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Specific [^{3}H]Ouabain Binding to Rat Heart and Skeletal Muscle: Effects of Thyroidectomy

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SUMMARY

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Administration of triiodothyronine increased specific [^{3}H]ouabain binding by 46% in gastrocnemius muscle and 54% in heart ventricle muscle microsomal suspensions derived from euthyroid rats. Thyroidectomy decreased specific [^{3}H]ouabain binding by 37% and 56% in skeletal and heart muscle microsomes, respectively. Administration of triiodothyronine to thyroidectomized rats increased specific [^{3}H]ouabain binding by 69% in gastrocnemius muscle and 425% in cardiac muscle membrane preparations. Scatchard analysis revealed that regulation of specific [^{3}H]ouabain binding sites by triiodothyronine is mediated by alterations of dissociation constants and not by changes in the maximal number of binding sites. These observations are incompatible with the hypothesis that induction of (Na^+ + K^+)-ATPase of cardiac and skeletal muscle membrane is the molecular mechanism for the thermogenic action of thyroid hormones.

INTRODUCTION

Thyroid hormone administration has been shown to increase the rate of oxygen consumption of the liver, heart, kidney, and skeletal muscle, which is believed to be due to increased (Na^+ + K^+)-ATPase activity (1-4). Evidence has been presented in support of the hypothesis that thyroid hormone stimulates energy expended in active Na^+ transport in three of the primary thermogenic target tissues: liver, kidney, and skeletal muscle (5-11). According to Edelman (6, 7, 9), in these tissues this effect mediates a significant portion—and in some circumstances virtually all—of the thermogenic effect. The Na^+-dependent fraction of thyroid hormone-induced thermogenesis appears to involve direct augmentation of (Na^+ + K^+)-ATPase activity, in that the Na^+_i:K^+_i ratio tends to fall (5-9). The physiological significance of the increase in the transmembrane electrochemical gradient for Na^+ has not yet been explored. Furthermore, in view of the evidence advanced by Tata and his associates (12, 13), induction of mRNA and protein synthesis has been considered the underlying mechanism in the activation of (Na^+ + K^+)-ATPase (5-9). Nevertheless, the molecular mechanisms that mediate thyroidal augmentation of active Na^+ transport remain to be elucidated.

In the present investigation attempts have been made to determine whether the

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augmentation of $(Na^+ + K^+)$-ATPase activity by thyroid hormone could result from activation of a fixed number of enzyme sites or from an increase in the total number of sites, by measuring specific [^{3}H]ouabain binding to microsomal suspensions derived from heart and gastrocnemius muscle of euthyroid and thyroidectomized rats. Although the present results are in agreement with earlier observations of augmentation of $(Na^+ + K^+)$-ATPase activity by thyroid hormones (1–11), the molecular mechanism for increased $(Na^+ + K^+)$-ATPase activity does not appear to involve induction of this enzyme system.

MATERIALS AND METHODS

Male Sprague-Dawley rats (120–160 g) were divided into four groups, each containing nine rats. One group each of thyroidectomized (5 weeks after surgery) and euthyroid rats received injections of 50 μg of triiodothyronine per 100 g of body weight on alternate days for a total of three doses. Animals were killed 24 hr after the last dose of triiodothyronine. The other two groups, one of thyroidectomized and one of euthyroid rats, received no triiodothyronine. The procedure for the preparation of the microsomal fractions of heart ventricle and gastrocnemius muscle was similar to that of Schwartz *et al.* (14) as described before (15).

The assay for specific [^{3}H]ouabain binding to microsomal fractions has been described before (15). Briefly, the binding assay consisted of incubation of the microsomal suspension (0.7–1.5 mg of protein per milliliter) at 37° for 20 min in 1 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing various concentrations of [^{3}H]ouabain, 4 mM $MgCl_2$, and 1 mM Tris-P_i. After incubation, 3 ml of ice-cold 0.05 M Tris-HCl buffer were added to each tube, and the mixture was filtered and washed over glass fiber filter papers (Reeve Angel) as described previously (15). Corrections were made for nonspecific accumulation of [^{3}H]-ouabain by assaying parallel incubations in which Mg^{++} and inorganic phosphate were replaced by 0.2 M NaCl. Specific binding was obtained by subtracting from the total radioactivity the 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) and counted in a Packard Tri-Carb 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. Ouabain-sensitive ATP hydrolytic activity was measured by the method of Post and Sen (16) as described before (17), except that 1 mM [^{32}P]ATP was used instead of 3 mM unlabeled ATP. Triiodothyronine was purchased from Sigma Chemical Company, and [^{3}H]ouabain (10 Ci/mmole) and [^{32}P]ATP (150 Ci/mmole) were obtained from New England Nuclear.

