Thyroid hormone downregulates the expression and function of sarcoplasmic reticulum-associated CaM kinase II in the rabbit heart

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Thyroid hormone downregulates the expression and function of sarcoplasmic reticulum-associated CaM kinase II (CaM kinase II) in the rabbit heart. The present study investigated the effects of L-thyroxine (T4) on transcription and translation of sarcoplasmic reticulum (SR) proteins. Recent studies have demonstrated that thyroid hormone affects cytosolic Ca2+ cycling at the level of the myofilaments and sarco(endo)plasmic reticulum (SERCA2) calsequestrin in hyperthyroid rabbits (2, 29, 30, 46), 2) overexpression of SR Ca2+-ATPase protein, with concomitant downregulation of phospholamban (PLN) protein level (27, 28, 30), 3) enhanced Ca2+ uptake by SR isolated from hearts with thyrotropic hypertrophy (25, 32, 50, 52), and 4) diminished PKA-mediated phosphorylation of PLN and decreased maximal inotropic and lusitropic responses to β-adrenergic agonist in hyperthyroid atria (47).

Despite the impressive evidence documenting direct regulation of cardiac SR Ca2+-cycling protein expression and function by thyroid hormone, no study has yet examined the influence of thyroid hormone on the regulation of SR function by Ca2+/calmodulin (CaM)-dependent protein kinase II (CaM kinase II) in the myocardium. Several studies have demonstrated direct phosphorylation of SERCA2 in vitro by endogenous CaM kinase II (10, 16, 41, 42, 55, 57–60), in addition to its previously characterized substrates PLN and ryanodine receptor (RyR)-Ca2+ release channel (RyR-CRC); Ca2+-ATPase, and phospholamban (PLN) was significantly lower (30–70%) in JSR and LSR vesicles from hyperthyroid than from euthyroid rabbit heart. Western immunoblotting analysis revealed significantly higher (40%) levels of sarco(endo)plasmic reticulum Ca2+-ATPase isofrom 2 (SERCA2) in JSR, but not in LSR, from hyperthyroid than from euthyroid rabbit heart. Maximal velocity of Ca2+ uptake was significantly increased in JSR (130%) and LSR (50%) from hyperthyroid compared with euthyroid rabbit hearts. Affinant affinity of the Ca2+-ATPase for Ca2+ did not differ between the two groups. Protein levels of PLN and CaM kinase II were significantly lower (30–40%) in JSR, LSR, and ventricular tissue homogenates from hyperthyroid rabbit heart. These findings demonstrate selective downregulation of expression and function of CaM kinase II in hyperthyroid rabbit heart in the face of upregulated expression and function of SERCA2 predominantly in the JSR compartment.

**CARDIAC HYPERTROPHY** following thyrotoxic administration to experimental animals is associated with enhancement of myocardial contractility, speed of systolic contraction and diastolic relaxation, cardiac output, and heart rate (13, 35, 44). On the other hand, decreases in these parameters were observed in the hypothyroid state. Available evidence suggests that mechanisms underly these changes include direct transcriptional regulation of cardiac genes by thyroid hormone, which in turn affects cytosolic Ca2+ cycling at the level of the myofilaments (6, 19) and the sarcoplasmic reticulum (SR) (5, 15, 37, 62).

Observations supporting thyroid hormone-induced alterations in SR target gene transcription and protein translation in the heart include: 1) increased levels of myocardial sarco(endo)plasmic reticulum Ca2+-ATPase isofrom 2 (SERCA2) mRNA and protein, with no changes in the mRNA level of calsequestrin in hyperthyroid rabbits (2, 29, 30, 46), 2) overexpression of SR Ca2+-ATPase protein, with concomitant downregulation of phospholamban (PLN) protein level (27, 28, 30), 3) enhanced Ca2+ uptake by SR isolated from hearts with thyrotropic hypertrophy (25, 32, 50, 52), and 4) diminished PKA-mediated phosphorylation of PLN and decreased maximal inotropic and lusitropic responses to β-adrenergic agonist in hyperthyroid atria (47).

