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Effect of thyroid hormone on the distribution and activity of Na, K-ATPase in ventricular myocardium

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Abstract

Employing detergent-free sucrose density gradient fractionation method we isolated cholesterol rich lighter membrane fractions containing ~ 10% of protein, ~ 30% of cholesterol in membranes of ventricular myocardium. Cholesterol- rich lighter membrane fractions contain > 70% of Na, K-ATPase, and caveolins 1 and 3, and < 10% of β -actin. Treatment of hypothyroid rats with T₃ increased the relative abundance of both α 1 and β 1 Na, K-ATPase subunits in total membranes by 4–5-fold (with no change in caveolin 3), and resulted in 1.9-fold increase in enzyme activity. T₃ induced Na, K-ATPase subunits were preferentially distributed to the lighter fractions (#s 4, 5 and 6); and increased abundance of α 1 and β 1 were 34% to 70% and 43% to 68% respectively. We conclude that the activity of Na, K-ATPase is not uniform in cardiac membranes, and while a significant amount of Na, K-ATPase is present in cardiac cholesterol-rich membrane fractions, the intrinsic activity is significantly less than the enzyme present in relatively cholesterol-poor membranes.

Keywords

Na; K-ATPase; cholesterol; rafts; detergent resistant membranes; membrane fractionation; caveolar membranes

Na, K-ATPase (Na, K- pump) is an integral membrane protein that maintains normal physiological gradient of Na⁺ and K⁺ by catalyzing the coupled active transport of these ions across the plasma membrane. The enzyme is comprised of α and β subunits, both of which are essential for ATPase and ion pumping function [1–6]. Additionally Na, K-ATPase acts as a signal transducer by interacting with neighboring membrane proteins. The binding of ouabain to the Na, K-pump evokes protein and lipid kinase cascades generating secondary messengers [3], [7].

Na, K-ATPase is located in plasma membranes that contain microdomains (lipid rafts) which are rich in cholesterol and sphingolipids and domains that are relatively cholesterol poor. Caveolae are specific forms of lipid rafts that contain caveolins and a host of signaling molecules [8–10]. Employing detergent-free fractionation procedures, Liu et al have reported the specific co-localization of Na, K-ATPase and caveolin-3 in cardiac tissue [4]. They have

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also reported that up to 75% of the total Na, K-ATPase activity resides in caveolae and in rafts (also identified as detergent-resistant membranes, DRMs) [4–6,11]. These caveolar / raft structures segregate in lighter fractions in sucrose-density gradients and contain 5–10% of the total membrane protein and 25–30% of the cell cholesterol [5]. Na, K-ATPase molecules present in these lighter fractions play an important role in signaling and have been proposed to represent “non-pumping” Na, K-ATPase [3]. The potential signaling role of Na, K-ATPase and its localization in caveoli has been reviewed recently [12]. At concentrations well below those necessary to inhibit Na, K-ATPase, ouabain (and endogenous cardiac glycosides) stimulate signaling pathways involving Src and EGF receptor that play an important role in the hypertrophic response of the heart [12]. Given the physiological importance of localization of Na, K-ATPase in the plasma membrane domains, the relative enzymatic activity of Na, K-ATPase in and outside of the lighter fractions is one of the subjects addressed in the present study.

Rat ventricular myocardium expresses Na, K-ATPase $\alpha 1$, $\alpha 2$ and $\beta 1$ subunits. In the heart the $\alpha 1$ isoform constitutes > 90% of total α subunit content [1], [13]. It has been reported by this lab and others that treatment with triiodothyronine (T_3) increases Na, K-ATPase subunit abundance and activity [1], [14,15]. Indeed, T_3 induction of Na, K-ATPase and associated increase in energy consumption by cardiac and other thyroid hormone response tissues plays an important role in the calorogenic action of hormone (15, 17). Given that the expression and abundance of Na, K-ATPase is regulated by T_3 , and in light of the above reports on the distribution of Na, K-ATPase in the plasma membrane, we carried out a series of experiments to address the following questions: 1) What is the distribution and activity of Na, K-ATPase in cholesterol rich lighter fractions versus relatively cholesterol-poor heavier fractions? 2) What is the effect of T_3 on the distribution and activity of the enzyme in plasma membrane subdomains? and 3) What is the effect of T_3 on cholesterol / protein ratio in membranes isolated from ventricular myocardium.

To address these questions hypothyroid, euthyroid and hyperthyroid rats were used. Described and characterized detergent-free fractionation procedures for rat ventricular myocardium were employed [5]. The fractions were studied either individually or pooled for various experiments. The results demonstrate that there is significant difference in the Na, K-ATPase activity in the different fractions of euthyroid ventricular myocardium. Moreover, we report that the Na, K-ATPase molecules induced by thyroid hormone are preferentially distributed to the cholesterol-rich fractions, and still exhibit relatively lower enzymatic activity.