RESULTS

Initial experiments were performed with whole homogenates of rat heart and gastrocnemius muscle. Specific [^{3}H]ouabain binding to these two muscle preparations was relatively low and extremely variable. Since cardiac glycosides exhibit remarkable species and tissue selectivity (15, 18) and the non-neuronal tissue $(Na^+ + K^+)$-ATPase activity of rodents is relatively insensitive to inhibition by ouabain (16), these results are not surprising. Asano and his associates (8), however, were able to demonstrate augmentation of $(Na^+ + K^+)$-ATPase activity by administration of triiodothyronine to thyroidectomized rats in a partially purified microsomal suspension obtained following treatment with EDTA and deoxycholate. Therefore we decided to measure [^{3}H]ouabain binding in a partially purified membrane preparation, prepared by a slightly different method (14) involving the use of both deoxycholate and EDTA (15).

Specific [^{3}H]ouabain binding to heart and gastrocnemius muscle membrane preparations derived from euthyroid rats with and without triiodothyronine treatment is shown in Tables 1 and 2. Specific [^{3}H]ouabain binding to the microsomes of gastrocnemius muscle was found to be 378 fmoles/mg of protein, compared with 821

TABLE 1

Specific [^{3}H]ouabain binding to microsomal fraction obtained from gastrocnemius muscle of rats

The concentration of [^{3}H]ouabain was 80 nM. Values are means ± standard errors of nine determinations in three separate experiments. The procedure for measuring specific [^{3}H]ouabain binding is described in the text.

Thyroid status	Specific [^{3}H]ouabain binding	*p*
	fmoles/mg protein	
Euthyroid	378 ± 20	
Euthyroid + triiodothyronine	550 ± 30	<0.001
Thyroidectomized	240 ± 12	
Thyroidectomized + triiodothyronine	402 ± 30	<0.001
Euthyroid	378 ± 20	
Thyroidectomized	240 ± 12	<0.001

TABLE 2

Effect of triiodothyronine on specific [^{3}H]ouabain binding to heart ventricular microsomal fraction of thyroidectomized and euthyroid rats

The concentration of [^{3}H]ouabain was 80 nM. Values are means ± standard errors of nine determinations in three separate experiments. The procedure for measuring specific [^{3}H]ouabain binding is described in the text.

Thyroid status	Specific [^{3}H]ouabain binding	*p*
	fmoles/mg protein	
Euthyroid	821 ± 42	
Euthyroid + triiodothyronine	1263 ± 62	<0.001
Thyroidectomized	357 ± 14	
Thyroidectomized + triiodothyronine	1876 ± 119	<0.001
Thyroidectomized	357 ± 14	
Euthyroid	821 ± 42	<0.001

fmoles/mg of protein observed for the heart preparation derived from euthyroid rats. Administration of triiodothyronine enhanced specific [^{3}H]ouabain binding by 46% in skeletal muscle and 54% in heart muscle microsomal suspensions. Thus the increase in specific [^{3}H]ouabain binding due to administration of triiodothyronine was highly significant in heart as well as skeletal muscle preparations from euthyroid rats.

Thyroidectomy decreased specific [^{3}H]ouabain binding by 37% and 56% in gastrocnemius muscle and heart microsomes, respectively (Tables 1 and 2). Again, statistical analysis indicated a highly significant difference in specific [^{3}H]ouabain binding between euthyroid and thyroidectomized skeletal muscle preparations. With skeletal muscle microsomes, administration of triiodothyronine to thyroidectomized rats increased specific [^{3}H]ouabain binding to the level observed in euthyroid rats (Table 1). On the other hand, in cardiac microsomes, triiodothyronine administration to thyroidectomized rats doubled specific [^{3}H]ouabain binding compared with that observed in the euthyroid rat heart preparation (Table 2).