Despite the impressive evidence documenting direct regulation of cardiac SR Ca2+-cycling protein expression and function by thyroid hormone, no study has yet examined the influence of thyroid hormone on the regulation of SR function by Ca2+/calmodulin (CaM)-dependent protein kinase II (CaM kinase II) in the myocardium. Several studies have demonstrated direct phosphorylation of SERCA2 in vitro by endogenous CaM kinase II (10, 16, 41, 42, 55, 57–60), in addition to its previously characterized substrates PLN and ryanodine receptor (RyR)-Ca2+ release channel (RyR-CRC); this phosphorylation was shown to result in stimulation of ATP hydrolysis and Ca2+ transport (16, 57–60). Although some studies (41, 45) have questioned the physiological significance of SERCA2 phosphorylation, evidence from more recent studies strongly supports the view that SERCA2 phosphorylation is a physiological event that results in stimulation of the maximal velocity (Vmax) of Ca2+ pumping in native cardiac SR (58, 60, 61). The present study was undertaken to investigate the influence of thyroid hormone on 1) protein expression of SR CaM kinase II and its substrates, 2) endogenous CaM kinase II-mediated SR protein phosphorylation, and 3) SR Ca2+ pump function in the heart.

It is also noteworthy that all previous studies on the mechanistic basis of thyroid hormone-induced alterations in cardiac SR protein expression and function utilized heart homogenate or a heterogeneous population of membrane vesicles derived from the longitudinal and junctional SR (LSR and JSR, respectively) membrane network of the heart. In our studies, native SR membranes isolated from ventricular muscle of rabbit heart were subfractionated into LSR and JSR for evaluation of SR function. It has been well documented that, in the mammalian heart, the SR comprises two membrane systems: LSR, which represents the membrane network that surrounds the myofilaments, and JSR, which consists of the membrane of terminal cisternae, subsarcolemmal cisterns, and the corbular SR (4, 43, 51). The two SR membrane compartments are distinct not only morphologically, but also in their physiological functions re-
resulting from the protein composition. For example, the RyR-CRC, which is primarily responsible for releasing Ca\(^{2+}\) from the SR lumen, is localized in the junctional face membrane of the terminal cisternae and is absent from LSR (1, 17). Calsequestrin, a Ca\(^{2+}\) storage protein, is confined to the lumen of JSR but is absent from LSR (24). In contrast, the LSR and JSR contain a nearly identical amount of the Ca\(^{2+}\)-ATPase (18). PLN, an inhibitor of SR Ca\(^{2+}\)-ATPase, is uniformly distributed in LSR and JSR membrane (23). To our knowledge, the present study is the first to characterize thyroid state-dependent alterations in protein expression and functional properties of these two distinct SR compartments in the heart and their protein phosphorylation by SR membrane-associated CaM kinase II.

**METHODS**

**Animals.** Male New Zealand White rabbits were obtained from a local breeder at 12 wk of age and maintained on ordinary rabbit chow in the Health Sciences Center animal care facility of this institution. Hyperthyroidism was induced by injection of L-thyroxine (200 \(\mu\)g/kg body wt im) daily for 7 days (2). Age-matched untreated (euthyroid) rabbits but is absent from LSR (24). In contrast, the LSR and JSR PLN, an inhibitor of SR Ca\(^{2+}\) (<Golden, CO); and anti-PLN monoclonal antibody from Upstate phosphorylation by SR membrane-associated CaM kinase II. Two distinct SR compartments in the heart and their protein ratios in protein expression and functional properties of these two distinct SR compartments in the heart and their protein phosphorylation by SR membrane-associated CaM kinase II.

**Preparation of muscle homogenates and cytosol.** In addition to SR membranes, cardiac muscle homogenates and cytosol were used in some experiments. The homogenates were prepared by homogenization (three 10-s bursts at 30-s intervals at a setting of 8; Polytron PT-10, Brinkman Instruments) of the ventricular tissue in 10 vol (based on tissue wt) of 10 mM Tris maleate-100 mM KCl buffer (pH 6.8). The homogenates were filtered through four layers of cheesecloth and used for experiments. The cytosol fraction was derived by centrifugation of the homogenate at 105,000 \(g\) for 60 min.

**SDS-PAGE and immunoblotting of Ca\(^{2+}\)-ATPase, PLN, CaM kinase II, and calsequestrin.** The Western immunoblotting procedure was used to localize and quantify Ca\(^{2+}\)-ATPase, PLN, CaM kinase II, and calsequestrin in SR membrane vesicles and cardiac muscle homogenates according to the procedure described by Xu and Narayanan (59). Because no significant thyroid state-dependent alteration was observed in the cardiomycocyte calsequestrin content (see Fig. 3), calsequestrin served as an ideal protein-loading control for the Western blotting experiments. The Western blots used for the immunodetection of CaM kinase II protein were subsequently stained by incubation in stripping buffer [62.5 mM Tris-HCl (pH 6.7)-100 mM 2-mercaptoethanol-0.2% SDS] at 50°C for 30 min. The membranes were then washed with Tris-buffered saline-0.5% Tween 20, blocked with 3% milk, and rebotted with anti-calsequestrin antibody for the detection of calsequestrin protein.