MATERIALS AND METHODS

Materials

Chemicals of highest purity were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Protein assay kits and cholesterol reagents were purchased from Bio-Rad (Hercules, CA), Thermo Electron (Louisville, CO) respectively. Anti-Na, K-ATPase $\alpha 1$ monoclonal, and anti-Na, K-ATPase $\beta 1$ (rabbit antiserum) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-caveolin-3 mouse monoclonal (clone 26) was purchased from BD Transduction Labs (Lexington, KY). Anti-caveolin-1 rabbit polyclonal IgG was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti- β -actin mouse monoclonal was purchased from Sigma. Western blots were developed using chemiluminescence kit from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrocellulose membrane was purchased from Schleicher & Schuell (Keene, NH).

Treatment of rats

Rats were made hypothyroid by including 6-n-propyl -2-thiouracil in the drinking water (0.5 g/liter) for 3 weeks, at which time half of the rats (usually 6) were injected with diluent and the others received triiodothyronine (T_3) by intraperitoneal injection once every 48 h at a dose of 0.5 μ g/g body weight 3 times to induce hyperthyroidism [1,13,14] [16]. Euthyroid rats were used in some experiments. Rats were anesthetized using CO_2 and exanguinated following which cardiac ventricles were rapidly dissected and frozen in liquid nitrogen for no longer than 6 weeks. Rats were handled according to Institutional Animal Care and Use committee guidelines.

Fractionation of ventricular myocardium and membranes

The detergent- free procedure was carried out according to methods described by Liu and Askari [5] with only minor modifications. This method was characterized and validated by the above authors [3,5]. In brief, 75–80 mg of cardiac ventricular myocardium was minced, placed at 4° C in 1 ml of MES buffer [25 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer (pH 6.5) containing 0.5 M KCl, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM NaF, 1 mM, phenylmethyl-sulfonyl fluoride (PMSF), 1 mM DTT and 10 nM okadaic acid] and homogenized on ice for 15 seconds at setting 4 using a hand-held homogenizer (Type T8 homogenizer , IKA Labortechnik, Staufen , Germany). Homogenates were then sonicated on ice for 5 seconds at setting 4 using a Fisher Sonicator (Model 550 sonic dismembrator). All subsequent steps were at 4° C. 200 μ l of the homogenate was used to prepare total membranes by dilution with 800 μ l of 25 mM MES buffer. The diluted suspension was centrifuged at 1000 rpm for 2 min, and 900 μ l of the supernatant was diluted with 9.0 ml of MBS (25 mM MES pH 6.5, plus 150 mM NaCl) and was centrifuged at 100,000 x g for 1 h. The pellet was re-suspended in 100 μ l of TS buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM EGTA). This was labeled as total membrane (TM) preparation and was used in the indicated experiments.

The remaining 800 μ l of the homogenate was diluted to 2.0 ml with MES buffer. 2.0 ml of 90% sucrose prepared in MBS containing 250 mM KCl was added. This suspension was placed at the bottom of the 12 ml centrifuge tube and was overlaid with 4 ml of 35% sucrose, and then 4 ml of 5% sucrose both prepared in MBS buffer containing 250 mM KCl. Samples were centrifuged with a TH641 rotor (Sorval Ultra Pro80) at 39,000 rpm for 18 h, and twelve 1.0 ml fractions (numbered from top to bottom) were collected. Each fraction was diluted to 10 ml with MBS buffer and was centrifuged at 100,000 x g for 1 h. Pellets were re-suspended in 60 μ l of TS buffer and equal volumes from each fraction were subjected to SDS-PAGE. Alternatively, fractions 4, 5 and 6, and fractions 8, 9 and 10 were combined and are labeled as pooled fractions (4, 5 and 6) and pooled fractions (8, 9 and 10), respectively. Equal volumes of these pooled fractions were subjected to SDS-PAGE and were also used for Na, K-ATPase enzyme measurement. Pooled samples used for ATPase assays did not contain either Na_3VO_4 or NaF in MES buffer.

Analysis of immunoblots

Samples were subjected to 4–7.5% SDS-PAGE, transferred to nitrocellulose membrane and probed with appropriate antibodies. Immunoreactive bands of interest were developed and detected by enhanced chemiluminescence. Quantitative comparisons of scanned images were carried out by the publicly available software, Image J (1.37v), NIH, USA. In all instances multiple exposures were made to ensure that the bands were in the linear range of the film.

