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Serine Phosphorylation of the Sarcoplasmic Reticulum Ca^{2+}-ATPase in the Intact Beating Rabbit Heart

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Recent studies have demonstrated that Ca^{2+}/calmodulin-dependent protein kinase phosphorylates the Ca^{2+}-pumping ATPase of cardiac sarcoplasmic reticulum (SR) *in vitro.* Also, evidence from *in vitro* studies suggested that this phosphorylation, occurring at Ser^{38}, results in stimulation of Ca^{2+} transport. In the present study, we investigated whether serine phosphorylation of the SR Ca^{2+}-ATPase occurs in the intact functioning heart. Hearts removed from anesthetized rabbits were subjected to retrograde aortic perfusion of the coronary arteries with oxygenated mammalian Ringer solution containing $^{32}P_i$ and contractions were monitored by recording systolic left ventricular pressure development. Following 45–50 min of ^{32}P perfusion, the hearts were freeze-clamped, SR isolated, and analyzed for protein phosphorylation. SDS–polyacrylamide gel electrophoresis and autoradiography showed phosphorylation of several peptides including the Ca^{2+}-ATPase and Ca^{2+} release channel (ryanodine receptor). The identity of Ca^{2+}-ATPase as a phosphorylated substrate was confirmed by Western immunoblotting as well as immunoprecipitation using a cardiac SR Ca^{2+}-ATPase-specific monoclonal antibody. The Ca^{2+}-ATPase showed immunoreactivity with a phosphoserine monoclonal antibody indicating that the *in situ* phosphorylation occurred at the serine residue. Quantification of Ca^{2+}-ATPase phosphorylation *in situ* yielded a value of 208 ± 12 pmol ^{32}P/mg SR protein which corresponded to the phosphorylation of ~20% of the Ca^{2+} pump units in the SR membrane. Since this phosphorylation occurred under basal conditions (i.e., in the absence of any inotropic intervention), a considerable steady-state pool of serine-phosphorylated Ca^{2+}-ATPase likely exists in the normally beating heart. These findings demonstrate that serine phosphorylation of the Ca^{2+}-ATPase is a physiological event which may be important in the regulation of SR function.

In cardiac and skeletal muscle cells, the Ca^{2+}-pumping ATPase of the sarcoplasmic reticulum (SR) serves the critical function of promoting muscle relaxation by sequestering Ca^{2+} from the myoplasm at the expense of ATP hydrolysis (1–3). A well known mechanism for the regulation of the cardiac SR Ca^{2+}-ATPase involves phosphorylation of another intrinsic SR protein, phospholamban (4–7). In its unphosphorylated state, phospholamban is believed to interact with the Ca^{2+}-ATPase exerting an inhibitory effect; phosphorylation of phospholamban by c-AMP-dependent protein kinase (PKA) or Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) is thought to disrupt this interaction resulting in stimulation of Ca^{2+} pump activity (4–7).

Recent studies from this (8–13) and other laboratories (14–17) have demonstrated that in cardiac SR, a membrane-associated CaM kinase phosphorylates the Ca^{2+}-ATPase *in vitro,* in addition to its previously characterized substrates, phospholamban (4–7) and the ryanodine receptor-Ca^{2+} release channel (RYR-CRC) (18, 19). The phosphorylation occurred at a serine residue and was specific for the cardiac/slow twitch muscle-isoform (SERCA2a) of the Ca^{2+}-ATPase (9). Site-directed mutagenesis studies by Toyofuku *et al.* (14) resulted in the identification of Ser^{38} as the site in SERCA2a that is phosphorylated by CaM kinase. Studies using native cardiac SR vesicles (8), purified Ca^{2+}-ATPase preparations (8, 9) and SERCA2a expressed in HEK-293 cells (14) suggested that Ser^{38} phosphorylation of the Ca^{2+}-ATPase results in activation of the V_{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.* (15) showed CaM kinase mediated phosphorylation of the Ca^{2+}-ATPase in native cardiac SR as well as in 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.* (20) 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

