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STIMULATION OF SPECIFIC [^{3}H]-OUABAIN BINDING TO MICROSOMAL PREPARATIONS FROM RAT HEART AND SKELETAL MUSCLE BY THYROID HORMONES: EFFECTS OF 6-HYDROXYDOPAMINE

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1 Surgical thyroidectomy decreased specific [^{3}H]-ouabain binding to heart ventricular microsomes by 43% and gastrocnemius muscle microsomes by 34%. Administration of triiodothyronine to euthyroid rats enhanced specific [^{3}H]-ouabain binding to heart and skeletal muscle membrane by 60% and 33% respectively.

2 Treatment of thyroidectomized rats with triiodothyronine increased specific [^{3}H]-ouabain binding by 44% in skeletal muscle membrane preparation and 428% in cardiac microsomes.

3 Specific [^{3}H]-ouabain binding decreased by 55% in heart and 53% in gastrocnemius muscle preparations following chemical sympathectomy with 6-hydroxydopamine.

4 Treatment with triiodothyronine of euthyroid rats which had been sympathectomized did not significantly alter specific [^{3}H]-ouabain binding to heart or skeletal muscle membrane preparations.

5 Administration of triiodothyronine to thyroidectomized and sympathectomized rats increased specific [^{3}H]-ouabain binding by 80% in heart and 83% in skeletal muscle membrane preparations.

6 These results suggest that triiodothyronine may influence specific [^{3}H]-ouabain binding to thyroid hormone nonresponsive tissue such as sympathetic nerve endings. Therefore, the present observations are incompatible with the hypothesis that induction of ($Na^+ + K^+$)-adenosine triphosphatase of skeletal muscle membrane is the molecular mechanism for the calorigenic actions of thyroid hormones.

Introduction

Thyroid hormone administration has been shown to increase the activity of ($Na^+ + K^+$)-adenosine triphosphatase (($Na^+ + K^+$)-ATPase) in thermogenic organs such as liver, heart, kidney, skeletal muscle, smooth muscle, and intestinal epithelium (Ismail-Beigi & Edelman, 1970; 1971; 1973; 1974; Israel, Videla, MacDonald & Bernstein, 1973; Asano, Liberman & Edelman, 1976; Lo, August, Liberman & Edelman, 1976). This activation of ($Na^+ + K^+$)-ATPase by thyroid hormones is not observed in non-thermogenic organs such as brain (Ismail-Beigi & Edelman, 1971; Edelman & Ismail-Beigi, 1974). Thyroid hormone-induced enhancement of ($Na^+ + K^+$)-ATPase activity has been suggested to lead to increased utilization of ATP and a resultant drop in the phosphorylation potential (ATP/ADP × Pi) which normally regulates the rate of mitochondrial oxidation (Chance & Maitra, 1963). This drop in the phosphorylation potential stimulates mitochondrial respiration which is believed to be the biochemical basis for thermogenic action of thyroid hormone (Edelman, 1974; 1975; 1976; Israel, Videla & Bernstein, 1975). Since induction of mRNA synthesis at the transcriptional level has been shown to mediate the thermogenic response to thyroid hormone (Tata, 1963; Tata, Ernster, Arrhenius, Pederson & Hedman, 1963), Edelman (1975) proposed that the induced protein either may be an element of the ($Na^+ + K^+$)-ATPase and may be inserted into the plasma membrane or may activate the transport enzyme system found in the membrane.

The purpose of the present investigation was to explore further the hypothesis regarding induction of ($Na^+ + K^+$)-ATPase in the thermogenic response to thyroid hormone. Since ouabain exclusively binds to ($Na^+ + K^+$)-ATPase of a microsomal preparation Smith, Wagner & Young, 1974; Ruoho & Kyte, 1974; Schwartz, Lindenmayer & Allen, 1975; Hegyvary, 1975) specific [^{3}H]-ouabain binding to cardiac and skeletal muscle membrane preparations from euthyr-

