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Ca^{2+}/Calmodulin-Dependent Phosphorylation of the Ca^{2+}-ATPase, Uncoupled from Phospholamban, Stimulates Ca^{2+}-Pumping in Native Cardiac Sarcoplasmic Reticulum

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Recent studies have demonstrated phosphorylation of the cardiac and slow-twitch muscle isoform (SERCA2a) of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (at Ser^{38}) by a membrane-associated Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase). Analysis of the functional consequence of Ca^{2+}-ATPase phosphorylation in the native SR membranes, however, is complicated by the concurrent phosphorylation of the SR proteins phospholamban (PLN) which stimulates Ca^{2+} sequestration by the Ca^{2+}-ATPase, and the ryanodine receptor-Ca^{2+} release channel (RYR-CRC) which likely augments Ca^{2+} release from the SR. In the present study, we achieved selective phosphorylation of the Ca^{2+}-ATPase by endogenous CaM kinase in isolated rabbit cardiac SR vesicles utilizing a PLN monoclonal antibody (PLN AB) which inhibits PLN phosphorylation, and the RYR-CRC blocking drug, ruthenium red, which inhibits phosphorylation of RYR-CRC. Analysis of the Ca^{2+} concentration-dependence of ATP-energized Ca^{2+} uptake by SR showed that endogenous CaM kinase mediated phosphorylation of the Ca^{2+}-ATPase, in the absence of PLN and/or RYR-CRC phosphorylation, results in a significant increase (~50-70%) in the V_{max} of Ca^{2+} sequestration without any change in the $k_{0.5}$ for Ca^{2+} activation of the Ca^{2+} transport rate. On the other hand, treatment of SR with PLN AB (which mimics the effect of PLN phosphorylation by uncoupling Ca^{2+}-ATPase from PLN) resulted in ~2-fold decrease in $k_{0.5}$ for Ca^{2+} without any change in V_{max} of Ca^{2+} sequestration. These findings suggest that, besides PLN phosphorylation, direct phosphorylation of the Ca^{2+}-ATPase by SR-associated CaM kinase serves to enhance the speed of cardiac muscle relaxation. © 1999 Academic Press

By virtue of its ability to control myoplasmic free Ca^{2+} concentration, the sarcoplasmic reticulum (SR) plays a central role in the contraction-relaxation cycle of heart muscle. Following excitation, Ca^{2+} is released from the SR through a Ca^{2+}-release channel (the ryanodine receptor) and the consequent increase in myoplasmic Ca^{2+} produces myofilament activation and muscle contraction (1–3). Subsequently, a Ca^{2+}-pumping ATPase (Ca^{2+}-ATPase) present in the SR actively sequesters Ca^{2+} back into the SR lumen, thus lowering myoplasmic Ca^{2+} to promote muscle relaxation (2–4). A well known mechanism for the regulation of the cardiac SR Ca^{2+}-ATPase involves phosphorylation of another intrinsic SR protein, phospholamban (5–8). In its unphosphorylated state, phospholamban is thought to interact with the Ca^{2+}-ATPase exerting an inhibitory effect; phosphorylation of phospholamban by cAMP-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 (5–8). In cardiac SR, the ryanodine receptor-Ca^{2+}-release channel (RYR-CRC) also undergoes phosphorylation by CaM kinase (9–11). The phosphorylation of cardiac RYR-CRC by CaM kinase may result in stimulation of Ca^{2+} release from the SR (9, 12–14).