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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 effect 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. In addition, the presence of tightly bound calmodulin in isolated SR vesicles may mask the true potential of calmodulin CaM kinase-dependent regulation of SR Ca^{2+} pump function in *in vitro* experiments (12, 21). Recently, we achieved selective phosphorylation of the Ca^{2+}-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 (12). Under these conditions, Ca^{2+}-ATPase phosphorylation by endogenous CaM kinase *in vitro* resulted in enhanced V_{max} of Ca^{2+} transport without any change in the $K_{0.5}$ for Ca^{2+} activation of the transport rate (12). These observations from *in vitro* studies suggest that, in addition to phospholamban phosphorylation, direct phosphorylation of the Ca^{2+}-ATPase by CaM kinase may serve to regulate cardiac SR Ca^{2+} pump function. However, whether serine phosphorylation of the Ca^{2+}-ATPase is a physiological event occurring in the intact functioning heart has not been addressed in previous studies. The present study was undertaken to evaluate this possibility. The results presented here demonstrate serine phosphorylation of the SR Ca^{2+}-ATPase in the isolated perfused intact beating heart. These findings imply that serine phosphorylation of the SR Ca^{2+}-ATPase is a physiological event which may be important in regulating SR Ca^{2+} pump function in heart muscle.

MATERIALS AND METHODS

Chemicals. Reagents for electrophoresis were obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada), [γ-^{32}P]ATP was purchased from Amersham (Oakville, Ontario, Canada) and ^{32}P (as Na_2HPO_4 in water) from Dupont New England Nuclear (Montreal, PQ, Canada). Phosphoserine monoclonal antibody was obtained from Sigma Chemical (St. Louis, MO) and SERCA2 monoclonal antibody (IID8) from Affinity Bioreagents (Golden, CO). All other chemicals were from Sigma Chemical, or BDH Chemicals (Toronto, Ontario, Canada).

Heart perfusion, recording of contractions, and ^{32}P-labeling. Hearts from New Zealand White male rabbits (2.5–3 kg) were used for all experiments. The experimental protocols were approved by the Animal Care Committee established by the University of Western Ontario and conformed to the guidelines of the Canadian Council on Animal Care. The rabbits were anesthetized with sodium pentobarbital (35 mg/kg, iv). After anesthesia, the chest was rapidly opened, the hearts were removed and immediately cannulated for retrograde aortic perfusion of the coronary arteries with mammalian Ringer solution consisting of (in mmol liter^{-1}) NaCl 154, KCl 5, $CaCl_2$ 2.2, $NaHCO_3$ 6 and Dextrose 5.5. 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 18 ml/min using a peristaltic pump. A latex balloon-tipped cannula filled with degassed H_2O and attached to a calibrated COBE disposable transducer was inserted through the left atrium into the lumen of the left ventricle for obtaining systolic left ventricular pressure (LVP) development. LVP was assessed by a preamplifier (7P-1F) and recorded on a Grass 79D polygraph (Grass Instruments, Quincy, MA). For the initial 15–20 min, the hearts were perfused in a non-recirculating manner to allow the preparations to stabilize. Subsequently, the hearts were paced electrically (at 120 or 180 beats/min) with a Grass SD9 stimulator via a platinum wire electrode inserted into the epicardium, at double threshold voltage and a duration of 5 ms. The perfusion circuit was then switched to a recirculating flow containing the same Ringer solution supplemented with 4 mCi of $^{32}P_i$. The perfusion with ^{32}P was continued for 45–50 min. Following this, perfusion with nonradioactive Ringer solution was resumed for 5 min and then the hearts were freeze-clamped with Wollenberger clamps precooled in liquid nitrogen. The frozen tissue was powdered and used for the determination of the specific radioactivity of [γ-^{32}P]ATP and isolation of SR membranes and analysis of protein phosphorylation.