The above results clearly demonstrate that thyroid hormones may play an important role in the regulation of specific [^{3}H]ouabain binding sites on the plasma membrane of rat gastrocnemius and cardiac muscles. Thyroid hormones may influence specific [^{3}H]ouabain binding sites either by altering the apparent affinity of $(Na^+ + K^+)$-ATPase for this cardiac glycoside or by changing the number of specific binding sites. In order to determine the molecular mechanism for the regulation of specific [^{3}H]ouabain binding sites on the cell surface of rat striatal muscles, the equilibrium dissociation constant and maximal number of specific [^{3}H]ouabain binding sites were determined by Scatchard analysis (Figs. 1 and 2). Only a single type of specific [^{3}H]ouabain binding site was found in the microsomal fractions of gastrocnemius and heart muscle obtained from euthyroid as well as thyroidectomized rats, with or without triiodothyronine treatment. The most striking feature of Figs. 1 and 2 is the inability of triiodothyronine to alter the maximal number of binding sites for [^{3}H]ouabain in the microsomal suspensions derived from rat cardiac and gastrocnemius muscles. On the other hand, there were significant changes in the apparent affinity of

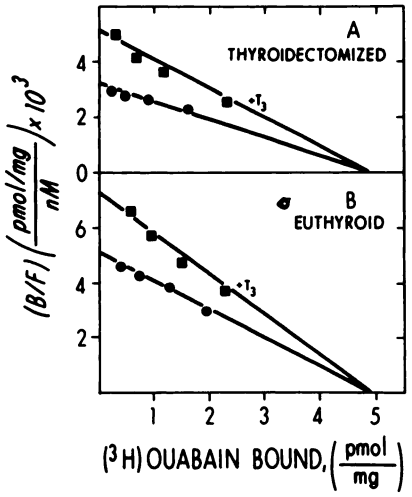

FIG. 1. *Scatchard plots of specific [³H]ouabain binding to microsomal suspensions derived from gastrocnemius muscle*

The procedure for the measurement of specific [³H]ouabain binding is described in the text. A. Scatchard plot obtained with microsomes derived from gastrocnemius muscle of thyroidectomized rats. B. Scatchard plot obtained with microsomes derived from gastrocnemius muscle of euthyroid rats. The equilibrium dissociation constants and maximal number of binding sites calculated from this figure are provided in Table 3. Values are averages of three separate experiments consisting of three determinations each. T_3, triiodothyronine.

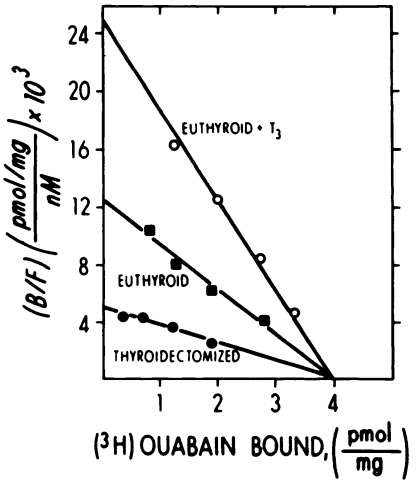

FIG. 2. *Scatchard plots of specific [³H]ouabain binding to microsomal preparation obtained from cardiac muscle*

Specific [³H]ouabain binding to microsomes derived from heart was measured as described in MATERIALS AND METHODS. The equilibrium dissociation constants calculated from this figure are shown in Table 3. Values are averages of three separate experiments consisting of three determinations each. T_3, triiodothyronine.

membrane-bound (Na^+ + K^+)-ATPase toward ouabain, depending upon the thyroid status of the animals (Table 3). The equilibrium dissociation constant of (Na^+ + K^+)-ATPase for ouabain was found to be highest with thyroidectomized rat skeletal muscle preparations: 1.54 μM, compared with 0.8 μM for the heart microsomal suspension. The dissociation constant of (Na^+ + K^+)-ATPase for ouabain markedly decreased following administration of triiodothyronine to either thyroidectomized or euthyroid rats (Table 3). These results clearly demonstrate that regulation of specific [³H]ouabain binding sites on cardiac and skeletal muscle membrane by thyroid hormones is mediated by changes in the apparent affinities of these binding sites for ouabain and does not involve alteration of the rate of net synthesis of this enzyme system *in vivo*.

The ability of triiodothyronine to modify

TABLE 3

Effects of thyroid hormones on specific [³H]ouabain binding to gastrocnemius muscle and heart ventricular microsomal fractions of thyroidectomized and euthyroid rats

The specific binding of various concentrations of [³H]ouabain to microsomal fractions from gastrocnemius muscle and heart of the rat was assayed as described in MATERIALS AND METHODS. The equilibrium dissociation constant (K_d) and maximal number of binding sites were estimated from Scatchard plots. Values are averages of three separate experiments consisting of three determinations each, which varied less than 10%.