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oid and thyroidectomized rats was measured to determine the effect of thyroid status on the number of $(Na^+ + K^+)$-ATPase molecules on thermogenic cell surfaces. Furthermore, a large proportion of $(Na^+ + K^+)$-ATPase found in cardiac and skeletal muscle microsomal suspensions has been shown to be located at the sympathetic nerve endings of these organs (Sharma, Dasgupta & Banerjee, 1977; Sharma & Banerjee, 1977b, c). Since neuronal cells are not believed to exhibit thermogenesis, the effect of thyroid hormones on $(Na^+ + K^+)$-ATPase located on sympathetic nerve endings of heart and skeletal muscle membrane preparations was estimated by examining the effects of these hormones before and after selective destruction of catecholaminergic nerve terminals to peripheral organs by 6-hydroxydopamine (Thoenen & Tranzer, 1973). Although the present results are in agreement with earlier observations of augmentation of $(Na^+ + K^+)$-ATPase activity by thyroid hormones, the molecular mechanism for increased $(Na^+ + K^+)$-ATPase activity does not appear to involve induction of this enzyme system.

Methods

Euthyroid and thyroidectomized male Sprague-Dawley rats (120 to 160 g) were divided into two major groups, A and B, each consisting of 36 rats. To destroy the catecholaminergic nerve endings effectively in Group A, the animals were treated with injections of 6-hydroxydopamine hydrobromide into the tail vein, 2 doses of 50 mg/kg on the first day and 2 doses of 68 mg/kg one week later, as described by Thoenen & Tranzer (1968). These animals were killed one week after the last dose of 6-hydroxydopamine. The rats of Group B, which served as controls, received equal volumes of 0.9% w/v NaCl solution (saline). Na^+-dependent [^{3}H]-noradrenaline uptake was measured in the saline- and 6-hydroxydopamine-treated heart and spleen slices, as described before (Sharma & Banerjee, 1977a). The velocity of [^{3}H]-noradrenaline uptake was 13.8 ± 1.3 and 39.7 ± 3.7 pmol g^{-1} 30 min^{-1} in control heart and spleen slices, respectively. Administration of 6-hydroxydopamine decreased the velocity of [^{3}H]-noradrenaline uptake to 1.4 ± 0.1 and 3.4 ± 0.5 pmol g^{-1} 30 min^{-1} in heart and spleen slices, respectively.

Each of the two major groups was divided into 4 subgroups of 9 rats each, as follows: control; thyroidectomized; control treated with triiodothyronine; and thyroidectomized treated with triiodothyronine. Thyroidectomized (5 weeks after surgery) or euthyroid rats were injected with 50 µg of triiodothyronine/100 g of body weight on alternate days for 3 days.

The procedure for the preparation of the microsomal fractions of heart ventricle and gastrocnemius muscle was similar to that of Schwartz, Nagano, Nakao, Lindenmayer, Allen & Matsui (1971), as described by Sharma & Banerjee (1977a).

The assay for specific [^{3}H]-ouabain binding to microsomal fractions has been described by Sharma & Banerjee (1977a) and Banerjee & Sharma (1978). Briefly, the assay consisted of incubation of microsomal suspensions (0.7 to 1.5 mg protein/ml) at 37°C for 20 min in 1 ml of 0.05 M Tris HCl buffer (pH 7.4) containing various concentrations (40 to 640 nM) of [^{3}H]-ouabain, 4 mM $MgCl_2$, and 1 mM inorganic phosphate. After incubation, 3 ml of ice-cold 0.05 M Tris HCl buffer was added to each tube, and the mixture was filtered and washed over glass fibre filter papers (Reeve-Angel) as described previously (Sharma & Banerjee, 1977a). Corrections were made for non-specific accumulation of [^{3}H]-ouabain by assaying parallel incubations in which Mg^{2+} and inorganic phosphate were replaced by 0.2 M Na^+. Specific binding was obtained by subtracting from the total radioactivity the number of counts per minute found in the presence of excess Na^+. The filter papers were dried and each filter paper was transferred to a counting vial containing 10 ml of Scintiverse (Fisher Co.) and counted in a Packard Tricarb liquid scintillation spectrometer (Model 3380) at 30% efficiency as determined with internal standards. Specific [^{3}H]-ouabain binding was over 90% of the total binding. Triiodothyronine was purchased from Sigma Chemical Co., St. Louis, Missouri, and [^{3}H]-ouabain (10 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Massachusetts.