Na, K-ATPase assay

Ouabain-inhibitable Na, K-ATPase activity was determined in triplicate according to methods described previously with minor modifications [14,17]. Membrane preparations (10 μ l containing ~10–30 μ g of protein) in TS buffer with protease inhibitors containing deoxycholate at a final concentration of 0.4 mg/ml were used for the assay. Incubation medium with the final concentrations of (all in mM) ATP (4.0), Tris (47), NaCl (77), NaN_3 (4.21), KCl (2.57), MgCl_2 (2.14), and EGTA (0.1) in 750 μ l with or without ouabain (1.6) was used in each set of incubations; blanks had no ouabain or membranes. Samples were mixed and were incubated at 37° C for 1 h. Reactions were stopped by addition of 200 μ l of TCA (31.25%). Membrane preparations were added to blanks after addition of TCA. Samples were placed on ice for 10 min and centrifuged at 500 x g for 10 min. The generated inorganic phosphate was estimated using Fiske and Subbarow's method [18]. The colorimetric determination of phosphorus was done by the method of Lowry and Lopez [19]. To both samples and standards, 200 μ l of 10% ascorbic acid and 1 ml of ammonium molybdate (0.5%) were added mixed well. Exactly after 1 min, 1 ml of citrate and arsenite reagent (mixture of 2% sodium citrate, 2% sodium arsenite in 2% glacial acetic acid) was added, mixed rapidly and optical density was measured after 30 min at 700 nm. Results are expressed as nmol/h or μ mol/mg protein/h. Standards were prepared using K_2HPO_4 .

Measurement of protein and cholesterol

Protein and cholesterol were measured using Bio-Rad kit and Infinity cholesterol liquid stable reagent of Thermo DMA, respectively. Cholesterol in total membranes was measured using Infinity cholesterol of Thermo DMA.

Data analysis

Results are expressed as means $\pm$ SE. Student's *t*-test was used and significance was accepted at $P < 0.05$.

RESULTS

Distribution of cholesterol, protein, Na, K-ATPase subunits, caveolin-1, caveolin-3, and β -actin in cardiac ventricular myocardium of euthyroid rats

Employing homogenization and density gradient centrifugation protocols not involving detergents, Liu et al have shown that a significant quantity of (~ 2/3) of Na, K-ATPase and its subunits are present in membrane fractions that are rich in cholesterol, also identified as rafts, caveoli and “detergent-resistant membranes” (DRMs) [4–6]. In accordance with their report, we found that a significant fraction of tissue cholesterol and ~10% of protein is present in lighter fractions of the sucrose gradient resulting in high cholesterol/protein ratio in these fractions (#s 4, 5, and 6) (Figure 1A).

To determine the distribution of Na, K-ATPase subunits and other proteins of interest, 1.0 ml aliquots were removed from the top of the gradient and diluted with 9.0 ml of buffer devoid of sucrose, and centrifuged at 100,000 x g for 1 h. The resulting pellets were resuspended in 100 μ l of buffer and equal volumes were loaded per lane (Figure 1B). It can be appreciated that a significant fraction of Na, K-ATPase α 1 and β 1 subunits in ventricular myocardium of euthyroid rats is present in these lighter fractions. Caveolin-1 and -3 which are concentrated in these lighter fractions are also somewhat distributed in the remaining fractions. In contrast, β -actin is mostly present in the heavier fractions #s 9–12. In repeated experiments we found that 65 to 70% of Na, K-ATPase subunits and less than 10% of β -actin is present in fractions 4, 5, and 6.

Na, K-ATPase activity in the different density fractions of euthyroid ventricular myocardium

Given the different cholesterol/protein ratio in fractions 4, 5, and 6 versus the heavier fractions, we tested the hypothesis that the activity of the enzyme is different among the various fractions. Because of the high cholesterol content of some of the fractions, and to ensure that maximal Na, K-ATPase activity was being measured in the assay, we first compared the activity of total membranes resuspended in buffer containing various concentrations of deoxycholate, saponin and alamethicin we then chose 0.04% deoxycholate, 0.1% saponin, and 0.1 mg/mg protein alamethicin for comparison between the agents. We found that the enzyme activity was highest in buffer containing 0.04% deoxycholate, and used this detergent in all subsequent assays of Na, K-ATPase activity (data not shown).

