

Stimulation of Na,K-ATPase by hypothyroidism in the thyroid gland

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Abstract

Although studies have documented the regulatory effects of thyroid hormones on the Na,K-ATPase in peripheral tissues, there is little information on the regulation of this transporter in the thyroid gland itself. Accordingly, we investigated the effects of thyroid status on Na,K-ATPase specific activity and the abundance of its constituent subunits in rat thyroid. Exogenous tri-iodothyronine (T_3) was administered daily to produce hyperthyroidism. 6n-propyl-2-thiouracil (PTU), an inhibitor of thyroid hormone synthesis, was used to induce hypothyroidism. There was a four-fold increase in Na,K-ATPase specific activity in the follicular membranes from PTU-treated

animals after 7 days. Enzymatic activities were not changed in the T_3 -treated glands. Immunoblotting of membranes from T_3 -treated rats revealed a 75% reduction in α_1 subunit abundance and a slight, but nonsignificant reduction in β_1 abundance. On the other hand, the membranes from PTU-treated rats displayed 136 and 567% increases in the abundance of the α_1 and β_1 subunits respectively. These data demonstrate that thyroid hormone status regulates Na,K-ATPase in the gland, but the effects are in direct contrast to those seen in the periphery.

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Introduction

Na,K-activated adenosine triphosphatase (Na,K-ATPase or Na,K-pump) is the membrane-spanning protein complex responsible for extrusion of Na^+ and absorption of K^+ by most animal cells. It consists of a catalytic α subunit (100 000 Da) and a glycosylated β subunit (50–60 000 Da) (Lingrel & Kuntzweiler 1994). In epithelia, active transport mediated by the pump provides the driving force for the movement of solutes and water between serosal and luminal fluids. The follicular epithelium of the thyroid gland is no exception, and the iodine required for hormone biosynthesis is accumulated within the gland through the combined actions of the Na,K-ATPase and the Na^+, I^- -cotransporter (Dai *et al.* 1996). Numerous studies have documented the regulation of Na,K-ATPase in nonthyroidal tissues by thyroid hormones (Ismail-Beigi & Edelman 1971, McDonough *et al.* 1988). However, the effects of hormonal status on the pump are less well studied in the thyroid gland itself. Accordingly, we examined enzymatic activity and the abundance of the constituent subunits of the pump complex in glands from hypo- and hyperthyroid rats. The results show that the Na,K-ATPase of the thyroid gland is stimulated dramatically by hypothyroidism, in direct contrast to the response in nonthyroidal tissues.

Materials and Methods

Animals

Male Sprague–Dawley rats (~200 g) were housed in a controlled environment (25 °C) and given standard rat chow and water unless otherwise specified. Animals were assigned randomly to one of three experimental groups. In the first group, hyperthyroidism was induced by daily intraperitoneal injection of tri-iodothyronine (T_3 , 1 μ g/g body weight) (Denereaz & Lemarchand-Bereaud 1995). In the second group, hypothyroidism was induced by including 6n-propyl-2-thiouracil (PTU) in the drinking water (1 g/l) (Veronikis *et al.* 1996). Control rats received no special treatment. Other than injection of the T_3 -treated animals, handling of the rats was kept to a minimum. Body weight data were recorded when treatment was initiated and again immediately before killing 1 week later.

Organ removal and preparation

Rats were euthanized by intraperitoneal injection of sodium pentobarbitol (2.0 μ l/g body weight; 50 mg/ml). A midline incision was made in the ventral side of the neck, and both lobes of the thyroid gland were removed.

