Effect of Thyroid Hormone on the Expression of mRNA Encoding Sarcoplasmic Reticulum Proteins

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The purpose of this study was to determine the expression of genes encoding various sarcoplasmic reticulum components that are functionally coupled with calcium release, uptake, and storage function during cardiac hypertrophy induced by thyroid hormone. Hyperthyroidism was induced in two groups of rabbits by the injection of 200 μg/kg l-thyroxine (T4) daily for 4 days (T4-4-day group) and 8 days (T4-8-day group). Hypothyroidism was induced in another group of rabbits by adding 0.8 mg/ml propylthiouracil to the drinking water for 4 weeks. The relative expression level of mRNA encoding different sarcoplasmic reticulum proteins was determined by RNA slot blot and Northern blot analysis. In hyperthyroid hearts, the steady-state level of cardiac ryanodine receptor mRNA and sarcoplasmic reticulum cardiac/slow-twitch Ca\(^{2+}\)-ATPase mRNA were both increased to 147% (T4-4-day group) and 186% (T4-8-day group) of control, respectively, but decreased to 71% and 75%, respectively, in hypothyroid ventricles. The mRNA level for phospholamban was decreased in both hyperthyroidism (T4-4-day group, 72%) and hypothyroidism (77%) in these hearts. On the other hand, calsequestrin mRNA levels did not change in hyperthyroid and hypothyroid ventricles. In accord with the changes in Ca\(^{2+}\)-ATPase mRNA levels, the Ca\(^{2+}\)-ATPase protein was increased to 199% (T4-8-day group) in hyperthyroid ventricles and decreased to 86% of control in hypothyroid ventricles. The expression levels of ryanodine receptor, Ca\(^{2+}\)-ATPase, phospholamban, and calsequestrin mRNAs were similarly altered in skeletal muscle tissues from hyperthyroid and hypothyroid rabbits. These results indicate that the mRNA levels of sarcoplasmic reticulum proteins responsible for calcium release and calcium uptake are coordinately regulated in response to changes in thyroid hormone level in both heart and skeletal muscle. These changes in mRNA level should lead to changes in protein levels and thus to altered calcium release and uptake in the chronic stages of hyperthyroidism and hypothyroidism. (Circulation Research 1991;69:266–276)

Thyroid hormone–induced cardiac hypertrophy is a well-defined experimental model used to investigate mechanisms altering cardiac function.\(^1,2\) Cardiac hypertrophy after thyroxine administration to experimental animals is associated with an increased rate of tension development and an enhanced velocity of fiber shortening.\(^3–6\) This contrasts with a depression of cardiac contractile velocity associated with hypothyroidism.\(^7\) Although some of the changes in contractile properties can be explained by changes in myosin expression,\(^8\) recent findings\(^9\) have indicated that the Ca\(^{2+}\) cycling function of the sarcoplasmic reticulum is altered in hypertrophic cardiac muscle. An increase in the rate of calcium uptake by the sarcoplasmic reticulum has been reported in hyperthyroidism,\(^9,10\) and myothermal measurements (tension-independent heat) using papillary muscles support this observation.\(^11,12\) Recently, we\(^13\) and others\(^14\) have reported that thyroid hormone markedly increases the mRNA levels of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, suggesting an increase in Ca\(^{2+}\)-ATPase pump sites in these hearts. Furthermore, intracellular Ca\(^{2+}\) transient measurements using calcium-sensitive bioluminescent assays have shown a rapid calcium release and reuptake...
without an alteration in the peak level of free cytoplasmic calcium during contraction in thyrotoxic hearts.\textsuperscript{15,16} These alterations of sarcoplasmic reticulum function are thought to play an important role in determining the contractile properties of the hypertrophied myocardium. The molecular basis for altered sarcoplasmic reticulum function, however, remains to be determined.

