglobulin G can be detected using Staphylococcus aureus protein A based reagents as an alternative to anti-rabbit IgG preparations. To overcome the nonspecific recognition of proteins in rabbit cardiac SR by our goat anti-rabbit peroxidase reagent, rabbit cardiac SR was treated with CaMKII and analyzed with antibody SERCA PS-38 using protein A-peroxidase detection techniques. With this approach, the phosphopeptide was detected in the calibration standard series at loadings in excess of 0.1 pmol, but SERCA was not detected (Fig. 5B) despite loading 10 μg of CaMKII treated SR, which would contain 10 pmol of SERCA2a (26). Careful examination of this experiment shows the recognition of a protein of ~70 kDa in control and CaMKII-treated rabbit SR. This band was not eliminated by inclusion of the phosphorylated epitope peptide, and was not seen in numerous additional experiments. It represents a minor, nonspecific band detected by the high sensitivity methods being deployed to observe trace amounts of SERCA2 phosphorylation.

Thr-17 phosphorylation of phospholamban in the samples presented in Fig. 4A is shown in Fig. 4D. These data show enhanced Thr-17 phosphorylation of phospholamban following exposure to CaMKII (Fig. 4D, lanes 2 and 3), which confirms that the kinase was active against SR targets despite the fact that SERCA phosphorylation on Ser-38 was not observed. Taken together, the data of Figs. 4 and 5B demonstrate that CaMKII was active (Fig. 4D), that the immunodetection of the Ser-38 phosphopeptide was performed with high sensitivity (Figs. 4A and 5B), but that SERCA was not detected by the phosphospecific antibody (Figs. 4A and 5B). This suggests that there is no basal (pre-existing) Ser-38 phosphorylated SERCA in SR vesicles, and that CaMKII is unable to phosphorylated
this residue. It is possible that SERCA phosphorylation occurred at levels below that detected by the antibody (0.1 pmol in this experiment). However, SR loadings of 10 μg contain at least 10 pmol SERCA (assuming SERCA is 10% of total SR protein, Ref. 26). Thus phosphorylation of <0.1 pmol represents phosphorylation of <1% of SERCA molecules.

**Cardiac SR Phosphoprotein of 100 kDa Is Not SERCA**—CaMKII phosphorylation of rabbit cardiac SR using radioactive ATP, employing the conditions of Hawkins et al. (26) results in the production of a phosphoprotein of ~100 kDa (Fig. 5A, lane 2). The use of high concentrations of ATP appeared important, as this phosphoprotein was not observed when 0.2 mM ATP was used (data not shown). To evaluate whether SERCA was the phosphoprotein of ~100 kDa, this membrane was blotted with antibody SERCA PS-38 (Fig. 5B). Immunodetection sensitivity in these experiments was shown to be high (0.1 pmol of calibration-38, Fig. 5B); however, SERCA was not detected. The radioactivity associated with the ~100 kDa protein was quantified at the end of the experiment, at 0.16 pmol, while 10 pmol SERCA would be expected at that location. The amount of phosphoprotein was within the sensitivity range of antibody SERCA PS-38, but was not detected by this antibody. Further experiments were performed with both rabbit and rat cardiac SR. The loading of protein was increased to 60 μg (60 pmol of SERCA) but antibody SERCA PS-38 did not detect SERCA (data not shown). This suggests that Ser-38 phosphorylation is not responsible for the incorporation of 32P into a protein of ~100 kDa in rabbit cardiac SR.
SERCA Remains Dephosphorylated in Isolated Rat Ventricular Myocytes—In a further attempt to evaluate SERCA phosphorylation on Ser-38, we analyzed the pattern of (selected) protein phosphorylation in isolated cardiac myocytes exposed to stimulants able to activate CaMKII. Others have shown that an increase in electrical stimulation frequency (45), exposure to \( \beta \)-H252-adrenergic agonists (46) and exposure to potent Ser/Thr phosphatase inhibitors (47) results in the activation of CaMKII in isolated rat ventricular myocytes. Rat myocytes treated for 5 min with each of the above intervention regimes (stimulated electrically at 0.5 Hz, unless otherwise stated) displayed enhanced CaMKII phosphorylation of phospholamban compared with control myocytes (Fig. 6B, lanes 2–5, see lane 1). The phosphorylation of SERCA on Ser-38 was examined in these myocyte samples using the most sensitive detection reagents available (SuperSignal West Femto; Pierce). This permitted the detection of 0.03 pmol of epitope in the calibration series using this antibody, but did not result in the immunodetection of SERCA with any of these treatments.