In one series of experiments, the recovered glands were processed for standard histologic examination. In the remaining experiments, each gland was weighed, submerged in cold homogenizing buffer (250 mM sucrose, 50 mM Tris-HCl, 1 mM EGTA pH 7.4), and sonicated (Fisher Scientific Sonic Dismembrator 60, Pittsburgh, PA, USA) for 2×15 s. Homogenates from this procedure were used in a preliminary series of immunoblotting experiments. However, most experiments utilized crude membrane fractions, prepared from whole gland homogenates by ultracentrifugation ($400\,000 \times g$; 4°C ; 15 min). The resulting pellets were resuspended in 150–200 μl homogenizing buffer and stored at -80°C . The protein concentration of each sample was determined by the method of Lowry *et al.* (1951). In some experiments, blood samples were obtained by direct aortic puncture.

Circulating hormone measurements

Plasma concentrations of thyrotropin were determined by radioimmunoassay using a rat-specific commercially-available kit (Amersham International, Amersham, Bucks, UK). Radioimmunoassay kits were also used to measure plasma concentrations of total T_3 and T_4 (Diagnostic Products, Los Angeles, CA, USA).

Na,K-ATPase enzyme assay

Specific activities of Na,K-ATPase in crude membranes from rat thyroid were measured as the difference in rates of phosphate release from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence and presence of 3 mM ouabain (Muallem & Karlish 1980, Pressley *et al.* 1986). Sodium azide (3 mM) was added to minimize ouabain-insensitive ATPase activity. Detergents such as sodium deoxycholate are often used to open any sealed vesicles in membrane preparations. However, preliminary experiments indicated that treating with different amounts of detergent had negligible effects.

Gel electrophoresis and immunoblotting

Abundance of the Na,K-ATPase subunits was evaluated by immunoblotting. Thyroid homogenates or crude membranes were electrophoresed through sodium dodecyl sulfate (SDS)-polyacrylamide gels, then electroblotted onto nitrocellulose (Laemmli 1970, Towbin *et al.* 1979, Higham *et al.* 1993). Gels intended for detection of the α isoforms were prepared with 7.5% polyacrylamide; those intended for β_1 were prepared with 10%. Prior to loading, equal amounts of protein from each sample were combined with loading buffer and were heated at 65°C for 10 min. Boiling was avoided to prevent the formation of subunit aggregates. Following protein transfer, the nitrocellulose blots were stained with a nonspecific protein dye, Ponceau S, to confirm successful protein separation and transfer, as well as even loading of samples. Before probing

with specific antibodies directed against Na,K-ATPase α and β isoforms, each blot was preincubated in phosphate-buffered saline containing 5% (w/v) nonfat dried milk and 0.05% Tween 20 to minimize nonspecific binding. The α_1 isoform was detected with affinity-purified anti-NASE (1:500), a site-directed polyclonal antibody that recognizes an isoform-specific region near the ATP binding site. The isoform-specific site-directed antibodies, anti-HERED (1:500) and anti-TED (1:1000) were used to evaluate the α_2 and α_3 isoforms respectively. The specificity of all three site-directed antibodies for their respective isoforms has been demonstrated by a variety of criteria (Pressley 1992). Their names are derived from the sequences of the target oligopeptides. For the detection of the β subunit, we used polyclonal antisera specific for the β_1 isoform. It was generated from a bacteria-expressed β_1 fusion protein (FP β_1) (Dr Alicia McDonough, University of Southern California) and it was diluted 1:500. Antibody-antigen interactions were visualized with peroxidase-conjugated goat anti-rabbit IgA (1:5000), followed by chemiluminescence and exposure to Hyperfilm ECL (Amersham, Arlington Heights, IL, USA). Care was taken to remain within the linear response of the film. The optical densities of the detected bands were compared using whole band densitometry software running on a SPARCstation 2 (Sun Microsystems, Mountain View, CA, USA) equipped with an image analysis system (Bio Image, Ann Arbor, MI, USA).

