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Regulation of myocardial Ca^{2+}-ATPase and phospholamban mRNA expression in response to pressure overload and thyroid hormone

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ABSTRACT **The sarcoplasmic reticulum (SR) and the contractile protein myosin play an important role in myocardial performance. Both of these systems exhibit plasticity—i.e., quantitative and/or qualitative reorganization during development and in response to stress. Recent studies indicate that SR Ca^{2+} uptake function is altered in adaptive cardiac hypertrophy and failure. The molecular basis (genetic and phenotypic) for these changes is not understood. In an effort to determine the underlying causes of these changes, we characterized the rabbit cardiac Ca^{2+}-ATPase phenotype by molecular cloning and ribonuclease A mapping analysis. Our results show that the heart muscle expresses only the slow-twitch SR Ca^{2+}-ATPase isoform. Second, we quantitated the steady-state mRNA levels of two major SR Ca^{2+} regulatory proteins, the Ca^{2+}-ATPase and phospholamban, to see whether changes in mRNA content might provide insight into the basis for functional modification in the SR of hypertrophied hearts. In response to pressure overload hypertrophy, the relative level of the slow-twitch/cardiac SR Ca^{2+}-ATPase mRNA was decreased to 34% of control at 1 week. The relative Ca^{2+}-ATPase mRNA level increased to 167% of control after 3 days of treatment with thyroid hormone. In contrast, in hypothyroid animals, the relative Ca^{2+}-ATPase mRNA level decreased to 51% of control at 2 weeks. The relative level of phospholamban mRNA was decreased to 36% in 1-week pressure overload. Hyperthyroidism induced a decrease to 61% in the phospholamban mRNA level after 3 days of treatment, while hypothyroidism had virtually no effect on phospholamban mRNA levels. These data indicate that the expression of SR Ca^{2+}-ATPase and phospholamban mRNA may not be coordinately regulated during myocardial adaptation to different physiological conditions.**

Myocardial hypertrophy is an adaptive response to sustained stress where survival depends on the increase in muscle mass. In addition to an increase in cell size there is significant reorganization of the myocardial cell that results in alteration in the function of the contractile proteins and the sarcoplasmic reticulum (SR). The major changes in the myocardial contractile proteins are attributable to altered phenotypic expression (1). The objective of the present study is to discover the basis for the change in myocardial SR function in hypertrophied hearts.

The SR of striated muscle is a highly organized intracellular membrane system that plays a critical role in the contraction–relaxation cycle of the myocardium by regulating the cytosolic Ca^{2+} level (2). Ca^{2+} pumping by the SR membranes is mediated by a Ca^{2+}-dependent ATPase. The Ca^{2+}-ATPase of skeletal muscle SR has been studied extensively with respect to structure and mechanism of action (3, 4). The structure and function of the Ca^{2+}-ATPase phenotype(s) expressed in the heart muscle, however, have been less well studied. Recently, MacLennan and co-workers have described the existence of four distinct Ca^{2+}-ATPase isoforms (5–8, 40). Two of these are alternatively spliced products of a single gene and are expressed only in fast-twitch skeletal muscle (6, 7, 40). The other two isoforms are alternatively spliced products of a second gene. One of these isoforms is expressed in cardiac and slow-twitch muscle (5, 7), while the other is expressed in nonmuscle cells (8, 9) and in smooth muscle (10).

Cardiac SR also contains a unique low molecular weight integral protein, phospholamban (11), for which cDNAs have been cloned and sequenced (12, 26). The phosphorylation of phospholamban by cAMP-dependent protein kinase increases Ca^{2+} uptake by increasing the turnover rate of the SR Ca^{2+}-ATPase (11).

A number of recent studies indicate that the calcium pumping function of SR is altered during cardiac hypertrophy as measured by Ca^{2+} uptake *in vitro* (13–18) and by thermal measurement of papillary muscles during isometric twitch (19–21). Decreased Ca^{2+} uptake by microsomal vesicles isolated from hearts with chronic pressure overload (PO) hypertrophy (16–18) has been observed, while increased uptake occurs in thyrotoxic hypertrophy (14, 15). The increase in Ca^{2+} uptake after thyroxine treatment may be due to increased synthesis of the Ca^{2+}-ATPase (15). A recent report indicated that the SR Ca^{2+} ATPase mRNA levels are highly influenced by thyroid hormone (22) in the rat heart.