Determination of specific radioactivity of [γ-^{32}P]ATP in the ^{32}P-labeled heart. The specific radioactivity of [γ-^{32}P]ATP of each heart was determined using the procedure of Hawkins *et al.* (22) with minor modifications. ATP was extracted from ^{32}P-labeled cardiac tissue as follows. Approximately 200 μg of frozen tissue was thawed and homogenized in 0.8 ml of 0.5 M $HClO_4$ using a polytron PT-10 homogenizer at setting 8, with 10 seconds burst for four times at 30 sec intervals. The homogenate was centrifuged at 39,000*g* for 20 min and the supernatant was neutralized with saturated K_2CO_3 in the presence of BDH Universal indicator (20 μl/ml). The mixture was kept ice-cold for 30 min and precipitated $KClO_4$ was removed by centrifugation at 39,000*g* for 20 min. To determine the specific radioactivity of the γ-phosphate group of [^{32}P]ATP, aliquots of the supernatant were used in a histone phosphorylation assay as follows. A 40-μl portion of the supernatant was added to a reaction mixture (total volume 100 μl) containing 50 mM Tris–HCl (pH 6.8), 0.4 mg of histone H2A/ml, 10 mM magnesium acetate, 30 mM dithiothreitol, 30 mM NaF and 5 μg of cAMP-dependent protein kinase (catalytic subunit). The reaction mixture was incubated at 30°C for 140 min. Under these conditions, histone phosphorylation reached a plateau within 90 min and remained stable at least until 140 min. The reaction was terminated by transferring 45 μl reaction mixture to a 4-cm^2 piece of filter paper (No. 1, Whatman International Ltd., Maidstone, England). The filter squares were washed four times for 15 min each in ice-cold 10% trichloroacetic acid, rinsed once in ethanol and dried, and the radioactivity was determined by liquid scintillation spectrometry. A sample of pure [γ-^{32}P]ATP with known specific radioactivity was included in the phosphorylation assay as a standard. The specific radioactivity of each heart was expressed as the ratio between the unknown and the standard.

Preparation of SR membranes. SR-enriched membranes vesicles were prepared from ventricular tissue from ^{32}P-perfused hearts as well as from hearts not subjected to the perfusion protocol, as described previously (23). 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 nitrogen. Membrane protein was determined by the method of Lowry *et al.* (24) using bovine serum albumin as standard.

Ca^{2+}/Calmodulin-dependent SR protein phosphorylation in vitro. *In vitro* phosphorylation of SR proteins by CaM kinase was performed as described previously (8). The incubation medium (total volume 50 μl) contained 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 200 μM $CaCl_2$, 200 μM EGTA, 1 μM calmodulin, 0.8 mM [γ-^{32}P]ATP (specific activity 200–300 cpm/pmol) and SR (25–35 μg protein). The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP following 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 μl of SDS sample buffer, and the samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 4–18% gradient gel. The gels were stained with Coomassie Brilliant Blue, dried and autoradiographed (8). Quantification of phosphorylation was carried out by liquid scintillation counting of the radioactive bands from gels (9).

SR membranes isolated from ^{32}P-perfused hearts were solubilized in SDS-sample buffer and subjected to SDS–PAGE and autoradiography as described above.

Immunoblotting of serine-phosphorylated peptides and immunoprecipitation of the Ca^{2+}-ATPase. Serine-phosphorylated peptides in the SR were detected by Western immunoblotting using a phosphoserine monoclonal antibody (Sigma). The identity of the 105-kDa phosphorylated peptide as the Ca^{2+}-ATPase was verified by Western immunoblotting and immunoprecipitation using a cardiac SR Ca^{2+}-ATPase (SERCA2)-specific monoclonal antibody (IID8, Affinity Bioreagents). For Western blotting, the SR proteins solubilized in SDS sample buffer were subjected to SDS–PAGE and then electroblotted to nitrocellulose membrane (9). In experiments designed to verify the identity of the 105-kDa peptide, this band was excised from the gel, subjected to SDS–PAGE on 10% mini gel and then electroblotted to nitrocellulose membrane (9). The nitrocellulose membrane was incubated with phosphoserine monoclonal antibody (dilution 1:500) or SERCA2-specific monoclonal antibody (dilution 1:3000) and then with a peroxidase-linked anti-mouse immunoglobulin (Ig)G as secondary antibody (dilution 1:5000). The immunoreactive peptides were visualized using the ECL detection system from Amersham. Nitrocellulose membranes incubated in the absence of the corresponding primary antibody served as control for these experiments. For immunoprecipitation of the Ca^{2+}-ATPase, the SR membrane (~2 mg protein/ml) was mixed with an equal volume of solubilizing buffer (10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100). Aliquots of the solubilized material were incubated with SERCA2 antibody in the presence of 0.3% bovine serum albumin at 4°C for 2 h. Subsequently, protein A-agarose (Bio-Rad) beads precoated with secondary antibody (anti-mouse IgG) were added to the reaction mixture for 1 h at 24°C, the beads were removed by centrifugation in a microfuge, washed with phosphate-buffered saline, digested in SDS sample buffer, and subjected to SDS–PAGE and autoradiography. The solubilized membrane samples that underwent the same incubation protocol in the absence of SERCA2 antibody served as control for this experiment.