Deglycosylation

In some experiments, proteins in the samples were deglycosylated prior to electrophoresis. Samples containing 200 μg protein in homogenizing buffer were incubated in 50 μl deglycosylation buffer (1 mM Tris-HCl; pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 1 $\mu\text{g}/\mu\text{l}$ soybean trypsin inhibitor and 1% CHAPS) for 1 h on ice. Then 50 mM sodium phosphate and 25 000 units/ml PNGase F were added to the sample. After incubation for 1 h at 37°C , an equal volume of loading buffer was added to stop the deglycosylation reaction. Proteins were then separated by electrophoresis as described above.

Statistical analysis

All data are expressed as means \pm s.e. with the number of samples shown in parentheses. Differences between groups were analyzed by Student's *t*-test or ANOVA, as appropriate, and were considered significant for $P < 0.05$.

Results

General animal observations

Various indices of thyroid status were monitored to verify hypo- and hyperthyroid conditions in treated animals

Table 1 Indices of thyroid status in treated rats. Rats were treated for 7 days, as described in Materials and Methods. Values are expressed as means \pm S.E. with the number of rats used in parentheses

	Body weight (g)		Thyroid weight (g)	Serum hormone concentrations		
	Pre-treatment	Post-treatment		T ₄ (μ g/ml)	T ₃ (ng/ml)	TSH (ng/ml)
Control	213.9 \pm 8.3 (10)	264.7 \pm 6.3 (10)	24.2 \pm 1.6 (10)	0.49 \pm 0.02 (5)	0.70 \pm 0.02 (5)	16.0 \pm 1.3 (5)
T ₃ -treated	203.4 \pm 4.7 (10)	215.7 \pm 2.1 (10)*	21.2 \pm 1.2 (10)	0.13 \pm 0.02 (5)*	110 \pm 21 (5)*	10.0 \pm 0.8 (5)*
PTU-treated	201.5 \pm 2.5 (12)	217.4 \pm 3.9 (12)*	34.2 \pm 2.3 (12)*	0.23 \pm 0.05 (6)*	0.34 \pm 0.05 (6)*	38.4 \pm 3.0 (6)*

* $P \leq 0.5$ when compared with control.

(Table 1). After 1 week, T₃-treated rats weighed significantly less than euthyroid controls, consistent with the increased metabolic rate of hyperthyroidism. Somewhat paradoxically, animals treated with PTU also weighed significantly less, suggesting that growth was minimal in the hypothyroid state. We limited treatment to 1 week to minimize any complications resulting from the depressed growth.

Examination of the glands provided further support for altered thyroid status. The PTU-treated rats exhibited obvious goiters, with a greater than 40% increase relative to controls. Histologic examination revealed an increase in number and size of the follicular cells and a profound decrease in the size of the lumen (data not shown). This hypertrophy of the thyroid gland is consistent with PTU-induced hypothyroidism and presumably results from an elevation of circulating thyroid-stimulating hormone (TSH) concentrations in response to the inhibition of thyroid hormone synthesis. This was confirmed by measurements of circulating hormone concentrations, which show the expected increase in TSH and corresponding decrease in T₄ with PTU treatment (Table 1). T₃ administration produced the expected increase in circulating T₃ and decrease in TSH, while T₄ concentrations were depressed.

Na,K-ATPase enzyme analysis

Having verified thyroid status in the three conditions, we determined if the activity of the Na,K-ATPase was altered within the thyroid gland (Fig. 1). Ouabain-sensitive hydrolysis of ATP in the follicular membranes from T₃-treated animals was not significantly different from controls. In contrast, the enzymatic activity in the PTU-treated samples was over four times greater than euthyroid controls. This hypothyroid-induced increase in enzymatic activity is in direct contrast to the effects observed in nonthyroidal tissues (e.g. McDonough *et al.* 1988).

