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Thyroidal Enhancement of Rat Myocardial Na,K-ATPase: Preferential Expression of $\alpha 2$ Activity and mRNA Abundance

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Summary. In hypothyroid rat myocardium, the low-ouabain-sensitivity Na,K-ATPase activity had a $K_I = 10^{-4}$ M and accounted for ~95% of the enzyme activity, while the high-ouabain-sensitivity activity contributed ~5% to the total activity, with a $K_I = 3 \times 10^{-7}$ M. $mRNA_{\alpha 1}$ was 7.2- and 5.5-fold more abundant than $mRNA_{\alpha 2}$ and $mRNA_{\beta}$, respectively, in hypothyroid ventricles while $mRNA_{\alpha 3}$ was undetectable. Administration of T_3 increased total Na,K-ATPase activity 1.6-fold; the low-ouabain-sensitivity activity increased 1.5-fold while high-ouabain-sensitivity activity was stimulated 3.2-fold. T_3 increased the number of high-affinity ouabain-binding sites 2.9-fold with no change in K_d (~2×10^{-7} M). The abundances of $mRNA_{\alpha 1}$, $mRNA_{\alpha 2}$, and $mRNA_{\beta}$ (per unit RNA) following T_3 treatment increased 3.6-, 10.6-, and 12.7-fold, respectively. The larger increments in subunit mRNA abundances than in Na,K-ATPase activity suggests the involvement of translational and/or post-translational regulatory steps in Na,K-ATPase biogenesis in response to T_3. It is concluded that T_3 enhances myocardial Na,K-ATPase subunit mRNA abundances and Na,K-ATPase activity, and that the expression of the high- and low-ouabain-sensitivity activities are probably a reflection of the abundances of the $\alpha 2$ and $\alpha 1$ isoforms, respectively. The physiological role played by the β subunit remains uncertain.

Key Words thyroid hormone · myocardium · Na,K-ATPase · Na,K-ATPase isoforms · Na,K-ATPase-mRNAs

Introduction

Na^+,K^+-activated adenosine triphosphatase (Na,K-ATPase; also identified as the Na,K-Pump) is a ubiquitous plasma-membrane enzyme that plays a fundamental role in animal cells. By means of its continuous function transmembrane gradients of Na^+ and K^+, resting membrane potential, and cell volume are maintained [19]. In addition, stimulation of Na,K-ATPase and active Na,K transport have been implicated as an important pathway in thyroid hormone-induced thermogenesis [16].

Mammalian Na,K-ATPase is a noncovalent oligomer of equimolar amounts of α and β subunits that make up the mature functional enzyme [19]. Three isoforms of the catalytic α subunit have been identified, designated α, α^+, and α_{III} in earlier reports and now designated as $\alpha 1$, $\alpha 2$, and $\alpha 3$, respectively [40, 44, 51]. Tissue-specific expression of α-subunit isoforms has been associated with the presence of various classes of Na,K-ATPase manifesting differing sensitivities to inhibition by cardiac glycosides [5, 14, 20, 44, 46, 49]. Rat liver and kidney cortex nearly exclusively express the $mRNA_{\alpha 1}$ isoform, whereas cardiac and skeletal muscle express both $\alpha 1$- and $\alpha 2$-mRNA isoforms [6, 15, 26, 40, 51]. In the adult rat brain, three Na,K-ATPase α-subunit-mRNAs and their respective polypeptides are expressed [38–40, 48, 51]. The expression of the various α-mRNA isoforms and β-mRNA has been recently described in several tissues of the rat during development [32]. The $\alpha 1$- and $\alpha 3$-mRNA isoforms are expressed in neonatal myocardium whereas $\alpha 1$- and $\alpha 2$-mRNAs, but not the $\alpha 3$-mRNA, are present in the adult atrial and ventricular muscle [32, 51]; $\alpha 2$-mRNA comprises a minor component of the α-mRNA isoforms in the normal adult rat heart [32, 51]. Expression of the $\alpha 2$-mRNA and Na,K-ATPase activity exhibiting a high sensitivity to ouabain has also been reported in a myocyte system undergoing differentiation in vitro [31]. Recently, a candidate isoform of the β subunit ($\beta 2$) of Na,K-ATPase has been cloned and sequenced; the corresponding $mRNA_{\beta 2}$ is expressed in rat myocardium [27], but the existence of $\beta 2$ peptide as a component of Na,K-ATPase awaits confirmation.

Thyroid hormone (T_3) stimulates the Na,K-ATPase activity of a variety of mammalian target

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tissues [2, 8, 16, 22, 33]. The enhancement of Na,K-ATPase activity by T_3 is associated with increased numbers of specific ouabain-binding of Na^+-dependent phosphorylation sites in plasma membranes isolated from target tissues [21, 22]. The T_3-induced increase in Na,K-ATPase abundance is mediated by increased synthesis of both subunits of the enzyme in rat renal cortex [23, 24], and is associated with increased abundances of $mRNA_{\alpha 1}$ and $mRNA_{\beta}$ [7, 28]. Recent evidence indicates that the T_3-induced increase in the abundances of $mRNA_{\alpha 1}$ and $mRNA_{\beta}$ in rat renal cortex is in part mediated by enhanced transcription of the corresponding genes [11].

Thyroid hormone induction of Na,K-ATPase in target tissues expressing more than one $mRNA_{\alpha}$ and enzyme isoform, such as the myocardium, raises the possibility of selective or preferential regulation of isoform-specific gene expression. We now report on the effect of thyroid status on myocardial Na,K-ATPase $\alpha 1$, $\alpha 2$, and $\alpha 3$ isoform expression as evaluated by ouabain-sensitivity of the enzyme, quantitation of the high-ouabain-affinity binding sites, and measurement of the abundances of $mRNA_{\alpha}$ isoforms and $mRNA_{\beta}$ by Northern blot analysis. The results indicate a preferential induction of the high-affinity ouabain-binding form of Na,K-ATPase and of the $\alpha 2$- and β-mRNAs by T_3 in the myocardium. The increase in $mRNA_{\alpha 2}$ is sufficient to account for the increase in high-affinity ouabain-binding form and is consistent with the interpretation of preferential induction of the $\alpha 2$ isoform of the enzyme. A preliminary report of some of those findings has been presented earlier [17].

Materials and Methods

Materials

Analytical grade 3,3′,5-triiodothyronine (T_3), Tris, ATP, deoxycholate, bovine serum albumin, ouabain and other standard compounds were obtained from Sigma Chemical. ^{3}H-ouabain (24.1 Ci/mmol) was purchased from New England Nuclear, and ^{32}P-α-TTP (3000 Ci/mmol) from Amersham. Cellulose acetate/nitrate filters (0.65 μm) was obtained from Millipore and nitrocellulose paper (BA-85) from Schleicher and Schuell. "Nick-translation" kits were purchased from Bethesda Research Laboratories.

Animals and Preparation of Membrane Fractions

Hypothyroidism was induced in male Sprague-Dawley rats (200–225 g) by maintenance on low-iodide diet plus 0.5% sodium perchlorate in the drinking water for four weeks [43]. Rats were injected subcutaneously once daily with either T_3 (100 μg/100 g body weight) or diluent alone and were sacrificed at various intervals thereafter. This dosage schedule ensures near-saturation of nuclear T_3-receptors during the entire 24-hr interval before the next injection of the hormone [30].