Recent studies from this (11, 15–18) and other (19–21) laboratories 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 found to be isoform-specific in that the Ca^{2+}-ATPase isoform expressed in cardiac muscle and slow twitch skeletal muscle (SERCA2a) but not that expressed in fast twitch skeletal muscle (SERCA1) underwent phosphorylation by endogenous and exogenous CaM kinase (15). Site-directed mutagenesis studies by Toyofuku *et al.* (22) resulted in the identification of Ser^{38} as the site in SERCA2a that is phorphorylated by CaM kinase. In experiments using microsomes containing SERCA2a expressed in HEK-293 cells, they also showed that Ser^{38} phosphorylation of SERCA2a

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resulted in activation of the V_{max} of Ca^{2+} transport (22). Some studies have, however, questioned the physiological role of Ca^{2+}-ATPase phosphorylation. Thus, a study by Odermatt *et al.* (19) showed CaM kinase mediated phosphorylation of Ca^{2+}-ATPase in native rabbit cardiac SR as well as of 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.* (23) reported failure to observe phosphorylation of the Ca^{2+}-ATPase in canine cardiac SR or of 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 effect of Ca^{2+}-ATPase phosphorylation on the 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 the present study, we achieved selective phosphorylation of the cardiac SR Ca^{2+}-ATPase by the membrane-associated CaM kinase by utilizing a phospholamban monoclonal antibody which inhibits phospholamban phosphorylation (and uncouples Ca^{2+}-ATPase from phsopholamban) (24), and the RYR-CRC blocking drug, ruthenium red, which was found to inhibit Ca^{2+} channel phosphorylation (18). The results reported here demonstrate that in native cardiac SR, endogenous CaM kinase mediated phosphorylation of the Ca^{2+}-ATPase, in the absence of phospholamban and/or RYR-CRC phosphorylation, results in activation of the V_{max} of Ca^{2+} pumping. These findings suggest that besides phospholamban phosphorylation, direct phosphorylation of the Ca^{2+}-ATPase by the SR-associated CaM kinase serves to enhance the speed of cardiac muscle relaxation.

MATERIALS AND METHODS

Chemicals. Reagents for electrophoresis were obtained from Bio-Rad Laboratories (Mississauga, ON, Canada), [γ-^{32}P]ATP was purchased from Amersham (Oakville, ON, Canada), and $^{45}CaCl_2$ was from NEN (Mississauga, ON, Canada). Anti-phospholamban monoclonal antibody (24) was obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were from Sigma chemical (St. Louis, MO).

Isolation of SR vesicles and treatment with anti-phospholamban monoclonal antibody. SR-enriched membrane vesicles were isolated from rabbit heart ventricles as described previously (25). Following isolation, the SR vesicles were suspended in 10 mM Tris-maleate (pH 6.8) containing 100 mM KCl, divided into small aliquots ($\sim$100 μl), quick-frozen in liquid N_2 and stored at -80°C. The membranes were thawed once on ice and used for experiments. Protein was determined by the method of Lowry *et al.* (26) using bovine serum albumin as standard.

Treatment of SR vesicles with anti-phospholamban monoclonal antibody (24) was performed as follows. The SR vesicles (60 μg protein) were incubated in a medium (total volume 110 μl) containing 10 mM Tris-maleate (pH 6.8), 120 mM KCl and 40 μg anti-phospholamban monoclonal antibody for 10 min at 24°C and for an additional 20 min at 4°C. Subsequently, the SR vesicles were recovered by centrifugation (at 15,000 rpm for 40 min in a microcentrifuge) and used for phosphorylation and Ca^{2+} uptake assays. SR vesicles subjected to the same experimental protocol in the absence of anti-phospholamban monoclonal antibody in the incubation medium served as control for these experiments.

Phosphorylation assay. The standard incubation medium (total volume 50 μl) for phosphorylation by endogenous CaM kinase 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 300-400 cpm/pmol) and SR (30 μg protein). The initial free Ca^{2+} concentration, determined using the computer program of Fabiato (27) was 5.4 μM. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP 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 μl 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 (11). Quantification of phosphorylation was carried out by liquid scintillation counting after excision of the radioactive bands from the gels (15). The Ca^{2+}- and calmodulin-dependence of phosphorylation was monitored in parallel assays in which Ca^{2+} and/or calmodulin was lacking in the assay medium.