In recent years, the major sarcoplasmic reticulum proteins controlling calcium release, reuptake, and storage have been isolated, and their primary structures have been determined.\textsuperscript{17} The calcium release channel (ryanodine receptor) has been identified as a tetramer made up of subunits of $M_r=565,000$ Da.\textsuperscript{18–23} Two distinct isoforms of the ryanodine receptor have been identified in cardiac and skeletal muscle.\textsuperscript{20–23} The cardiac isoform is expressed in heart and brain, whereas the skeletal muscle isoform is expressed in fast- and slow-twitch skeletal muscle.\textsuperscript{23,24} Similarly, Ca\textsuperscript{2+}-ATPase, the major protein controlling calcium uptake, has been well characterized in cardiac and skeletal muscle.\textsuperscript{17} Recent molecular cloning analysis has identified at least five distinct isoforms of the Ca\textsuperscript{2+}-ATPase: adult fast-twitch muscle isoform (SERCA1a),\textsuperscript{25} its alternatively spliced neonatal isoform (SERCA1b),\textsuperscript{26} cardiac/slow-twitch muscle isoform (SERCA2a),\textsuperscript{13,27} its alternatively spliced smooth/nonmuscle isoform (SERCA2b),\textsuperscript{28,29} and an isoform found in a broad variety of muscle and nonmuscle tissues (SERCA3).\textsuperscript{30} In addition to the calcium release channel and Ca\textsuperscript{2+}-ATPase, phospholamban and calsequestrin have an important role in sarcoplasmic reticulum function. The calcium pumping action of Ca\textsuperscript{2+}-ATPase has been shown to be inhibited by phospholamban.\textsuperscript{31–33} There are no isoforms for phospholamban, and the same protein is expressed in cardiac and slow-twitch muscle.\textsuperscript{34,35} The calcium exchanges between the cytosol and sarcoplasmic reticulum are influenced by calcium binding proteins located within the sarcoplasmic reticulum membranes. Calsequestrin is located within the lumen of sarcoplasmic reticulum and acts as a calcium buffer or store.\textsuperscript{36–38} Two isoforms, a cardiac muscle isoform\textsuperscript{39} and a fast-twitch muscle isoform,\textsuperscript{40,41} have been identified.

The present study was undertaken to examine the mechanisms altering sarcoplasmic reticulum function during cardiac hypertrophy, with the following objectives: 1) to determine the effect of thyroid hormone on the expression of mRNA encoding individual sarcoplasmic reticulum proteins that are responsible for calcium release, uptake, and storage in rabbit hearts, 2) to determine whether the expression of sarcoplasmic reticulum proteins controlling both calcium release and calcium uptake are regulated in a coordinate manner during cardiac hypertrophy, and 3) to determine whether thyroid hormone affects the expression of sarcoplasmic reticulum proteins to the same degree in skeletal muscle, since skeletal muscle is not subjected to the increased hemodynamic load imposed on the heart secondary to hyperthyroidism.

### Materials and Methods

#### Animal Models

New Zealand White male rabbits were obtained commercially (Charles River Laboratories, Inc., Montreal, Canada) and maintained on ordinary rabbit chow. Thyrotoxic cardiac hypertrophy was produced in 16-week-old rabbits by injecting L-thyroxine (T4) intramuscularly at 200 $\mu$g/kg body wt for 4 days ($n=4$) or 8 days ($n=4$).\textsuperscript{11,13} Age-matched untreated rabbits ($n=4$) were used as controls. Hypothyroidism was induced in 12-week-old rabbits by adding 0.8 mg/ml of propylthiouracil (PTU) to the drinking water for 4 weeks ($n=4$).\textsuperscript{42} All rabbits were placed under sodium pentobarbital anesthesia (30 mg/kg), and the heart and other tissues were quickly removed. The atria and ventricles were rinsed in cold physiological saline, blotted onto sterile paper towels, weighed, and frozen in liquid nitrogen. Other tissues were immediately immersed in liquid nitrogen.

#### Isolation of RNA

Total cellular RNA was isolated using the guanidine thiocyanate method.\textsuperscript{43} The final RNA pellet was resuspended in a solution containing 10 mM Tris (pH 7.5) and 1 mM EDTA (pH 7.5) and stored at $-70^\circ$C.

#### Probes

The following cDNA and genomic probes were used for Northern blot and RNA slot blot analyses:

1) For the cardiac ryanodine receptor, a 2.25-kb cDNA fragment corresponding to amino acids 2662–3413 of rabbit cardiac muscle ryanodine receptor was used.\textsuperscript{22} 2) For the skeletal ryanodine receptor, a 3-kb cDNA fragment corresponding to amino acids 2846–3873 of rabbit skeletal muscle ryanodine receptor was used.\textsuperscript{22} 3) For Ca\textsuperscript{2+}-ATPase, a 1.7-kb cDNA fragment containing the protein coding region and the 3' untranslated region of rabbit cardiac/slow-twitch muscle Ca\textsuperscript{2+}-ATPase (BamHI–3' end) was used.\textsuperscript{27} This probe cross-hybridizes to fast-twitch and smooth muscle/nonmuscle Ca\textsuperscript{2+}-ATPase mRNA isoforms.\textsuperscript{26,29,30} Cardiac/slow-twitch muscle Ca\textsuperscript{2+}-ATPase mRNA gives a signal around the 4.6-kb position on Northern blot analysis. The fast-twitch muscle isoform is detectable at $\sim 4.0$ kb. 4) For phospholamban, a 1.3-kb cDNA fragment of the rabbit cardiac phospholamban containing the entire coding sequence (5' EcoRI linker–EcoRI) was used.\textsuperscript{34,35} 5) For fast-twitch muscle calsequestrin, a 109-bp genomic fragment (Sst II–Pvu II) containing the last exon of the fast-twitch muscle calsequestrin gene was used.\textsuperscript{41} 6) For cardiac/slow-twitch muscle calsequestrin, cDNA (2.5 kb) encoding the rabbit cardiac/slow-twitch muscle calsequestrin was used (M. Arai and M. Periasamy, manuscript in preparation). 7) For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1.65-kb chicken GAPDH cDNA cloned from chicken breast muscle library (generous gift from Dr. Robert J. Schwartz, Baylor College of Medicine, Houston, Tex.) was used.\textsuperscript{44} GAPDH mes-
sage estimation was used as an internal standard to verify that a constant amount of RNA was loaded onto each lane during Northern blot and RNA slot blot analyses.