Crude plasma membrane fractions were prepared by methods described previously with minor modifications [33]. Hearts were excised and ventricles isolated by sharp dissection and frozen immediately in liquid nitrogen. After 24 hr, individual ventricles were minced and disrupted using a Polytron homogenizer (Brinkmann Instruments) two times for 20 sec each on the maximal setting in a buffer containing 250 mM mannitol, 30 mM histidine, 5 mM EDTA, 5 mM Tris, 0.1% sodium deoxycholate (pH 6.8) in a volume of 10 ml/g of tissue at 4°C. Samples were centrifuged at 8,000 × *g* for 10 min. The supernatants were frozen in liquid nitrogen and thawed to room temperature, and centrifuged at 100,000 × *g* for 30 min at 25°C. The resulting pellets were resuspended (~3.5 mg protein/ml) in a buffer (pH 7.6) containing 250 mM sucrose, 50 mM Tris, 1.25 mM [ethylene-*bis*(oxyethylenenitrilo)]tetraacetic acid (EGTA) (STE buffer) and dispersed with a Potter-Elvehjem homogenizer. These crude membrane fractions were stored at −80°C for up to six weeks prior to assay.

Ouabain Sensitivity of Na,K-ATPase

Membrane fractions were thawed and diluted in the STE buffer. Triplicate 100-μl samples of membrane suspensions containing 50 μg protein from hypothyroid or 25 μg protein from hyperthyroid rats were preincubated for 2 hr at 37°C in 0.9 ml of buffer containing Na^+ and K^+ (*see below*) and varying concentrations of ouabain. The ATPase reaction was started by the addition of 100 μl of ATP to the 0.9-ml suspensions to yield final concentrations of (all in mM) 5 ATP, 105 Na^+, 6 K^+, 5 Mg^{2+}, 144 Cl^-, 5 azide, 25 sucrose, 0.125 EGTA, and 50 Tris (pH 7.4), and the incubation continued for 60 min at 37°C. Reactions were terminated by the addition of trichloro-acetic acid (5%, final concentration) and phosphate was assayed colorimetrically [3]. In preliminary experiments, the ATPase reaction was linear with respect to time and concentration of protein; the reaction conditions were chosen to ensure less than 10% hydrolysis of ATP during the assay. The 2-hr preincubation period was based upon preliminary studies demonstrating no further inhibition of ATPase activity by 3×10^{-7} M ouabain after 1.5 hr of incubation. Ouabain-sensitive ATPase activity accounted for ~90% of the ATPase activity in membrane fractions isolated from either the hypothyroid or hyperthyroid myocardium. Total, Na,K-ATPase activity was calculated as the difference in ATPase activity in the absence and presence of 10^{-2} M ouabain. The ouabain-sensitive ATPase activity at each ouabain concentration was calculated as the fraction of that activity to the total Na,K-ATPase activity. Inhibition of Na,K-ATPase activity was analyzed using one-site and two-site models by a derivative-free nonlinear regression program, as adapted by Pressley and Edelman [34].

^{3}H-Ouabain Binding to Myocardial Membrane Fractions

Five concentrations of ouabain were used: 0.50, 1.0, 3.0, 6.0 and 12 (each $\times 10^{-7}$) M prepared by diluting ^{3}H-ouabain with nonradioactive ouabain to 7.0 Ci/mmol. The assays were carried out by a minor modification of the method of Wallick et al. [50]. The medium contained (final concentrations, all in mM): 0.8 EDTA, 40 Tris, 4 MgCl, 4 sodium phosphate, 100 sucrose and 0.7 EGTA (pH 7.4). Duplicate samples of diluted membranes (50 μg protein) were incubated at 37°C for 2 hr with the above concentra-

Table 1. Effect of T_3 on protein, DNA and RNA content of rat ventricular myocardium

Thyroid status	mg protein/g wet weight	mg protein/mg DNA	RNA/DNA[a]
Hypothyroid	195 ± 6	117 ± 5	0.95 ± 0.03
Hypo + T_3 (24 hr)	178 ± 8	117 ± 4	1.21 ± 0.10[b]
Hypo + T_3 (72 hr)	205 ± 5	137 ± 9[b]	1.50 ± 0.07[b]

Hypothyroid rats (Hypo) were injected daily with either diluent or T_3 (100 μg/100 g body weight) and sacrificed at 24 or 72 hr of treatment; $n = 10$ for the diluent-injected rats (Hypo) and $n = 5$ for each of the 24- and 72-hr groups. Results are expressed as means ± SEM.

[a] The RNA/DNA ratios have been reported previously [7].

[b] Denotes $P < 0.05$ as compared to diluent-injected controls.

tions of ^{3}H-ouabain in the absence and presence of 3×10^{-3} M nonradioactive ouabain. Unbound ouabain was removed by rapid filtration, under vacuum, through 0.65-μm Millipore filters. The filters were washed four successive times with 6 ml of ice-cold water, in less than 15 sec, and counted in a liquid scintillation spectrometer (Packard Instrument). Specific ^{3}H-ouabain binding was calculated as the difference in radioactivity in the absence and presence of 3×10^{-3} M nonradioactive ouabain. Nonspecific binding of radioactive ouabain to membranes was linearly proportional to the concentration of the radiolabel and accounted for <30% of the total radioactivity at all concentrations of ouabain employed. The moles of ouabain bound by the low-affinity site ($K_d = 10^{-4}$ M) was calculated for each ouabain concentration employed in the binding assay and subtracted from the observed total binding at the given ouabain concentration to derive the moles bound to the high-affinity site. This minor correction, which accounted for <4% of the total binding in both hypo- and hyperthyroid membranes at all ouabain concentrations employed, is based on several reasonable assumptions including noninteraction between the sites, one ouabain-binding site per molecule, and equivalence between the K_i and K_d of the low-affinity site. The resulting binding data were analyzed by the method of Scatchard [37].

Determination of Protein and DNA

Protein concentrations were determined by the method of Lowry et al. [25], using bovine serum albumin as a standard. DNA was assayed as previously described [7].

Isolation of RNA; Quantitation of $mRNA_\alpha$ Isoforms and $mRNA_\beta$ Abundances

Freshly isolated ventricular tissue was frozen in liquid nitrogen and total RNA was isolated from individual ventricles by homogenization in guanidine thiocyanate and ultracentrifugation, as described previously [7]. The relative abundances of the $mRNA_\alpha$ isoforms and $mRNA_\beta$ were determined by Northern blot analysis [7, 47]. Aliquots of total RNA (20 μg) from hypothyroid and hyperthyroid rats were electrophoresed in adjacent lanes in replicate agarose gels, as previously described [7]. The resulting blots were hybridized with $\sim 5 \times 10^7$ cpm of full-length rat $cDNA_{\alpha 1}$, $cDNA_{\alpha 2}$, $cDNA_{\alpha 3}$, and $cDNB_\beta$ [40, 41, 52], nick-translated to near-equivalent specific activity of $\sim 5 \times 10^8$ cpm/μg DNA. Blots were washed under high stringency and quantified as described previously [7]. In cases where blots were probed for a second time, filters were washed ×2 for 15 min each in a solution containing (in mM): 15 NaCl, 1 NaH_2PO_4, and 0.1 EDTA at 95°C, and exposed to film before use.