Thus we conclude that SERCA phosphorylation, if occurring at all, results in the generation of less than 0.03 pmol of Ser-38 phosphoprotein in the cells studied (10,000 viable myocytes). In previous studies, Ser-16 phosphorylation of phospholamban in myocytes following similar interventions was quantified at 8.5 pmol/1,000 cells (46), indicating the presence of at least 85 pmol of phospholamban in the 10,000 cells of the present study. As phospholamban and SERCA are expressed in similar amounts in cardiac muscle (2 phospholamban per SERCA, Ref. 16), we might expect 42 pmol of SERCA in the experiments performed. Our failure to detect Ser-38 phosphoprotein with the antibody described herein suggests that less than 0.1% of SERCA is phosphorylated in rat cardiac myocytes treated with CaMKII stimulants.

DISCUSSION

In the present study we have described a polyclonal antibody wholly specific for a phosphorylated Ser-38 epitope on SERCA2. The antibody was able to detect the phosphorylated epitope in a calibration standard with high sensitivity (0.03–0.1 pmol). However, it failed to recognize SERCA2 in cardiac SR samples from a variety of animal species, despite the presentation of large amounts of SERCA (10–60 pmol) and the presence of a phosphoprotein of 100 kDa. This indicates that either SERCA is not phosphorylated on Ser-38, or that only a minor fraction of SERCA molecules (i.e. less than ~1%) are phosphorylated on Ser-38. CaMKII activation in isolated car-
Ser-38 Phosphorylation of SERCA2

A cardiac phosphoprotein of 100 kDa is not recognized by SERCA2 antibody. Rabbit cardiac SR vesicles (1 μg/ml) were phosphorylated by exogenous CaMKII (6 μg/ml) as described under “Experimental Procedures.” Phosphorylation reactions were initiated by the addition of 2 mM [γ-32P]ATP (specific activity 400 cpm/pmol). After 2 min at 37 °C the reactions were terminated by the addition of Laemmlli sample buffer (37) and SR samples (10 μg) plus a series of calibration-38 samples (0.06–4 pmol) were subjected to SDS-PAGE and transferred to PVDF membrane. Lane 1 contains control SR (1 mM EGTA, no Ca2+, CaM, or exogenous CaMKII) and lane 2 CaMKII phosphorylated SR. A, autoradiograph of the PVDF membrane. B, blot probed with antibody SERCA PS-38 (1:5000) detected using protein A-peroxidase and an enhanced chemiluminescent substrate (SuperSignal West Femto, Pierce).

Fig. 5.

A cardiac phosphoprotein of 100 kDa is not recognized by SERCA2 antibody. Rabbit cardiac SR vesicles (1 μg/ml) were phosphorylated by exogenous CaMKII (6 μg/ml) as described under “Experimental Procedures.” Phosphorylation reactions were initiated by the addition of 2 mM [γ-32P]ATP (specific activity 400 cpm/pmol). After 2 min at 37 °C the reactions were terminated by the addition of Laemmlli sample buffer (37) and SR samples (10 μg) plus a series of calibration-38 samples (0.06–4 pmol) were subjected to SDS-PAGE and transferred to PVDF membrane. Lane 1 contains control SR (1 mM EGTA, no Ca2+, CaM, or exogenous CaMKII) and lane 2 CaMKII phosphorylated SR. A, autoradiograph of the PVDF membrane. B, blot probed with antibody SERCA PS-38 (1:5000) detected using protein A-peroxidase and an enhanced chemiluminescent substrate (SuperSignal West Femto, Pierce).