The present study was undertaken with the objectives of determining the types of Ca^{2+}-ATPase transcripts expressed in the heart muscle and of quantitating both the Ca^{2+}-ATPase and phospholamban transcripts in heart muscle subjected to functional and hormonal stress. Since changes in transcription usually parallel changes in translation, changes in mRNA content might provide insight into the basis for functional modifications in the SR of stressed hearts.

MATERIALS AND METHODS

Animal Models. Pressure overload hypertrophy was produced in 8-week-old New Zealand White rabbits by a 60% constriction of the pulmonary artery, for 1 week ($n = 5$) and 4 weeks ($n = 5$) (23). Sham-operated animals 1 week ($n = 5$) and 4 weeks ($n = 5$) after surgery were also prepared for comparison. Thyrotoxic cardiac hypertrophy was produced (20) in 12-week-old rabbits by injecting thyroxine (T_4) intramuscularly at 200 μg/kg of body weight for 3 days ($n = 6$) or 1 week ($n = 6$). Untreated rabbits of the same age ($n = 6$) were used as controls. Hypothyroidism was induced (24) in 12-

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Abbreviations: SR, sarcoplasmic reticulum; PO, pressure overload; PTU, propylthiouracil; T_4, thyroxine.

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week-old rabbits by the addition of propylthiouracil (PTU) in the drinking water (0.8 g/liter) for 2 weeks ($n = 6$) or 4 weeks ($n = 5$). Age-matched, untreated rabbits, 14 weeks old ($n = 5$) and 16 weeks old ($n = 5$), were studied as controls for each group of 2- and 4-week hypothyroid rabbits.

Screening of a Cardiac cDNA Library. A rabbit cardiac cDNA library constructed in the λgt10 vector by using poly(A)$^+$ mRNA from adult heart (a kind gift of J. Lytton, University of Toronto) was screened, using the coding and 3′ noncoding regions of the slow-twitch Ca^{2+}-ATPase cDNA as probes.

RNase A Protection Assay. For analysis of Ca^{2+}-ATPase mRNA expression, antisense RNA probes were prepared from the 3′ untranslated region of the slow-twitch/cardiac (nucleotides 3176–3400) and the adult fast-twitch Ca^{2+}-ATPase cDNA (nucleotides 3359–3716) clones (5, 7). These probes would produce fragments of 224 and 357 nucleotides, respectively, when fully protected from RNase A digestion (7). Conditions for the antisense RNA riboprobe synthesis, annealing with RNA, and RNase A digestion were as described previously (7). The precipitated RNA was dissolved in a solution containing 90% (vol/vol) formamide and 0.8 mM EDTA, denatured by heating, and separated electrophoretically on a denaturing acrylamide gel.

RNA Blot Analysis. Total RNA was extracted from heart samples by the hot phenol method (ref. 25, p. 194). Ten micrograms of total RNA was size-fractionated in 1% agarose gels containing 1 M formaldehyde. The agarose gel was blotted onto a nitrocellulose membrane (Schleicher & Schuell) (ref. 25; p. 202) and hybridized with nick-translated cDNA probes from the 3′ untranslated region [a 224-base-pair (bp) *Rsa* I–*Rsa* I restriction endonuclease fragment containing residues 3176–3400 (5)], from the 5′ translated region [a 657-bp *Nco* I–*Nco* I restriction endonuclease fragment containing residues −1 to 656 (5)] of the slow-twitch/cardiac Ca^{2+}-ATPase cDNA, or a 377-bp cDNA fragment containing the entire coding region [5′ *Eco*RI linker (position −177) to *Alu* I (position 200)] of the rabbit cardiac phospholamban (26).

Slot Blot Assay. Samples (2, 5, and 10 μg) of total RNA from individual hearts were loaded onto the nitrocellulose membranes of a slot blot apparatus (Schleicher & Schuell). The nitrocellulose filter was hybridized with ^{32}P-labeled cDNA probes under the conditions described for RNA blot analysis. Filters were washed at 65°C with 30 mM NaCl/3 mM sodium citrate and 0.1% SDS followed by exposure to Kodak X-Omat-AR films. The slot blot assay was performed at least twice for each sample. Autoradiograms in which the densities of bands were linearly increased with loading of 2, 5, and 10 μg of RNA were quantitated by densitometer scanning.