Results

Specific [^{3}H]-ouabain binding to rat heart ventricle and gastrocnemius muscle microsomal fractions is shown in Table 1. When the concentration of [^{3}H]-ouabain was 160 nM, specific binding to euthyroid rat heart membrane preparations was 1.26 ± 0.06 pmol/mg protein as compared to 0.7 ± 0.03 pmol/mg protein obtained with skeletal muscle microsomal fractions. Thyroidectomy decreased specific [^{3}H]-ouabain binding to heart microsomes by 43% and to gastrocnemius muscle microsomes by 34%. Administration of triiodothyronine to euthyroid rats enhanced specific [^{3}H]-ouabain binding to heart and skeletal muscle membranes by 60% and 33% respectively. The effect of triiodothyronine on specific [^{3}H]-ouabain binding to thyroidectomized rat tissue preparations was even more pronounced. In skeletal muscle membrane preparations, specific binding increased 44% and in cardiac microsomes, 428%. All

these differences were statistically highly significant ($P < 0.001$; Table 1). The percentage increases in specific [^{3}H]-ouabain binding to heart microsomes in response to triiodothyronine treatment were higher than those noted with the gastrocnemius muscle membrane preparations. This observation is consistent with the findings of Ismail-Beigi & Edelman (1973), who reported that administration of triiodothyronine resulted in a fall in intracellular Na^+ concentration and a 7% rise in intracellular K^+ concentration, leading to a significant depression (20%) of intracellular Na^+/K^+ ratio in thyroidectomized rat skeletal muscle. In contrast, the effect was more marked in cardiac tissue of thyroidectomized rats, where triiodothyronine evoked a 29% fall in intracellular Na^+ and a 7% rise in intracellular K^+ concentrations. The intracellular Na^+/K^+ ratio decreased by 34% (Ismail-Beigi & Edelman, 1973). Thus the results shown in Table 1 demonstrate a specific influence of triiodothyronine in the regulation of ouabain binding sites, which provides strong support for Edelman's original hypothesis on the role of thyroid hormones in the regulation of the ($Na^+ + K^+$)-ATPase activity of thermogenic muscle cell plasma membrane.

We measured the distribution of specific [^{3}H]-ouabain binding in sympathetic nerve endings of several peripheral organs of cats (Sharma *et al.*, 1977; Sharma & Banerjee, 1977b, c). Specific [^{3}H]-ouabain binding to ($Na^+ + K^+$)-ATPase in sympathetic nerve endings was estimated by measuring the difference between specific [^{3}H]-ouabain binding to microsomes of peripheral organs of control and 6-hydroxydopamine-treated cats. Since 6-hydroxydopamine is selectively accumulated in sympathetic nerve endings by a high affinity uptake mechanism for noradrenaline and is highly toxic to these cells, intravenous administration of this drug causes a complete chemical sympathectomy (Thoenen & Tranzer, 1968; 1973). We decided to examine the effects of triiodothyronine and thyroidectomy on specific [^{3}H]-ouabain binding to sympathectomized rat heart and skeletal muscle membrane preparations (Table 2). Specific [^{3}H]-ouabain binding to chemically sympathectomized euthyroid rat heart and gastrocnemius muscle membrane preparations was 0.57 ± 0.03 and 0.33 ± 0.01 pmol/mg protein, respectively. Administration of triiodothyronine to euthyroid rats that had previously been sympathectomized did not significantly alter specific [^{3}H]-ouabain binding to heart or skeletal muscle membrane preparations (Table 2). However, chemical sympathectomy of thyroidectomized rats significantly decreased specific [^{3}H]-ouabain binding by 32% in heart and 63% in skeletal muscle microsomes. Administration of triiodothyronine to thyroidectomized and sympathectomized rats increased specific [^{3}H]-ouabain binding by 80% in heart and 83% in skeletal muscle membrane preparations. These changes were statistically highly significant.

