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

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Jiang, Mao, Ande Xu, and Njanoor Narayanan. Thyroid hormone downregulates the expression and function of sarcoplasmic reticulum-associated CaM kinase II in the rabbit heart. Am J Physiol Heart Circ Physiol 291: H1384–H1394, 2006. First published April 14, 2006; doi:10.1152/ajpheart.00875.2005—Phosphorylation of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-cycling proteins by a membrane-associated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) is a well-documented physiological mechanism for regulation of transmembrane Ca\(^{2+}\) fluxes and the cardiomyocyte contraction-relaxation cycle. The present study investigated the effects of l-thyroxine-induced hyperthyroidism on protein expression of SR CaM kinase II and its substrates, endogenous CaM kinase II-mediated SR protein phosphorylation, and SR Ca\(^{2+}\) pump function in the rabbit heart. Membrane vesicles enriched in junctional SR (JSR) or longitudinal SR (LSR) isolated from euthyroid and hyperthyroid rabbit hearts were utilized. Endogenous CaM kinase II-mediated phosphorylation of ryanodine receptor-Ca\(^{2+}\) release channel (RyR-CRC), Ca\(^{2+}\)-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 Ca\(^{2+}\)-ATPase isoform 2 (SERCA2) in JSR, but not in LSR, from hyperthyroid than from euthyroid rabbit heart. Maximal velocity of Ca\(^{2+}\) uptake was significantly increased in JSR (130%) and LSR (50%) from hyperthyroid compared with euthyroid rabbit hearts. Affinent affinity of the Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) 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 (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)-Ca\(^{2+}\) release channel (RyR-CRC); this phosphorylation was shown to result in stimulation of ATP hydrolysis and Ca\(^{2+}\) 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 (\(V_{\text{max}}\)) of Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 myofibrils, 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-

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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\(^{2+}\) storage protein, with 3% milk, and reblotted with anti-calsequestrin antibody for the detection of calsequestrin protein.

**Preparation of SR membranes.** SR membranes were prepared by the procedure of Jones and Narayanan (22) and then subjected to sucrose density gradient fractionation according to the procedure of Feher and Davis (12) to yield membrane vesicles enriched in JSR and LSR. Briefly, the ventricular tissue was minced and homogenized (3 bursts of 15-s duration at 30-s intervals at a speed 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 homogenate were then washed with Tris-buffered saline-0.5% Tween 20, blocked with 3% milk, and reblotted with anti-calsequestrin antibody for the detection of calsequestrin protein.

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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 immunosay 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 i.-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-isoform 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 served as an ideal protein-loading control for the Western blotting experiments, and the ratio of CaM kinase II to calsequestrin did not differ between the euthyroid and hyperthyroid groups (Fig. 3, A and B). Therefore, 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>
<thead>
<tr>
<th>Group</th>
<th>BW, kg</th>
<th>VW, g</th>
<th>VW/BW, g/kg</th>
<th>T₄, pmol/l</th>
<th>T₃, 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>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>2.75±0.04*</td>
<td>6.50±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>
</tbody>
</table>

Values are means ± SE (n = 5) BW, body weight; VW, ventricular weight; T4, free thyroxine; T3, triiodothyronine; TSH, thyroid-stimulating hormone.

*p < 0.05 vs. euthyroid.
Fig. 1. Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II (CaM kinase II)-mediated phosphorylation of Ca\(^{2+}\)-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 \(^{32}\)P incorporation into peptide bands representing ryanodine receptor-Ca\(^{2+}\) release channel (RyR-CRC), Ca\(^{2+}\)-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\(^{2+}\)-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\(^{2+}\)-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 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. \(V_{max}\) 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 7 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 Table 2. Heart rate, \(+dP/dt_{max}\), and \(-dP/dt_{max}\) 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 JSR 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\(^{2+}\) 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\(^{2+}\) 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

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**Fig. 6.** Relative amounts of SERCA2 and PLN in cardiac muscle homogenate of euthyroid and hyperthyroid rabbits. Identical amounts of homogenate protein (25 μg) from euthyroid and hyperthyroid hearts were subjected to Western blotting analysis using antibodies specific for SERCA2 and PLN. Representative immunoblots were obtained using separate homogenate preparations for SERCA2 and PLN (4 groups, boiled sample) each from euthyroid and hyperthyroid hearts. Bar graphs depict relative amount of immunoreactive protein as determined by densitometric scanning of Western blots. Values are means ± SE; n = 5. *P < 0.05 vs. euthyroid.

**Fig. 7.** Time course of ATP-dependent Ca\(^{2+}\) uptake (A) and effects of varying Ca\(^{2+}\) concentrations on Ca\(^{2+}\) uptake (B) by cardiac JSR and LSR from euthyroid and hyperthyroid rabbits. Ca\(^{2+}\) uptake reactions were carried out under standard assay conditions. Values are means ± SE of 5 experiments using separate JSR and LSR preparations. *P < 0.05 vs. euthyroid. Kinetic parameters derived from the data (B) are shown in Table 2.
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 \(V_{\text{max}}\) binding capacity of calsequestrin (36–50 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**

<table>
<thead>
<tr>
<th></th>
<th>JSR</th>
<th>LSR</th>
<th>JSR</th>
<th>LSR</th>
<th>(n_H) JSR</th>
<th>(n_H) LSR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Euthyroid</strong></td>
<td>194±8.4</td>
<td>252.4±4.8</td>
<td>1.5±0.2</td>
<td>1.3±0.2</td>
<td>1.2±0.2</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td><strong>Hyperthyroid</strong></td>
<td>451.7±19*</td>
<td>381.3±5.1*</td>
<td>1.7±0.3</td>
<td>1.6±0.4</td>
<td>1.4±0.2</td>
<td>1.4±0.2</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; \(K_{0.5}\) for Ca\(^{2+}\), Ca\(^{2+}\) concentration giving half of \(V_{\text{max}}\); \(n_H\), Hill’s coefficient; \(*\) \(P < 0.05\) vs. euthyroid.

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**Fig. 8.** Ca\(^{2+}\)-stimulated ATP hydrolysis by cardiac JSR (A) and LSR (B) from euthyroid and hyperthyroid rabbits. Reaction was carried out under standard assay conditions. Values are means ± SE of 5 experiments using separate JSR and LSR membrane preparations. \(*\) \(P < 0.05\) 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...

*Fig. 9. Typical contractions recorded from isolated perfused spontaneously beating euthyroid (A) and hyperthyroid (B) rabbit hearts. Results from analysis of contractile function parameters are summarized in Fig. 10.*

*Fig. 10. Analysis of recordings shown in Fig. 9: effects of thyroid status on rate of pressure development (+dP/dt, A), rate of relaxation (−dP/dt, B), and heart rate (C). Values are means ± SE of contractions recorded from 5 isolated heart preparations each from euthyroid and hyperthyroid rabbits. *P < 0.05 vs. euthyroid.*
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 overriding 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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