Statistical Analysis. The statistical tests were the standard two-group t tests. The P values reported were not adjusted for multiple comparisons.

RESULTS

Effect of Pressure Overload and Thyroid Hormone on Ventricular Weight. Right ventricular weight normalized for body weight or total heart weight was increased significantly in 1-week and 4-week PO compared to 1-week sham and 4-week shams ($P < 0.01$) (Table 1). The ratio of right ventricle weight to body weight was increased significantly in both 3-day and 1-week thyrotoxicosis ($P < 0.01$) (Table 1). These data are consistent with the development of cardiac hypertrophy in PO and thyrotoxic hearts. The PTU-treated hearts were not significantly different from controls (Table 1).

The Ca^{2+}-ATPase Phenotype in the Heart Muscle. Two different approaches were undertaken in the present study to confirm earlier observations (7) that only the slow-twitch isoform is expressed in the heart. Initially, a cardiac cDNA library of 1×10^6 recombinant phages cloned in the vector λgt10 was screened, using the entire coding and 3′ noncoding regions of the slow-twitch Ca^{2+}-ATPase cDNA as probes. Eleven positive clones were isolated and analyzed by restriction mapping and Southern blotting analysis. All of the clones contained the complete 3′ nontranslated region and had restriction maps identical to those of the slow-twitch Ca^{2+}-ATPase cDNA (5). Three of these clones were found to be full length [4.2 kilobases (kb)] (results not shown). Then we analyzed the phenotypic expression of the Ca^{2+}-ATPase in normal and functionally stressed myocardium using complementary RNA probes (riboprobes) specific to fast-twitch and slow-twitch muscle isotypes (5, 6). We wanted to determine specifically whether the fast-twitch isoform appeared in the heart under conditions of remodeling associated with PO or manipulation of the thyroid hormone axis. Antisense RNA probes specific to each isoform were synthesized and hybridized to heart RNA samples and then treated with RNase A to reveal the DNA·RNA-protected hybrids. Fig. 1*a* shows the results of such an experiment, using the slow-twitch Ca^{2+}-ATPase probe, which is composed of 224 nucleotides of 3′ untranslated region and the 40 bp of polylinker sequence of the vector pTZ. The results demonstrate that a 224-nucleotide fragment is fully protected by fetal and adult heart mRNAs and by mRNAs from hypertrophied (PO and thyrotoxic) and hypothyroid hearts. When the same experiment was performed using the fast-twitch-specific riboprobe con-

Table 1. Body weight and right ventricular weight

Treatment	n	BW, kg	RV/BW, g/kg	RV/(LV + RV) × 100
1-week sham	5	2.01 ± 0.07	0.41 ± 0.06	25 ± 1
1-week PO	5	1.95 ± 0.03	0.81 ± 0.02*	38 ± 2*
4-week sham	5	2.29 ± 0.09	0.34 ± 0.02	21 ± 1
4-week PO	5	2.26 ± 0.09	0.77 ± 0.07*	34 ± 2*
12-week-old control	6	2.10 ± 0.12	0.32 ± 0.03	
3-day thyroxine	6	1.96 ± 0.14	0.56 ± 0.05†	
1-week thyroxine	6	1.66 ± 0.10	0.60 ± 0.06†	
14-week-old control	5	2.17 ± 0.14	0.40 ± 0.03	
2-week PTU	6	2.36 ± 0.15	0.38 ± 0.02	
16-week-old control	5	2.99 ± 0.08	0.31 ± 0.01	
4-week PTU	5	2.59 ± 0.06	0.30 ± 0.02	

BW, body weight; RV, right ventricle weight; LV, left ventricle weight. Ventricular ratios were not calculated for thyroxine and PTU treatments because both LV and RV are affected. The values are mean ± SEM.

*$P < 0.01$; PO vs. sham for the same treatment period.

†$P < 0.01$; thyroxine vs. control.