**Northern Blot Analysis**

The mRNA level of Ca\(^{2+}\)-ATPase, phospholamban, calsequestrin, and GAPDH were quantitated using Northern blot analysis. Fifteen micrograms of total RNA was denatured at 65°C for 5 minutes, fractionated on 1% agarose gel containing 2.2 M formaldehyde, blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.), and hybridized to random primed cDNA probes as described for Northern blot analysis. The membranes were washed with 2X standard saline citrate and 0.1% sodium dodecyl sulfate (SDS) at 55°C for 15 minutes (Ca\(^{2+}\)-ATPase, phospholamban, and calsequestrin) or with the same solution at 48°C for 10 minutes (GAPDH), air-dried, and exposed to Kodak X-Omat-AR film using an intensifying screen at −70°C.

After autoradiography the individual mRNA bands were quantitated using a laser densitometer. The optical density of each of the Ca\(^{2+}\)-ATPase, phospholamban, and calsequestrin bands was divided by the GAPDH band optical density. The relative level of these messages corrected against the GAPDH value in each sample was calculated as a percentage of the mean value of the corresponding message level in the control group.

**RNA Slot Blot Analysis**

The RNA slot blot system was used to analyze the expression level of the ryanodine receptor mRNA after confirmation that each of the ryanodine receptor probes hybridized specifically to its mRNA and showed a single band on Northern blot. Total RNA was denatured in 50% formamide, 7% formaldehyde, and 1× standard saline citrate solution by heating at 65°C for 15 minutes and cooling on ice. Serial dilutions (20, 10, 5, and 2.5 μg) of samples were loaded onto the nitrocellulose filter of a slot blot apparatus (Schleicher & Schuell). The same amount of tRNA was also loaded to detect nonspecific binding of probes. The nitrocellulose filter was hybridized with \(^{32}P\)-labeled cDNA probes (ryanodine receptors and GAPDH) as described for Northern blot analysis. Autoradiograms in which the densities of bands were linearly increased with loading of 2.5, 5, 10, and 20 μg RNA were quantitated by densitometer scanning. The optical density measured using the ryanodine receptor probe was divided by the optical density obtained using the GAPDH probe. The relative expression level of ryanodine receptor mRNA over GAPDH mRNA in each sample was calculated as a percentage of the mean value observed for the control group.

**Isolation of Crude Sarcoplasmic Reticulum**

Crude sarcoplasmic reticulum was isolated using a modified method of Jones et al.\(^{45}\) One gram of frozen ventricular tissue was ground into fine powder by mortar and pestle and homogenized three times for 10 seconds in 4 vol of 10 mM NaHCO\(_3\) (pH 7.1) with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). A small aliquot of the homogenate was saved as a crude muscle homogenate for the assay of myosin heavy chain (MHC) and actin proteins. The remaining homogenate was spun for 10 minutes at 1,000 g (maximum), and the supernatant was mixed with 3 M KCl to make up 0.6 M KCl in homogenate solution. This homogenate was then centrifuged at 14,000 g (maximum) for 20 minutes, and the resulting supernatant fraction was sedimented at 100,000 g (maximum) for 45 minutes. The pellet was resuspended in 5 ml of 150 mM KCl and 1 mM HEPES (pH 7.1) and recentrifuged at 100,000 g (maximum) for 45 minutes. The pellet was resuspended in 200 μl of 50 mM NaCl and 1 mM HEPES (pH 7.1) and was used as a crude sarcoplasmic reticulum preparation. The yield was approximately 500 μg crude sarcoplasmic reticulum protein/g heart tissue. The protein concentration of crude muscle homogenate and crude sarcoplasmic reticulum was measured by a modified biuret reaction using bichoninic acid (micro BCA protein assay kit, Pierce Chemical Co., Rockford, Ill.).\(^{46}\)

**Protein Gel Electrophoresis and Western Blot Analysis**

SDS–polyacrylamide gel electrophoresis was performed with a slab gel apparatus according to the procedure of Laemmli.\(^{47}\) Acrylamide gel of 13.5% (0.75-mm thickness) was used for the separation of sarcoplasmic reticulum proteins, and 11% gel was used for the analysis of crude muscle homogenate. Ten and five micrograms of sarcoplasmic reticulum protein and muscle homogenate protein were separated by electrophoresis. MHC and actin bands were identified on the basis of their abundance and mobility with molecular weight markers. A strong band corresponding to albumin was used as an internal standard to quantitate the total amount of myosin and actin.

