Specific Binding Sites for Triiodothyronine in the Plasma Membrane of Rat Thymocytes

CORRELATION WITH BIOCHEMICAL RESPONSES

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ABSTRACT As a prerequisite to studies of whether the plasma membrane of the rat thymocyte contains specific, saturable binding sites for the thyroid hormone 3,5,3'-triiodothyronine (T_3), a method was developed for the isolation of a plasma membrane fraction from these cells. As judged from both electron microscopic and marker enzyme studies, the fraction was composed principally of plasma membrane vesicles, was free of nuclear contaminants, and was only slightly contaminated with other subcellular components. At 37°C and pH 7.4, binding of [^{125}I]T_3 by the fresh membrane preparation was rapid, reaching a maximum at 5 min and then declining with time, so that by 60 min binding was virtually nil. Decreased binding with time was due to a loss of functional binding sites, but did not reflect desensitization, since the decrease in binding activity with time was independent of the presence or absence of T_3. Scatchard analysis of saturation studies revealed the presence of two binding sites, one with an apparent dissociation constant (K_d) of 0.95 nM and a maximum capacity of 5.3 × 10^{10} sites/100 μg protein, and the other with an apparent K_d of 25 nM and a binding capacity of 1.4 × 10^{12} sites/100 μg protein. Measurement of the ability of several thyronine analogues to inhibit the binding of [^{125}I]T_3 revealed the following rank order of potency: L-T_3 > L-T_4 > D-T_3 = D-T_4 > L-3,5-T_2 > rT_3 > D,L-thyronine. Binding of T_3 was inhibited by the omission of calcium from the medium or by the addition of the beta adrenergic antagonist alprenolol. As judged from studies of the lower affinity binding site, these manipulations decreased the affinity, but not the number, of binding sites for T_3. The relative potencies of thyronine analogues to inhibit the binding of [^{125}I]T_3 were generally parallel to their previously reported potencies in stimulating the uptake of the sugar analogue 2-deoxy-glucose (2-DG) in intact rat thymocytes in vitro. Further, the inhibition of T_3-binding produced by L-alprenolol or by excluding calcium from the medium resembled the previously reported inhibition that these manipulations produce with respect to T_3-induced enhancement of 2-DG uptake. These findings suggest that the binding sites for T_3 present in the plasma membrane of rat thymocytes act as functional receptors linked to the stimulation of 2-DG uptake that T_3 induces in these cells.

INTRODUCTION

We have previously demonstrated that 3,5,3'-triiodothyronine (T_3)[1] enhances the uptake of the glucose analogue 2-deoxy-D-glucose (2-DG) in rat thymocytes in vitro (1). Several lines of evidence indicate that T_3 induces its effect by acting at the level of the plasma membrane. Thus, the effect is rapid, is independent of new protein synthesis, and is mediated via an increase in cellular cyclic (c)AMP concentration, as a consequence of an increase in the activity of adenylate cyclase (2–4), an enzyme that resides within the plasma membrane. Furthermore, epinephrine, which is known to initiate its action upon binding to its

[1] *Abbreviations used in this paper*: T_3, 3,5,3'-triiodothyronine; T_4, 3,5,3',5'-tetraiodothyronine (thyroxine); rT_3, 3,3',5'-triiodothyronine; 3,5-T_2, 3,5-diiodothyronine, 2-DG, 2-deoxy-D-glucose.

plasma membrane receptors and thence activating adenylate cyclase, produces with T_3 a synergistic increase in the 2-DG uptake (5). If T_3 does indeed act at the level of the thymocyte plasma membrane, there should be specific saturable binding sites for the hormone present in the plasma membrane of these cells. We have now shown that the plasma membrane of rat thymocytes does contain specific binding sites for T_3, and have found several properties of the binding of T_3 to these sites that strengthen the likelihood that they are linked to the actions of T_3 described above.