**Measurement of CaM kinase II-mediated SR protein phosphorylation.** Phosphorylation of SR proteins by endogenous CaM kinase II was determined by the procedure of Xu et al. (57). The standard phosphorylation assay medium (total volume 50 \(\mu\)l) contained 50 mM HEPES (pH 7.4), 10 mM MgCl\(_2\), 2 \(\mu\)M CaCl\(_2\), 2 \(\mu\)M calmodulin, 0.8 mM \([\gamma\(^{32}\)P\)]ATP (specific activity 300–400 cpm/pmol), and SR (25 \(\mu\)g of protein). In some experiments, the standard phosphorylation assay medium was supplemented with the protein phosphatase inhibitors microcystin-LR (10 mM) and sodium pyrophosphate (1 mM). The phosphorylation reaction was initiated by addition of \([\gamma\(^{32}\)P\)]ATP after preincubation of the remaining assay components for 3 min at 37°C. The reaction was allowed to proceed for 2 min and then terminated by the addition of 15 \(\mu\)l of SDS sample buffer. The samples were subjected to SDS-polyacrylamide gel (4–18% gradient) electrophoresis (21), stained with Coomassie brilliant blue, dried, and autoradiographed. Phosphorylation was quantified by liquid scintillation counting after excision of the radioactive bands from the gel (21). The Ca\(^{2+}\)/CaM dependence of phosphorylation was monitored in parallel assays without Ca\(^{2+}\) (with 1 mM EGTA) and/or calmodulin in the assay medium. The SR protein phosphorylation is due to the activation of endogenous CaM kinase by Ca\(^{2+}\) and calmodulin (57, 60). The identity of the phosphorylated peptide bands designated RyR, Ca\(^{2+}\)-ATPase, and PLN has been confirmed by Western blotting analysis of the peptide bands excised from the gels with use of antibodies specific for each of the proteins (40, 57).

**Determination of Ca\(^{2+}\) uptake and Ca\(^{2+}\)-ATPase activity.** ATP-dependent oxalate-facilitated Ca\(^{2+}\) uptake was measured in cardiac JSR and LSR by a Millipore filtration technique as described previously by Jones and Narayanan (22). The standard Ca\(^{2+}\) uptake assay medium (total volume 1 ml) contained 50 mM Tris maleate (pH 6.8), 5 mM MgCl\(_2\), 5 mM ATP, 120 mM KCl, 5 mM potassium oxalate, 5 mM sodium azide, 0.1 mM EGTA, 0.1 mM \(4\text{-CaCl}_2\) (specific activity 12,000–15,000 cpm/mmol), and LSR or JSR membranes (30 \(\mu\)g of protein). The assays were performed at 37°C; the Ca\(^{2+}\) uptake reaction was initiated by addition of the membrane fraction after preincubation of the rest of the assay components for 3 min. The initial free Ca\(^{2+}\) concentrations in the assay medium were determined using the computer program of Fabiato (11).
The Mg\(^{2+}\)-dependent Ca\(^{2+}\)-ATPase activity was determined as described previously (38). The basal ATPase activity was subtracted from the enzyme activity measured in the presence of Ca\(^{2+}\) to obtain the Ca\(^{2+}\)-ATPase activity.

Measurement of blood hormone levels. Blood samples were collected by cardiac puncture when the rabbits were killed and then centrifuged. The serum samples were treated with polyethylene glycol to precipitate any endogenous antibodies (31), and the hormones were assayed by a fully automated chemiluminescent immunoassay analyzer (model ACS-180, Chiron, Walpole, MA).

Contractile performance of the isolated perfused heart. Isolated perfused rabbit heart preparations were utilized to assess the influence of thyroid status on contractile performance of the heart as described previously (61). The contractions were recorded on a personal computer (Biopac TCI/MP WSW 100 system) and analyzed by Acqknowledge software (Biopac, Santa Barbara, CA) for the following parameters: heart rate, developed force, rate of force development (+dP/dt), and rate of relaxation (−dP/dt).

Data analysis. Statistical analysis was performed using SigmaPlot scientific graph program (Systat) run on an IBM-compatible personal computer, with Student’s t-test used for unpaired data. P < 0.05 was taken as the level of significance. Results were averaged and are expressed as means ± SE of experiments for which separate preparations were used. The n values denote the number of independent determinations for which separate SR or homogenate preparations were used.