To analyze the amount of Ca\(^{2+}\)-ATPase, proteins in the gel were transferred electrophoretically to a nitrocellulose membrane at 150 mA for 2 hours in a buffer containing 40 mM Tris (pH 8.5), 0.25 M glycine, 0.1% SDS, and 20% ethanol.\(^{48}\) After blotting, the molecular weight marker lane was cut out and stained with 1% amido black, and sample lanes were processed for immunoreaction. After blocking with 3% bovine serum albumin in 0.03% Tween 20 phosphate buffered saline, the membrane was incubated with a polyclonal anti-rabbit Ca\(^{2+}\)-ATPase antibody for 2 hours. Then, the membrane was rinsed, reacted with \(^{125}I\)-labeled protein A for 1 hour, rinsed again, dried, and exposed to Kodak X-Omat-AR film at −70°C overnight. The intensity of each signal was quantitated by laser densitometer, and each intensity of the experimental group was calculated as a percentage of the mean value of the control group.
statistical analyses

Overall differences within groups were determined by the Kruskal-Wallis test, which is equivalent to an analysis of variance of the ranks.\textsuperscript{49,50} When this test indicated that differences existed, individual experimental groups were compared with the control group using the Wilcoxon U test. The test was considered significant at $p<0.05$.

Results

Effect of Thyroid Hormone on Atrial and Ventricular Weight

Atrial and ventricular weight, both as raw values and values normalized for body weight, were increased significantly in the groups that were injected with T4 for 4 days (T4–4-day group) and 8 days (T4–8-day group) but decreased in the group in which PTU was administered for 4 weeks (PTU group) (Table 1). The blood T4 level in the PTU group was measured using a standard clinical radioimmunoaassay kit. Blood T4 level fell from 2.7 μg/dl (average titer before PTU treatment) to <1.0 μg/dl after 4 weeks of PTU treatment, indicating the development of hypothyroidism in the rabbits. These data indicate the development of cardiac hypertrophy in thyrotoxic heart and cardiac atrophy in hypothyroid heart.

Expression of Cardiac Ryanodine Receptor mRNA and Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase mRNA Are Coordinately Upregulated in Hyperthyroid Hearts

First, we examined the expression level of mRNA for cardiac ryanodine receptor in the ventricle (Figure 1A) and atrium using an RNA slot blot method. In ventricular muscle, the cardiac ryanodine receptor mRNA level was increased in hyperthyroid rabbits (T4–4-day group: 147%, $p<0.05$; T4–8-day group: 120%, $p<0.05$) and decreased in hypothyroid rabbits (PTU group: 71%, $p<0.05$) (Figure 2). Values listed are calculated as a percentage of the mean of control rabbit values (e.g., 147% represents an increase of 47% from control, and 71% indicates a decrease of 29% from control). Similar analysis on the atrial muscle showed that the cardiac ryanodine receptor mRNA was also increased in hyperthyroidism (T4–4-day group: 151%, $p<0.05$; T4–8-day group: 132%, $p<0.05$) and decreased in hypothyroidism (PTU group: 85%, $p<0.05$) (Figure 2).

The expression level of the mRNA for sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase was also examined in these heart preparations (Figure 1B). The cardiac/slow-twitch muscle Ca\textsuperscript{2+}-ATPase mRNA level was increased in hyperthyroid ventricle (T4–4-day group: 174%, $p<0.05$; T4–8-day group: 186%, $p<0.05$) and decreased to 75% in hypothyroid ventricle (PTU group: $p<0.05$) (Figure 2). In the atrium, the cardiac/slow-twitch muscle Ca\textsuperscript{2+}-ATPase mRNA was also increased to 128% (T4–4-day group: $p<0.05$) and to 133% (T4–8-day group: $p<0.05$) and decreased to 58% in hypothyroidism (PTU group: $p<0.05$) (Figure 2).

In addition to the Ca\textsuperscript{2+}-ATPase, the mRNA levels of phospholamban were examined using Northern blots (Figure 1B). In thyrotoxic ventricle, the phospholamban mRNA level was decreased (T4–8-day group: 72%, $p<0.05$) as opposed to an increased level of Ca\textsuperscript{2+}-ATPase mRNA (Figure 2). In PTU-treated hypothyroid ventricle, the phospholamban mRNA level was decreased (77%, $p<0.05$) as noted for Ca\textsuperscript{2+}-ATPase mRNA. The phospholamban mRNA level was similarly decreased in the atrial muscle of hyperthyroid rabbits (T4–4-day group: 85%, $p<0.05$; T4–8-day group: 64%, $p<0.05$), but in hypothyroid rabbit atrium, the relative level of phospholamban mRNA did not change (PTU group: 96%, $p=\text{NS}$) (Figure 2).