METHODS

Materials used were purchased from the following sources: L-alprenolol bitartrate, epinephrine bitartrate, sodium tartrate, Tris-ATP, ascorbic acid, disodium 5′-AMP, DNA, 2,6-dichlorophenol indophenol, diphenylamine, disodium D-glucose-6-phosphate, B-glycerophosphate, Hepes, phenazine methosulfate, and DL-thyronine from Sigma Chemical Co., St. Louis, MO; ammonium molybdate, paraformaldehyde, lead nitrate, sodium succinate, and uranyl acetate from Fisher Scientific Co., Pittsburgh, PA; L-T_4, D-T_4, L-T_3, D-T_3, and L-3,5-T_2 from Henning Berlin GMBH, West Germany; rT_3 from Calbiochem-Behring Co. La Jolla, CA; sodium cacodylate (sodium dimethyl arsenate) from ICN Pharmaceutical Inc., Cleveland, OH; 50% glutaraldehyde solution from Electron Microscopy Sciences, Fort Washington, PA; Epon 812 from Ted Pella, Inc.; propylene oxide from the Eastman Kodak Co., Rochester, NY; and [^{125}I]T_3 (sp act ranging from 1,106 to 1,250 μCi/μg) from New England Nuclear, Boston, MA.

Female Sprague-Dawley rats of the CD strain, 26–28 d old and purchased from Charles River Breeding Laboratories (Wilmington, MA), served as the source of thymocytes. Thymocytes were isolated according to a method previously described (1).

Preparation of plasma membranes. Plasma membranes were prepared at 0–4°C by the following technique. Isolated thymocytes were resuspended in hypotonic homogenization buffer (10 mM Tris-HCl; 2.5 mM MgCl$_2$; 1 mM EGTA), pH 7.4, 1 ml/5 thymus glands, and were allowed to stand for 15 min to permit cells to become swollen, thereby facilitating their disruption. Cells were then transferred into a glass homogenizer and were homogenized until >95% were disrupted. This usually required 40–50 strokes. The homogenate was centrifuged at 300 g for 10 min, the supernate was collected, and the pellet was resuspended in homogenization buffer and recentrifuged at 300 g for 10 min. The two supernatants were combined and centrifuged at 4,000 g for 15 min. The supernate was collected and centrifuged at 20,000 g for 30 min. The yellow pellet obtained was suspended in 40% sucrose-10 mM Tris-HCl, pH 7.4, and was transferred into a cellulose-nitrate tube. An equivalent volume of 30% sucrose-10 mM Tris-HCl, pH 7.4, was carefully overlayered on the 40% sucrose-membrane fraction, and the sucrose-gradient was centrifuged at 80,000 g for 4 h, during which time plasma membranes were concentrated in a narrow milky band at the interphase of the 30–40% sucrose solutions. The plasma membranes were harvested and transferred into a centrifugation tube into which 10–20 vol of Tris-HCl, pH 7.4, were added. The suspension was then centrifuged at 20,000 g for 60 min. The pellet containing the plasma membranes was collected and kept on ice until samples were taken for electron microscopic studies, assays for cellular markers, and measurements of T_3-binding.

Measurement of [^{125}I]T_3binding. Freshly prepared membranes were always used in the T_3-binding assay. The plasma membranes were suspended in Krebs-Ringer-Tris buffer, (20 mM Tris-HCl, 5 mM Tris-base, 120 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1.5 mM NaH$_2$PO$_4$, 2.5 mM MgCl$_2$), pH 7.4. This is the same buffer as that used in studies on the effects of T_3 in the intact cell, except that Hepes is omitted (Hepes was omitted from the buffer because it interferes with the measurement of protein concentration.). In some cases, where so indicated, membranes were suspended in Ca^{2+}-free medium, i.e., the standard medium from which calcium salts were omitted. Ca^{2+}-free medium contained 5 μM Ca^{2+} because of contamination by calcium in the other buffer salts. The suspended membranes were transferred into a glass homogenizer and 10 strokes were then applied to obtain a homogenous membrane suspension. Membrane suspensions were then transferred into plastic tubes (12-ml capacity) containing buffer, placed in a shaking water bath, and equilibrated for 10 min at 37°C in air. Thereafter, 50 μl of buffer containing 75 nCi of [^{125}I]T_3 was added. As appropriate to the experiment, varying concentrations of unlabeled T_3 or other thyronine analogues were added at the same time, keeping the final volume of the reaction mixture constant. Nonspecific binding of [^{125}I]T_3 was assessed in specimens containing unlabeled T_3 in a concentration of 10 μM, and values for [^{125}I]T_3 found therein were subtracted from total binding to determine the saturable binding of [^{125}I]T_3.

At the end of the incubation period, triplicate 250-μl aliquots of reaction mixture were quickly removed and applied onto 0.3-μm Millipore filters (Millipore Corp., Bedford, MA) under vacuum. The membranes were then quickly washed three times with 2.5 ml of ice-cold buffer. The filters were then dried, transferred into tubes, and were counted in a gamma counter.