To evaluate the effect of endogenous CaM kinase mediated phosphorylation on Ca^{2+} uptake, phosphorylated SR vesicles were prepared by incubating the membranes in the phosphorylation assay medium described above with the exception that the total volume of the reaction mixture was 250 μl and non-radioactive ATP was used instead of [γ-^{32}P]ATP. Also, in experiments where blockade of phosphorylation of RYR-CRC was desired, the phosphorylation assay medium was supplemented with 25 μM ruthenium red (18). Following the phosphorylation reaction, aliquots of the SR vesicles were transferred to a Ca^{2+} uptake assay medium for measurement of ATP-dependent Ca^{2+} uptake (Ca^{2+} pump) activity (see below). Unphosphorylated SR used as control for these experiments were prepared by subjecting the SR vesicles to the same experimental protocol with the exception that the phosphorylation reaction medium lacked calmodulin and contained 10 μM W7, a calmodulin antagonist, so as to preclude activation of endogenous CaM kinase by SR-associated calmodulin.[2]

Determination of Ca^{2+} uptake. ATP-dependent, oxalate facilitated Ca^{2+} uptake was determined in cardiac SR using the Millipore filtration technique as detailed elsewhere (28). The incubation medium (total volume 250 μl) contained 50 mM Tris-maleate (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 5 mM NaN_3, 120 mM KCl, 5 mM potassium oxalate, 25 μM ruthenium red, 0.1 mM EGTA, SR vesicles (4 μg protein) and differing amounts of $^{45}CaCl_2$ ($\sim$15,000 cpm/pmol) to yield the desired free Ca^{2+} concentration. The initial free Ca^{2+} in the assay medium was determined using the computer program of Fabiato (27). The Ca^{2+} uptake reaction was initiated by the addition of SR to the rest of the assay components preincubated for 3 min at 37°C. The reaction was allowed to proceed for 1 min following which aliquots (200 μl) of the reaction mixture were filtered through Millipore filters (45 μm pore size). The filters were washed with 3 ml of 10 mM Tris-maleate buffer (pH 6.8) containing 10 mM $MgCl_2$ and 10 μM ruthenium red. Ca^{2+} retained by the filters was measured by liquid scintillation counting.

Data analysis. Results are presented as mean $\pm$ SE. Statistical significance was evaluated with the Student's *t*-test. $P < 0.05$ was taken as the level of significance.

[2] Western blotting analysis using an anticalmodulin antibody showed the presence of appreciable amounts of calmodulin in isolated cardiac SR vesicle. Also, addition of 10 μM W7 to the Ca^{2+} uptake assay medium was found to cause $\sim$8-12% inhibition of Ca^{2+} uptake.