Last, we analyzed the expression of calsequestrin mRNA, a major Ca\textsuperscript{2+} storage protein in sarcoplasmic reticulum. In contrast to other calcium regulatory proteins of the sarcoplasmic reticulum studied, the expression level of calsequestrin mRNA did not change in hyperthyroid and hypothyroid ventricular muscle (T4–4-day group: 114%, $p=\text{NS}$; T4–8-day group: 118%, $p=\text{NS}$; and PTU group: 109%, $p=\text{NS}$) (Figure 2). Similarly, the calsequestrin mRNA levels in the atrium were not altered by T4 or PTU treatment (T4–4-day group: 110%, $p=\text{NS}$; T4–8-day group: 94%, $p=\text{NS}$; and PTU group: 140%, $p=\text{NS}$).

The Ratio of Ca\textsuperscript{2+}-ATPase Protein to Myosin Heavy Chain and Actin Proteins Is Increased in Hyperthyroid Ventricular Muscle

To determine whether changes in the expression level of Ca\textsuperscript{2+}-ATPase mRNA is reflected in the amount of protein in the tissue, we quantitated the relative amounts of Ca\textsuperscript{2+}-ATPase protein in these tissues using Western blot analysis (Figure 3A). The relative amount of Ca\textsuperscript{2+}-ATPase protein was increased in hyperthyroidism as noted for mRNA (T4–8-day group: 199%, $p<0.05$) (Figure 4A).

In addition to Ca\textsuperscript{2+}-ATPase, the total amount of MHC proteins (V\textsubscript{1}+V\textsubscript{2}+V\textsubscript{3}) and actin in the ventricle from hyperthyroid and hypothyroid rabbits was

### Table 1. Comparison of Body Weight, Atrial Weight, and Ventricular Weight in Hyperthyroid and Hypothyroid Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW (kg)</th>
<th>A (g)</th>
<th>A/BW (g/kg)</th>
<th>V (g)</th>
<th>V/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>2.656</td>
<td>0.758</td>
<td>0.29</td>
<td>4.697</td>
<td>1.77</td>
</tr>
<tr>
<td>T4–4-day</td>
<td>4</td>
<td>2.374*</td>
<td>0.930*</td>
<td>0.39*</td>
<td>5.001</td>
<td>2.11</td>
</tr>
<tr>
<td>T4–8-day</td>
<td>4</td>
<td>2.119*</td>
<td>0.867</td>
<td>0.41*</td>
<td>5.667*</td>
<td>2.68*</td>
</tr>
<tr>
<td>PTU</td>
<td>4</td>
<td>2.708</td>
<td>0.513*</td>
<td>0.19*</td>
<td>3.874*</td>
<td>1.44*</td>
</tr>
</tbody>
</table>

Values are means of each group. n, Number of rabbits; BW, body weight; A, atrial weight; A/BW, atrial weight/body weight ratio; V, ventricular weight; V/BW, ventricular weight/body weight ratio; Control, untreated rabbits; T4–4-day and T4–8-day, rabbits with thyroidism induced by injection of thyroxine for 4 days and 8 days, respectively; PTU, rabbits with hypothyroidism induced by intake of propylthiouracil for 4 weeks. Overall differences within groups were determined by the Kruskal-Wallis test, and every parameter showed a significant difference ($p<0.001$).

*p<0.05 vs. corresponding value for control group by the Wilcoxon U test.
FIGURE 1. Analysis of cardiac ryanodine receptor, cardiac/slow-twitch muscle sarcoplasmic reticulum Ca\(^2+\)-ATPase, cardiac calsequestrin, and phospholamban mRNA using RNA slot blot and Northern blot analysis. Control, untreated rabbits; T4-4days and T4-8days, rabbits with hyperthyroidism induced by injection of thyroxine for 4 days and 8 days, respectively; PTU, rabbits with hypothyroidism induced by intake of propylthiouracil for 4 weeks. Panel A: RNA slot blot analysis of the cardiac muscle ryanodine receptor mRNA in the ventricular muscle from control, hyperthyroid, and hypothyroid rabbits. Total RNA of 2.5, 5, 10, and 20 µg was loaded onto each slot. Same serial amounts of tRNA were also loaded as a control for nonspecific binding of probes. Panel B: Northern blot analyses of cardiac/slow-twitch Ca\(^2+\)-ATPase, phospholamban (Pho.), cardiac calsequestrin (Cals.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in hyperthyroid and hypothyroid ventricles. GAPDH message estimation was used as an internal standard.

determined on SDS gel (Figure 3B). The ratio of MHC protein/total protein was increased in the T4–8-day group (114%, \(p<0.05\)) and was decreased in the PTU group (91%, \(p<0.05\)) (Figure 4A). The ratio of actin protein/total protein was also increased in hyperthyroidism (T4–8-day group: 128%, \(p<0.05\)) (Figure 4A).