Thyroid status	K_d	B_{max}
	μM	pmoles/mg
Skeletal muscle		
Euthyroid	0.96	4.9
Euthyroid + triiodothyronine	0.68	4.9
Thyroidectomized	1.54	4.9
Thyroidectomized + triiodothyronine	0.96	4.9
Heart		
Euthyroid	0.32	4.0
Euthyroid + triiodothyronine	0.16	4.0
Thyroidectomized	0.80	4.0

the apparent affinity of (Na^+ + K^+)-ATPase toward [^{3}H]ouabain would suggest that IC_{50} values of ouabain for the inhibition of ouabain-sensitive ATP hydrolytic activity may be modified by alterations of thyroid status. To test this possibility, log dose-response curves for the inhibition of (Na^+ + K^+)-dependent ATP hydrolytic activity of thyroidectomized and triiodothyronine-treated thyroidectomized rat heart preparations by unlabeled ouabain were plotted (Fig. 3). The IC_{50} values of ouabain for inhibition of (Na^+ + K^+)-ATPase obtained from thyroidectomized and triiodothyronine-treated thyroidectomized rat cardiac microsomes were found to be 146 and 120 μM, respectively. The apparent affinity of (Na^+ + K^+)-ATPase for ouabain is decreased in the presence of K^+ (18). In our hands, the equilibrium dissociation constant of cardiac (Na^+ + K^+)-ATPase for ouabain is approximately 200–500 times less than the IC_{50} values of the glycoside obtained from Fig. 3 (see Table 3 also). Thus a markedly lower sensitivity due to a many hundred times lower apparent affinity of (Na^+ + K^+)-dependent ATP hydrolytic activity toward ouabain would explain our inability to detect significant changes in IC_{50} values following triiodothyronine administration, despite detectable changes observed in the dissociation constants by Scatchard analysis of an apparently several hundred fold higher affinity system.

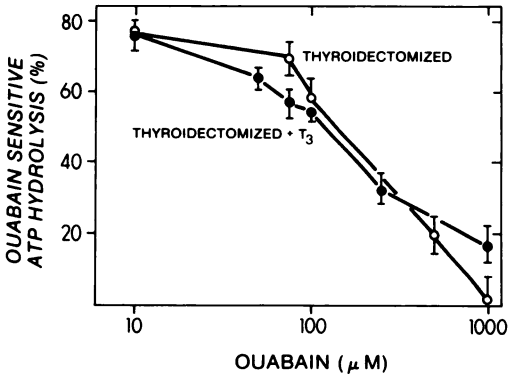

FIG. 3. *Inhibition of (Na^+ + K^+)-ATPase activity of cardiac microsomes by ouabain*

The (Na^+ + K^+)-dependent ATP hydrolytic activity was measured as described in MATERIALS AND METHODS, with and without the indicated concentrations of ouabain. The mean ouabain-sensitive ATP hydrolytic activities of cardiac microsomes obtained from thyroidectomized and triiodothyronine (T_3)-treated thyroidectomized rats were 2.8 and 8.1 μmoles/mg of protein per hour, respectively. The IC_{50} values for the inhibition of ouabain-sensitive ATP hydrolytic activity by ouabain were found to be 146 μM for thyroidectomized and 120 μM for triiodothyronine-treated thyroidectomized rat heart preparations. Values were obtained from three rats in each group and are averages of six determinations for each point.

To determine whether the observed changes in the apparent affinity of (Na^+ + K^+)-ATPase for ouabain in the various thyroid states could be seen by other kinetic methods, the displacement curves for reduction of specific [^{3}H]ouabain binding by nonradioactive ouabain were plotted (Fig. 4). The IC_{50} values of ouabain for inhibition of specific [^{3}H]ouabain binding to cardiac microsomes obtained from thyroidectomized and triiodothyronine-treated rats were 0.36 and 0.64 μM, respectively. These values are similar to those obtained by Scatchard analysis (Table 3), confirming that thyroid hormones regulate the apparent affinity of (Na^+ + K^+)-ATPase toward ouabain.