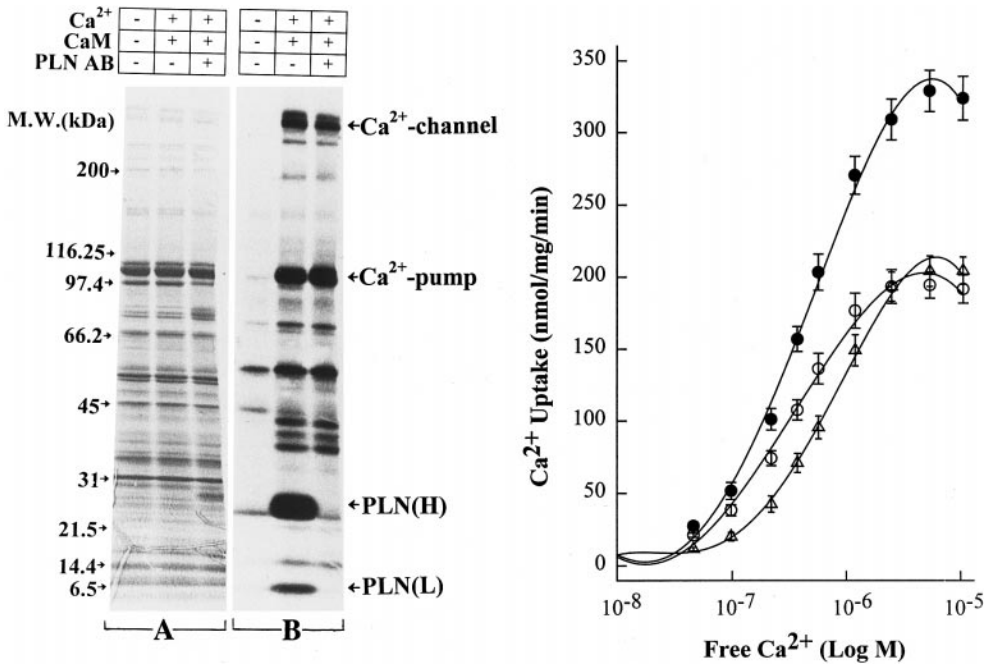

FIG. 1. Left panel: The effect of phospholamban monoclonal antibody (PLN AB) on Ca^{2+}/calmodulin (CaM)-dependent protein phosphorylation in rabbit cardiac SR. Control SR (denoted "−PLN AB") and PLN AB-treated SR (denoted "+PLN AB") were prepared and the phosphorylation reaction was carried out as descried in Materials and Methods. Coomassie blue-stained SDS-PAGE gel depicting SR protein profile (panel A) and an autoradiogram of the same gel depicting protein phosphorylation (panel B) are shown. The absence (−) or presence (+) of Ca^{2+} and CaM in the phosphorylation reaction medium is indicated on the top. Note that phosphorylation of phospholamban (PLN; H, high molecular weight form; L, low molecular weight form) is selectively abolished in PLN AB-treated SR. Quantitative data from several experiments on the effect of PLN AB-treatment on SR protein phosphorylation are summarized in Table 1. Right panel: Comparison of the Ca^{2+} concentration dependence of ATP-energized Ca^{2+} uptake by control unphosphorylated SR (△), PLN AB-treated unphosphorylated SR (○) and PLN AB-treated phosphorylated SR (●). The phosphorylation reaction and subsequent Ca^{2+} uptake assays were performed as described in Materials and Methods. The data represent mean ± SE of 7 experiments in each case. The kinetic parameters of Ca^{2+} uptake derived from these data are summarized in Table 2.

RESULTS AND DISCUSSION

Treatment of cardiac SR membranes with phospholamban monoclonal antibody is known to result in abolition of the inhibitory interaction of phospholamban with the Ca^{2+}-ATPase, apparently due to the ability of the antibody to mimic the effect of phospholamban phosphorylation by PKA and CaM kinase; the antibody also blocks phosphorylation of phospholamban by PKA and CaM kinase (24). Therefore, in a series of experiments, we utilized phospholamban monoclonal antibody to uncouple Ca^{2+}-ATPase from phospholamban prior to inducing activation of endogenous CaM kinase in isolated rabbit cardiac SR vesicels. The autoradiogram shown in Fig. 1 (left panel) compare the Ca^{2+}/calmodulin-dependent, endogenous CaM kinase mediated protein phosphorylation in control and phospholamban monoclonal antibody-treated SR. It can be seen that phospholamban antibody treatment of SR abolished phospholamban phosphorylation. Phosphorylation of the Ca^{2+}-ATPase was unaffected by the antibody treatment whereas phosphorylation of RYR-CRC was slightly (~25%) depressed. Quantification of data from several experiments corroborating these observations are summarized in Table 1.