Table 1 Specific [^{3}H]-ouabain binding to microsomal fractions obtained from cardiac and gastrocnemius muscles of thyroidectomized and euthyroid rats

Muscle and thyroid status	*Specific [^{3}H]-ouabain binding (pmol/mg protein)*
Heart	
Euthyroid	1.26 ± 0.06
Euthyroid + triiodothyronine	2.02 ± 0.11*
Thyroidectomized	0.72 ± 0.05
Thyroidectomized + triiodothyronine	3.08 ± 0.15*
Thyroidectomized	0.72 ± 0.05*
Euthyroid	1.26 ± 0.06
Skeletal	
Euthyroid	0.70 ± 0.03
Euthyroid + triiodothyronine	0.93 ± 0.05*
Thyroidectomized	0.46 ± 0.02
Thyroidectomized + triiodothyronine	0.66 ± 0.03*
Thyroidectomized	0.46 ± 0.02
Euthyroid	0.70 ± 0.03*

The concentration of [^{3}H]-ouabain was 160 nM. Values are means ± s.e. of 9 determinations of 3 separate experiments.

* Significantly different from the preceding value, $P < 0.001$.

Table 2 Specific [^{3}H]-ouabain binding to muscle microsomal fractions of euthyroid and thyroidectomized rats following chemical sympathectomy

Muscle and thyroid status	*Specific [^{3}H]-ouabain binding (pmol/mg protein)*
Heart	
Euthyroid	0.57 ± 0.03
Euthyroid + triiodothyronine	0.65 ± 0.03
Thyroidectomized	0.39 ± 0.02
Thyroidectomized + triiodothyronine	0.70 ± 0.04*
Thyroidectomized	0.39 ± 0.02
Euthyroid	0.57 ± 0.03*
Skeletal	
Euthyroid	0.33 ± 0.01
Euthyroid + triiodothyronine	0.29 ± 0.01
Thyroidectomized	0.12 ± 0.01
Thyroidectomized + triiodothyronine	0.22 ± 0.01*
Thyroidectomized	0.12 ± 0.01
Euthyroid	0.33 ± 0.01*

Euthyroid and thyroidectomized rats were chemically sympathectomized by administration of 6-hydroxydopamine, as described in Methods. The concentration of [^{3}H]-ouabain was 160 nM. Values are means ± s.e. of 9 determinations of 3 separate experiments.
* Significantly different from preceding value, $P < 0.001$.

In order to evaluate the effects of thyroid status on specific [^{3}H]-ouabain binding to sympathetic nerve endings, the values for specific binding in Table 2 were subtracted from those in Table 1. The results, which provide an estimate of specific [^{3}H]-ouabain binding to sympathetic nerve endings (Table 3), indicate that thyroid status may play an important role in the regulation of specific [^{3}H]-ouabain binding sites located at the peripheral nerve endings.

Table 3 Estimated specific [^{3}H]-ouabain binding to sympathetic nerve endings of rat cardiac and gastrocnemius muscles

Muscle and thyroid status	*Specific [^{3}H]-ouabain binding (pmol/mg protein)*
Heart	
Euthyroid	0.69 ± 0.02*
Euthyroid + triiodothyronine	1.37 ± 0.04
Thyroidectomized	0.33 ± 0.02*
Thyroidectomized + triiodothyronine	2.38 ± 0.10
Skeletal	
Euthyroid	0.37 ± 0.02*
Euthyroid + triiodothyronine	0.64 ± 0.03
Thyroidectomized	0.34 ± 0.01*
Thyroidectomized + triiodothyronine	0.44 ± 0.02

The concentration of [^{3}H]-ouabain was 160 nM. Specific [^{3}H]-ouabain binding to sympathetic nerve endings was estimated by subtracting specific binding observed in the muscle microsomal preparations obtained from 6-hydroxydopamine-treated rats from those found in microsomal fractions derived from heart and skeletal muscles of rats not so treated.
* $P < 0.001$.

Discussion

The primary objective of this work was to explore the molecular mechanism for activation of $(Na^+ + K^+)$-ATPase by triiodothyronine and to evaluate the importance of this activation of the transport enzyme system in the thermogenic actions of thyroid hormones. Since ouabain is a highly specific inhibitor of $(Na^+ + K^+)$-ATPase (Ruoho & Kyte, 1974; Smith *et al.*, 1974; Schwartz *et al.*, 1975; Hegyvary, 1975), we measured specific [^{3}H]-ouabain binding to rat cardiac and skeletal muscle membrane preparations under different thyroid status to determine the effects of this hormone on the number of $(Na^+ + K^+)$-ATPase molecules on surface membranes. Again $(Na^+ + K^+)$-ATPase in cardiac and skeletal muscle microsomes appears to be predominantly located at the sympathetic nerve endings (Sharma *et al.*, 1977; Sharma & Banerjee, 1977b, c). Since $(Na^+ + K^+)$-ATPase located on peripheral nerve cells is not believed to contribute to the thermogenic actions of thyroid hormones, the effects of thyroid status on $(Na^+ + K^+)$-ATPase located on sympathetic nerve endings of heart and skeletal muscle membrane preparations were also evaluated.