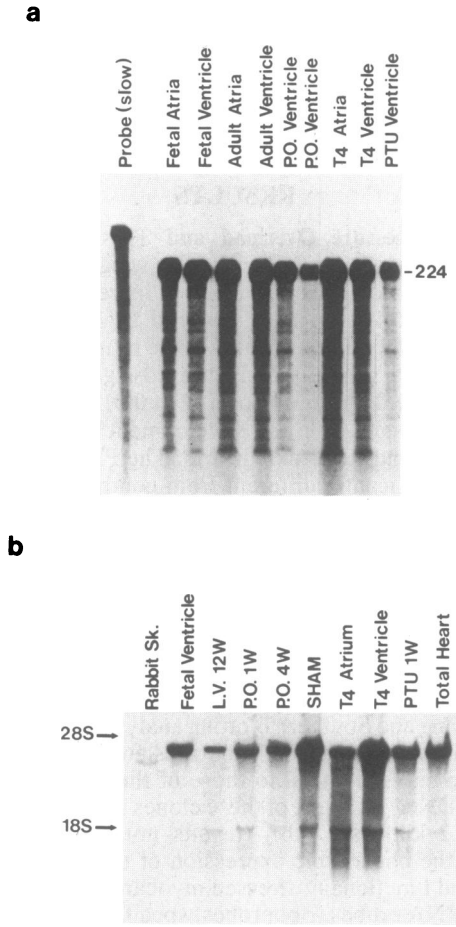

FIG. 1. (*a*) RNase protection assay. A riboprobe from the 3′ untranslated region of the slow-twitch cardiac Ca^{2+}-ATPase [a 224-bp *Rsa* I–*Rsa* I restriction endonuclease fragment containing residues 3176–3400 (5)] was used to hybridize with mRNA from fetal atrium and right ventricle, normal adult atrium and ventricle, PO right ventricle (1-week and 4-week), hyperthyroid (T_4) atrium and ventricle, and hypothyroid (PTU) ventricle. The presence of a band of 224 nucleotides represents full protection of the RNase digestion. (*b*) RNA blot analysis. cDNA probe from 5′ coding region of the slow-twitch cardiac Ca^{2+}-ATPase [a 657-bp *Nco* I–*Nco* I restriction endonuclease fragment containing residues −1 to 656 (5)] was nick-translated and hybridized to an RNA blot with total RNA from rabbit skeletal muscle, fetal ventricle, left ventricle 12-week, PO 1-week, PO 4-week, sham-operated, T_4 atrium, T_4 right ventricle, PTU 1-week, and total heart.

sisting of 254 nucleotides of 3′ untranslated region, none of the mRNAs protected the probe either fully or partially (results not shown). Moreover, the absence of the fast-twitch Ca^{2+}-ATPase mRNA was also confirmed by RNA blot analysis, as shown in Fig. 1*b*. A single band of 4.2 kb corresponding to the size of the slow-twitch/cardiac mRNA was found in all of the samples, but the fast-twitch mRNA band, corresponding to a size of about 3.7 kb, was absent from all samples.

The results of cDNA cloning and RNase A mapping analyses, taken together, demonstrate that the cardiac atrial and ventricular muscle transcribe only the slow-twitch fiber Ca^{2+}-ATPase gene, in both normal and functionally stressed myocardium.

Ca^{2+}-ATPase mRNA Expression in the Experimental Models of Cardiac Hypertrophy and Hypothyroidism. Previous data from several laboratories indicate that Ca^{2+} uptake *in vitro* by SR vesicle preparations from PO hearts is decreased, while uptake of Ca^{2+} by SR vesicles from thyrotoxic hypertrophied hearts is increased (13–18). These results, in conjunction with the finding that only the slow-twitch/cardiac SR calcium pump is present in heart muscle, raise the possibility that Ca^{2+} uptake might be linked to a change in the number of Ca^{2+} pumps. To examine this possibility, we measured the steady-state levels of the Ca^{2+}-ATPase mRNA, using the slow-twitch/cardiac-specific probe in experimentally induced models of cardiac hypertrophy (PO by aortic constriction and thyroid hormone administration) and in hypothyroidism.