Since phospholamban antibody treatment of SR resulted in selective abolition of phospholamban phosphorylation and retention of Ca^{2+}-ATPase phosphorylation, in subsequent experiments, phospholamban antibody-treated SR vesicles were used to determine

TABLE 1

Effects of Phospholamban Monoclonal Antibody (PLN AB) and Ruthenium Red (RR) on Endogenous CaM Kinase Mediated Protein Phosphorylation in Cardiac SR

	Phosphorylation (pmol ^{32}P/mg protein)		
Treatment	RYR-CRC	Phospholamban	Ca^{2+}-ATPase
None (Control)	24 ± 2	310 ± 69	57 ± 9
PLN AB-treated	18 ± 1.4*	5 ± 1*	54 ± 8
RR-treated	2 ± 0.7*	300 ± 66	40 ± 6*
PLN AB + RR-treated	1.2 ± 0.2*	7 ± 0.9*	40 ± 8*

Note. Values are mean ± SE of 5 experiments in each case.
* Significantly different from control.

TABLE 2

Effect of Activation of Endogenous CaM Kinase on the Kinetic Parameters of Ca^{2+} Uptake in Cardiac SR Treated with Phospholamban Monoclonal Antibody (PLN AB) and Ruthenium Red (RR)

Treatment	Phosphorylation status	Kinetic parameter	
		V_{max} (nmol Ca^{2+}/mg protein/min)	$K_{0.5}$ for Ca^{2+} (μM)
None (Control)	Unphosphorylated	215 ± 5	0.64 ± 0.04
PLN AB-treated	Unphosphorylated	200 ± 4	0.31 ± 0.02†
PLN AB-treated	Phosphorylated	337 ± 5*	0.41 ± 0.02†
PLN AB + RR-treated	Unphosphorylated	296 ± 4	0.30 ± 0.01†
PLN AB + RR-treated	Phosphorylated	436 ± 12**	0.31 ± 0.03†

Note. Values are mean ± SE of 4–7 experiments and were derived from the data shown in Fig. 1 and Fig. 2.
* Significantly different when compared with control unphosphorylated or PLN AB-treated unphosphorylated.
** Significantly different from PLN AB + RR-treated unphosphorylated.
† Significantly different from control unphosphorylated.

the influence of endogenous CaM kinase activation on Ca^{2+}-ATPase function independent of phospholamban phosphorylation. The results presented in Fig. 1 (right panel) compare the Ca^{2+} concentration-dependence of ATP-energized Ca^{2+} uptake activity (Ca^{2+} pump function of Ca^{2+}-ATPase) of control unphosphorylated SR, phospholamban antibody-treated unphosphorylated SR, and phospholamban antibody-treated phosphorylated SR. It can be seen that phospholamban antibody treatment of SR vesicles resulted in moderate stimulation of Ca^{2+} uptake at subsaturating but not at saturating Ca^{2+} concentrations. On the other hand, activation of endogeous CaM kinase in phospholamban antibody-treated SR vesicles resulted in stimulation of Ca^{2+} uptake at the wide range of subsaturating and saturating Ca^{2+} concentrations tested (compare phospholamban antibody-treated unphosphorylated SR *versus* phospholamban antibody-treated phosphorylated SR). Analysis of the kinetic paramenters of Ca^{2+} uptake revealed that phospholamban antibody treatment of SR vesicles resulted in a significant increase (~2 fold) in the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+} whereas activation of endogenous CaM kinase in phospholamban antibody-treated SR vesicles resulted in a significant increase (~60-70%) in the V_{max} of Ca^{2+} uptake (Table 2). These findings imply that activation of endogenous CaM kinase leads to enhanced Ca^{2+} pump activity in cardiac SR vesicle independently of phospholamban phosphorylation.