Kinetic parameters of T_3 binding were obtained in saturation studies using Scatchard plots and the iterative technique described by Minneman (6).

Electron microscopy. Plasma membrane fractions were prepared for examination under the electron microscope according to the technique described by Stemerman (7).

Cellular markers. DNA content in homogenates and membrane preparations was measured according to a method described by Burton (8); 5′-nucleotidase activity according to the method of Bosmann (9); succinate dehydrogenase activity by the method of Hatefi (10); Na$^+$,K$^+$-ATPase, activity by the method described by Wallach and Kamat (11); acid phosphatase activity by the method of Baudhuin (12); and glucose-6-phosphatase activity, as well as inorganic phosphate content, by methods described by Ames (13).

Purity of [^{125}I]T_3. Purity of preparations of [^{125}I]T_3 was assessed by descending filter paper chromatography in a hexane-tertiary amyl alcohol-N-ammonia (1:10:11) solvent system according to a technique described by Borges et al. (14).

Statistical analysis. The following statistical methods were used: Dunnet's test for comparisons between multiple groups and a single control; analysis of variance, followed by the Newman-Keuls multiple range test, for comparisons of multiple groups with each other; and analysis of covariance, followed by Dunnet's test, for comparisons of multiple regression lines with a single control line (15).

RESULTS

Purity of plasma membrane preparations. Both ultramicroscopic appearance and biochemical markers indicated that plasma membrane preparations were

highly purified. Electron microscopic studies revealed a homogenous plasma membrane preparation, comprising closed vesicles of varying sizes, ranging from ~0.25 to 1.2 μm Diam. Small quantities of ribosomes were present, but nuclear, mitochondrial, and lysosomal contaminants were not evident.

A comparison of the relative concentrations of specific cellular markers in the initial homogenates and the membrane preparations also indicated that the preparations were highly enriched in plasma membranes and were essentially devoid of other cellular components (Table I). DNA content was virtually nil, indicating the absence of nuclei. The plasma membrane markers, 5'-nucleotidase and Na^+,K^+-ATPase, were enriched by 15- and 12.5-fold, respectively. The preparation contained a small amount microsomes, as indicated by glucose-6-phosphatase; smaller amounts of mitochondria, as indicated by succinate dehydrogenase; and negligible amounts of lysosomes, as indicated by acid phosphatase. In terms of both the yield of protein (~0.43% of initial total protein) and of the relative enrichment of specific marker enzymes, the present preparations of rat thymocyte plasma membranes were quite comparable to those derived from thymocytes of calf and rabbit, as described by others (16–19).

Binding of [^{125}I]T$_3$: effect of membrane concentration and time. Binding of [^{125}I]T$_3$ to various concentrations of plasma membranes was linear between 20 and 240 μg protein/ml in the reaction medium (data not shown). In the ensuing experiments, plasma membranes were used in a concentration of 100–200 μg protein/ml.

TABLE I
Biochemical Characterization of Thymocyte Plasma Membrane Preparations *

Marker‡	Homogenate	Plasma membrane§ fraction
DNA	348.8±28.5	0.000
5'-nucleotidase	0.34±0.01	5.13±0.05
Na^+,K^+-ATPase	0.90±0.02	11.26±0.74
Glucose-6-phosphatase	1.33±0.17	0.51±0.03
Acid phosphatase	2.11±0.20	0.48±0.04
Succinate dehydrogenase	1.06±0.01	0.03±0.01

* Homogenates and plasma membrane fractions derived therefrom were prepared as described in Methods. Values for the various subcellular markers are the mean±SD of those obtained in three separate preparations.
‡ DNA micrograms per milligram protein; enzymes, micrograms product per milligram protein/60 min.
§ Plasma membranes fractions contained 83.7±11.7 ng protein/10^9 original thymocytes (mean±SD, n = 19), representing 0.43% of the original protein content.

Specific binding of T$_3$ to the plasma membranes was vary rapid and reached 70% of maximum values at 1 min of incubation. Maximum binding (~1.5 times the nonspecific binding) was evident at 5 min of incubation, and then gradually declined to almost nil at 60 min (Fig. 1). Incubation times of 5 min were chosen for use in subsequent experiments.

Nonspecific binding of T$_3$ reached maximum values at 7 min of incubation. Thus, as expected, the binding of T$_3$ to its specific binding sites was more prompt than its binding to the nonspecific sites.