In Fig. 2 the difference in the steady-state level of Ca^{2+}-ATPase mRNA between control and PO hearts (right ventricle) is presented. RNA blot analysis showed that in both normal and PO hearts only the slow-twitch/cardiac mRNA was expressed (as revealed by a 4.2-kb mRNA) (Fig. 2*a*); however, the relative level of the Ca^{2+}-ATPase mRNA was markedly decreased in 1-week PO hypertrophy. A more quantitative slot blot analysis (Fig. 2*b* and Table 2) and densitometer scanning revealed that the Ca^{2+}-ATPase mRNA levels in 1-week PO group decreased to 34% of control ($P < 0.01$), and in the 4-week PO group, the Ca^{2+} ATPase mRNA decreased to 56% of control ($P < 0.05$).

Since thyroid hormone is known to produce effects opposite to those seen in PO, cardiac hypertrophy was induced by thyroxine administration to rabbits. Fig. 3*a* shows a slot blot analysis using RNA samples from thyrotoxicosis and control hearts. Densitometer scanning analysis showed that SR Ca^{2+}-ATPase mRNA was increased significantly: at 3 days to 167% of control values ($P < 0.02$) and at 7 days to 150% of control values ($P < 0.05$) (Table 2). The observed increase in Ca^{2+}-ATPase mRNA correlated with the reported increase in Ca^{2+}-ATPase activity (13, 15) in thyrotoxic hearts.

Finally, we investigated the effects of hypothyroidism on the Ca^{2+}-ATPase mRNA. In hypothyroidism (Fig. 3*b*), the cardiac Ca^{2+}-ATPase mRNA decreased significantly: at 2 weeks to 51% of control ($P < 0.01$) and at 4 weeks to 64% of control ($P < 0.05$) compared to the age-matched, untreated controls consisting of 14- and 16-week-old rabbits (Table 2).

Cardiac Phospholamban mRNA Expression in Experimental Models of Cardiac Hypertrophy and Hypothyroidism. The

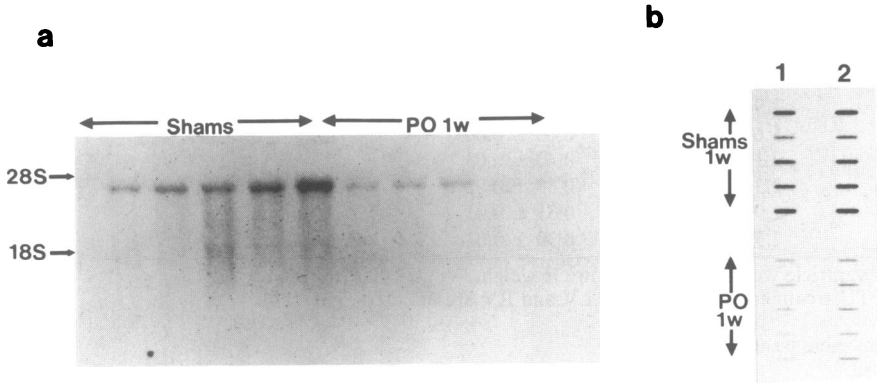

FIG. 2. Analysis of Ca^{2+}-ATPase mRNA in PO hearts. (*a*) RNA blot analysis. Total RNA from 1-week sham-operated and 1-week PO hypertrophy rabbits (each lane represents one rabbit) was separated electrophoretically (ref. 25, p. 202) and hybridized with a full-length slow-twitch/cardiac Ca^{2+}-ATPase cDNA. (*b*) Slot blot assay to quantitate slow-twitch/cardiac Ca^{2+}-ATPase mRNA. Five (lane 1) and 10 (lane 2) μg of total RNA from five 1-week sham-operated and five 1-week PO rabbits were loaded and hybridized with the slow-twitch/cardiac cDNA 3′ nontranslated fragment (a 224-bp *Rsa* I–*Rsa* I restriction endonuclease fragment) (5).