Subunit abundance

Given the striking changes in Na,K-ATPase activity within the thyroid gland, we next examined the effects

of altered hormonal status on the abundance of the Na,K-pump's constituent subunits. Samples from whole gland lysates and isolated membranes were separated by SDS electrophoresis and transferred to nitrocellulose. Preliminary immunoblots of protein from whole gland homogenates probed with an α_1 -specific antibody, anti-NASE, displayed bands with a mobility appropriate for the subunit (~ 100 kDa) (Fig. 2A). Moreover, the mobility of these bands was indistinguishable from those in a rat kidney sample included as a control for antibody binding. The intensities of the α_1 bands in the samples from PTU-treated animals were significantly greater than those of euthyroid controls, while those in the T₃-treated samples were less (Table 2). Similar changes were observed when the blots were probed with a β_1 -specific antibody, FP β 1. Diffuse bands corresponding to ~ 55 kDa were detected in the gland homogenates (Fig. 2B). The intensities of these bands also increased in the PTU-treated samples and decreased in the T₃-treated samples. A kidney sample included as a control revealed a band of slightly different mobility, presumably reflecting tissue-specific variations in glycosylation. These results suggest that hypothyroidism produced an increase in Na,K-ATPase subunit abundance within the gland.

Such an analysis of whole gland homogenates might be compromised if there were substantial shifts in overall

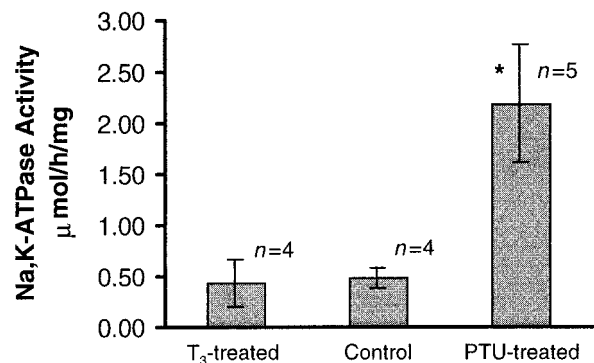


Figure 1 Effect of thyroid status on Na,K-ATPase specific activity in isolated membranes from thyroid gland. Rats were treated for 7 days as described in Materials and Methods. * $P < 0.05$ when compared with control.

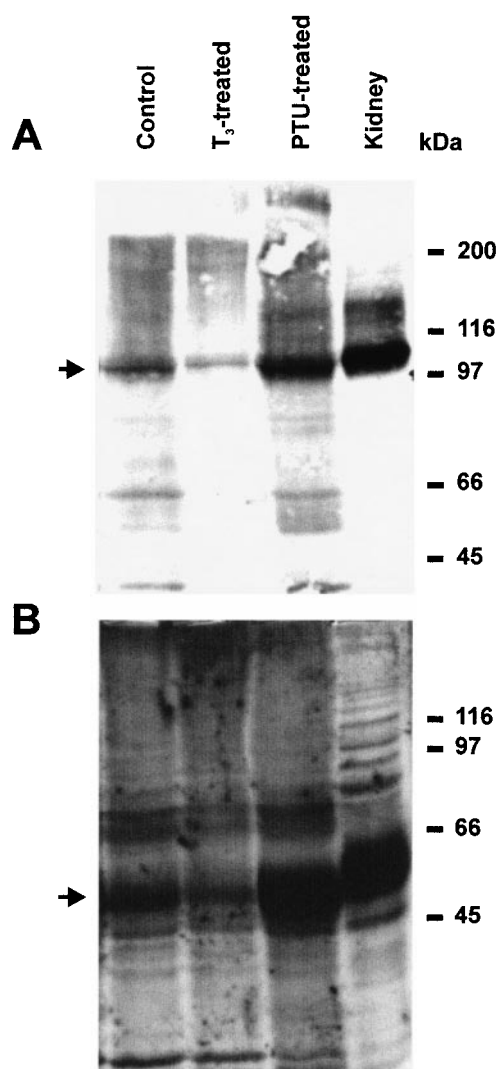


Figure 2 Effect of thyroid status on abundance of Na,K-ATPase subunits in gland homogenates. After 7-day treatment, proteins (100 μ g) were separated through denaturing polyacrylamide gels and then blotted onto nitrocellulose. Representative immunoblots were probed with anti-NASE, an antibody directed against the α_1 subunit (A), or FP β 1, an antibody directed against the β_1 subunit (B). The arrows indicate bands of the expected mobility. Rat kidney homogenate served as a positive control for antibody binding.