We have shown previously that ruthenium red, a RYR-CRC blocking drug, strongly inhibits the phosphorylation of cardiac RYR-CRC by endogenous CaM kinase (18). In additional experiments we exploited this property of ruthenium red, and the ability of phospholamban antibody to inhibit phospholamban phosphorylation (cf. Fig. 1, left panel), to achieve selective phosphorylation of the cardiac SR Ca^{2+}-ATPase by endogenous CaM kinase. The results from these experiments are summarized in Fig. 2 (left panel) and Table 1. It can be seen that in cardiac SR not treated with phospholamban antibody, phosphorylation of RYR-CRC was virtually abolished in the presence of 25 μM ruthenium red in the phosphorylation assay medium. In agreement with our previous findings (18), phospholamban phosphorylation was unaffected by ruthenium red whereas phosphorylation of the Ca^{2+}-ATPase was reduced about 25%. When the phosphorylation reaction was carried out using phospholamban antibody-treated SR in the presence of ruthenium red (25 μM), Ca^{2+}-ATPase, but not phospholamban and RYR-CRC, underwent phosphorylation by endogenous CaM kinase. Having established the experimental conditions for selective phosphorylation of the Ca^{2+}-ATPase, it was then possible to determine the effect of Ca^{2+}-ATPase phosphorylation on Ca^{2+} pump function in the absence of phosphorylation of phospholamban and RYR-CRC. The results presented in Fig. 2 (right panel) compare the Ca^{2+} concentration-dependence of ATP-energized Ca^{2+} uptake activity of phospholamban antibody-treated SR phosphorylated in the presence of 25 μM ruthenium red which permitted selective phosphorylation of Ca^{2+}-ATPase but not phospholamban and RYR-CRC, and the corresponding unphosphorylated control SR. It can be seen that the velocity of Ca^{2+} uptake is greater in the phosphorylated, compared with the unphosphorylated, phospholamban antibody *plus* ruthenium red-treated SR at the wide range of subsaturating and saturating Ca^{2+} concentrations used. Analysis of the kinetic parameters of Ca^{2+} uptake revealed a significant increase in V_{max} (~50%) and no change in $k_{0.5}$ for Ca^{2+} as a result of selective phosphorylation of the Ca^{2+}-ATPase by endogenous CaM kinase (Table 2).

In contrast to earlier reports (11, 15–17, 21, 22) and the findings presented here, a study by Odermatt *et al.* (19) did not observe stimulation of Ca^{2+} uptake in cardiac SR upon phosphorylation of the Ca^{2+}-ATPase by CaM kinase II. Odermatt *et al.* (19) suggested that