Statistical Analysis

The data are presented as the mean ± SEM. P values were calculated by Student's two-tailed unpaired t test [42]. In time-course experiments where more than one set of measurement was made, statistical significance was determined by analysis of variance [42].

Results

In previous studies using the hypothyroid rat model, we found that T_3 increased total RNA (expressed per gram of tissue) and RNA/DNA ratios in liver, kidney cortex, and ventricular myocardium [7, 11]. In the case of the ventricle, T_3 increased the RNA/g wet weight and decreased the DNA/g wet weight such that the RNA/DNA ratios increased by 1.3- and 1.6-fold at 24 and 72 hr, respectively [7]. To establish a common reference base for comparisons of Na,K-ATPase activity, we measured the protein and DNA content of ventricular myocardium (Table 1). The protein content (expressed per g wet weight) showed no significant change at either 24 or 72 hr after T_3. There was, however, a 1.5-fold increase in the mass of the isolated ventricle 72 hr after treatment of hypothyroid rats with the hormone (from 0.40 ± 0.03 to 0.60 ± 0.04 g wet weight; $P < 0.05$, $n = 6$). The protein to DNA ratio was unchanged at 24 hr after T_3 injection, and increased 1.2-fold at 72 hr. The previously reported increases in myocardial RNA/DNA ratios following 24 and 72 hr of T_3 treatment [7] are also included in Table 1 for comparison.

Administration of maximal doses of T_3 for 72 hr elicited a 1.6-fold increase in total Na,K-ATPase activity in ventricular membrane fractions from 9.3

Table 2. Effect of T_3 on high- and low-ouabain-sensitivity Na,K-ATPase activity

Thyroid status	Total activity	High-sensitivity		Low-sensitivity	
		V_{max}	$K_I \times 10^{-7}$	V_{max}	$K_I \times 10^{-4}$
Hypo	9.3 ± 0.4	0.47 ± 0.05	3 ± 0.7	8.8 ± 0.4	1 ± 0.1
Hypo + T_3	15.0 ± 1.1[a]	1.50 ± 0.20[a]	3 ± 0.5	13.5 ± 1.0[a]	0.9 ± 0.1

Hypothyroid rats were treated daily with diluent or T_3 for 72 hr. Na,K-ATPase activities were determined as described in Materials and Methods, and are expressed as μmol P_i/hr/mg protein (mean ± SEM; $n = 6$).

The K_I values were derived by nonlinear curve fitting of the data shown in Fig. 1.

[a] Denotes $P < 0.05$ as compared to diluent-injected hypothyroid (Hypo) controls.

to 15 activity units[1] (Table 2). Expressed per unit DNA, the increment in Na,K-ATPase activity is 1.9-fold, assuming that the recovery of microsomal membrane protein is independent of thyroid status. Although the recovery of membrane fractions were not quantitated in the present study, the above assumption is supported by the finding of equivalent T_3-induced augmentation of Na,K-ATPase activity in crude homogenates and isolated membranes of rat heart [33].

The effect of T_3 on expression of the enzyme activity exhibiting "high" and "low" K_I values towards cardiac glycosides was then assessed. The sensitivity of Na,K-ATPase to inhibition by ouabain in hypo- and hyperthyroid membrane fractions was analyzed by curve fitting to a two-site inhibition model, as previously described [34]; the results are shown in Fig. 1. In hypothyroid ventricles the two-site model gave a minor component (5 ± 2%) exhibiting a ouabain K_I of 3×10^{-7} M and a major component with a K_I of 10^{-4} M. Administration of T_3 increased the proportion of the high-sensitivity (low K_I) form of the enzyme to 10 ± 2% without any change in either of the K_I's. Also shown in Fig. 1 are the predicted inhibition profiles for a single-site inhibition model with a K_I of 10^{-4} M (dotted lines). The derived numerical values of the Na,K-ATPase activities associated with each site and their respective K_I's from hypo- and hyperthyroid membranes are summarized in Table 2. T_3 elicited a 3.2-fold increase in the high-ouabain-sensitivity Na,K-ATPase activity (i.e., from 0.47 to 1.50 units) while the low-ouabain-sensitivity enzyme increased by 1.5-fold (i.e., from 8.8 to 13.5 units) and accounted for most of the absolute increment in Na,K-ATPase activity. Although there was a 1.2-fold increase in the tissue protein/DNA ratio after 72 hr of T_3 treatment, this change does not affect the relative activities of the high- and low-sensitivity forms of the enzyme in the hypo- and hyperthyroid ventricles.

[1] Activity unit = μmol P_i/hr/mg protein.

To ascertain whether stimulation of the high-sensitivity Na,K-ATPase is due to an increase in the abundance of enzyme sites, ouabain-binding assays were performed on hypo- and hyperthyroid myocardial membrane fractions (Table 3). To count only the high-ouabain-affinity sites, [^{3}H]ouabain concentrations were used (up to 1.2×10^{-6} M) that would occupy only a maximum of 1% of the low-affinity sites. In the ouabain concentration range of 0.5 to 12×10^{-7} M, linear Scatchard plots (single-site dominance) were obtained. The 2.9-fold increment in maximal high-affinity ouabain-binding sites was similar to the 3.2-fold increase in high-sensitivity Na,K-ATPase activity (Table 2). Based on the Na,K-ATPase activity and ouabain-binding data, the catalytic turnover number of the high-ouabain-sensitivity site was 4100 and 4500/min for hypo- and hyperthyroid myocardium, respectively.

To assess the relative abundance of Na,K-ATPase α-subunit mRNA isoforms and β-subunit mRNA, full-length rat α-subunit isoform-specific cDNAs and $cDNA_\beta$ were used in Northern blot analysis [40, 41, 52]. In hypothyroid ventricle $mRNA_{\alpha 1}$ was the predominant α-isoform expressed (migrating at ~27 S); much smaller amounts of α2-mRNA with its characteristic bands migrating at ~35 and ~26 S were detected (Fig. 2). No $mRNA_{\alpha 3}$ was detectable in the adult rat myocardium, in accordance with previous reports [32, 51]. $mRNA_\beta$ was expressed in the hypothyroid myocardium in lesser quantities than $mRNA_{\alpha 1}$. Because of the lower abundances of $mRNA_{\alpha 2}$ and $mRNA_\beta$, longer exposure times were necessary to visualize the corresponding bands. The relative abundances of α-subunit-mRNA isoforms and $mRNA_\beta$ in the hypothyroid ventricle are summarized in Table 4. $mRNA_{\alpha 1}$ abundance was ~sevenfold greater than that of $mRNA_{\alpha 2}$ with the latter comprising 12% of the $mRNA_\alpha$ pool. The abundance of $mRNA_\beta$ was ~16% of the combined abundances of the α-mRNA isoforms.