RESULTS

Establishment of the hyperthyroid state and cardiac hypertrophy. Hyperthyroidism and cardiac hypertrophy were induced in rabbits by an L-thyroxine treatment protocol established previously (20). Briefly, the data demonstrated that we had successfully developed the hyperthyroid state, with attendant cardiac hypertrophy, in this animal model, as evidenced by a significant increase in ventricular weight-to-body weight ratio, blood levels of thyroxine and triiodothyronine, and markedly lower levels of thyroid-stimulating hormone in the hyperthyroid than in the euthyroid rabbit (Table 1).

CaM kinase II-mediated phosphorylation of SR proteins. A δ-isof orm of CaM kinase II (δ-CaM kinase II), associated with the SR, is known to play an important role in regulating the Ca\(^{2+}\) uptake function of cardiac SR through phosphorylation of PLN (9, 26, 49, 54) and Ca\(^{2+}\)-ATPase (16, 42, 55, 57–60). In the present study, endogenous CaM kinase II-mediated protein phosphorylation was determined in LSR and JSR vesicles isolated from the cardiac muscle of euthyroid and hyperthyroid rabbits. The isolated JSR membranes were highly enriched in RyR-CRC, whereas the LSR membranes contained only trace amounts of RyR-CRC (Fig. 1A). In the presence of Ca\(^{2+}\) and calmodulin, the SR-associated CaM kinase II catalyzed the phosphorylation of RyR-CRC, Ca\(^{2+}\)-ATPase, and PLN in JSR from euthyroid and hyperthyroid animals; the major substrates undergoing phosphorylation in the LSR included Ca\(^{2+}\)-ATPase and PLN (Fig. 1A). The CaM kinase II-mediated substrate phosphorylation of RyR-CRC, Ca\(^{2+}\)-ATPase, and PLN, quantified per unit amount of total JSR or LSR protein, was significantly lower in the hyperthyroid than in the euthyroid rabbits (Fig. 1B). Because protein levels of substrates changed in the hyperthyroid state (see Fig. 5), CaM kinase II-mediated phosphorylation of these proteins was quantified per unit amount of each of the immunoreactive substrates. A significantly lower substrate phosphorylation of RyR-CRC, Ca\(^{2+}\)-ATPase, and PLN by CaM kinase II was evident in JSR and LSR from the hyperthyroid than from the euthyroid hearts (Fig. 1C). The diminished CaM kinase II-mediated phosphorylation was more pronounced in the case of RyR-CRC (−65%) and Ca\(^{2+}\)-ATPase (−70%) than in the case of phospholamban (−30%). Virtually similar decrements in the phosphorylation of SR substrates by endogenous CaM kinase II in the hyperthyroid heart could also be observed when the phosphorylation reaction was carried out in the presence of the protein phosphatase inhibitors microcystin-LR and sodium pyrophosphate (Fig. 2). Therefore, it is unlikely that alteration of protein phosphatase activity in the hyperthyroid state contributes to the observed decline in substrate phosphorylation. It is also noteworthy that the phosphorylation level of individual substrates was not enhanced appreciably in the presence of phosphatase inhibitors, indicating minimal substrate dephosphorylation under the phosphorylation assay conditions employed (Fig. 2).

Expression of SR Ca\(^{2+}\)-cycling proteins and CaM kinase II. To understand the mechanisms underlying the altered phosphorylation status of SR Ca\(^{2+}\)-cycling proteins in the hyperthyroid heart, the expression levels of the Ca\(^{2+}\)-cycling proteins and CaM kinase II were determined in homogenates and JSR/LSR preparations from euthyroid and hyperthyroid rabbit hearts by quantitative immunoblotting with antibodies specific for RyR-CRC, SERCA2, PLN, δ-CaM kinase II, and calsequestrin.

The relative amount of CaM kinase II was significantly lower (−40%) in heart homogenates from hyperthyroid than from euthyroid rabbits (Fig. 3A). When the nitrocellulose membranes used for Western blotting of CaM kinase II were subsequently reprobed for calsequestrin, the relative amount of calsequestrin did not differ between the euthyroid and hyperthyroid groups (Fig. 3, A and B). Therefore, calsequestrin served as an ideal protein-loading control for the Western blotting experiments, and the ratio of CaM kinase II to calsequestrin was significantly diminished in the hyperthyroid group (Fig. 3C). Western blotting analysis also revealed a decrease (−30%) in CaM kinase II protein content that was similar in JSR, LSR, and cytosol fraction from the hyperthyroid and euthyroid hearts (Fig. 4). There was no significant difference in the relative amount of CaM kinase II between LSR and JSR.

The density of SERCA2 was markedly higher (40%) in JSR vesicles of hyperthyroid than euthyroid rabbit heart (Fig. 5A).