protein expression within the various thyroid states. A major change in thyroglobulin abundance induced by TSH, for example, would alter the mix of total protein that served as a basis for standardization. To minimize the contribution of thyroglobulin and other soluble proteins, we examined the abundance of pump subunits in crude membrane fractions from the glands of treated and control rats. Immunoblots of membrane protein probed with the α_1 - and β_1 -specific antibodies displayed bands similar to those in total homogenates (Fig. 3). Moreover, the

Table 2 Effect of thyroid status on Na, K-ATPase subunit abundance in gland homogenates. Values are expressed as means \pm S.E. with the number of rats used in parentheses. Abundance of subunits is expressed relative to control

	α -subunit	β -subunit
Control	1.00 \pm 0.02 (5)	1.00 \pm 0.06 (5)
T ₃ -treated	0.32 \pm 0.08 (5)*	0.36 \pm 0.10 (5)*
PTU-treated	2.62 \pm 0.20 (6)*	2.71 \pm 0.40 (6)*

* $P \leq 0.5$ when compared with control.

increases in intensity observed with PTU treatment were also evident (Table 3). An additional band of faster mobility was sometimes detected with the α_1 -specific antibody (\sim 50 kDa), but its identity remains unknown. Similarly, the β_1 -specific antibody detected a slower band (\sim 100 kDa). It is possible that this band contains β

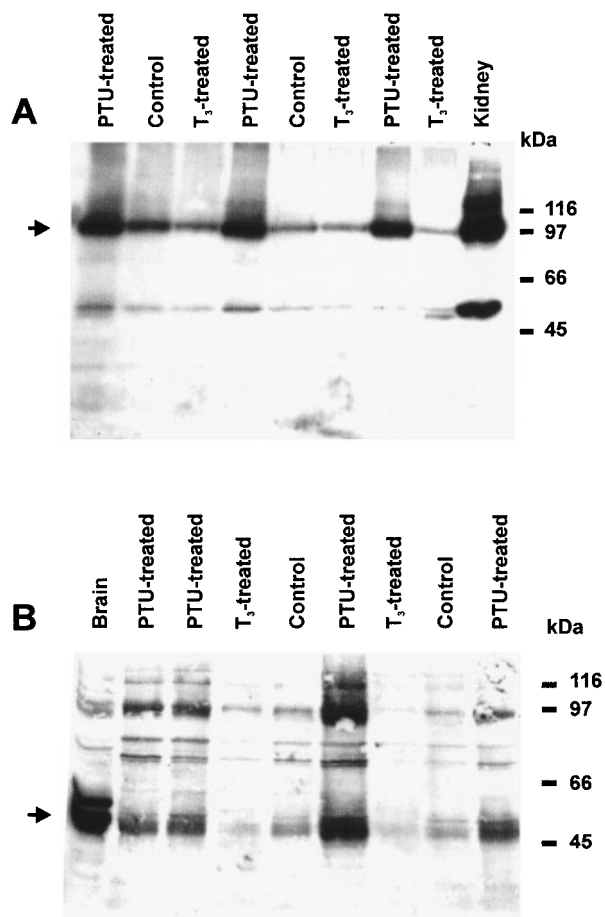


Figure 3 Effect of thyroid status on abundance of Na,K-ATPase subunits in isolated gland membranes. Details were as described in Fig. 1. Rat kidney and brain homogenates served as positive controls for antibody binding. The arrows indicate bands of the expected mobility.