Administration of T_3 for 72 hr increased the

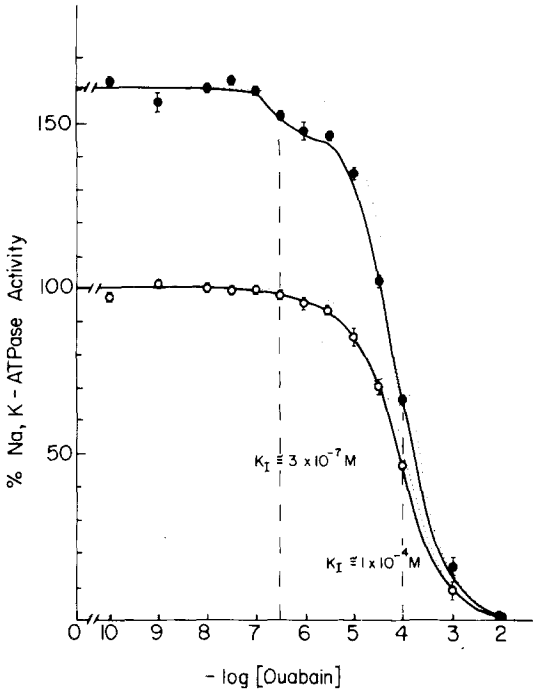

Fig. 1. Effect of thyroid status on the sensitivity of ventricular Na,K-ATPase activity to ouabain. Ventricular membrane fractions isolated from hypothyroid (○—○) and 72-hr T_3-treated (●—●) rats were preincubated in triplicate at various concentrations of ouabain for 2 hr and then assayed for Na,K-ATPase activity in the presence of these concentrations of ouabain. ATPase activity, at each ouabain concentration, was calculated as the fraction of total Na,K-ATPase activity. The activity in the absence and presence of 10^{-10} M ouabain were not different from each other and were averaged and normalized to 1.00 (the reference Na,K-ATPase activity was that of the hypothyroid myocardium). Total Na,K-ATPase activity of hypothyroid myocardium was 9.3 ± 0.4 μmol P_i/hr/mg protein. T_3 administration increased Na,K-ATPase activity by 61% (i.e., to 15.1 ± 1.1 μmol P_i/hr/mg protein; normalized to 161% in the figure). The dotted lines represent the activity profile for a single-site inhibition model with a K_I of 10^{-4} M. The data are expressed as mean ± SEM (n = 6)

abundances of $mRNA_{\alpha 1}$, $mRNA_{\alpha 2}$, and $mRNA_{\beta}$ to varying extents, while $mRNA_{\alpha 3}$ remained undetectable (Fig. 2). Of note was that the increments in $mRNA_{\alpha 2}$ and $mRNA_{\beta}$ both exceeded the increase in $mRNA_{\alpha 1}$ abundance. The abundances of $mRNA_{\alpha 1}$, $mRNA_{\alpha 2}$, and $mRNA_{\beta}$ (expressed per unit RNA) measured from replicate Northern blots increased significantly by 3.6-, 10.6-, and 12.7-fold, respectively, following T_3 treatment (Table 5); the respective T_3-induced values are 1.6-fold greater if the mRNA abundances are corrected for the increase in tissue RNA/DNA ratio after 72 hr of T_3 treatment [7] (*see* Table 1). In the hyperthyroid ventricle $mRNA_{\beta}$ (expressed per unit RNA or per unit DNA) increased to ~45% of the combined abundances of α-subunit-mRNAs (as compared to ~16% in the hy-

Table 3. Effect of thyroid status on the number and affinity of high-affinity ^{3}H-ouabain-binding sites in rat ventricular membranes

Thyroid status	N_{max} (pmol/mg protein)	$K_d \times 10^{-7}$ [M]
Hypo	1.9 ± 0.2	1.9 ± 0.3
Hypo + T_3	5.5 ± 0.3[a]	1.6 ± 0.3

Ventricular membranes isolated from hypothyroid and 72-hr T_3-treated rats were incubated for 2 hr in duplicate in buffer containing various concentrations of [^{3}H]-ouabain (0.5 to 12 × 10^{-7} M). Specific ouabain-binding was determined as the difference in the radioactivity bound in the absence and presence of 10^{-3} M nonradioactive ouabain. N_{max} and K_d represent maximal binding capacity and dissociation constant, respectively, and are given as mean ± SEM (n = 5).
The number of sites (N_{max}) was corrected for binding of ^{3}H-ouabain to the low-affinity-sites (*see* Materials and Methods).
[a] Denotes $P < 0.05$ as compared to diluent-injected hypothyroid (Hypo) controls.

Table 4. Relative abundance of $mRNA_{\alpha}$ isoforms and $mRNA_{\beta}$ in hypothyroid rat ventricle

Na,K-ATPase subunit mRNA	Relative abundance[a]
$mRNA_{\alpha 1}$	7.2 ± 1.0
$mRNA_{\alpha 2}$	1.0 ± 0.1
$mRNA_{\alpha 3}$	undetectable
$mRNA_{\beta}$	1.3 ± 0.2

[a] The relative abundance of the Na,K-ATPase subunit mRNAs was normalized to that of $mRNA_{\alpha 2}$. Twenty-five μg of total RNA isolated from hypothyroid ventricles was fractionated on agarose gels in duplicate lanes and the resulting blots hybridized with subunit-specific cDNAs, as described under Materials and Methods. Values represent ± SEM; n = 5.

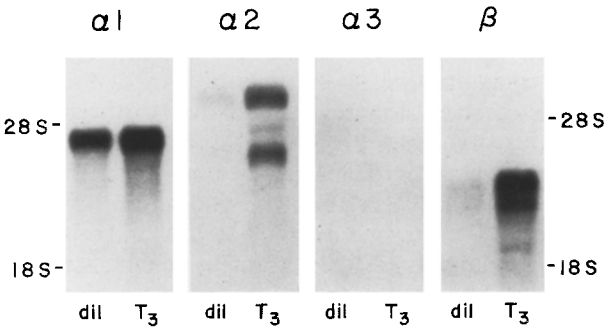

Fig. 2. Na,K-ATPase $mRNA_{\alpha}$ isoforms and $mRNA_{\beta}$ in ventricular myocardium of hypo- and hyperthyroid rats. RNA was isolated from heart ventricles of rats treated either with diluent (*dil*) or T_3 for 72 hr. Equal amounts of total RNA (20 μg) were loaded in each lane, and the resulting blots hybridized with full-length $cDNA_{\alpha 1-3}$ and $cDNA_{\beta}$ nick-translated to equivalent specific activity (~5 × 10^8 cpm/μg DNA). The positions of 28- and 18-S ribosomal RNA are shown

Table 5. Effect of thyroid status on the abundance of myocardial Na,K-ATPase $mRNA_\alpha$ isoforms and $mRNA_\beta$

Thyroid status	Na,K-ATPase subunit mRNA content			
	$mRNA_{\alpha 1}$	$mRNA_{\alpha 2}$	$mRNA_{\alpha 3}$	$mRNA_\beta$
Hypo	1.0 ± 0.1	1.0 ± 0.2	Undetectable	1.0 ± 0.1
Hypo + T_3	3.6 ± 0.4[a]	10.6 ± 2.4[a]	Undetectable	12.7 ± 1.7[a]

RNA was isolated from hypothyroid rats injected daily with either diluent (Hypo) or T_3 for 72 hr. Total RNA was fractionated in agarose gels, hybridized with full-length $cDNA_{\alpha 1}$ and $cDNA_\beta$ and quantitated by densitometry. The blots were washed, as described under Materials and Methods, and then hybridized with full-length $cDNA_{\alpha 2}$ and $cDNA_{\alpha 3}$. The effect of T_3 on each mRNA species is expressed as the ratio to the mean of the hypothyroid values normalized to 1.0. Values are given as mean ± SEM; n = 12.