Table 1. Body weight, ventricular weight, and hormone level in euthyroid and hyperthyroid rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, kg</th>
<th>VW, g</th>
<th>VW/BW, g/kg</th>
<th>T(_{4}), pmol/l</th>
<th>T(_{3}), pmol/l</th>
<th>TSH, mU/l</th>
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<tr>
<td>Euthyroid</td>
<td>3.06±0.07</td>
<td>5.63±0.19</td>
<td>1.85±0.08</td>
<td>14.05±1.34</td>
<td>8.05±0.51</td>
<td>1.08±0.24</td>
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<tr>
<td>Hyperthyroid</td>
<td>2.75±0.04*</td>
<td>5.60±0.19*</td>
<td>2.37±0.08*</td>
<td>146.1±8.10*</td>
<td>33.4*</td>
<td>0.09±0.05*</td>
</tr>
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Values are means ± SE (n = 5) BW, body weight; VW, ventricular weight; T\(_{4}\), free thyroxine; T\(_{3}\), triiodothyronine; TSH, thyroid-stimulating hormone. *P < 0.05 vs. euthyroid.
Fig. 1. Ca\textsuperscript{2+}/calmodulin (CaM)-dependent protein kinase II (CaM kinase II)-mediated phosphorylation of Ca\textsuperscript{2+}-cycling proteins in longitudinal and junctional sarcoplasmic reticulum (LSR and JSR) of euthyroid and hyperthyroid rabbits. Phosphorylation reaction was carried out for 2 min in the absence (−) and presence (+) of CaM. Sarcoplasmic reticulum (SR) proteins were fractionated by SDS-PAGE, and \textsuperscript{32}P incorporation into peptide bands representing ryanodine receptor-Ca\textsuperscript{2+} release channel (RyR-CRC), Ca\textsuperscript{2+}-ATPase, and phospholamban [high- and low-molecular weight forms: PLN(H) and PLN(L)] was determined by liquid scintillation counting. A: Coomassie blue-stained SDS-polyacrylamide gel showing SR protein profiles (left) and autoradiogram of the same gel (right). B and C: phosphorylation of RyR-CRC, Ca\textsuperscript{2+}-ATPase, and PLN in LSR and JSR from euthyroid (open bars) and hyperthyroid (filled bars) rabbits. CaM kinase II-mediated phosphorylation of each substrate was quantified per unit amount of total SR protein (B) and per unit amount of each immunoreactive protein (phosphorylation-to-immunoreactive substrate protein ratio, C). Relative amount of each immunoreactive substrate was determined by laser scanning densitometry of Western immunoblots (cf. Fig. 5). Values are means ± SE of 8 experiments using separate preparations. *P < 0.05 vs. euthyroid.

Fig. 2. Endogenous CaM kinase II-mediated phosphorylation of Ca\textsuperscript{2+}-cycling proteins in LSR and JSR of euthyroid (EU) and hyperthyroid (Hyper) rabbits in the presence and absence of protein phosphatase inhibitors. Phosphorylation reaction was carried out under standard assay conditions in the presence (+) and absence (−) of phosphatase inhibitors (10 nM microcystin-LR and 1 mM sodium pyrophosphate). A: Coomassie blue-stained SDS-polyacrylamide gel depicting SR protein profiles (left) and autoradiogram of the same gel depicting protein phosphorylation (right). B: CaM kinase II-mediated phosphorylation of each substrate in the presence of protein phosphatase inhibitors. Values are means ± SE of 3 experiments using separate preparations. *P < 0.05 vs. euthyroid. See Fig. 1 for quantitative data on substrate phosphorylation in the absence of protein phosphatase inhibitors.
Interestingly, the amount of SERCA2 was essentially similar in LSR vesicles from hyperthyroid and euthyroid rabbits. As reported in our previous study (20), the protein level of RyR-CRC was significantly greater (~2-fold) in JSR from the hyperthyroid rabbit heart; RyR-CRC content was minimal in LSR from euthyroid and hyperthyroid groups (results not shown).

The density of PLN was significantly lower in JSR (25%) and LSR (30%) from hyperthyroid than from euthyroid rabbit hearts (Fig. 5B). The ratio of PLN to Ca\(^{2+}\)-ATPase, a criterion normally used to evaluate the regulation of the rate of SR Ca\(^{2+}\) uptake and the relaxation properties of the cardiomyocyte (28, 30), was significantly decreased in JSR and LSR from hyperthyroid rabbit hearts compared with euthyroid groups (Fig. 5C).