Specific [^{3}H]-ouabain binding was significantly increased in thyroidectomized and euthyroid rat cardiac and skeletal muscle microsomal suspensions following administration of triiodothyronine (Table 1). Similarly, there was a significant difference between thyroidectomized and euthyroid rats in specific [^{3}H]-ouabain binding to cardiac and skeletal muscle microsomes (Table 1). These observations are in agreement with earlier reports on the effects of thyroid status on $(Na^+ + K^+)$-ATPase of cardiac and skeletal muscle (Ismail-Beigi & Edelman, 1973; Asano *et al.*, 1976; Curfman, Crowley & Smith, 1977; Sharma & Banerjee, 1978; Lin & Akera, 1978).

Administration of 6-hydroxydopamine decreased specific [^{3}H]-ouabain binding by 55% in heart and 53% in gastrocnemius muscle preparations (Tables 1 and 2). Since neuronal cells are not recognized as target tissue of thyroid hormones (Barker, 1964), we measured the effect of thyroid status on specific [^{3}H]-ouabain binding on chemically sympathectomized rat cardiac and skeletal muscle preparations. There is considerable evidence that sympathetic innervation of the thyroid gland plays an important role in the regulation of thyroid hormone secretion (Melander, Ericson & Sundler, 1974; Sterling & Lazarus, 1977). Therefore, it is possible that ablation of sympathetic innervation will decrease thyroid hormone secretion, which would lead to a decrease in $(Na^+ + K^+)$-ATPase activity. However, Melander *et al.* (1974) have reported that the changes in the slope of the blood radioiodine curve that followed sympathectomy were transient, persisting for only 1 or 2 days before a normal slope was re-established. In addition, sympathectomy performed a week before blood radioiodine studies were initiated did not affect the slope of the blood radioiodine curves subsequently obtained. Thus, these findings suggest that the presumed reduction in thyroid hormone secretion caused by sympathectomy is short-lived, probably because of a compensatory increase in the secretion of thyroid stimulating hormone.

Specific [^{3}H]-ouabain binding estimated on sympathetic nerve endings to heart and gastrocnemius muscle blood vessels was found to be significantly influenced by thyroid status (Table 3). Since sympathetic nerves are not considered to be thermogenic cells (Barker, 1964), activation of $(Na^+ + K^+)$-ATPase does not appear to be the molecular basis for biochemical actions of thyroid hormones. This observation is inconsistent with an earlier hypothesis of Edelman (1976) that induction of $(Na^+ + K^+)$-ATPase of skeletal muscle membrane is the molecular mechanism for the thermogenic action of thyroid hormones.

Recently, Lin & Akera (1978) reported that thyroid hormone may influence $(Na^+ + K^+)$-ATPase activity by regulating the density of these enzyme molecules. These results are in conflict with our observations (Sharma & Banerjee, 1978). They used euthyroid rats, comparing untreated and triiodothyronine-treated animals. But since the rat is a hyperthyroid animal (Yazaki & Raben, 1975), further administration of triiodothyronine would not significantly alter the thyroid status. In fact, in these two groups of animals, there is no significant difference in the density of β-adrenoceptors (Banerjee & Kung, 1977) or noradrenaline-stimulated adenylate cyclase activity (Levey, Skeleton & Epstein, 1969; McNeill, Muschek & Brody, 1969). Administration of triiodothyronine to thyroidectomized rats increased ouabain binding by about 400% (Sharma & Banerjee, 1978). Only in these two groups could we detect significant alteration in the apparent affinity for ouabain by displacement curves. Lin & Akera (1978) also adopted this procedure. Since displacement curves are drawn on a log scale, this method is not efficient for detecting small changes in affinity constants. It is hence not surprising that, more recently, Chubb, Akera & Brody (1978) found that triiodothyronine does alter the affinity constant for ouabain, in agreement with our previous work.

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