[a] Denotes $P < 0.05$ as compared to diluent-injected hypothyroid controls.

pothyroid heart), and $mRNA_{\alpha 2}$ comprised 29% of the $mRNA_\alpha$ pool.

The time course of the increases in myocardial Na,K-ATPase mRNAs following T_3 injection are shown in Fig. 3. Although both $mRNA_{\alpha 2}$ and $mRNA_\beta$ abundances were augmented 3 hr after T_3 injection, only the increment in $mRNA_{\alpha 2}$ abundance (+74%) was statistically significant. The abundances of all three mRNA species were significantly increased both at 12 and 24 hr after T_3 treatment.

Because more than one mRNA species (differing in the 5′-upstream and 3′-downstream untranslated regions) encodes each of the $\alpha 2$ and β subunits of the Na,K-pump in the myocardium [40, 41, 51, 52], it was of interest to examine whether T_3 increases the abundance of the multiple forms of these mRNAs to the same extent. The mRNA encoding the $\alpha 2$ isoform of Na,K-ATPase migrates in two bands, as described previously [40, 51], with the lower ~26-S mRNA band comprising 41 ± 2% of the total $mRNA_{\alpha 2}$ pool (n = 4) (Fig. 4). Twelve hr after injection of the hormone, the abundance of $mRNA_{\alpha 2}$ was increased 2.1-fold without significantly changing the relative abundance of the two mRNAs species. Figure 5 shows the Northern blot of myocardial RNA probed with $cDNA_\beta$. $mRNA_\beta$ in hypothyroid myocardium exhibits three bands migrating at ~22, ~20, and ~17 S, that account for 31 ± 3%, 38 ± 4%, and 32 ± 2% of the total $mRNA_\beta$ pool, respectively. Also seen in the figure is some cross-hybridization with a ~28 S band, presumably that of ribosomal RNA (this additional band is observed in blots washed at lower stringency [35]). T_3 treatment for 12 hr produced a 5.9-fold increase in the abundance of total $mRNA_\beta$ pool. The increments in the abundances of the three bands were not proportionate: the 22, 20, and 17 S bands now accounted for 47 ± 2%, 26 ± 2%, and 27 ± 1% of the total $mRNA_\beta$ pool, respectively (n = 4); the changes in the proportions of the ~22 and ~20 S bands were statistically significant ($P < 0.5$). Although the different $mRNA_\beta$ species encode the same β-polypeptide [52], the physiological significance of differential regulation of each species by T_3 remains unclear.

Discussion

Recent evidence indicates that expression of Na,K-ATPase $mRNA_{\alpha 1}$ and $mRNA_{\alpha 2}$ isoforms is associated, respectively, with low- and high-sensitivity of the enzyme to ouabain [26, 44, 45, 51]. Tissues of the rat that nearly exclusively express the $mRNA_{\alpha 1}$ isoform of the enzyme (e.g., renal cortex, liver and liver-derived cells in culture) manifest a single class of low-sensitivity sites with K_I's of 2 × 10^{-5} to 10^{-4} M, while tissues expressing both $mRNA_{\alpha 1}$ and $mRNA_{\alpha 2}$ isoforms exhibit biphasic inhibition of Na,K-ATPase activity; the K_I of the high-ouabain-sensitivity site is ~2 × 10^{-7} M [15, 18, 26, 31, 44, 45]. Transfection of rat $cDNA_{\alpha 1}$ into ouabain-sensitive primate cells in culture conferred the ouabain-resistance trait to the recipients [20], consistent with expression of the low-ouabain-sensitivity of the $\alpha 1$ isoform of the enzyme. And, gene transfer and expression of an avian $cDNA_\alpha$ (associated with a high affinity towards ouabain) in ouabain-insensitive mouse L cells elicited ouabain-sensitive Na,K transport in the transfectants [46]. Na,K-ATPases containing $\alpha 1$ or both $\alpha 1$ and $\alpha 2$ isoforms, exhibit correlative differences in sensitivity to ouabain [14, 15, 26, 32, 44, 46, 48, 51]. Our findings indicate that T_3 induction of $mRNA_{\alpha 2}$ (>10-fold) is more than sufficient to account for the ~threefold increment in the high-ouabain-sensitivity sites. Alpha isoform composition alone, however, is not an absolute determinant of the K_I for

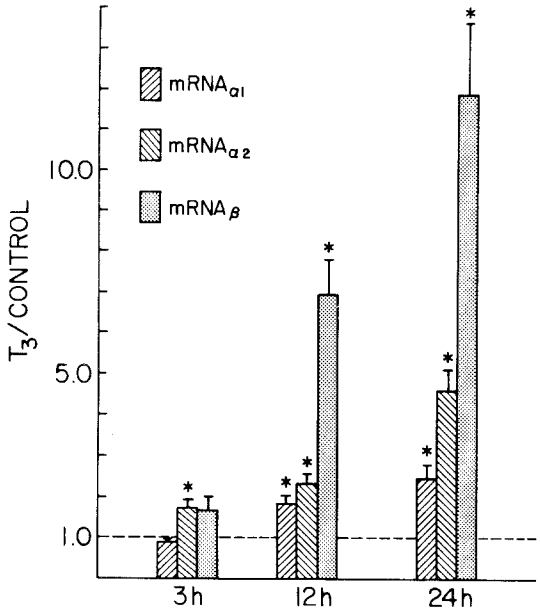

Fig. 3. Time course of the effect of T_3 on myocardial mRNA$_{\alpha 1}$, mRNA$_{\alpha 2}$ and mRNA$_{\beta}$ abundances. Three, 12 and 24 hr following treatment with either T_3 or diluent, rats were sacrificed and RNA isolated from ventricular myocardium. The abundances of the corresponding mRNAs were measured by electrophoretic fractionation and Northern blotting of total RNA samples and probed with the respective cDNAs. The abundances were determined by densitometry, corrected for time of exposure to the film [7], and are expressed as the ratio of the value for each sample from T_3-treated rats to the mean abundance of the samples obtained from diluent-treated rats at the indicated time points. $n = 5$ for each of the groups at 3 hr; $n = 4$ and 6 for diluent- and T_3-treated groups, respectively, at 12 hr; and $n = 9$ for the 24-hr groups (means ± SEM). The abundance of mRNA$_{\alpha 2}$ was significantly increased at 3 hr, while the abundances of all three subunit mRNAs were increased at 12 and 24 hr. Statistical significance is denoted by the asterisks (*)

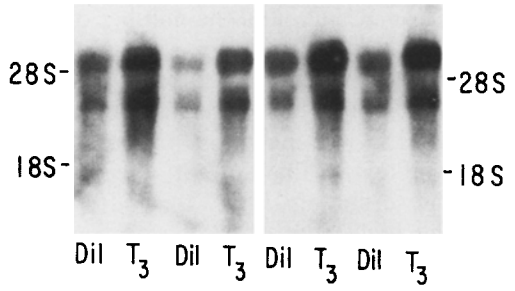

Fig. 4. Expression of myocardial mRNA$_{\alpha 2}$ in diluent- and T_3-treated hypothyroid rats 12 hr following T_3 injection. RNA was isolated from ventricles 12 hr after diluent or T_3 administration. Twenty μg of total RNA was loaded per lane, and the resulting blot hybridized with full-length cDNA$_{\alpha 2}$. *Dil* and T_3 denote diluent- and T_3-treated rats. The positions of the 28 and 18 S ribosomal RNA are shown. The experiment was performed two times in duplicate as shown in the left and right panels