To determine whether Na, K-ATPase activity is different among the fractions, pellets derived from fractions 4, 5, and 6 were pooled and Na, K-ATPase activity and relative enzyme subunit content were compared to those in pooled samples derived from fractions 8, 9, and 10 (Figure 2 and Table 1). The specific activity of Na, K-ATPase in total membranes of euthyroid ventricular myocardium averaged 2.43 ± 0.59 $\mu\text{mol/h/mg}$ protein (Table 1); the specific activity was 6.55 ± 0.8 and 1.12 ± 0.25 $\mu\text{mol/h/mg}$ protein in fractions 4, 5 and 6 and fraction 8, 9 and 10, respectively. The relatively higher specific activity of the enzyme in fractions 4, 5 and 6 in large part reflects the low abundance of protein in this pooled fraction. Consistent with results shown in Figure 1A, the bulk of Na, K-ATPase $\alpha 1$ and $\beta 1$ subunits are present in pooled fractions 4, 5 and 6. Because the protein content differs significantly among the different fractions, we also expressed Na, K-ATPase activity (expressed as nmol Pi produced/h) in different fractions. This enables the expression of enzyme activity to be normalized against the relative abundance of the subunits of the enzyme present in the fractions. Hence, Na, K-ATPase activity (expressed as nmol/h) measured in equal volumes of the reconstituted pellets was 2.3-fold higher in fractions 4, 5 and 6 compared to fractions 8, 9 and 10. However, when the enzyme activity was expressed per relative abundance of the $\alpha 1$ (catalytic) subunit present in the two pooled fractions, we found that the activity of the enzyme is significantly higher in pooled sample 8, 9 and 10; in five independent experiments the activity of the enzyme was 4.5 ± 0.2 -fold higher in the heavier pooled fractions compared to the lighter pooled fractions 4, 5 and 6.

Effect of T₃ treatment on thyroid status, Na, K-ATPase activity, and Na, K-ATPase subunit expression in rat ventricular myocardium

Hypothyroid rats were injected with three every-other-day doses of T₃ or diluent, as previously described [1,13,14]. Heart (ventricles) to body weight ratio (times 100) was 1.91 ± 0.07 and 3.46 ± 0.19 in hypothyroid and T₃-treated animals, respectively ($n = 6$, $P < 0.05$). Similarly, kidney to body weight ratio ($\times 100$) increased from 6.56 ± 0.27 to 10.01 ± 0.34 in T₃-treated animals. The above changes (all $P < 0.05$) are consistent with the induction of hyperthyroid state in the T₃-treated group [1,14].

We then determined the effect of thyroid status on Na, K-ATPase activity. Similar to previous reports [14,20,21] we found that the activity of the enzyme was 1.9-fold higher in total membranes isolated from the myocardium of T₃-treated compared to diluent-treated hypothyroid rats (Figure 3A). The effect of T₃ on the relative abundance of Na, K-ATPase subunits was also determined in the same total membrane isolates (Figure 3B). We found that the relative abundance of Na, K-ATPase $\alpha 1$ and $\beta 1$ subunits increased significantly in total membranes isolated from T₃-treated animals compared to those treated with diluent alone; in contrast, the relative abundance of caveolin-3 remained unchanged. Results of repeated Western blots determining the effect of T₃ on the relative abundance of Na, K-ATPase subunits in total membrane fractions is summarized in Figure 3C. T₃ treatment resulted in a 4 to 5-fold increase in the relative abundance of both subunits. It can be appreciated that the T₃-induced increase in the abundance of Na, K-ATPase subunits significantly exceeds the ~2-fold increase

in Na, K-ATPase enzyme activity. This finding raises the possibility that some of the T₃-induced enzyme is inactive, or that heterogeneity exists in the molecular activity of the enzyme depending on the localization of the enzyme within the plasma membrane.

Effect of thyroid status on the distribution of Na, K-ATPase subunits in ventricular myocardium

Given that the bulk of Na, K-ATPase in ventricular myocardium of euthyroid rats is localized in the “lighter” fractions, we examined whether the same is the case in hypothyroid heart, and whether the distribution is altered in the hyperthyroid state. We hence performed sucrose density gradient centrifugation in homogenates prepared from hypothyroid and hyperthyroid cardiac ventricles (Figure 4). Unlike the distribution observed in the euthyroid heart, less than half of Na, K-ATPase α 1 and β 1 subunits in ventricles of hypothyroid rats are present in the lighter fractions. Treatment of hypothyroid rats with T₃, which as noted above results in an overall increase in the abundance of both subunits, led to a disproportionate increase in the abundance of both subunits in fractions 4, 5, and 6. In six independent experiments we found $34 \pm 6\%$ and $43 \pm 5\%$ of the Na, K-ATPase α 1 and β 1 subunits in the hypothyroid heart was present in fractions 4, 5 and 6; these percentages increased to $70 \pm 7\%$ and $68 \pm 9\%$, respectively, in ventricles of hyperthyroid animals.