Table 2. Ca^{2+}-ATPase and phospholamban mRNA levels estimated by densitometric scanning of slot blot assays

Treatment	*n*	Ca^{2+}-ATPase mRNA			Phospholamban mRNA		
		mRNA*	*P*	% of sham or control	mRNA*	*P*	% of sham or control
1-week sham	10	4.14 ± 1.77		100	2.50 ± 0.68		100
1-week PO	10	1.39 ± 0.39	<0.01	34	0.89 ± 0.18	<0.001	36
4-week sham	5	4.40 ± 1.57		100	2.98 ± 1.16		100
4-week PO	5	2.45 ± 0.51	<0.05	56	2.81 ± 0.39	NS	94
Control	6	1.81 ± 0.58		100	5.75 ± 0.95		100
3-day thyroxine	6	3.03 ± 0.78	<0.02	167	3.53 ± 1.22	<0.02	61
7-day thyroxine	6	2.71 ± 0.79	<0.05	150	3.33 ± 1.21	<0.02	58
Control	5	4.94 ± 1.65		100	1.92 ± 0.90		100
2-week PTU	6	2.52 ± 0.55	<0.01	51	1.87 ± 0.83	NS	97
Control	5	4.13 ± 1.17		100	4.64 ± 1.68		100
4-week PTU	5	2.65 ± 0.76	<0.05	64	4.32 ± 0.62	NS	93

P, two-tailed value for *t* test vs. appropriate sham or control. NS, not significant.
*Relative absorbance units of densitometric scanning at 600 nm. The values are mean ± SD.

expression of phospholamban mRNA was assessed in view of the involvement of phospholamban in the regulation of slow-twitch Ca^{2+}-ATPase activity (11). Slot blot analyses were performed, using a rabbit cardiac cDNA probe for phospholamban (26). A significant decrease in the phospholamban mRNA to 36% ($P < 0.001$) was observed in the 1-week PO group compared with sham-operated animals (Table 2). Thyroxine treatment induced a decrease in the phospholamban mRNA, to 61% of control ($P < 0.02$), after 3 days, and to 58% control ($P < 0.02$) after 1 week of treatment, while hypothyroidism induced by PTU did not show statistically different levels of this mRNA compared with the respective controls (Table 2).

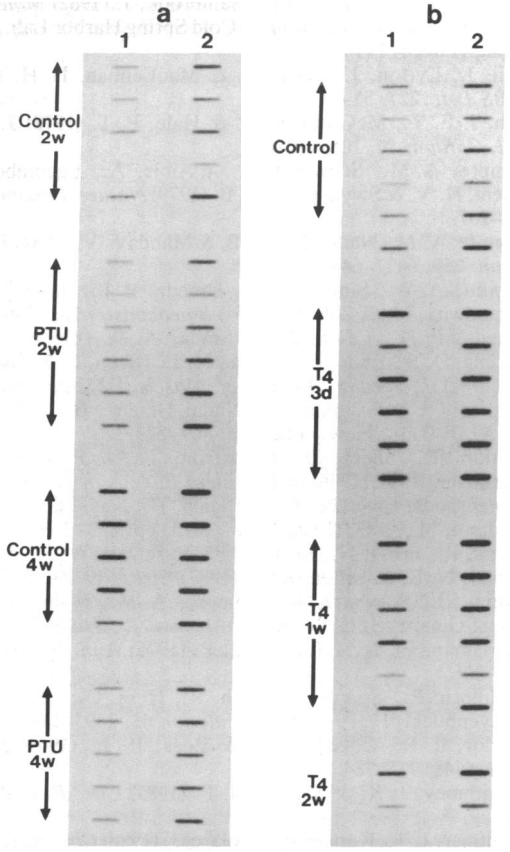

FIG. 3. Analysis of Ca^{2+}-ATPase mRNA levels in hypo- and hyperthyroid hearts. (*a*) Slot blot analysis from five 2-week untreated control, six 2-week hypothyroid (PTU), five 4-week untreated control, and five 4-week hypothyroid (PTU) rabbits. (*b*) Slot blot assay from six untreated control, six 3-day thyrotoxicosis, six 1-week thyrotoxicosis, and two 2-week thyrotoxicosis. Five (lane 1) and 10 (lane 2) μg of total RNA were loaded into both slot blots and hybridized with the slow-twitch/cardiac probe as described in the legend to Fig. 2.