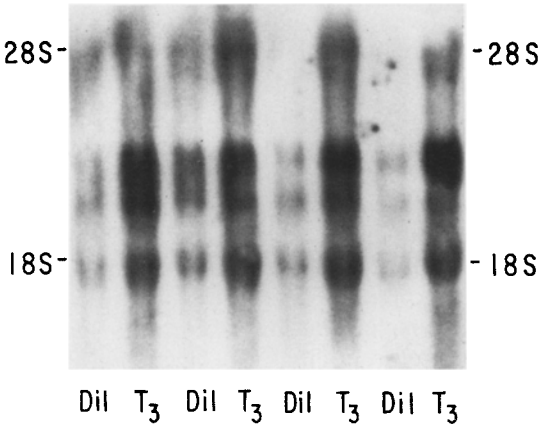

Fig. 5. Expression of myocardial mRNA$_{\beta}$ in diluent- and T_3-treated hypothyroid rats 12 hr following T_3 injection. Conditions are those described in the legend to Fig. 4, except that electrophoresis was continued for a longer period to obtain further separation of the RNA bands, and the resulting blot was probed with cDNA$_{\beta}$. The positions of 28 and 18 S ribosomal bands are shown. Some cross-hybridization is seen with an RNA band migrating at 28 S (presumably that of ribosomal RNA). *Dil* and T_3 denote diluent- and T_3-treated rats

ouabain. For example, the rat renal cortex has a K_I of $\sim 2 \times 10^{-5}$ M, as compared to a K_I of $\sim 10^{-4}$ M in rat liver, although both contain the $\alpha 1$ isoform almost exclusively [15, 19, 51].

The expression of both mRNA$_{\alpha 1}$ and mRNA$_{\alpha 2}$, and the corresponding polypeptides of Na,K-ATPase, in normal adult rat ventricle [5, 6, 51] raised the possibility of differential regulation of these isoforms by T_3. In analyzing the response to T_3, we elected to examine the full transition from hypo- to hyperthyroidism. T_3 treatment for 72 hr increased total Na,K-ATPase activity of the ventricular myocardium 1.6-fold, in accordance with results reported previously [7, 33]. The low-ouabain-sensitivity form increased 1.5-fold, while the high-ouabain-sensitivity enzyme increased 3.2-fold (Fig. 1 and Table 2). Preferential stimulation of the high-sensitivity activity, presumably that of the $\alpha 2$ isoform, correlated with an increased number of high-affinity ouabain-binding sites (2.9-fold), yielding no significant change in the calculated catalytic turnover of ~4000–4500/min for this site (Table 3). These turnover numbers are similar to the value of ~4500/min reported for the high-ouabain-sensitivity site in diaphragms of hypothyroid and T_3-treated rats [13]. In a recent study, Haber and Loeb reported a selective induction of the high-ouabain-affinity isoform of Na,K-ATPase in rat diaphragm following T_3 treatment [13]. They noted that while the high-ouabain-affinity enzyme activity (presumably $\alpha 2$) accounted for ~$\frac{1}{3}$ of the total activity in hypo-

thyroid muscle, the activity and the number of ouabain-binding sites attributable to this site was selectively increased some ~2.5-fold in the hyperthyroid state. In the developing rat neonatal brain, however, hypothyroidisim reduced the expression of both the high- and low-ouabain-affinity forms of the enzyme [38]. These findings imply that differential regulation of the high-affinity form by T_3 may be tissue specific.

The abundance of $mRNA_{\alpha 1}$ (expressed per unit RNA) in hypothyroid rats treated with T_3 for 72 hr increased 3.6-fold while that of $mRNA_{\alpha 2}$ increased by greater than 10-fold (Table 5). We have also observed a preferential induction of $\alpha 2$ in rat skeletal muscle Na,K-ATPase mRNAs [17]. Of interest is the finding that in rat tissues expressing nearly exclusively a single α isoform (such as liver and renal cortex expressing $\alpha 1$), T_3 did not appear to induce the expression of other $mRNA_{\alpha}$ isoforms (*unpublished observations*). Moreover, the Na,K-ATPase $mRNA_{\alpha 3}$ isoform which is not present in the adult rat ventricle remained undetectable following T_3 treatment. Thus in contrast to the effects of T_3 on the expression of the myosin heavy chains [36], T_3 did not act as an absolute molecular "switch" in the expression of the Na,K-ATPase α-subunit isoforms. In the heart, liver, kidney, and skeletal muscle, our results indicate that T_3 augmented only the constitutively expressed α isoforms, and in cardiac and skeletal muscle, the $\alpha 2$ isoform is induced markedly and preferentially by T_3 (Table 5) [11, 17].

In this study no attempt was made to separate the various cell types present in the myocardium prior to analysis. Both the $\alpha 1$ and $\alpha 2$ Na,K-ATPase isoforms are present in preparations of adult rat cardiac myocytes isolated by tissue disruption and differential centrifugation [5]. It is thus probable that the preferential stimulation of the $\alpha 2$-isoform described herein reflects the enhanced expression of this enzyme isoform in cardiac myocytes. Recent studies have implicated the role of high-ouabain-sensitivity Na,K-ATPase sites in the positive isotropic effect of digitalis glycosides in rat myocardium [1, 12, 29], while inhibition of the low-ouabain-sensitivity site correlates with toxic effects of these agents [4, 10]. If we postulate that the number of uninhibited high-sensitivity ouabain-binding sites is of importance in the inotropic response, then the preferential induction of the $\alpha 2$ Na,K-ATPase isoform by T_3 would necessitate a higher concentration of ouabain to leave free the same number of uninhibited sites, and could explain in part the increased sensitivity of hypothyroid and the decreased sensitivity of hyperthyroid myocardium to the therapeutic effects of cardiac glycosides [4, 10].

Na,K-ATPase isolated to near-homogeneity from various sources is composed of equivalent amounts of α and β peptide subunits [19]. One of the striking findings of the present study is the lack of equivalence in the abundances of $mRNA_{\alpha}$ and $mRNA_{\beta}$ under the various thyroid states examined. In the hypothyroid steady state, the ventricular myocardium contained ~sixfold more $mRNA_{\alpha}$ (total of $\alpha 1$ and $\alpha 2$) than $mRNA_{\beta}$. The increments in both the enzyme activity and $mRNA_{\alpha 1}$ abundance in myocardial tissue appear to reach near-steady-state values at 72 hr after daily administration of receptor-saturating doses of T_3 [7, 30, 33]. In the hyperthyroid ventricle, despite the very large (12.7-fold) increment in $mRNA_{\beta}$ content, the abundance of total $mRNA_{\alpha}$ still exceeded $mRNA_{\beta}$ by ~twofold.

Orlowski and Lingrel [32] recently reported that in the euthyroid adult rat myocardium the abundance of $mRNA_{\beta}$ exceeds the combined abundances of the $mRNA_{\alpha}$ isoforms by ~55%. These findings taken in the context of our results, raise the possibility of discontinuities in the transition from the hypo- to eu- to hyper-thyroid states in the myocardium, i.e., differential induction of the β subunit (relative to $\alpha 1$ and $\alpha 2$) in the transition from hypo- to euthyroidism, and differential induction of the α isoforms relative to that of β in the transition from eu- to hyperthyroid state. Alternatively, technical errors in quantifying the relative abundances of the α and β subunit RNAs may also have to be considered. Of note is that Orlowski and Lingrel used subunit-specific molecular fragments as probes while we used full-length cDNA probes. In other related experiments (*unpublished observations*) and as is also seen in Fig. 2, using our hybridization and wash conditions no cross-hybridization between the probes and the related isoform mRNAs was detected.