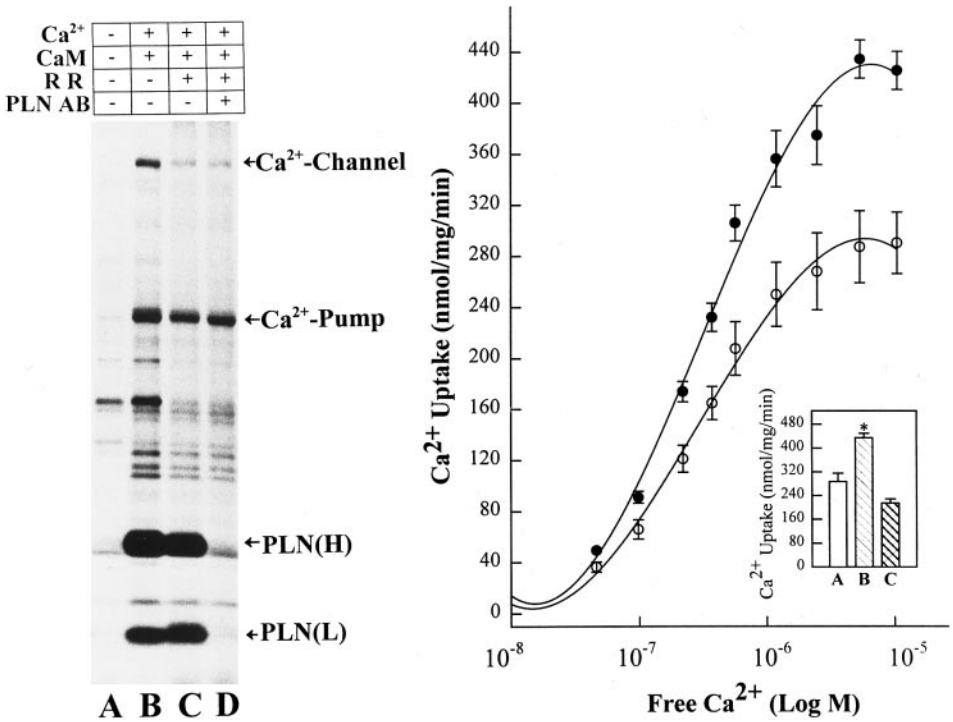

FIG. 2. Left panel: Autoradiogram showing selective phosphorylation of Ca^{2+}-ATPase (Ca^{2+} pump) by endogenous CaM kinase in rabbit cardiac SR. Control SR (denoted "−PLN AB") and phospholamban monoclonal antibody (PLN AB)-treated SR (denoted "+PLN AB") were prepared and the phosphorylation reaction was carried as described in METHODS. The absence (−) or presence (+) of Ca^{2+}, calmodulin (CaM) and ruthenium red (RR) in the phosphorylation reaction medium is indicated on the top of the autoradiogram. Note that RR selectively abolishes phosphorylation of the ryanodine receptor-Ca^{2+} release channel (Ca^{2+}-channel, lane C); when PLN AB-treated SR is phosphorylated in the presence of RR, Ca^{2+}-ATPase (Ca^{2+} pump) but not phospholamban (PLN, H and L forms) or Ca^{2+} channel undergoes phosphorylation by endogenous CaM kinase (lane D). Quantitative data from several experiments on the effects of PLN AB and RR on SR protein phosphorylation are summarized in Table 1. Right panel: Comparison of the Ca^{2+} concentration dependence of ATP-energized Ca^{2+} uptake by PLN AB-treated SR phosphorylated in the presence of 25 μM RR (●) and the corresponding unphosphorylated control SR (○). The phosphorylation reaction and subsequent Ca^{2+} uptake assays were performed as described in METHODS. The bar graph in the inset shows Ca^{2+} uptake activities measured at a saturating Ca^{2+} concentration (5.4 μM) using PLN AB-treated unphosphorylated SR (A), PLN AB-treated SR in which Ca^{2+}-ATPase was selectively phosphorylated by including 25 μM RR in the phosphorylation reaction medium (B), and PLN AB-treated SR incubated in the phosphorylation reaction medium supplemented with 25 μM CaM kinase inhibitor peptide (peptide fragment corresponding to amino acid reisdues 290-309 of CaM kinase II, cf. Ref. 36) to prevent Ca^{2+}-ATPase phosphorylation (C). The data represent mean ± SE of 4 experiments in each case. The kinetic parameters of Ca^{2+} uptake derived from these data are summarized in Table 2. *Significantly different from PLN AB-treated unphosphorylated.