Data presentation. Results from typical experiments are presented in the figures. The experiments were repeated using six ^{32}P-perfused rabbit heart preparations and the results obtained were similar.

RESULTS

Phosphorylation of SR proteins in intact beating hearts. Figure 1 shows the rhythmic contractions recorded from two isolated heart preparations perfused with mammalian Ringer solution containing $^{32}P_i$. The contraction records shown were taken just prior to freeze clamping of the hearts for the isolation of SR; the frequency of contractions differed in the two preparations (~120 and 180 beats/min). Following 45–50 min of perfusion with ^{32}P, the hearts were freeze-clamped, SR membranes were isolated and analyzed for protein phosphorylation. As shown in Fig. 2, a number of peptides were found to be phosphorylated *in situ*, and these included the 105-kDa band designated "Ca^{2+} pump" (Ca^{2+}-ATPase) and the ~446-kDa band designated "Ca^{2+} channel" (ryanodine receptor). Phosphorylation of phospholamban was barely detectable under the basal conditions (i.e., in the absence of adrenergic stimulation) employed. To ascertain the identity of the 105-kDa band as the Ca^{2+} pump protein, this band was excised from the gel, homogenized and electrophoresed again, and analyzed. The band could be detected using cardiac SR Ca^{2+}-ATPase (SERCA2)-specific antibody (Fig. 2C) suggesting that the 105-kDa band was SERCA2. The 105 kDa band was also detected by autoradiography, indicating that it was phosphorylated (Fig. 2D). Phosphorylation of SERCA2 *in situ* was further verified in an experiment where the SR membranes were solubilized and immunoprecipitated using SERCA2-specific antibody, and the immunoprecipitate was analyzed by SDS–PAGE and autoradiography. The antibody immunoprecipitated the 105-kDa peptide band corresponding to SERCA2 (Fig. 2E), and it was phosphorylated (Fig. 2F). No immunoprecipitation of the 105-kDa band was observed when the primary antibody was excluded from the immunoprecipitation step (Figs. 2E and 2F). The identity of the 446-kDa band as the Ca^{2+} release channel was inferred from the immunoreactivity of this band with a cardiac ryanodine receptor-specific antibody (a generous gift from Drs. M. Shigekawa and T. Imagawa, National Cardiovascular Center Research Institute, Suita, Japan) in Western blots (results not shown, but see Ref. 11).

To quantify the *in situ* phosphorylation of SR Ca^{2+} pump and Ca^{2+} release channel, the corresponding peptide bands from the gels were excised and the ^{32}P

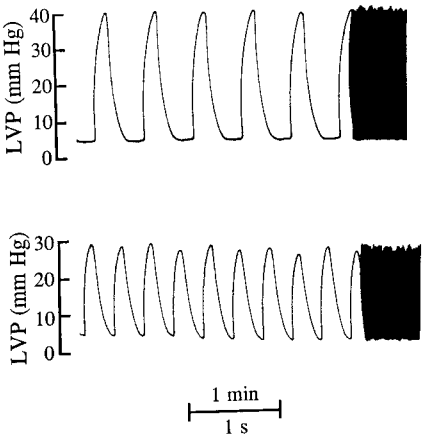

FIG. 1. Contractile function of isolated ^{32}P-perfused intact beating rabbit hearts. The hearts were perfused with mammalian Ringer solution containing 4 mCi ^{32}Pi and contractions were recorded as described under Materials and Methods. Shown are the contractions recorded for two separate isolated heart preparations beating at different frequencies (upper trace, 120 beats per min; lower trace, 180 beats per min). These contraction were recorded just prior to freeze-clamping of the hearts for isolation of SR. The horizontal time bar indicates interval at either fast or slow recorder speed. Data on the phosphorylation of SR proteins in the same hearts are presented in Fig. 2.