In addition to the inequality in the abundances of subunit mRNAs, there is also a striking discrepancy between the T_3-induced increment in mRNA abundance *versus* increases in enzyme activity. Both types of discrepancies have been noted in previous studies in various rat tissues as well as in cultured cells, suggesting translational and/or post-translational control of α and β subunit contents [7, 9, 11, 35]. Recently a putative isoform of the β subunit of Na,K-ATPase has been cloned and sequenced, and the corresponding cDNA hybridizes to RNA isolated from rat myocardium [27]. The expression of this putative isoform may account for some of the nonequivalence between the α and β subunit mRNA abundances in the myocardium, but not for the discrepancy between increases in mRNA abundances *versus* enzyme content.

The abundances of $mRNA_{\alpha 1}$, $mRNA_{\alpha 2}$, and

$mRNA_\beta$ significantly increased between 3 and 12 hr following injection of the hormone (Fig. 3); at 3 hr, $mRNA_{\alpha 2}$ content was higher than that of diluent-treated controls, while at 12 hr, all three mRNAs were increased in abundance. It is important to note that the T_3-induced augmentation in myocardial Na,K-ATPase subunit mRNA abundances shown in Fig. 3 precedes the increment in cardiac Na,K-ATPase activity and changes in Na^+ and K^+ concentrations reported previously in the same hypothyroid rat model [33]. The fractional increments in the three mRNAs were not equal at 12 and 24 hr, perhaps reflecting the approach of each mRNA to a different "steady-state" value at 72 hr of T_3 treatment (Table 4). A complete analysis of the kinetics of accumulation of the mRNAs would require a knowledge of the rates of synthesis and degradation of each species during the approach to the new steady state. Previous studies on thyroidal regulation of Na,K-ATPase gene expression in rat renal cortex and liver implied that the T_3-induced increments in mRNA abundance are mediated both at transcriptional and post-transcriptional levels [11]. The roles of gene transcription and post-transcriptional processing of α isoform and β mRNAs in response to T_3 in the heart remain to be defined.

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References

1. Akera, T., Ng, Y.-C., Hadley, R., Katano, Y., Brody, T.M. 1986. High affinity and low affinity ouabain binding sites in the rat heart. *Eur. J. Pharmacol.* **132:**137–146
2. Asano, Y., Liberman, U.A., Edelman, I.S. 1976. Relationships between Na-dependent respiration and $Na^+ + K^+$-adenosine triphosphatase activity in rat skeletal muscle. *J. Clin. Invest.* **57:**368–379
3. Baginski, E., Zak, B. 1960. Microdetermination of serum phosphate and phospholipids. *Clin. Chim. Acta* **5:**834–838
4. Buccino, R.A., Spann, J.F., Pool, P.E., Sonnenblink, E.H., Braunwald, E. 1967. Influence of thyroid state on the intrinsic contractile properties and energy stores of myocardium. *J. Clin. Invest.* **44:**1669–1682
5. Charlemagne, D., Maixent, J.-M., Preteseille, M., Lelievre, L.G. 1986. Ouabain binding sites and (Na^+, K^+)-ATPase activity in rat cardiac hypertrophy. *J. Biol. Chem.* **261:**185–189
6. Charlemagne, D., Mayoux, E., Poyard, M., Oliviero, P., Geering, K. 1987. Identification of two isoforms of the catalytic subunit of Na,K-ATPase in myocytes isolated from adult rat heart. *J. Biol. Chem.* **262:**8941–8943
7. Chaudhury, S., Ismail-Beigi, F., Gick, G.G., Levenson, R., Edelman, I.S. 1987. Effect of thyroid hormone on the abundance of Na,K-adenosine triphosphatase α-subunit messenger ribonucleic acid. *Mol. Endocrinol.* **1:**83–89
8. Curfman, G.D., Crowley, T.J., Smith, T.W. 1977. Thyroid induced alterations in myocardial sodium- and potassium-activated adenosine triphosphatase, monovalent cation active transport, and cardiac glycoside binding. *J. Clin. Invest.* **59:**586–590
9. Emanuel, J.R., Garetz, S., Stone, L., Levenson, R. 1987. Differential expression of Na,K-ATPase α and β subunit mRNA's in rat tissues and cell lines. *Proc. Natl. Acad. Sci. USA* **84:**9030–9034
10. Frye, R.L., Braunwald, E. 1961. Studies on digitalis. III. The influence of triiodothyronine on digitalis requirements. *Circulation* **23:**376–382
11. Gick, G.G., Ismail-Beigi, F., Edelman, I.S. 1988. Thyroidal regulation of rat renal and hepatic Na,K-ATPase gene expression. *J. Biol. Chem.* **263:**16610–16618
12. Grupp, I., Im, W.-B., Lee, C.O., Lee, S.W., Pecker, M.S., Schwartz, A. 1985. Relation of sodium pump inhibition to positive inotropy at low concentrations of ouabain in rat heart muscle. *J. Physiol. (London)* **360:**149–160
13. Haber, R.S., Loeb, J.N. 1988. Selective induction of high-ouabain-affinity isoform of Na^+-K^+-ATPase by thyroid hormone. *Am. J. Physiol.* **255:**E912–E919
14. Hara, Y., Nikamoto, A., Kojima, T., Matsumoto, A., Nakao, M. 1988. Expression of sodium pump activities in BALB/c 3T3 cells transfected with cDNA encoding the α_3-subunits of rat brain Na^+, K^+-ATPase. *FEBS Lett.* **238:**27–30
15. Hubert, J.J., Schenk, D.B., Skelly, H., Leffert, H.L. 1986. Rat hepatic (Na^+, K^-)-ATPase α-subunit isolation by immunoaffinity chromatography and structural analysis by peptide mapping. *Biochemistry* **25:**4156–4163
16. Ismail-Beigi, F., Edelman, I.S. 1971. The mechanism of the calorigenic action of thyroid hormone. *J. Gen. Physiol.* **57:**710–722
17. Ismail-Beigi, F., Edelman, I.S., Gick, G.G. 1988. Greater induction of Na,K-ATPase alpha-2 *versus* alpha-1 mRNA abundance by thyroid hormone in rat skeletal muscle and ventricular myocardium. *Clin. Res.* **36:**542 (*Abstr.*)
18. Ismail-Beigi, F., Pressley, T.A., Haber, R.S., Gick, G.G., Loeb, J.N., Edelman, I.S. 1988. Kinetic analysis of Na,K-activated adenosine triphosphatase induced by low external K^+ in a rat liver cell line. *J. Biol. Chem.* **263:**8162–8167
19. Jorgensen, P.L. 1982. Mechanism of the Na^+, K^+ pump. Protein structure and conformations of the pure ($Na^+ + K^+$)-ATPase. *Biochim. Biophys. Acta* **694:**27–68
20. Kent, R.B., Fallows, D.A., Geissler, E., Glaser, T., Emanuel, J.R., Lalley, P.A., Levenson, R., Housman, D.E. 1987. Genes encoding α and β subunits of Na,K-ATPase are located on three different chromosomes in the mouse. *Proc. Natl. Acad. Sci. USA* **84:**5369–5373
21. Lin, M.H., Akera, T. 1978. Increased ($Na^+ + K^+$)-ATPase concentrations in various tissues of rats caused by thyroid hormone treatment. *J. Biol. Chem.* **253:**723–726
22. Lo, C.-S., August, T., Liberman, U.A., Edelman, I.S. 1976. Dependence of renal (Na^+, K^+)-adenosine triphosphatase activity on thyroid status. *J. Biol. Chem.* **251:**7826–7833
23. Lo, C.-S., Edelman, I.S. 1976. The effect of triiodothyronine on the synthesis and degradation of renal cortical ($Na^+ + K^+$)-adenosine triphosphatase. *J. Biol. Chem.* **251:**7834–7840
24. Lo, C.-S., Lo, T.N. 1980. Effect of triiodothyronine on the synthesis and degradation of the small subunit of renal corti-