The interpretation of negative data requires careful consideration, to ensure that technical shortcomings do not underlie the observations made. Such technical shortcomings would include: 1) deployment of an experimental format unsuitable for the antibody in question; 2) presentation of insufficient antigen (phospho-SERCA2); or 3) presentation of an epitope inaccessible to the antibody. Each of these can be discounted using experimental evidence or logical argument. The data derived from experiments using the peptide calibration standard (calibration-38) address the first two potential shortcomings directly. It is clear that Western blotting is a suitable format for the immunodetection of an epitope by this antibody, as detection of 0.1 pmol of epitope was seen in all experiments. Furthermore these experiments confirm that sufficient SERCA was presented in each experiment, as 10–60 pmol of SERCA was analyzed in the various experiments performed.

Although we do not have experimental data which address the accessibility of the Ser-38 epitope directly, two separate lines of evidence suggest that it is accessible. Firstly, the three-dimensional structure of SERCA1 shows that the equivalent segment of this isoform is solvent accessible in the enzyme (11). Furthermore this region undergoes significant movement as the enzyme undergoes conformational change in its reaction cycle (44). This infers that the segment is solvent accessible and highly mobile, both of which would assist antibody access to the site. In this study we are dealing with SERCA2 not SERCA1. The structure of SERCA2 is not available, however the primary sequence of the two enzymes is well conserved in this region particularly from residue 39 onwards (43). Thus it is likely that they share a similar structure in this region, and that the antibody epitope on SERCA2 is in a solvent-exposed, highly mobile segment of the protein.

A highly mobile, solvent accessible structure is a prerequisite for a kinase substrate. Generally, a protein segment subject to phosphorylation interacts with the active site of the kinase in an extended conformation (48) and thus the phosphorylation motif is located in a flexible loop, or at the N or C terminus of a protein (48). Therefore if we assume Ser-38 is a phosphorylation site, this segment of SERCA2 must be surface exposed and mobile. This would make the segment accessible to antibody binding, as has been described for many hundreds of other phosphoproteins.

SERCA2 Is Not Phosphorylated on Ser-38 by CaMKII—In this study we have examined the phosphorylation of SERCA on Ser-38 in isolated SR vesicles and isolated cardiac myocytes. In each case interventions that activate CaMKII have been deployed, using the optimal conditions described in the literature (26, 45–47). In experiments using cardiac SR vesicles and those using isolated rat myocytes, Thr-17 phosphorylation of phospholamban has demonstrated high levels of CaMKII activity focused on SR targets. Furthermore large amounts of sample...
have been analyzed in an attempt to identify the SERCA phosphoprotein (10–60 pmol). Nevertheless, Ser-38 phosphorylation of SERCA has not been observed with the specific antibody.

In the SR experiments, a phosphoprotein of 100kDa was observed, when phosphorylation reactions were performed at high ATP concentration. This protein was not recognized by the SERCA PS-38 antibody. As such, we do not believe that this phosphoprotein is SERCA. Reddy et al. (14) has previously reported an SR phosphoprotein of 100 kDa, which was not SERCA. This phosphoprotein was restricted to junctional SR, whereas SERCA is distributed throughout junctional and longitudinal SR. The identity of the 100 kDa phosphoprotein was not described (14).

SERCA phosphorylation has been observed by others (24–30). These studies employ a variety of approaches (in particular immunoprecipitation and heterologous expression of SERCA mutants) to establish the identity of the phosphoprotein critically (24–26, 30). In other cases the description of SERCA phosphorylation is made on the basis of coincident migration of SERCA and the phosphoprotein in a one-dimensional SDS-PAGE gel (27–29). This criterion is not sufficiently critical to enable identification of the phosphoprotein, and does not establish whether SERCA is a phosphoprotein.

Where the identification of SERCA as a phosphoprotein has been made using more rigorous criteria, the stoichiometry of SERCA phosphorylation reported is low (0.12 mol/mol, Ref. 26). It has been suggested that this might be explained in part due...
to significant existing phosphorylation (as is the case for RYR2, Ref. 38). Phosphorylation to these levels would result in the presentation of measurable amounts of Ser-38 phosphorylation in experiments employing 10 μg of SR, Figs. 4 and 5). Such amounts would be readily detected in the present experiments, where detection sensitivity of 0.1 pmol (and lower) was observed. Our failure to observe SERCA recognition by the antibody described here, challenges previous conclusions that phosphorylation of Ser-38 of SERCA is a potent regulatory step of Ca2⁺ transport (24–26).

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