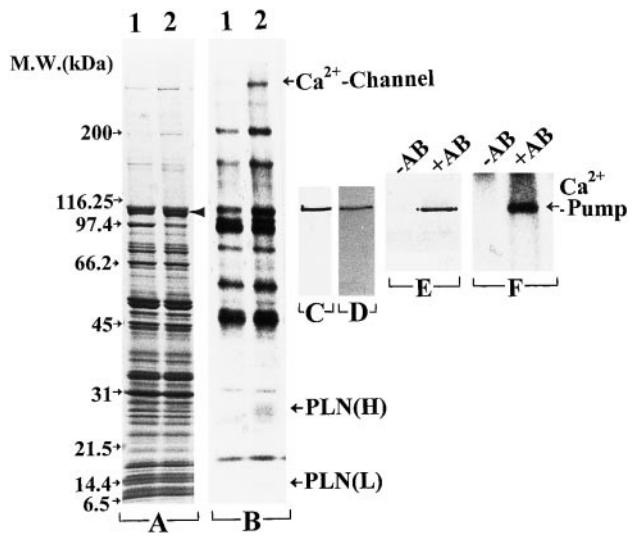

FIG. 2. Phosphorylation of Ca^{2+}-ATPase (Ca^{2+} pump), ryanodine receptor (Ca^{2+} channel) and phospholamban (PLN) in isolated perfused intact beating rabbit hearts. SR membranes derived from the two isolated heart preparations described in the legend to Fig. 1 were subjected to SDS–PAGE and autoradiography to monitor protein phosphorylation (for experimental details, see Materials and Methods). A shows the Coomassie Blue-stained gel showing SR protein profile. B shows autoradiogram of the same gel. The lanes marked 1 and 2 represent SR isolated from hearts beating at 120 (upper trace in Fig. 1) and 180 (lower trace in Fig. 1) beats/min, respectively. Lane C is a Western blot of the 105-kDa phosphorylated peptide band (excised from lane 2 of the gel) showing immunoreactivity with SR Ca^{2+}-ATPase-specific antibody; lane D is the autoradiogram of the Western immunoblot. Presented in E is a Coomassie blue-stained gel showing specific immunoprecipitation of the Ca^{2+}-ATPase by the antibody, and F shows autoradiogram of the same gel. Note that both Ca^{2+} pump and Ca^{2+} channel undergo phosphorylation. Under these experimental conditions (i.e., in the absence of adrenergic stimulation) no appreciable phosphorylation of phospholamban [PLN: high (H) or low (L) molecular weight forms] is evident. −AB, primary antibody absent; +AB, primary antibody present.

radioactivity associated with these bands was determined by liquid scintillation counting. Also the specific radioactivity of [γ-^{32}P]ATP in the ^{32}P-perfused hearts was determined as detailed under Materials and Methods. From these data, the *in situ* phosphorylation of the SR Ca^{2+} pump and Ca^{2+} release channel was estimated to be 208 ± 12 and 50 ± 8 pmol ^{32}P/mg SR protein, respectively (mean ± SE of triplicate determinations using two separate SR preparations from ^{32}P-perfused hearts).

Detection of serine-phosphorylated peptides in in vitro phosphorylated SR. Previous studies have demonstrated that *in vitro* phosphorylation of the cardiac SR Ca^{2+} pump and Ca^{2+} release channel by CaM kinase occurs at a serine residue (8, 9, 18), identified as Ser^{38} in the case of Ca^{2+} pump (14) and Ser^{2809} in the case of Ca^{2+} channel (18). We performed Western immunoblotting analysis using a phosphoserine monoclonal antibody (Sigma) in an attempt to detect serine-phosphorylated peptides in the SR. SR membranes subjected to incubation in the phosphorylation assay medium containing Ca^{2+} and calmodulin (*in vitro* phosphorylated SR) were used for this experiment. As shown in Fig. 3, phosphoserine immunoreactivity could be observed in peptide bands corresponding to the Ca^{2+} pump and Ca^{2+} release channel. No immunoreactive peptides were detected in control experiments performed in the absence of the primary anybody (Fig. 3).

Serine phosphorylation of the SR Ca^{2+} pump in the intact beating heart. Having established the specificity of the phosphoserine antibody to detect serine-phosphorylated peptides in the SR (Fig. 3), we utilized this antibody to investigate whether phosphorylation of the SR Ca^{2+} pump observed in the isolated perfused beating heart also occurred at a serine residue, as would be expected from the *in vitro* findings. Thus, *in situ* phosphorylated SR membranes isolated from ^{32}P-perfused beating hearts as well as SR membranes phosphorylated *in vitro* were subjected to SDS–PAGE (4–18% gradient gel) and autoradiography. The 105-kDa peptide band was excised from the gel, eluted and electrophoresed again on 10% mini gel, and electroblotted to nitrocellulose membranes, and probed with the phosphoserine, monoclonal antibody as well as the SERCA2-specific monoclonal antibody. As shown in Fig. 4, the 105-kDa band from both *in situ*-phosphorylated SR and *in vitro* phosphorylated SR showed immunoreactiv-