To determine the relative amounts of Ca\(^2+\)-ATPase protein to MHC or actin protein in hyperthyroid and hypothyroid hearts, we calculated the Ca\(^2+\)-ATPase protein/MHC protein ratio and the Ca\(^2+\)-ATPase protein/actin protein ratio (Figure 4B). The mean ratio in the control group was defined as 100%, and ratio in the experimental groups was expressed as the percentage of change compared with values obtained from the control group. As shown in Figure 4B, the Ca\(^2+\)-ATPase protein/MHC protein ratio was increased in thyrotoxic hearts (T4–8-day group: 174%, \(p<0.05\)). A similar increase in the Ca\(^2+\)-ATPase protein/actin protein ratio was seen in the thyrotoxic heart (T4–8-day group: 155%, \(p<0.05\)).

**Thyroid Hormone Increases the Expression of Ryanodine Receptor mRNA in Skeletal Muscles**

To examine whether the calcium release process in skeletal muscle is also regulated by thyroid hormone levels, we have analyzed the mRNA level of skeletal muscle ryanodine receptor in soleus and plantaris muscle from the same rabbits used for the analysis of cardiac muscle (Figure 5). It is of note that both slow- and fast-twitch skeletal muscle tissues express only the skeletal muscle specific ryanodine receptor isoform.\(^{23}\)

In hyperthyroid rabbits, the relative level of skeletal muscle ryanodine receptor mRNA was increased
FIGURE 2. Bar graphs showing quantitation of mRNA for various calcium transport proteins in sarcoplasmic reticulum in normal, hyperthyroid, and hypothyroid hearts. Euthyroid, control untreated rabbits; T4-4days and T4-8days, rabbits with hyperthyroidism induced by injection of thyroxine for 4 days and 8 days, respectively; PTU-4wks, rabbits with hypothyroidism induced by intake of propylthiouracil for 4 weeks. The expression level of cardiac muscle ryanodine receptor, cardiac/slow-twitch Ca\(^{2+}\)-ATPase, phospholamban, and cardiac calsequestrin mRNA from control, hyperthyroid, and hypothyroid rabbits was quantitated, corrected with glyceraldehyde-3-phosphate dehydrogenase mRNA level, and calculated as a percentage of the mean of the control group. Each dot superimposed on the column represents the value from an individual rabbit, and vertical columns represent the mean value from four rabbits in each group. Listed p values are results of the Kruskal-Wallis test, a test for overall differences within groups. The difference between each experimental group and the control group was tested using the Wilcoxon U test. The p value of each comparison is indicated in text. NS, no statistically significant difference.

FIGURE 3. Polyacrylamide gel analysis of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, myosin heavy chain (MHC), and actin proteins from hyperthyroid and hypothyroid ventricles. Control, untreated rabbits; T4-4days and T4-8days, rabbits with hyperthyroidism induced by injection of thyroxine for 4 days and 8 days, respectively; PTU, rabbits with hypothyroidism induced by intake of propylthiouracil for 4 weeks. Panel A: Crude ventricular sarcoplasmic reticulum protein (10 μg) from control, hyperthyroid, and hypothyroid rabbits was electrophoresed, blotted, and incubated with polyclonal anti-rabbit sarcoplasmic reticulum Ca\(^{2+}\)-ATPase antibody and then reacted with \(^{125}\)I-labeled protein A. Panel B: Crude ventricular muscle homogenate (10 μg) from control and experimental rabbits was analyzed on 11% sodium dodecyl sulfate–polyacrylamide gel. In this gel system, isoforms of ventricular MHC (V1, V2, and V3) and actin cannot be separated. The gel was stained with Coomassie blue. A strong band corresponding to albumin (⁎) was used as an internal standard to quantitate the relative amounts of MHC and actin protein.
in soleus muscle (T4-4-day group: 140%, p<0.05; T4-8-day group: 128%, p<0.05) and in plantaris muscle (T4-4-day group: 135%, p<0.05; T4-8-day group: 135%, p<0.05) (Figure 5). In hypothyroidism the expression level of skeletal muscle ryanodine receptor mRNA was not changed.

**Thyroid Hormone Increased the Expression of Ca\(^{2+}\)-ATPase mRNA in Skeletal Muscles**

To understand whether the thyroid hormone has a similar effect on the Ca\(^{2+}\)-ATPase mRNA levels of skeletal muscle, we quantitated the Ca\(^{2+}\)-ATPase mRNA levels in soleus and plantaris muscle using Northern blot analysis (Figure 6). Adult skeletal muscle expresses two different Ca\(^{2+}\)-ATPases: 1) the cardiac/slow-twitch muscle isoform (SERCA2) and 2) the fast-twitch muscle isoform (SERCA1). These Ca\(^{2+}\)-ATPases can be identified on Northern blot analysis, since their mRNAs are of different sizes.