Table 3 Effect of thyroid status on Na, K-ATPase subunit abundance in isolated gland membranes. Values are expressed as means \pm s.e. with the number of rats used in parentheses. Abundance of subunits is expressed relative to control

	α -subunit	β -subunit
Control	1.00 \pm 0.20 (7)	1.00 \pm 0.20 (7)
T ₃ -treated	0.25 \pm 0.06 (8)*	0.71 \pm 0.24 (8)
PTU-treated	2.36 \pm 0.48 (9)*	5.67 \pm 1.07 (9)*

* $P \leq 0.5$ when compared with control.

dimers. However, we know of no reports describing such aggregation among β subunits, and the identity of this band remains unknown.

Alternative isoforms of the α subunit

The α_1 isoform is the most ubiquitous of at least three forms of the catalytic subunit expressed in rat. In the thyroid, there was always the possibility that the α_1 isoform might be accompanied by α_2 and α_3 . Immunoblots of membrane proteins from thyroid glands probed with anti-HERED, an antibody specific for α_2 , displayed negligible binding, yet a band corresponding to 100 kDa was easily detected in a brain sample included as a positive control (data not shown). Anti-TED, an antibody specific for the α_3 isoform, revealed faint 100 kDa bands, but the intensities showed no systematic changes among the three treatment regimes (Fig. 4). A brain sample included as a positive control was greatly overexposed under the conditions necessary for detection in the thyroid samples, suggesting that α_3 is a relatively minor component of the Na,K-ATPase in the gland. Specificity of the anti-TED

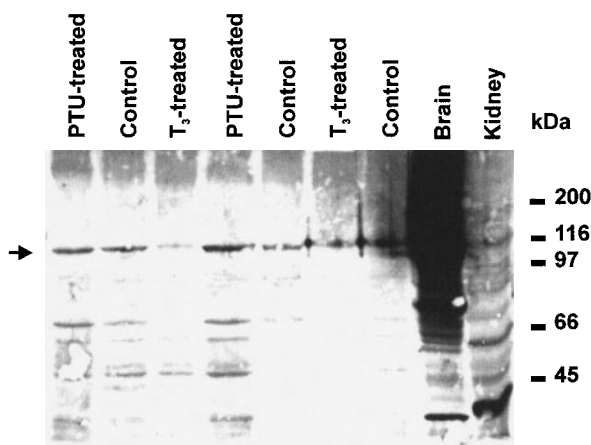


Figure 4 Effect of thyroid status on abundance of Na,K-ATPase α_3 subunit in isolated gland membranes. Details were as described in Fig. 1, except that anti-TED, an antibody that recognizes the α_3 subunit, was used as probe. Rat kidney and brain homogenates served as positive controls for antibody binding. The arrows indicate bands of the expected mobility.

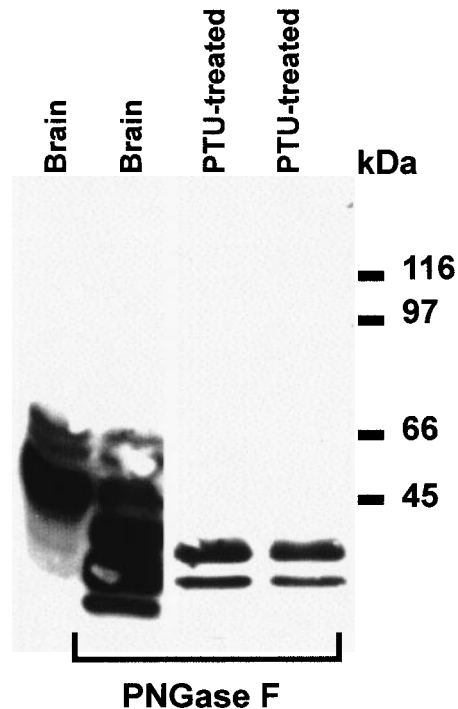


Figure 5 Deglycosylation of β_1 subunit in thyroid gland membranes. Proteins from PTU-treated animals were incubated in the presence of PNGase F prior to electrophoresis. Representative immunoblot was probed with FB β 1. PNGase-treated and nontreated brain homogenates served as positive controls.

was confirmed by the negligible binding to kidney, which expresses very little α_3 .