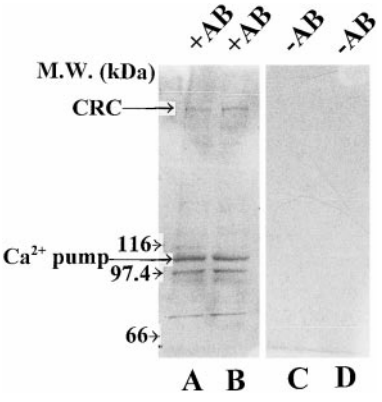

FIG. 3. Detection of serine-phosphorylated peptides in SR membranes phosphorylated *in vitro.* A monoclonal phosphoserine antibody was used to perform Western immunoblotting analysis of SR membranes subjected to incubation in the phosphorylation assay medium ("*in vitro*-phosphorylated SR"). Shown is a Western immunoblot obtained using two separate SR preparations (lanes A and C represent one SR preparation; lane B and D represent another SR preparation) phosphorylated *in vitro.* Phosphoserine immunoreactivity is observed in peptide bands corresponding to Ca^{2+} release channel (CRC) and Ca^{2+} pump. No immunoreactive peptides were detected in control experiments performed in the absence of the primary antibody. +AB, primary antibody present; −AB, primary antibody absent.

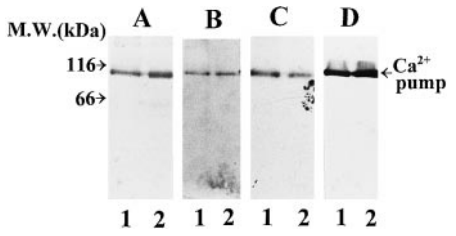

FIG. 4. Detection of serine phosphorylation of the SR Ca^{2+}-ATPase in the intact beating heart. *In situ* phosphorylated SR membranes isolated from ^{32}P-perfused intact beating heart as well as SR membranes phosphorylated *in vitro* were subjected to SDS–PAGE (4–18% gradient gel) and autoradiography (for experimental details, see Materials and Methods). The 105-kDa phosphorylated peptide band was excised from the gel, subjected to SDS–PAGE on 10% mini gel and electroblotted to nitrocellulose membranes. The membranes were probed with phosphoserine monoclonal antibody or Ca^{2+}-ATPase (SERCA2)-specific antibody. A, Coomassie blue-stained gel; B, Western immunoblot obtained with phosphoserine antibody; C, autoradiogram of the Western immunoblot shown in B; D, Western immunoblot obtained with SERCA2 antibody. For each panel, lanes 1 and 2 represent SR membranes phosphorylated *in vitro* and *in situ*, respectively.

ity with the SERCA2 antibody and the phosphoserine antibody.