The observed decrease in T$_3$-binding with time could have resulted from degradation of the T$_3$ or from a decrease in the number or affinity of the T$_3$ binding sites, or both. To determine which of these mechanisms was responsible, the following experiments were performed. First, [^{125}I]T$_3$, with and without 10 μM unlabeled T$_3$, was incubated in the presence or absence of plasma membranes. After various periods, samples were removed and analyzed chromatographically. [^{125}I]T$_3$ was not significantly degraded during the 60-min incubation period (data not shown).

In other experiments, membranes were divided into four groups. In the first two groups, one of which contained 0.10 nM T$_3$ and one of which did not, [^{125}I]T$_3$ was added at zero time. Samples were removed after 5, 35, and 65 min of incubation, and binding of [^{125}I]T$_3$ to the membranes was assessed. In the second two groups, membranes were preincubated with or without
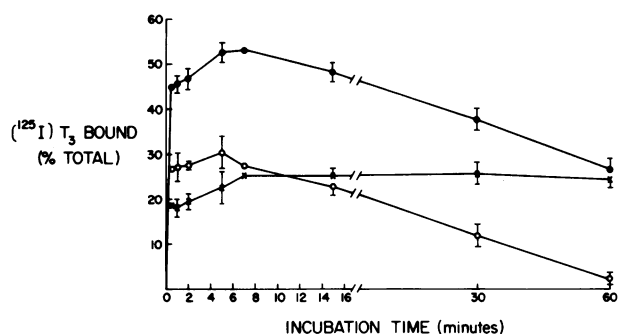
FIGURE 1 Binding of [^{125}I]T$_3$ by rat thymocyte plasma membranes as a function of incubation time. Plasma membranes (200 μg/protein per ml) were suspended in Krebs-Ringer-Tris buffer, pH 7.4, and were allowed to equilibrate in a shaking water bath at 37°C for 15 min. [^{125}I]T$_3$ (45 pM) was then added. After various periods of time, triplicate 250-μl samples were quickly removed and were applied to 0.30-μm millipore filters under vacuum. Binding of [^{125}I]T$_3$ to the membranes was assessed as described in Methods. Nonspecific binding of [^{125}I]T$_3$ was measured in the presence of 10 μM unlabeled L-T$_3$. ●——●, total binding of [^{125}I]T$_3$; ×-----×, nonspecific binding; O——O, specific binding. Single points represent the results of single experiments. Points and brackets represent mean±SD of results in three experiments. Values shown are expressed as percent total [^{125}I]T$_3$ added.

Effect of Incubating Rat Thymocyte Plasma Membranes for
Varying Periods, with and without T_3 (0.1 nM),
on the Binding of $[^{125}I]T_3$.[*]

Group	Time of adding $[^{125}I]T_3$	Time of adding T_3 (0.1 nM)	Time of sampling	$[^{125}I]T_3$ bound
	min	min	min	% Group A
A	0	—	5	100.0
B	0	—	35	37.0±3.4
C	30	—	35	33.3±6.7
D	0	—	65	16.1±2.8
E	60	—	65	19.8±4.6
F	0	0	5	104.4±6.5
G	0	0	35	32.5±6.4
H	30	0	35	35.1±5.0
I	0	0	65	18.2±2.6
J	60	0	65	17.5±3.8

[*] Plasma membranes were equilibrated for 15 min at 37°C in air. Experiments were then begun (zero time). $[^{125}I]T_3$ and unlabeled T_3 (0.1 nM) were added, and the binding of $[^{125}I]T_3$ was assessed at the differing intervals shown. Binding in Group A. (16.3±18 fmol T_3/100 μg protein), in which $[^{125}I]T_3$ was added at zero time and its binding measured 5 min later, was considered as 100%. Values shown are the mean±SD of those obtained in four separate experiments.

0.10 nM T_3 for 30 and 60 min. $[^{125}I]T_3$ was then added, and 5 min later its binding was assessed. Values for the percentage of $[^{125}I]T_3$ bound were an inverse function of the total duration of incubation of the membranes in all four groups, whether or not $[^{125}I]T_3$, unlabeled T_3, or both, were present during that period (Table II).

Kinetic analysis of $[^{125}I]T_3$ binding. Binding of $[^{125}I]T_3$ was studied in the presence of concentrations of unlabeled T_3 ranging from 45 pM to 1 μM. Percentage binding of $[^{125}I]T_3$ was greatest, ~14%, at the lowest concentrations of T_3 tested, and was progressively decreased by increasing concentrations of unlabeled T_3. The configuration of the displacement curve suggested that more than one binding site was present (Fig. 2).