Na, K-ATPase activity in the different density fractions of hypothyroid and hyperthyroid ventricular myocardium

In the euthyroid heart, we noted that Na, K-ATPase activity per relative α 1 subunit abundance was significantly lower in the “lighter” density fractions raising the question of whether a similar situation is present in the hypothyroid heart. Moreover, while it appears that the subunits induced by T₃ are differentially partitioned into the light fractions, it is not known whether thyroid hormone affects a differential change in the molecular activity of the enzyme that are localized in the “light” versus “heavier” fractions. We hence fractionated homogenates of ventricular myocardium derived from hypothyroid and hyperthyroid rats using sucrose density gradients and measured the effect of T₃ on Na, K-ATPase activity normalized against the relative abundance of the α 1-subunit in pooled samples of fractions 4, 5 and 6 and fractions 8, 9 and 10. A typical blot of the effect of T₃ on the relative abundance of Na, K-ATPase α 1 and β 1 subunits in equivalent samples from the two pooled fractions is shown in Figure 5. As noted above, treatment with T₃ resulted in an obvious differential distribution of the subunits to the lighter fractions (#s 4, 5, and 6). In six independent experiments, T₃ resulted in 2.3 ± 0.4 and 3.5 ± 0.6 -fold increases in the relative abundance of α 1 and β 1 subunits in pooled fractions 4, 5 and 6. In contrast, T₃ treatment resulted in 1.0 ± 0.2 and 1.3 ± 0.4 -fold change in α 1 and β 1 subunit abundance in pooled fractions 8, 9 and 10.

We then measured Na, K-ATPase activity in each of the two pooled fractions and normalized the enzymatic activity against the relative abundance of the α 1 subunit in the pooled fractions. The average results of repeated experiments are summarized in Table 2. The specific activity of Na, K-ATPase in total membranes of hypothyroid and hyperthyroid ventricles was 1.47 ± 0.28 and 2.61 ± 0.38 $\mu\text{mol/h/mg}$ protein, respectively. Similar to the euthyroid heart, the specific activity of the enzyme was higher in fractions 4, 5 and 6 compared to 8, 9 and 10 in both hypothyroid and hyperthyroid heart, mostly reflecting the lower abundance of total protein in the lighter fractions. We also expressed the activity of the enzyme per relative abundance of enzyme in the fractions, similar to the analysis performed on euthyroid heart (Table 1). As can be appreciated, Na, K-ATPase activity normalized against the relative abundance of the α 1 subunit is significantly higher in the heavier fractions of both hypothyroid and hyperthyroid hearts, suggesting that a significant amount of the Na, K-ATPase molecules induced by triiodothyronine (T₃) that are distributed to the lighter fractions are either inactive or only partially active.

Effect of thyroid hormone on cholesterol, protein, and their ratio in membranes isolated from ventricular myocardium

Finally, excess thyroid hormone has been reported to cause a decrement in the cholesterol content of membranes, largely reflecting the decrements in cholesterol content of plasma membranes; the decreased levels of cholesterol increases the phospholipids/cholesterol ratio and “fluidity” of membranes [22–25]. To verify this observation in our experimental system, we isolated total membranes from homogenates of ventricular myocardium of hypothyroid and hyperthyroid rats. Membrane pellets were re-suspended in buffer and analyzed for cholesterol and protein content (Table 3). We found that the content of cholesterol was significantly decreased in membranes derived from T_3 -treated rats while the content of protein remained unchanged. This led to a $26 \pm 3\%$ decrease in the cholesterol/protein ratio in the T_3 -treated group.

DISCUSSION

Na, K-ATPase expression and function is critical to intracellular ionic homeostasis. The expression of the enzyme is regulated by number of hormones and by the ionic environment. Among these thyroid hormone is an important positive regulator of Na, K-ATPase expression in heart and other thyroid hormone responsive tissues (1, 15, 17, 32). The central questions addressed in this study were two-fold: 1) whether the activity of Na, K-ATPase in the euthyroid heart ventricles, expressed per $\alpha 1$ -subunit abundance, is a function of the enzymes localization in cholesterol-rich versus cholesterol-poor regions of membranes, and 2) whether Na, K-ATPase subunits that are induced by thyroid hormone (T_3) are preferentially distributed to cholesterol-rich versus -poor regions. We adapted and employed a recently reported and well-characterized method for fractionation of heart membranes using euthyroid, hypothyroid and hyperthyroid rats [3,5]. In addressing the above questions we have made a number of highly novel and physiologically important observations. These include: a) although $\sim 2/3$ of Na, K-ATPase in ventricular myocardium of euthyroid rats is present in cholesterol-rich membranes (also labeled rafts, caveoli, and detergent resistant membranes), the activity of the enzyme, expressed per $\alpha 1$ -subunit abundance, present in the same region of the membrane is significantly lower than the activity of enzyme molecules present in relatively cholesterol-poor heavier regions. b) T_3 induction of Na, K-ATPase $\alpha 1$ and $\beta 1$ subunits is far greater than the measured stimulation of enzyme activity. c) The increase in cellular abundance of Na, K-ATPase $\alpha 1$ and $\beta 1$ subunits induced by T_3 are preferentially accumulated in the cholesterol-rich fractions. And, d) the activity of Na, K-ATPase localized to the cholesterol-rich regions is lower than the enzyme present in cholesterol-poor regions, irrespective of thyroid status. We have also confirmed previous findings that T_3 -treatment of hypothyroid rats leads to a decrease in membrane cholesterol/protein ratio, consistent with the reported increase in the “fluidity” of membranes in heart and other target tissues of hyperthyroid state [26,27].