DISCUSSION

The results of the present study demonstrate that the SR Ca^{2+} pump and Ca^{2+} release channel undergo serine phosphorylation in the isolated perfused intact beating hearts in the absence of any inotropic intervention. Thus, serine phosphorylation of these SR Ca^{2+} cycling proteins is a physiological event which may be important in the regulation of SR function. The major focus of the present study was on serine phosphorylation of the SR Ca^{2+} pump. Under the experimental conditions used, quantification of the *in situ* phosphorylation of the Ca^{2+} pump yielded a value of ~200 pmol ^{32}P incorporation/mg SR protein. Since the Ca^{2+}-ATPase comprised of ~10% of the protein content in the SR preparations used (see Fig. 2), the observed *in situ* phosphorylation corresponds to the phosphorylation of about 20% of the Ca^{2+} pump units in the SR, assuming a single phosphorylation site (14). Our previous studies on CaM kinase-catalyzed phosphorylation of the cardiac SR Ca^{2+} pump *in vitro* yielded low levels (~12–13%) of phosphorylation (9). The low levels of Ca^{2+} pump phosphorylation measured *in vitro* and *in situ* is likely due to high levels of pre-existing steady state phosphorylation of the Ca^{2+} pump. The *in situ* phosphorylation of the Ca^{2+} pump observed under basal condition (i.e., in the absence of any inotropic intervention) supports this possibility. Furthermore, we have observed that treatment of isolated SR with protein phosphatase results in enhancement of Ca^{2+} pump phosphorylation by CaM kinase *in vitro* (9). High level of pre-existing phosphorylation of substrates having a single phosphorylation site (such as the Ca^{2+} pump) may have contributed to the failure to observe phosphorylation of substrates other than phospholamban in previous studies. It should noted that a molar ratio of 2:1 phospholamban pentamer/Ca^{2+}-ATPase has been described in the SR membrane (25). This would mean that even when all Ca^{2+} pump units in the membrane undergo phosphorylation, the magnitude of phosphate incorporation into the membrane Ca^{2+} pumps would be 10% relative to phospholamban as the latter gets phosphorylated at each of the five Thr^{17} in the pentameric unit. Furthermore, pre-existing phosphorylation (which may be substantial) would limit *de novo* phosphorylation of the Ca^{2+} pump measured in phosphorylation assays performed *in vitro* or *in situ* making it difficult to readily detect Ca^{2+} pump phosphorylation. Our success in demonstrating the phosphorylation of the SR Ca^{2+} pump (and Ca^{2+} release channel) in the intact beating heart is due, at least in part, to the use of a phosphate-free buffer such as mammalian Ringer as this would be expected to enhance the specific radioactivity of the intracellular ATP pool in the ^{32}P-perfused hearts. Under the experimental conditions reported here the specific radioactivity of ATP in the ^{32}P-perfused hearts was 16–20 cpm/pmol; in our experience, this is about 4- to 5-fold higher than that generally obtained in rat hearts perfused with ^{32}P (2 mCi) in phosphate buffer.

The identity of the protein kinase responsible for serine phosphorylation of the SR Ca^{2+} pump and Ca^{2+} release channel in the intact beating heart was not ascertained in the present study. However, based on the observations from *in vitro* phosphorylation studies (8–13, 18, 19) it seems likely that the observed serine phosphorylation of the Ca^{2+} pump and Ca^{2+} release channel was mediated by CaM kinase. The involvement of CaM kinase may explain the apparent high level of pre-existing steady state substrate phosphorylation under basal condition, suggested by this study. Ca^{2+} and calmodulin required for the activation of CaM kinase is readily available to the myocyte—i.e., the cytoplasmic Ca^{2+} rises during each heart beat, and the concentration of calmodulin in the myocyte is sufficiently high (2–3 μM) (26). Furthermore, phosphorylation of SR substrates by CaM kinase is very rapid whereas their dephosphorylation by protein phosphatase is relatively slow (8), thus permitting the maintenance of a high level of phosphorylated substrate pool. The physiological significance of high steady state level of substrate phosphorylation remains enigmatic at this time. In the case of the Ca^{2+} pump, $Serine^{38}$ phosphorylation was found to result in an increased V_{max} of Ca^{2+} transport (12). Also, single mutation of $Serine^{38}$ to alanine in SERCA2a results in about 50% loss in V_{max} of Ca^{2+} transport (14). It is possible that the optimal Ca^{2+} sequestering activity of the SR may be determined, in

part, by the serine-phosphorylated state of the Ca^{2+} pump. Alteration in the V_{max} of SR Ca^{2+}-ATPase, attributed to the phosphorylation status of phospholamban (or Ca^{2+}-ATPase-phospholamban interaction), has been observed in some studies (27–31), while in other studies no effect on V_{max} could be observed (15, 32–34). Conceivably, differences in the serine-phosphorylation status of the Ca^{2+} pump units in the SR membrane may have contributed to such disparate observations.

While phospholamban is readily phosphorylated *in vitro* in the presence of Ca^{2+} and calmodulin (4–12), no appreciable phosphorylation of phospholamban was observed in the SR isolated from hearts perfused with ^{32}P under the basal conditions used in the present study. Although intriguing, this observation is consistent with previous studies showing that physiological increases in intracellular Ca^{2+} concentration do not stimulate phospholamban phosphorylation in the intact functioning heart unless accompanied by an increase in intracellular cAMP level (35, 36). Phosphorylation of phospholamban by cAMP-dependent protein kinase and CaM kinase occur at Ser^{16} and Thr^{17}, respectively (37). The mechanistic basis of the requirement for a cAMP-dependent step for the phosphorylation of phospholamban by CaM kinase *in vivo*, but not *in vitro*, has not been resolved.

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