the V_{max} effect of Ca^{2+}-ATPase phosphorylation reported in earlier studies (11, 15–17, 21, 22) may reflect differences in the stability of the cardiac SR Ca^{2+}-ATPase owing to the presence or absence of Ca^{2+} in incubation medium used for phosphorylation reaction prior to Ca^{2+} uptake assay. In the present study, the phosphorylation reaction was performed by incubating SR vesicles in the presence of Ca^{2+}, with calmodulin (to obtain phosphorylated SR), or without calmodulin (to obtain unphosphorylated control SR). Therefore, the V_{max} effect of Ca^{2+}-ATPase phosphorylation reported here is calmodulin-dependent, and cannot be attributed to differential stability of the Ca^{2+}-ATPase owing to the presence or absence of Ca^{2+} in the phosphorylation assay medium. Moreover, the V_{max} effect of phosphorylation on Ca^{2+} uptake under these conditions could be fully blocked by a peptide inhibitor of CaM kinase II (Fig. 2, inset). In this context, it is also noteworthy that mutation of Ser^{38} in SERCA2a (i.e. the site in cardiac SR Ca^{2+}-ATPase phosphorylated by CaM kinase II) to Ala results in 50% decrease in V_{max} of Ca^{2+} transport (22). Thus, Ser^{38} and its phosphorylation by CaM kinase II seem to play a critical role in controlling the velocity of Ca^{2+} transport by the cardiac SR Ca^{2+}-ATPase. Conceivably, in the study by Odermatt *et al.* (19), one or more of the following factors may have contributed to the failure to observe stimulation of Ca^{2+} uptake upon Ca^{2+}-ATPase phosphorylation by CaM kinase. (i) The SR isolation procedure used in their study (19) involved exposure of the membranes to protease inhibitors and flouride; this may result in altered Ca^{2+}-ATPase conformation adversely affecting its catalytic and ion transport properties (29), and consequently, imparied susceptibility to regulation by

phosphorylation. (ii) Phosphorylation of RYR-CRC by endogenous CaM kinase (occurring concurrently with the phosphorylation of Ca^{2+}-ATPase and phospholamban) likely results in enhanced Ca^{2+} release from the SR (9, 12–14). This may obscure the stimulatory effect of Ca^{2+}-ATPase phosphorylation on Ca^{2+} uptake especially when assays are performed in the absence of a Ca^{2+} release channel blocker (such as ruthenium red) as was the case in the study by Odermatt *et al*. (19). (iii) Our recent findings indicate that isolated cardiac SR vesicles contain significant amounts of firmly bound calmodulin as well as varying amounts serine phosphorylated Ca^{2+}-ATPase and autophosphorylated CaM kinase (A.Xu, T. Netticandan, D.L. Jones and N. Narayanan, manuscript in preparation). All of these factors would influence the extent of Ca^{2+}/calmodulin-dependent Ca^{2+}-ATPase phosphorylation and the stimulatory effect of phosphorylation on Ca^{2+} uptake measured in isolated SR vesicles. The use of a calmodulin antagonist (such as W7) to prevent activation of CaM kinase by endogenous calmodulin under control conditions (as done in the present study) may aid, at least in part, in discerning the regulatory potential of Ca^{2+}-ATPase phosphorylation on Ca^{2+} sequestration in isolated cardiac SR vesicles. The reports questioning the physiological significance of serine phosphorylation of the Ca^{2+}-ATPase (19, 23) did not take into account the various factors described above.

In conclusion, the results presented here demonstrate that selective phosphorylation of the Ca^{2+}-ATPase by endogenous CaM kinase in native cardiac SR results in an increase in the V_{max} of Ca^{2+} sequestration without any change in the $K_{0.5}$ for Ca^{2+} activation of the Ca^{2+} transport rate. This effect occurs independently of phospholamban phosphorylation, and is kinetically distinct from the effect of phospholamban phosphorylation. Activation of Ca^{2+}-ATPase by phospholamban phosphorylation involves mainly a decrease in $k_{0.5}$ for Ca^{2+} (7, 8 and references therein), but an increase in V_{max} may also occur (30–32). Thus, the positive V_{max} effect of Ca^{2+}-ATPase phosphorylation and the positive $k_{0.5}$ effect of phospholamban phosphorylation may provide a powerful and mutually complementary mechanism for the stimulation of Ca^{2+} sequestration in cardiac muscle SR. 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 tropinin C (33). This site has much lower affinity for Ca^{2+} than the Ca^{2+} binding sites in the Ca^{2+}-ATPase (33). 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_{max}. Consistent with this view, is the recent finding that acceleration of cardiac muscle relaxation caused by CaM kinase-mediated increase in SR Ca^{2+} pump activity occurs independently of phospholamban phosphorylation (34, 35).

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