DISCUSSION

The data presented here clearly demonstrate that cardiac SR Ca^{2+}-ATPase mRNA levels undergo significant changes in hypertrophied and hypothyroid hearts. However, the regulation is different from that seen for myosin or total myocardial protein. In response to both PO and thyrotoxic stress the total myocardial protein synthesis is increased. In PO, along with an increase in myocardial mass there is a switch from the α- to the β-myosin heavy chain mRNA and the predominant expression of β-myosin heavy chain (27–34). In thyrotoxicosis similar changes occur, with the switch being from the β- to the α-myosin heavy chain mRNA (27–34). The regulation of the Ca^{2+}-ATPase mRNA is under a different type of control from that seen for myosin. In PO, where the calcium pumping rate is decreased (16–18), there is a paradoxical reduction in the SR Ca^{2+}-ATPase mRNA despite the increase in total mRNA and the specific myosin mRNA. Thus control—i.e., slowing calcium uptake—is accomplished by reducing the message for the calcium pump. This occurs while total protein synthesis is increasing. In thyrotoxic hypertrophy SR Ca^{2+}-ATPase mRNA is increased. This is consistent with the increase in the rate of calcium uptake (14, 15) by the SR calcium pump in these hearts. The result in both experiments indicates that there is only one type of SR Ca^{2+}-ATPase in heart muscle and that this is identical to that found in slow-twitch muscle. The regulation of the calcium pump under these conditions is accomplished by changing the SR calcium pump number in response to the quantity of the specific message. The decrease in pumping rate in PO and increase under thyrotoxic conditions is coordinated with the synthesis of slower β-myosin heavy chain and faster α-myosin heavy chain. Two entirely different mechanisms of genetic control are used to accomplish this end.

Altered SR Ca^{2+} uptake activity may account for several aspects of cardiac function in PO, thyrotoxicosis, and hypothyroidism, especially such contractile properties as the time to peak tension or relaxation rate (35–37). Gwathmey *et al.* (38) demonstrated abnormalities in Ca^{2+} transients in PO hypertrophy in ferrets. They showed that elevation of free cytosolic Ca^{2+} lasted longer in hypertrophied heart than in control, which is in accordance with the longer time to peak tension in PO heart. Alpert *et al.* (19, 20) have measured heat liberation from papillary muscle where muscle contraction is blocked *in vitro*. The heat liberated under this condition is

referred to as "tension-independent heat," and the major source is considered to be SR Ca^{2+}-ATPase. The rate of this heat production is depressed approximately 50% in 4-week PO hypertrophy and increased 25% in 1- to 2-week hyperthyroidism in rabbits (19–21). Recently, Whitmer *et al.* (39) demonstrated that the major defect in dilated cardiomyopathy may be a decrease in the volume or number of SR Ca^{2+} pumps. Our present study suggests that those changes in Ca^{2+} transport activity can also be explained by a decrease or an increase in the Ca^{2+} pump number in SR.

Phospholamban has been proposed to serve as a modulator of SR Ca^{2+} -ATPase (11); however, its definite physiological function remains to be established. The nonphosphorylated form is thought to inhibit SR Ca^{2+}-ATPase activity by raising the K_m for calcium. This inhibition is relieved when phospholamban is phosphorylated following β-adrenergic stimulation by a cAMP-dependent protein kinase (11). The phosphorylated form significantly increases SR Ca^{2+} uptake by augmenting the turnover rate of the Ca^{2+}-ATPase.

Our data suggest that the phospholamban mRNA expression does not show a coordinate regulation with that of Ca^{2+} ATPase. In PO hypertrophy Ca^{2+}-ATPase and phospholamban mRNA levels decrease in parallel. However, in thyrotoxicosis the phospholamban mRNA level decreases while the Ca^{2+}-ATPase mRNA increases. On the other hand, PTU-treated (hypothyroidic) hearts did not show significant changes in the phospholamban mRNA. The functional significance of these changes is not understood. They may play a role in the differential sensitivity to β-adrenergic action. In our opinion, the significance of these data will become more obvious once the phospholamban's role is clearly defined.

In conclusion, we propose that altered SR calcium uptake function in stressed hearts is caused mainly by an increase or a decrease in SR Ca^{2+}-ATPase gene transcription and/or changes in mRNA stability. Studies of the rates of protein synthesis and turnover of the SR Ca^{2+}-ATPase and phospholamban are required.

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