In soleus muscle the level of cardiac/slow-twitch Ca\(^{2+}\)-ATPase mRNA was increased in hyperthyroidism (T4-4-day group: 187%, p<0.05; T4-8-day group: 143%, p<0.05) but decreased in hypothyroidism (PTU group: 75%, p<0.05) (Figure 6A). In addition to an increase in the cardiac/slow-twitch Ca\(^{2+}\)-ATPase mRNA isoform, the fast skeletal iso-
form (not detectable in euthyroid soleus muscle) was induced after T4 treatment (data not shown).

Plantaris muscle expresses both the fast-twitch isofrom and the cardiac/slow-twitch isofrom. In hyperthyroid rabbits, the two isofroms were positively upregulated in this muscle (fast-twitch isofrom for T4-4-day group: 201%, p<0.05; fast-twitch isofrom for T4-8-day group: 239%, p<0.05; cardiac/slow-twitch isofrom for T4-4-day group: 164%, p<0.05; cardiac/slow-twitch isofrom for T4-8-day group: 169%, p<0.05), whereas in hypothyroid rabbits, the mRNA for the two Ca\textsuperscript{2+}-ATPase isofroms did not change (fast-twitch isofrom for PTU group: 92%, p=NS; cardiac/slow-twitch isofrom for PTU group: 106%, p=NS) (Figure 6A). The magnitude of induction of Ca\textsuperscript{2+}-ATPase mRNA in hyperthyroidism was greater for the fast-twitch isofrom than for the cardiac/slow-twitch isofrom. The relative proportion of fast-twitch isofrom mRNA (fast/fast+slow) was increased from 48% (control) to 55% in the T4-4-day group (p<0.05) and to 57% in the T4-8-day group (p<0.05) but decreased to 42% in the PTU group (p<0.05) (Figure 6B).

**Thyroid Hormone Effect on the Expression of Phospholamban mRNA and Calsequestrin mRNA in Skeletal Muscle is Very Similar to Cardiac Muscle**

We also examined the expression levels of phospholamban and calsequestrin mRNA in soleus muscle. In skeletal muscle, phospholamban is expressed only in slow-twitch muscle (soleus muscle) but not in fast-twitch muscle (plantaris muscle). In soleus muscle, the phospholamban mRNA level was reduced to 43% in hyperthyroidism (T4-4-day group: p<0.05) (Figure 7).

Two types of calsequestrin, the cardiac/slow-twitch isofrom and the fast-twitch isofrom, are expressed in soleus muscle. As shown in Figure 7, the mRNA levels for the two calsequestrin isofroms did not change in soleus muscle from hyperthyroid and hy-
Discussion

**Messenger RNAs of Sarcoplasmic Reticulum Protein Responsible for Calcium Release and Uptake Are Coordinately Upregulated in Hyperthyroidism and Downregulated in Hypothyroidism**

Previous work from our laboratory has indicated that chronic administration of thyroid hormone up-regulates the Ca\(^{2+}\)-ATPase mRNA in rabbit hearts.\(^{13}\)

In the present study we have demonstrated that the expression of the mRNA coding for both the cardiac ryanodine receptor and cardiac Ca\(^{2+}\)-ATPase is up-regulated in hyperthyroidism and downregulated in hypothyroidism. Furthermore, we showed that thyroid hormone–induced changes in the mRNA level for sarcoplasmic reticulum proteins are very similar in both cardiac and skeletal muscle tissues. We also found that an increase in the amount of Ca\(^{2+}\)-ATPase mRNA was paralleled by an increase in the Ca\(^{2+}\)-ATPase protein in cardiac muscle. These data indicate that in chronic hyperthyroid and hypothyroid conditions regulation of calcium release and re-uptake is accomplished primarily by increasing or decreasing the number of functional protein molecules for the calcium release channel and the Ca\(^{2+}\)-ATPase protein. More important, these alterations in calcium release and re-uptake (controlling muscle contraction and relaxation) are coordinately regulated. Although the data presented support our postulate that these changes are occurring at the mRNA level, it remains to be determined whether the regulation is occurring at the transcriptional or posttranscriptional level.

By contrast, there was no significant change in the mRNA levels of calsequestrin, the predominant calcium storage protein in sarcoplasmic reticulum.\(^{51}\) in either hyperthyroid or hypothyroid hearts. Myother- mal (tension-independent heat) measurements indicate that there are no differences between euthyroid and hyperthyroid hearts in the amount of Ca\(^{2+}\) cycled during contraction and relaxation.\(^{11,12}\) Suko\(^{7}\) has shown that the maximal amount of calcium stored inside vesicles of the sarcoplasmic reticulum fraction isolated from euthyroid, hyperthyroid, and hypothyroid hearts is not different. Our present observations on calsequestrin mRNA further support the idea that the calcium storage capacity of the sarcoplasmic reticulum is unaltered, even though the rate of calcium release and uptake is increased in thyrotoxic hearts and decreased in hypothyroid hearts.