DISCUSSION

Ismail-Beigi and Edelman (1-4) first demonstrated an increase in ouabain-sensitive (Na^+ + K^+)-ATPase in several primary thermogenic target tissues, such as liver, kidney, heart, and skeletal muscle, following administration of thyroid hormones. To determine whether the increase in (Na^+ + K^+)-ATPase activity represents recruitment of new Na^+ pump units or activation of a fixed number of pump sites, Asano and his associates (8) examined the effects of triiodothyronine on kinetic properties of rat skeletal muscle (Na^+ + K^+)-ATPase activity. Triiodothyronine elicited a significant increase (40%) in maximal velocity with no change in apparent affinity for ATP (8). To distinguish further between thyroid activation of a fixed number of (Na^+ + K^+)-ATPase sites and an increase in the number of sites per renal cell, Edelman and his co-workers estimated the number of enzyme active sites

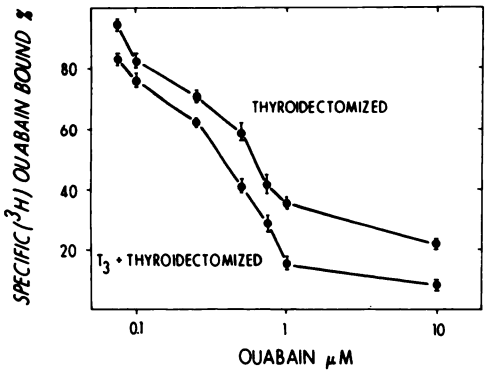

FIG. 4. *Inhibition of specific [³H]ouabain binding to cardiac microsomes by unlabeled ouabain*

The procedure for the measurement of specific [³H]ouabain binding is described in the text. The values for specific [³H]ouabain binding to cardiac microsomes obtained from thyroidectomized and triiodothyronine (T_3)-treated thyroidectomized rats were 401 and 1185 fmoles/mg of protein, respectively. The IC_{50} values for the inhibition of specific [³H]ouabain binding by unlabeled ouabain were found to be 0.64 μM for thyroidectomized and 0.36 μM for triiodothyronine-treated thyroidectomized rat heart preparations. Values were obtained from three rats in each group and are averages of three determinations for each point. Similar results were obtained when total binding was measured in the presence of 1 mM $MgCl_2$, 1 mM Na_2ATP, and 0.1 M NaCl, indicating that binding ligand conditions do not influence the effects of triiodothyronine on the apparent affinity of [³H]ouabain binding for (Na^+ + K^+)-ATPase.

by measuring Na^+- and Mg^{++}-dependent incorporation of ^{32}P from [γ-^{32}P]ATP. In this series of experiments, triiodothyronine elicited a 70% increase in renal cortical (Na^+ + K^+)-ATPase activity and a 79% increase in ^{32}P incorporation (7). These results led Edelman to suggest that thyroid hormones increase the number of transport enzyme sites rather than activate a fixed number of sites (6).

To investigate further possible involvement of protein synthesis in the activation of (Na^+ + K^+)-ATPase by thyroid hormones, we examined the effects of triiodothyronine on specific [³H]ouabain binding to microsomal fractions derived from rat heart and skeletal muscle. Since ouabain is a highly specific inhibitor of (Na^+ + K^+)-ATPase, specific [³H]ouabain binding provides a convenient method of estimating the number of (Na^+ + K^+)-ATPase molecules (18). Specific [³H]ouabain binding was significantly increased in thyroidectomized and euthyroid rat cardiac and skeletal muscle microsomal suspensions following administration of triiodothyronine (Tables 1 and 2). This increase in specific [³H]ouabain binding may be due to alteration in the apparent affinity of the microsomal preparation for ouabain or to an increase in the number of (Na^+ + K^+)-ATPase macromolecules following administration of triiodothyronine. Analysis by Scatchard plots indicated that the maximal number of specific [³H]ouabain binding sites is not modified by triiodothyronine (Figs. 1 and 2). The apparent affinity of cardiac and skeletal muscle (Na^+ + K^+)-ATPases for ouabain is markedly increased by administration of triiodothyronine to thyroidectomized and euthyroid rats (Table 3). Thus these results are not consistent with the hypothesis of induction of (Na^+ + K^+)-ATPase by thyroid hormones.