One of the novel findings of the present study is that Na, K-ATPase molecules that reside in the cholesterol-rich regions of membranes are significantly less active (per unit enzyme) than those distributed in relatively cholesterol-poor regions. In these studies we have employed fractionation methods described and validated recently [5]. The protocol employs a 0.5 M KCl buffer solution devoid of detergents at pH 6.5 for homogenization and sonication followed by sucrose-density gradient centrifugation (also without detergents) [5]. Since we used the protocols with no modifications and the fractionation has been adequately characterized [5], we did not repeat those validation studies in an extensive manner. In agreement with the previous reports [4–6], we found that $\sim 2/3$ of Na, K-ATPase present in ventricular myocardium is localized to cholesterol-rich fractions which contain $\sim 10\%$ and $\sim 30\%$ of cell protein and cholesterol, respectively (fractions 4, 5 and 6; Fig. 1A and 1B). Though treatment with T_3 did not change the abundance of caveolin3 in heart membranes, there are several reports indicating

that caveolin exists as large oligomers in association with other proteins including Na, K-ATPase in special microdomains identified as caveoli [28], [29], [30,31]. It is likely that cardiac caveolar Na, K-ATPase exists as a large aggregate of caveolins and related signaling proteins. It has also been reported that caveolar and noncaveolar pools of Na, K-ATPase may both be involved in signaling via different signaling partners [5], [12]. Certainly, cardiac Na, K-ATPase appears to involve the SRC/EGF receptor signaling pathway [5], [12].

The specific activity of Na, K-ATPase measured in hypothyroid, euthyroid, and hyperthyroid ventricular muscle (~ 1.5 to $2.6 \mu\text{mol P}_i/\text{h/mg protein}$) is well within activities measured in several previous reports (13, 23, 29), and lower than in some others (1, 34); the variation in specific activity probably reflects tissue homogenization techniques and assay conditions. Na, K-ATPase specific activity (expressed per unit protein) was higher in fractions 4, 5 and 6 compared to fractions 8, 9 and 10 in large part due to the distribution of proteins in these fractions. However, when we compared the activity of the enzyme expressed as nmol P_i generated/h per relative abundance of the $\alpha 1$ subunit present in pooled cholesterol-rich fractions versus fractions that are relatively cholesterol-poor (pooled fractions 8, 9, and 10), we noted that the activity of the enzyme present in the cholesterol-rich fractions is significantly less. While the exact explanation for this differential activity is not known and is under current investigation, some plausible explanations can be advanced. One possibility that has been proposed is that much of the Na, K-ATPase molecules present in cholesterol-rich domains do not function as Na-pumps and instead function as signaling molecules, *i.e.*, they are “non-pumping” Na-pumps [3]. This is in keeping with multiple previous reports demonstrating important signaling functions of Na, K-ATPase that may be involved in hypertrophic responses of the heart to various physiological and pathological perturbations including thyroid hormone [14,15,32–35]. Alternatively, it is possible that the high cholesterol composition (and high cholesterol/phospholipid ratio) of the membrane domain in which the Na-pumps are localized makes the membrane more “rigid” and impedes the enzymatic turnover by requiring higher energies of activation. This explanation is given credence based on previous observations that the “fluidity” of membranes is reduced and their melting temperature (T_m) increases as the cholesterol/phospholipid ratio is increased [22–25]. It is known that Na, K-ATPase activity is affected by the cholesterol and phospholipid content of the membrane as well as the length of phospholipid acyl chains [36,37]. In addition, increased cholesterol content of membranes induced by excess cholesterol feeding has been associated with decreases in Na, K-ATPase activity [38]. An additional possibility is the potential interaction of Na, K-ATPase molecules with FXYD proteins. This family of polypeptides, whose prototype represents the γ -subunit of Na, K-ATPase, are transmembrane proteins that appear to have multiple functions [39–41]. Two of these proteins, FXYD1 and FXYD5, are present in the heart and can modulate the activity of Na, K-ATPase [42,43]. This raises the possibility that these peptides are differentially distributed in the membrane and might interact with Na, K-ATPase molecules to alter their activity depending on their localization. Further studies are necessary to examine the above (and other) possibilities to more fully understand the basis of the differential “intrinsic” activity of Na, K-ATPase residing in the different membrane regions.