Consistent with the data obtained with isolated JSR/LSR vesicles (Fig. 5), Western blotting analysis using unfractionated cardiac muscle homogenates revealed significantly higher (~30%) expression of SERCA2 (Fig. 6A) and significantly lower (~40%) expression of PLN (Fig. 6B) in hyperthyroid than in euthyroid rabbit hearts.

**SR Ca\(^{2+}\) sequestration function in euthyroid and hyperthyroid hearts.** The influence of the hyperthyroid state on cardiac muscle SR Ca\(^{2+}\) sequestration function was studied by determining the ATP-dependent, oxalate-facilitated Ca\(^{2+}\) uptake (Ca\(^{2+}\) transport function) and Ca\(^{2+}\)-activated ATP hydrolysis (energy transduction function). Figure 7A shows the time course of Ca\(^{2+}\) uptake by cardiac muscle SR from euthyroid and hyperthyroid rabbits. The rate of Ca\(^{2+}\) uptake, measured in the presence of 8.2 \(\mu\)M free Ca\(^{2+}\) in the assay medium (which was found to be saturating; see below), was significantly higher in JSR (~2-fold) and LSR (~50%) vesicles from the hyperthyroid than from the euthyroid rabbit hearts \((P < 0.05)\). Figure 7B shows the effects of varying Ca\(^{2+}\) concentrations on ATP-supported Ca\(^{2+}\) uptake by cardiac JSR and LSR from the hyperthyroid and euthyroid rabbits. When the free Ca\(^{2+}\) concentration in the assay medium was varied from 0.01 to 8.2 \(\mu\)M, JSR and LSR from hyperthyroid rabbits showed significantly higher rates of Ca\(^{2+}\) uptake than the JSR and LSR from euthyroid rabbits at all Ca\(^{2+}\) concentrations. The kinetic parameters derived from these data are shown in Table 2. \(V_{\text{max}}\) of Ca\(^{2+}\) uptake was significantly higher in the hyperthyroid than in the euthyroid group. The apparent affinity of Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) did not differ significantly between the two groups.

Figure 8 compares the Ca\(^{2+}\)-ATPase activity of cardiac muscle JSR and LSR vesicles from hyperthyroid and euthyroid rabbits. The Ca\(^{2+}\)-ATPase activity was significantly higher in JSR (35%) and LSR (60%) from hyperthyroid than from euthyroid rabbit hearts \((P < 0.05)\). The efficiency of coupling ATP hydrolysis to Ca\(^{2+}\) transport (Ca\(^{2+}\) uptake-to-ATP hydrolysis ratio at saturating free Ca\(^{2+}\)) was significantly increased in JSR (0.5 ± 0.06 for euthyroid and 0.76 ± 0.07 for hyperthyroid) but not in LSR (0.67 ± 0.11 for euthyroid and 0.56 ± 0.09 for hyperthyroid) from the hyperthyroid compared with the euthyroid rabbit heart \((P < 0.05)\).

**Thyroid hormone-induced alteration of the contractile properties of the heart.** The influence of thyroid hormone on contractile performance was evaluated using isolated perfused heart preparations. Figure 9 shows typical contractions recorded from spontaneously beating euthyroid and hyperthyroid rabbit hearts. Contractile function parameters, including heart rate, \(+dP/dt\), and \(-dP/dt\), were assessed, and the results are presented in Fig. 10. Heart rate, \(+dP/dt\) \(\text{max}\), and \(-dP/dt\) \(\text{max}\) were significantly greater in the heart from hyperthyroid than from euthyroid rabbits \((P < 0.05)\). The developed force was not compromised in the spontaneously beating hyperthyroid
heart, despite its markedly higher beat frequency. In other experiments where the hearts were paced electrically at 260 beats/min, the developed force was 80% greater in hyperthyroid than in euthyroid heart (results not shown).

DISCUSSION

The results presented here demonstrate selective downregulation of the expression and function of CaM kinase II in the hyperthyroid rabbit heart in the face of upregulated expression and function of SERCA2. Furthermore, thyroid hormone-induced overexpression of SERCA2 molecules was confined to the morphologically and functionally distinct JSR compartment, whereas downregulation of the SERCA2 inhibitor protein PLN by thyroid hormone was observed in the JSR and LSR compartments. The implications of these novel observations, in the context of current knowledge of cardiac excitation-contraction coupling and regulation of SERCA2 function, are discussed below.

The overexpression of SERCA2 in hyperthyroid rabbit heart reported here is in agreement with previous studies showing increased steady-state mRNA level (2, 46) and protein expression (27, 30) of Ca\textsuperscript{2+}-ATPase in hyperthyroidism. The novel aspect of our observations, however, is that the overexpressed SERCA2 molecules in the hyperthyroid rabbit heart are specifically targeted to the JSR, but not the LSR. Consequently, there is a relative abundance of SERCA2 in JSR as opposed to the near equilibrium of SERCA2 molecules in JR and LSR in the euthyroid state (18).