Our data on the expression of phospholamban mRNA shows that there is no coordinate regulation between phospholamban and Ca\(^{2+}\)-ATPase. In hyper-thyroidism, the phospholamban mRNA level decreased while the Ca\(^{2+}\)-ATPase mRNA level increased. On the other hand, hypothyroid muscle showed a decrease of phospholamban mRNA level in ventricle and no change in atrium and soleus muscle while the Ca\(^{2+}\)-ATPase mRNA level decreased. The functional significance of these changes is not understood. Because the Ca\(^{2+}\)-ATPase pump activity can be modulated by cAMP- and calmodulin-dependent phosphorylation of phospholamban,\(^{31–33}\) it remains to be determined to what extent changes in phospholamban levels are involved in modulating Ca\(^{2+}\)-ATPase. Therefore, the significance of these data will become more obvious once the extent of phosphorylation in a different thyroid state is examined precisely.

The demonstration that thyroid hormone affects sarcoplasmic reticulum protein expression in skeletal muscle in the same manner as observed for the heart is unexpected. The thyroid hormone effects on skeletal muscle are primarily hormonal, whereas for cardiac muscle there is a humoral as well as a secondary hemodynamic effect, resulting from the elevated metabolic rate associated with hyperthyroidism.\(^{52}\) Nevertheless, the ryanodine receptor, Ca\(^{2+}\)-ATPase, phospholamban, and calsequestrin mRNA levels changed to similar extents in both skeletal and cardiac muscle. These results would suggest that the thyroid hormone effect on the expression level of sarcoplasmic reticulum proteins in the heart is likely to be direct rather than secondary.

**The Modulation of Sarcoplasmic Reticulum Protein Expression Plays an Important Role in the Altered Functional State of Muscle Induced by Thyroid Hormone Stress**

Both the sarcoplasmic reticulum and the contractile protein myosin play a major role in controlling muscle contractile properties in response to stress. It is well known that the MHC isozyme pattern in each muscle varies widely to meet diverse functional requirements, even in the euthyroid state.\(^{53–55}\) For example, fast-twitch skeletal muscle expresses the high ATPase activity MHC isoforms predominantly (fast IIA and fast IIB isoforms), and hyperthyroidism changes the composition of two isoforms whose ATPase activities are both high.\(^{53,54,56}\) Nevertheless, T4 treatment does cause a further increase in the velocity of contraction and relaxation in fast-twitch muscle.\(^{56}\) These changes in contractile properties are unlikely to result from MHC fiber-type changes since the thyroid hormone effect on MHC is the interconversion between two types of fast fibers (from fast IIA to fast IIB). In the absence of a significant change in myosin ATPase activity, alterations in the rate of calcium release and uptake by sarcoplasmic reticulum could contribute significantly to accelerated mechanical properties in hyperthyroidism. In this study, we have demonstrated that the ryanodine receptor mRNA and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase mRNA were both increased in fast-twitch
muscle (plantaris muscle) in hyperthyroidism, supporting this hypothesis.

In addition to fast-twitch muscle, we also observed an increase in the expression of both cardiac ryanodine receptor and cardiac Ca\textsuperscript{2+}-ATPase mRNA in atrium, a muscle tissue expressing exclusively the fast type of myosin in euthyroidism and hyperthyroidism.\textsuperscript{57} Our data suggest that hyperthyroidism can further increase the contractile velocity of atrial muscle by accelerating calcium release and uptake.

In summary, we offer the following conclusions: 1) The thyroid hormone-inducend changes in the levels of mRNAs encoding Ca\textsuperscript{2+} pump and Ca\textsuperscript{2+} release channel proteins are coordinately regulated in both cardiac and skeletal muscle. 2) The increased expression of the Ca\textsuperscript{2+} release channel mRNA and Ca\textsuperscript{2+}-ATPase mRNA should lead to an increase in corresponding proteins and thus to an increased calcium release and reuptake capacity in hyperthyroidism. Hypothyroidism should produce the opposite changes. 3) The expression of mRNA for calsequestrin is not altered by thyroid hormone, indicating that there is likely to be little effect of the hormone on the calcium storage capacity. 4) Thyroid hormone-induced changes in the level of mRNA for sarcoplasmic reticulum proteins appear to be a result of the direct action of thyroid hormone on the muscle and not of hemodynamic overload, since parallel results were obtained in both cardiac and skeletal muscle. 5) The calcium regulatory capacity of sarcoplasmic reticulum plays an important role in altering muscle properties in the absence of a significant change of myosin ATPase activity.

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