The second significant finding of the present study is that the induction of Na, K-ATPase subunits by thyroid hormone far exceeds the known stimulation of Na, K-ATPase activity in heart. The stimulation of Na, K-ATPase activity by T_3 in various target tissues, including the heart, is 1.5-2.5-fold [1,2], [14,20]. Here we found that the 1.9-fold increase in enzyme activity in cardiac membranes of T_3 -treated versus diluent-treated hypothyroid rats was associated with 4.5- and 5.5-fold increases in the abundance of $\alpha 1$ and $\beta 1$ subunits, respectively. While this finding is consistent with thyroidal induction of “non-pumping” Na, K-ATPase, it also suggests the possibility that the increase in the abundance of Na, K-ATPase subunits is differentially distributed between the cholesterol-rich and -poor regions of the membrane.

To test whether T₃-induced Na, K-ATPase subunits are differentially distributed in membranes from ventricular myocardium, we performed fractionation experiments in hearts of hypothyroid and hyperthyroid rats. We found that the relative abundance of both subunits in the cholesterol-rich fractions was significantly lower in ventricular myocardium of hypothyroid versus euthyroid rats. In addition, we noted that the increase in the abundance of Na, K-ATPase subunits was associated with their differential distribution into the cholesterol-rich fractions (Fig. 4A and 4B). This result might help explain the discrepancy between the T₃-induced induction of the subunits and enzyme activity noted above, if the T₃ induced increase in enzyme abundance in the cholesterol-rich fractions is also relatively less active.

This latter possibility was examined in pooled fractions derived from ventricular myocardium of hypothyroid and hyperthyroid animals (Fig. 5 and Table 2). The results demonstrated that while the induced subunits are preferentially localized in the cholesterol-rich fractions, their relative activity remains significantly less than the Na, K-ATPase molecules that are present in relatively cholesterol-poor fractions. Hence in all thyroid conditions examined, namely hypothyroid, euthyroid, and hyperthyroid states, the activity of Na, K-ATPase molecules present in relatively cholesterol-poor fractions is higher than those present in cholesterol-rich membranes.

It is noteworthy that the above changes in Na, K-ATPase abundance and activity induced by thyroid hormone are associated with significant changes in the composition of membranes. The ~26% decreases in membrane cholesterol content and in cholesterol/protein ratio in membranes derived from T₃-treated animals is in keeping with previous reports in heart and other thyroid target tissues [22,44]. Whether the cholesterol composition of the membrane domain plays a critical role in the activity of the enzyme or is merely associated with Na, K-ATPases with different intrinsic activities that are localized to specific domains of the membranes requires further detailed analysis.

In conclusion, we have found that the distribution and activity of Na, K-ATPase is not uniform in cardiac membranes. We also noted that T₃-treatment results in higher induction of Na, K-ATPase subunits compared to the stimulation of enzyme activity, and that much of the induced Na, K-ATPase molecules are distributed to cholesterol-rich domains of the membrane that exhibit relatively less enzymatic activity and might function as signaling molecules. Our results also raise the possibility that the Na, K-ATPase molecules that are induced in response to thyroid hormone, are predominantly localized in the cholesterol-rich domains and play an important role in the dramatic heterotrophic response of the heart to the hormone.

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Figure 1. Distribution of protein, cholesterol and specific proteins in density fractions of membranes from euthyroid ventricular myocardium

A. Samples of homogenates were fractionated by detergent-free KCl-based procedure and 12 fractions of equal volume (1 ml each) were collected (see Materials and Methods). The resulting fractions were assayed for protein and cholesterol. Fractions 1–3 did not contain protein.

B. Typical blots of fractionated preparations. Each 1ml fraction was diluted to 10 ml of buffer (devoid of detergent) and pelleted at 100,000 x g for 1 h. Pellets from each fraction were resuspended in 60 μ l of buffer and an equal volume of each fraction sample was used for Western blot analyses with specific antibodies.

Figure 2. Relative abundance of Na, K-ATPase $\alpha 1$ and $\beta 1$ subunits in pooled fractions of euthyroid myocardium

Equal volumes of resuspended membrane pellets from fractions 4, 5 and 6 were combined and are labeled as pooled fractions 4, 5, 6 the lighter fractions. In a similar way, pooled fractions 8, 9 and 10 were prepared (heavier fractions). Equal volumes of each pooled sample were subjected to Western blot analysis with specific antibodies as described in Materials and Methods. Results of six independent experiments are summarized in Table 1.