In a previous study, we showed thyroid hormone-induced overexpression of the RyR2 isoform of RyR-CRC in the rabbit heart, and the overexpressed RyR2 molecules are also targeted to their membrane locus in the JSR (20). This compartment-
specific de novo expression of RyR2 and SERCA2 under the influence of thyroid hormone appears to be of considerable pathophysiological significance, because JSR serves as the specialized primary site where the molecular events associated with excitation-contraction coupling unfold (53). Thus, according to the current concept, a small number of L-type Ca$^{2+}$/H$^{+}$ channels in the sarcolemma and a cluster of directly proximal RyR in the adjacent JSR serve as discrete Ca$^{2+}$ release units producing spatially localized transient elevations of intracellular Ca$^{2+}$ (Ca$^{2+}$ sparks) on myocyte excitation (8); the recruitment of these events, as a function of Ca$^{2+}$ channel activation, underlies the whole cell Ca$^{2+}$ transient induced by transsarcolemmal Ca$^{2+}$ influx (7, 33). On the one hand, the observed overexpression of RyR2 in the JSR would ensure their spatial proximity to the L-type Ca$^{2+}$/H$^{+}$ channels and, therefore, efficient coupling between the two molecular components of the Ca$^{2+}$ release unit. On the other hand, the JSR compartment-specific overexpression of SERCA2 would ensure immediate and efficient reuptake of Ca$^{2+}$ from the Ca$^{2+}$ release sites to prevent an excessive rise in intensity and duration of the Ca$^{2+}$ signal in the myoplasm, which would be detrimental to survival.

The JSR compartment-specific overexpression of SERCA2 in the hyperthyroid state was accompanied by significant downregulation of the protein levels of the SERCA2 inhibitor PLN in JSR and LSR. The consequent decrease in the PLN-to-SERCA2 ratio implies that more SERCA2 units can operate free of the inhibitory control exerted by PLN. Despite the intensive studies spanning the last three decades (26, 49), the molecular mechanism of regulation of SERCA2 by PLN remains unclear. According to the view that has prevailed until recently, a physical interaction of dephosphorylated PLN with SERCA2 causes inhibition of Ca$^{2+}$ pump activity, and phosphorylation of PLN (by PKA or CaM kinase II) disrupts this protein-protein interaction, thereby diminishing the inhibitory action of PLN. This view has been dispelled by the recent observation that Ca$^{2+}$ causes dissociation of the PLN-SERCA2 complex, but not PLN phosphorylation (3). Recent work in our laboratory on PLN regulation of SERCA2 in cardiac SR has
suggested that dissociation of the PLN-Ca\(^{2+}\)-ATPase complex is not an autonomous function of Ca\(^{2+}\); rather, it is governed by the Ca\(^{2+}\)-calmodulin interaction (39). The potential interplay of calmodulin-dependent processes other than phosphorylation on the regulation of SERCA2 function remains to be evaluated.

Analysis of the kinetic properties of the SR Ca\(^{2+}\) transport system revealed markedly enhanced \(V_{\text{max}}\) of Ca\(^{2+}\) sequestration with unaltered apparent affinity of the transport system for Ca\(^{2+}\) in the hyperthyroid rabbit. The enhanced \(V_{\text{max}}\) can be attributed to an increase in Ca\(^{2+}\) pump units due to the overexpression of SERCA2 in JSR vesicles as well as diminished inhibitory control by PLN in JSR and LSR. It is noteworthy that the hyperthyroid state is characterized by a disproportionately greater increase in the Ca\(^{2+}\)-sequestering activity in the JSR than in the LSR: 130% vs. 50% increase in \(V_{\text{max}}\) of Ca\(^{2+}\) uptake. Also, an increase in the efficiency of coupling ATP hydrolysis to Ca\(^{2+}\) transport was apparent in the hyperthyroid JSR. This may be due, in part, to the diminished PLN expression, inasmuch as PLN is reported to cause a decrease in the energetic efficiency of the SR Ca\(^{2+}\) pump (48).

Interestingly, the enhanced expression of RyR2 and SERCA2 in JSR in the hyperthyroid heart was not accompanied by a concomitant change in expression of the intraluminal Ca\(^{2+}\)-binding protein calsequestrin. The large intrinsic Ca\(^{2+}\)-binding capacity of calsequestrin (40–50 mol Ca\(^{2+}\)/mol protein) (36) appears to be adequate to handle Ca\(^{2+}\) buffering within the SR lumen without the need for enhanced expression of this protein, even when the Ca\(^{2+}\) uptake and release mechanisms undergo marked upregulation.