Specificity of the β_1 antisera

Immunoblots are only as good as the antibodies used for their analysis. Admittedly, the banding pattern using the anti- β_1 antisera was complex, raising questions about the specificity of the antibody. This issue was explored further with deglycosylation experiments. If the diffuse bands detected with the antisera were indeed β_1 , we would expect to see their mobility increase after removal of the N-linked polysaccharide side-chains. Immunoblots of thyroid gland samples treated with PNGase F did not reveal a band at 55 kDa, but instead generated bands between 32 and 40 kDa (Fig. 5), a size consistent with deglycosylated β_1 . A similar shift in mobility was observed for the detected bands in the brain control. These results confirm the presence of glycosylation in the antigen detected with the anti- β_1 antisera, as would be expected if the bands were genuine β_1 .

Discussion

These results strongly suggest that hypothyroidism increases the number of Na,K-ATPase subunits in the rat

thyroid gland. The abundance of the pump's constituent subunits, as measured with specific antibodies, was clearly altered by treatments that produce profound changes in circulating hormone concentrations. To our knowledge, this is the first such assessment of pump abundance in the thyroid gland. That the effects were seen in both whole gland homogenates and crude membrane preparations argues that the changes are not an artifact of the shifts in overall protein composition that accompany the different thyroid regimes. For example, a major component of total protein in the gland is thyroglobulin. One might have argued that the degradation of thyroglobulin to its constituent amino acids is stimulated by TSH, producing a decline in the amount of thyroglobulin that could yield an overestimate of pump subunit abundance when standardized to total protein. Although this might occur in whole gland homogenates, it seems unlikely to be a problem in isolated membranes.

Accompanying the increase in pump subunit abundance with hypothyroidism was a dramatic increase in Na,K-ATPase specific activity. The four-fold elevation in enzymatic activity that we observed with PTU treatment is nearly identical to the increase seen in rats treated with methimazole, another inhibitor of iodide organification (Chow *et al.* 1982). In the earlier work, the specific activities were standardized to DNA content rather than total protein. The consistency of the results, despite the differences in standardization, suggests that the apparent changes in activity are not an artifact of cell growth or proliferation. Moreover, the four-fold changes seen in the thyroid gland may be one of the more robust regulatory responses of the pump, at least in differentiated tissue. The activity of the renal enzyme in response to glucocorticoids, for example, increases by a more modest 100–150% (Sinha *et al.* 1981).

The glandular changes in pump activity and abundance are in direct contrast to those seen in the periphery. Hyperthyroidism in organs such as the liver and kidneys results in an increased pump activity and abundance (Gick *et al.* 1988). In the thyroid gland, however, it is hypothyroidism that produces such an increase. Although this might reflect a negative regulatory effect of thyroid hormones on the gland, it seems more likely that TSH is the direct mediator. The changes in circulating hormone levels, gland weight and histology produced by treatment with T_3 or PTU were consistent with the expected feedback regulation of pituitary thyrotropin secretion elicited by the hyperthyroid and hypothyroid conditions respectively. Moreover, a response by the pump to thyrotropin would have physiologic significance. The increase in thyroid hormone synthesis after stimulation by TSH requires an elevated uptake of iodide, which, in turn, depends on the Na^+ electrochemical gradient. Dissipation of that gradient by aggressive iodide transport would be minimized if there was an accompanying increase in the Na,K-pump.