Figure 3. Effect of T₃ treatment on Na, K-ATPase activity and $\alpha 1$ and $\beta 1$ subunit expression. A. Na, K-ATPase activity

Total membranes were prepared from hypothyroid and hyperthyroid myocardium. Results are expressed as mean $\pm$ SE; n = 5. * denotes p < 0.05. **B and C. Western blot analysis of relative abundance of Na, K-ATPase subunit and caveolin 3.** Total membranes from myocardium of T₃-treated and diluent-treated hypothyroid rats were used for Western blot analyses. Results are expressed as mean $\pm$ SE; n = 5. * denotes p < 0.05.

Figure 4. Effect of T_3 treatment on the distribution of Na, K-ATPase subunits

Membrane preparations from hearts of T_3 -treated and diluent-treated rats were processed as described in Materials and Methods and were subjected to Western blot analyses. **A. Western blot.** Typical blots are shown.

B. Relative abundance of Na, K-ATPase subunits in lighter pooled membrane fractions 4, 5 and 6. Results from 6 independent preparations from hypothyroid and T_3 -treated rats are summarized (mean $\pm$ SE). * denotes $p < 0.05$.

Figure 5. Effect of T₃ treatment on Na, K-ATPase subunits abundance in lighter and heavier membrane fractions

Membranes were prepared and analysed as described in Materials and Methods. A typical blot is shown. The results of 6 independent experiments are summarized in Table 2.

Table 1

Na, K-ATPase activity and relative subunit abundance in pooled fractions of membranes from euthyroid rat ventricular myocardium

	Fractions 4, 5 and 6	Fractions 8, 9 and 10
Na, K-ATPase ($\mu\text{mol/h/mg protein}$)	6.55 ± 0.8	$1.12 \pm 0.25^*$
Na, K-ATPase (nmol/h)	130 ± 45	$57 \pm 17^*$
α_1 - subunit (relative abundance)	1.0	$0.11 \pm 0.02^*$
β_1 - subunit (relative abundance)	1.0	$0.12 \pm 0.03^*$
Na, K-ATPase (nmol/h/ α_1 - subunit)	130 ± 45	$599 \pm 227^*$

Na, K-ATPase activity in total membranes was $2.43 \pm 0.59 \mu\text{mol/h/mg protein}$. Na, K-ATPase activity in pooled lighter (4, 5, 6) and heavier (8, 9, 10) fractions of euthyroid myocardium was also determined. In each experiment relative abundance of Na, K-ATPase α_1 and β_1 subunits in the pooled fraction 8, 9, 10 was calculated by dividing subunit band intensity by the value in lighter 4, 5 and 6; values are mean $\pm$ SE. Na, K-ATPase activity per α_1 subunit abundance was calculated by dividing the enzyme activity in the pooled samples by the relative abundance of α_1 subunit in the same pooled sample.

Values are mean $\pm$ SE (n= 6)

* denotes $p < 0.05$.

Table 2

Effect of T₃ treatment on Na, K-ATPase activity expressed per relative subunit abundance in different pooled density fractions of membranes

	Hypo		T ₃	
	4, 5 and 6	8, 9 and 10	4, 5 and 6	8, 9 and 10
Na, K-ATPase (μmol/h/mg protein)	3.41 ± 0.86	1.10 ± 0.22 *	10.27 ± 2.8	0.84 ± 0.15 *
Na, K-ATPase (nmol/h)	27 ± 5	27 ± 6	72 ± 12	21 ± 6 *
α ₁ - subunit (relative abundance)	1.0	0.42 ± 0.10 *	1.0	0.16 ± 0.02 *
β ₁ - subunit (relative abundance)	1.0	0.76 ± 0.20	1.0	0.22 ± 0.07 *
Na, K-ATPase (nmol/h/α ₁ -subunit)	27 ± 5	71 ± 18 *	72 ± 12	130 ± 29 *

Na, K-ATPase activity in total membranes of hypothyroid and hyperthyroid ventricles was 1.47 ± 0.28 and 2.61 ± 0.38 μmol/h/mg protein, respectively. Na, K-ATPase activity in pooled lighter (4, 5, 6) and heavier (8, 9, 10) fractions was calculated according to the method described in Table 1, but separately for hypothyroid and hyperthyroid myocardium. Results are from 6 independent experiments in each treatment group. Values are mean ± SE

* denotes p < 0.05.

Table 3Effect of T₃ on cholesterol & protein content in total membranes of ventricular myocardium

	Hypo	T ₃
Cholesterol (ng)	3946 ± 248	2909 ± 250 *
Protein (μg)	101 ± 9	99 ± 8
Cholesterol (ng) / Protein (μg)	410 ± 48	302 ± 47 *

Total membrane preparations from ventricular myocardium of hypothyroid and hyperthyroid rats were used for measuring protein and cholesterol. Results are mean ± SE; n = 6

* denotes $p < 0.05$.