The thyroid hormone-induced changes in the expression and function of Ca\(^{2+}\)-cycling proteins in LSR and JSR reported here correlate well with the altered intrinsic contractile properties of the heart in the hyperthyroid state (Fig. 10). Thus the enhanced Ca\(^{2+}\)-sequestering activity of LSR and JSR underlies the enhanced rate of cardiac muscle relaxation, whereas the enhanced RyR2 activity in the JSR underlies the enhanced speed of cardiac contraction in the hyperthyroid state. Previous studies have described enhanced SR Ca\(^{2+}\)-ATPase expression and function in the hyperthyroid heart; to our knowledge, the present study is the first to delineate JSR and LSR compartment-specific alterations in the expression and function of Ca\(^{2+}\)-cycling proteins in the hyperthyroid heart.

Endogenous CaM kinase II-mediated phosphorylation of Ca\(^{2+}\)-cycling proteins was found to be more pronounced in JSR than in LSR in euthyroid and hyperthyroid states. Because no appreciable difference in the relative distribution of CaM kinase II between JSR and LSR was evident, the reason for the disparity in substrate phosphorylation remains unclear. The phosphorylation of RyR2, SERCA2, and PLN was markedly diminished in the hyperthyroid state. This is due, at least in part, to the diminished protein level of CaM kinase II in JSR and LSR of the hyperthyroid group. Interestingly, however, a striking disparity was evident in the degree to which phosphor-

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**Table 2. Kinetic parameters of ATP-dependent Ca\(^{2+}\) uptake by cardiac JSR and LSR from euthyroid and hyperthyroid rabbits**

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<th>JSR</th>
<th>LSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}}) nmol Ca(^{2+})/mg protein(^{-1}) min(^{-1})</td>
<td>194±8.4</td>
<td>252.4±4.8</td>
</tr>
<tr>
<td>(K_{0.5}) for Ca(^{2+}), (\mu)M</td>
<td>1.5±0.2</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>(n_H)</td>
<td>1.2±0.2</td>
<td>1.4±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5). JSR and LSR, junctional and longitudinal sarcoplasmic reticulum; \(V_{\text{max}}\), maximum velocity of Ca\(^{2+}\) uptake; \(K_{0.5}\), Ca\(^{2+}\) concentration giving half of \(V_{\text{max}}\); \(n_H\), Hill’s coefficient. *\(P < 0.05\) vs. euthyroid.
ylation of individual substrates was altered in the hyperthyroid state. Thus endogenous CaM kinase II-mediated phosphorylation of PLN was diminished by 30%, whereas phosphorylation of RyR2 and SERCA2 was decreased by 65–70%. Such substrate-specific differences in the magnitude of phosphorylation are seen even when phosphorylation is normalized to the level of immunoreactive substrate protein. It is possible that the membrane-associated CaM kinase II molecules in the SR are segregated into target-dedicated pools, and thyroid status may impact strongly on the RyR2/SERCA2-dedicated CaM kinase II pools and weakly on the PLN-dedicated CaM kinase II pools.

It is intriguing that diminished expression and function of SR CaM kinase II accompany upregulated expression and function of RyR2 and SERCA2 in the hyperthyroid state. On the one hand, thyroid hormone-induced alterations in RyR2 and SERCA2 function result in marked enhancement in the rate and magnitude of Ca\(^{2+}\) release and uptake by the SR, thus augmenting the SR Ca\(^{2+}\) transient and the speed of contraction and relaxation of the heart. On the other hand, thyroid hormone-induced downregulation of the expression and function of CaM kinase II may impact negatively on the RyR2 and SERCA2 function, because CaM kinase II-mediated phosphorylation of RyR2 is recognized to stimulate Ca\(^{2+}\) release (14, 56), whereas phosphorylation of SERCA2 and its regulatory protein PLN stimulates Ca\(^{2+}\) sequestration (16, 26, 49, 57–60) by the SR. It appears that, in the hyperthyroid state, attenuation of the inhibitory control of SERCA2 by PLN is balanced by...
attenuation of the positive regulation by CaM kinase. Thus the diminished expression and function of CaM kinase II in the face of upregulated expression and function of RyR2 and SERCA2 may subserve to prevent overdrive of the SR Ca2+-cycling apparatus in the hyperthyroid heart. Future studies are needed to delineate the mechanisms by which thyroid hormone downregulates CaM kinase II protein expression in the heart.

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