Recently Lo, Edelman, and their colleagues (19, 20) demonstrated an increased rate of synthesis of (Na^+ + K^+)-ATPase in rat kidney following administration of triiodothyronine, by measuring incorporation of radioactive methionine into the larger polypeptide of the (Na^+ + K^+)-ATPase molecule. Several workers have shown that aldosterone regulates Na^+ transport through DNA-dependent synthesis of mRNA (21-24). Furthermore, Knox and Sen (25) have reported that one of the proteins induced by aldosterone in rat kidney appears to be the larger polypeptide of the (Na^+ + K^+)-ATPase molecule. There is considerable evidence that thyroid hormone regulates secretion as well as aldosterone-target tissue interactions. Fregly *et al.* (26) have shown that the secretion of aldosterone in thyroidectomized rats is decreased by 69% from that in euthyroid controls. In addition, Taylor and Fregly (27) reported a remarkable reduction (about 90%) in renal tubular sensitivity to aldosterone in rats treated with polythiouracil. Thus the synthesis of (Na^+ + K^+)-ATPase observed by Lo and Edelman (20) may not be a direct consequence of

triiodothyronine administration but could be mediated through aldosterone. Kidneys, intestines, sweat glands, and salivary glands are aldosterone-sensitive organs in mammals. Heart and skeletal muscles are not target organs for aldosterone (28). This difference in tissue sensitivity to aldosterone would help to explain our inability to observe increased synthesis of $(Na^+ + K^+)$-ATPase in rat muscles following triiodothyronine treatment.

Moreover, the demonstration by Lo and Edelman of induction of $(Na^+ + K^+)$-ATPase by triiodothyronine is controversial. For example, Katz and Lindheimer (29) concluded that decreased tubular Na^+ transport is a major determinant of the reduction in renal $(Na^+ + K^+)$-ATPase in thyroid deficiency, and not lack of thyroid hormone per se. Lo and Edelman (20) questioned this conclusion on the basis of their observation that alterations in thyroid status do not alter serum Na^+ levels (8). This observation is in conflict with several previous reports. For example, Roche *et al.* (30) found that thyroidectomized rats had lower serum Na^+ and higher serum K^+ levels than euthyroid controls. Finally, triiodothyronine has been shown to regulate other membrane receptor proteins, such as *alpha* and *beta* adrenergic receptors (31–33) and glucagon receptors (34). These effects of triiodothyronine on plasma membrane proteins may not involve synthesis of mRNA and could be due to some other effects on plasma membranes, as suggested by Tata (35).

Recently Curfman *et al.* (36) examined the effects of triiodothyronine on myocardial $(Na^+ + K^+)$-ATPase. They did not perform kinetic analyses of their ouabain binding data and assumed that 0.2 μM ouabain would saturate all the binding sites in guinea pig myocardial homogenates. Akera *et al.* (37) found the IC_{50} value of ouabain for the $(Na^+ + K^+)$-ATPase activity of a partially purified guinea pig heart microsomes to be 2.5 μM. We measured the IC_{50} of unlabeled ouabain for specific [^{3}H]ouabain binding to guinea pig heart microsomes and found it to be 2.3 μM (15). Therefore it appears very unlikely that 0.2 μM [^{3}H]ouabain would saturate ouabain binding sites in guinea pig myocardial homogenates.

An intriguing feature of the present results and previous observations (1–11) is the simultaneous increase in specific [^{3}H]ouabain binding and $(Na^+ + K^+)$-ATPase activity due to administration of thyroid hormones. A strict quantitative relationship between specific [^{3}H]ouabain binding and $(Na^+ + K^+)$-ATPase activity, however, has been found for a number of preparations with widely different ATP hydrolytic activities (38–40). Furthermore, Jørgensen and Skou (41) examined the relationship between specific [^{3}H]ouabain binding and the hydrolytic activity of $(Na^+ + K^+)$-ATPase in an untreated microsomal fraction and in preparations obtained by differential and zonal gradient centrifugation of microsomal fractions treated with deoxycholate and found a linear relationship between the two parameters for these preparations. Therefore there is considerable evidence for an intimate relationship between ATP hydrolytic activity and specific [^{3}H]ouabain binding in various $(Na^+ + K^+)$-ATPase enzyme systems.

In conclusion, administration of triiodothyronine to thyroidectomized or euthyroid rats increases [^{3}H]ouabain binding to microsomal fractions derived from cardiac and gastrocnemius muscles, in agreement with earlier observations of augmentation of $(Na^+ + K^+)$-ATPase activity (1–11). Triiodothyronine, however, influences specific [^{3}H]ouabain binding by regulating the apparent affinity of $(Na^+ + K^+)$-ATPase for ouabain without affecting the maximal number of binding sites. Although the present observations leave the possibility open for synthesis of a specific protein for activation of $(Na^+ + K^+)$-ATPase as the molecular mechanism for the thermogenic action of triiodothyronine, they are inconsistent with the hypothesis that induction of $(Na^+ + K^+)$-ATPase is the biochemical basis for the action of thyroid hormones.

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