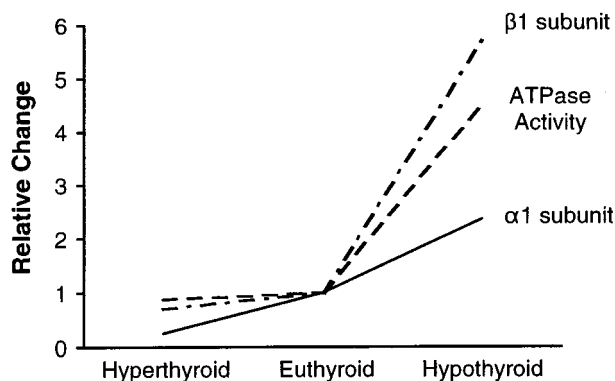


Figure 6 Relative changes in α_1 and β_1 subunit abundance and enzyme activity with altered thyroid status. This graph combines data from T_3 -treated, control, and PTU-treated rats. All data were standardized to control values.

Of course, one cannot dismiss the possibility that it is not TSH, but some additional factor responsive to thyroid status that is controlling pump abundance. Regulation of the follicular epithelium is mediated by an extensive autocrine/paracrine system that includes several key cytokines. Tumor necrosis factor α , interferon- γ , and interleukin-1 α have all been shown to decrease the abundance of mRNAs encoding thyroid-specific enzymes (Pekary *et al.* 1994, Ajjan *et al.* 1998, Pekary & Hershman 1998). A potential intermediary in this signaling system may be transforming growth factor β_1 (TGF- β_1), whose expression is modulated by both cytokines and thyrotropin in cell culture models (Pekary *et al.* 1995, Gärtner *et al.* 1997). Moreover, TGF- β_1 appears to have potent inhibitory effects on Na,K-ATPase expression (Pekary *et al.* 1997). It follows that modulation of the Na,K-pump within the gland may be the end result of multiple signaling pathways.

Regardless of the identity of the mediator(s) responsible for the changes in enzymatic activity, it is apparent that the response of the constituent subunits is discoordinate (Fig. 6). The abundance of the α_1 subunit did not vary as widely as the β_1 subunit, which increased nearly six-fold in the PTU-treated animals. Changes in the specific activity of the Na,K-ATPase were distinct from the changes in either subunit, although of the two, the enzymatic activity followed β_1 abundance more closely. Discrepancies between the subunits and enzymatic activity have been observed in other regulatory models. For example, differentiation of 3T3 L1 fibroblasts into adipocytes in culture is associated with an increase in the α_2 isoform, yet there is little relation among the changes in mRNA abundance, the α_2 polypeptide, and enzymatic activity attributable to the α_2 isoform (Russo *et al.* 1990). Similarly, stimulation of cultured cardiomyocytes with thyroid hormone produces discoordinate changes in Na,K-ATPase mRNA and polypeptide abundance (Hensley *et al.* 1992).

Although we hesitate to extrapolate too far based on measurements from only three regulatory states, the dis-coordinate regulation observed in the thyroid suggests that the β subunit is controlling pump abundance. The α_1 subunit may always be overexpressed relative to β_1 , such that large increases are not needed to augment the number of $\alpha\beta$ dimers. To achieve the four-fold increase in enzymatic activity, the amount of β_1 subunit must also increase four-fold or more. A slightly larger increase in β may compensate for inefficiencies in the assembly process. The β subunit has been implicated as a major determinant of pump number in several other regulatory systems, including heterologous expression (Ackermann & Geering 1990, Shanbaky & Pressley 1995), development (MacPhee *et al.* 1994) and response to hormones (Gick *et al.* 1990, Hensley *et al.* 1992).

The subunits of the Na,K-pump exist in multiple forms, and a focus on α_1 and β_1 might ignore the contributions of other isoforms. The negligible binding of an α_2 -specific antibody and the barely detectable binding of an α_3 -specific antibody to thyroid samples in the current study would seem to rule out an important role for alternative catalytic isoforms. The contribution of additional β isoforms, if present, would exaggerate the dis-coordinate regulation that we observed. Additional studies will be necessary to determine if the lack of coordination extends to the level of the mRNAs encoding the subunits. Moreover, it remains to be seen whether the increases in subunit abundance are the result of increased translation, decreased degradation, or both.

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