cal (Na^+ + K^+)-adenosine triphosphatase. *J. Biol. Chem.* **255:**2131–2136
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
26. Lytton, J., Lin, J.C., Guidotti, G. 1985. Identification of two molecular forms of (Na^+,K^+)-ATPase in rat adipocytes. *J. Biol. Chem.* **260:**1177–1184
27. Martin-Vasallo, P., Dackowski, W., Emanuel, J.R., Levenson, R. 1989. Identification of a putative isoform of the Na,K-ATPase β subunit. Primary structure and tissue-specific expression. *J. Biol. Chem.* **264:**4613–4618
28. McDonough, A.A., Brown, T.A., Horowitz, B., Chiu, R., Schlotterbeck, J., Bowen, J., Schmitt, C.A. 1988. Thyroid hormone coordinately regulates Na^+- K^+-ATPase α- and β-subunit mRNA levels in the kidney. *Am. J. Physiol.* **254:**C323–C329
29. Noel, F., Godfraind, T. 1984. Heterogeneity of ouabain specific binding sites and (Na^+ + K^+)-ATPase inhibition in microsomes from rat heart. *Biochem. Pharmacol.* **33:**47–53
30. Oppenheimer, H.H. 1983. The nuclear receptor-triiodothyronine complex: Relationship to thyroid hormone distribution, metabolism, and biological action. *In:* Molecular Basis of Thyroid Hormone Action. J.H. Oppenheimer and H.H. Samuels, editors. pp. 1–34. Academic, New York
31. Orlowski, J., Lingrel, J.B. 1988. Differential expression of the Na,K-ATPase α1 and α2 subunit genes in a murine myogenic cell line. Induction of the α2 isozyme during myocyte differentiation. *J. Biol. Chem.* **263:**17817–17821
32. Orlowski, J., Lingrel, J.B. 1988. Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic α isoform and β subunit mRNAs. *J. Biol. Chem.* **263:**10436–10442
33. Philipson, K.D., Edelman, I.S. 1977. Thyroid hormone control of Na^+ + K^+-ATPase and K^+-dependent phosphatase. *Am. J. Physiol.* **232:**C196–C201
34. Pressley, T.A., Edelman, I.S. 1986. Reduced ouabain inhibition of Na,K-activated adenosine triphosphatase in cultured cell recipients of the ouabain-resistance gene. *J. Biol. Chem.* **261:**9779–9786
35. Pressley, T.A., Ismail-Beigi, F., Gick, G.G., Edelman, I.S. 1988. Increased abundance of Na,K-ATPase mRNAs in response to low external K^+. *Am. J. Physiol.* **255:**C252–C260
36. Russell, S.D., Cambon, N., Nadal-Ginard, B., Whalen, R.G. 1988. Thyroid hormone induces a nerve-independent precocious expression of fast myosin heavy chain mRNA in rat hindlimb skeletal muscle. *J. Biol. Chem.* **263:**6370–6374
37. Scatchard, G. 1949. Attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51:**660–672
38. Schmitt, C.A., McDonough, A.A. 1988. Thyroid hormone regulates α and α^+ isoforms of Na,K-ATPase during development in neonatal rat brain. *J. Biol. Chem.* **263:**17643–17649
39. Schneider, J.W., Mercer, R.W., Gilmore-Herbert, M., Utset, M.F., Lai, C., Greene, A., Benz, E.D., Jr. 1988. Tissue specificity, localization in brain, and cell-free translation of mRNA encoding the A3 isoform of Na^+,K^+-ATPase. *Proc. Natl. Acad. Sci. USA* **85:**284–288
40. Shull, G.E., Greeb, J., Lingrel, J.B. 1986. Molecular cloning of three distinct forms of the Na^+, K^+-ATPase α-subunit form from brain. *Biochemistry* **25:**8125–8132
41. Shull, G.E., Lane, L.K., Lingrel, J.B. 1986. Amino-acid sequence of the β subunit of the (Na^+ + K^+)ATPase deduced from a cDNA. *Nature (London)* **321:**429–431
42. Snedecor, G.W., Cochran, W.G. 1976. Statistical Methods, Iowa University Press, Ames, IA
43. Somjen, D., Ismail-Beigi, F., Edelman, I.S. 1981. Nuclear binding of T_3 and effects on Q_{O2}, Na/K-ATPase, and α-GPDH in liver and kidney. *Am. J. Physiol.* **240:**E146–E154
44. Sweadner, K.J. 1979. Two molecular forms of (Na^+ + K^+)-stimulated ATPase in brain. *J. Biol. Chem.* **254:**6060–6067
45. Sweadner, K.J., Farshi, S.K. 1987. Rat cardiac ventricle has two Na^+, K^+-ATPases with different affinities for ouabain: Developmental changes in immunologically different catalytic subunits. *Proc. Natl. Acad. Sci. USA* **84:**8404–8407
46. Takeyasu, K., Tamkun, M.M., Renaud, K.J., Fambrough, D.M. 1988. Ouabain-sensitive (Na^+,K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α-subunit of an avian sodium pump. *J. Biol. Chem.* **263:**4347–4354
47. Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77:**5201–5205
48. Urayama, O., Shutt, H., Sweadner, K.J. 1989. Identification of three isozyme proteins of the catalytic subunit of the Na,K-ATPase in rat brain. *J. Biol. Chem.* **264:**8271–8280
49. Urayama, O., Sweadner, K.J. 1988. Ouabain sensitivity of alpha 3 isozyme of rat Na,K-ATPase. *Biochem. Biophys. Res. Commun.* **156:**796–800
50. Wallick, E.T., Pitts, B.J.R., Lane, L.K., Schwartz, A. 1980. A kinetic comparison of cardiac glycoside interactions with Na^+, K^+-ATPase from skeletal and cardiac muscle and from kidney. *Arch. Biochem. Biophys.* **202:**442–449
51. Young, R.M., Lingrel, J.B. 1987. Tissue distribution of mRNAs encoding the alpha isoforms and beta subunit of rat Na^+, K^+-ATPase. *Biochem. Biophys. Res. Commun.* **145:**52–58
52. Young, R.M., Shull, G.E., Lingrel, J.B. 1987. Multiple mRNAs from rat kidney and brain encode a single Na^+, K^+-ATPase β subunit protein. *J. Biol. Chem.* **262:**4905–4910

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