Transformation of values from percentage binding to absolute quantities of T_3 bound revealed that the binding of T_3 to the membrane preparation was saturable, a plateau of T_3 binding being achieved at total T_3 concentrations of 20–35 nM. Scatchard analysis revealed the presence of two apparent classes of binding sites, one a higher affinity, low capacity site, and the other a lower affinity, high capacity site (Fig. 3). Values for the apparent K_d of the two sites were 0.95 and 25 nM, respectively, and for binding capacities (sites/100 μg protein) were 5.3×10^{10} and 1.4×10^{12}, respectively. The slope of a Hill plot was 0.97 ($r = 0.994$), indicating a lack of cooperativity in the binding of $[^{125}I]T_3$.
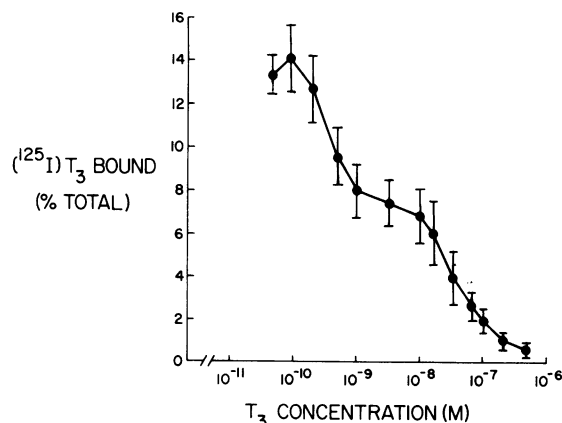

FIGURE 2 Binding of T_3 by rat thymocyte plasma membranes. $[^{125}I]T_3$ (45 pM) together with various concentrations of nonlabeled T_3 was added to the plasma membranes (100 μg protein/ml), and 5 min later its binding by the membranes was assessed. Values shown are expressed as percent total $[^{125}I]T_3$ added and are the mean±SD of those obtained in four separate experiments.

Effect of thyronine analogues. To evaluate the hormone specificity of T_3-binding, the ability of standard concentrations (1 nM and 100 nM) of T_3 itself and of various other thyronine analogues to inhibit the binding of tracer concentrations (77 pM) of $[^{125}I]T_3$ to the plasma membrane was assessed. On the basis of the kinetic analyses described above, it can be calculated that at this concentration of added T_3 >70% of the T_3 bound was associated with the higher affinity
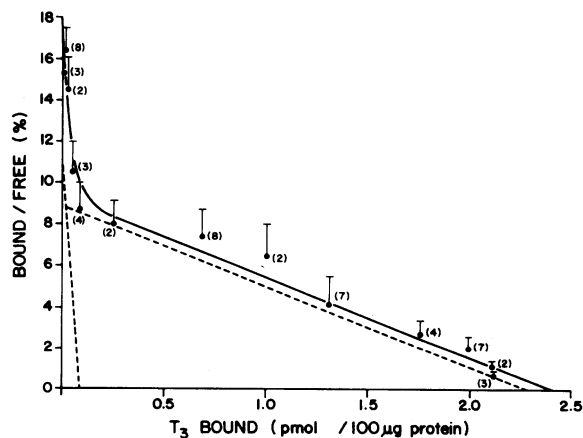

FIGURE 3 Scatchard plot of T_3 binding by plasma membranes from rat thymocytes. The solid line represents the unresolved Scatchard plot derived from the original binding data. Individual values and brackets are the mean±SD of data shown in Fig 2. The broken lines represent the Scatchard plot resolved into plots for the high affinity sites and the low affinity sites, according to the technique described by Minneman and co-workers (6).

binding sites. In rank order of decreasing inhibitory potency, the following sequence was obtained: L-T_3 > L-T_4 > D-T_3 = D-T_4 > L-3,5-T_2 > rT_3 > DL-thyronine (Table III). This rank-order agreed quite closely with that of the potency of these agents to increase the uptake of 2-DG in the intact cell (1).

Effect of calcium. To ascertain the role, if any, of Ca^{2+} in the binding of T_3, experiments were performed in which membranes were suspended in standard or calcium-free media, in some cases with or without 1 mM EGTA. Some experiments were performed at tracer concentrations of [^{125}I]T_3, as above, while other experiments were performed at T_3 concentrations of 10 nM–1 μM, over which range >90% of T_3-binding occurs at the low affinity site.

In the presence of tracer concentrations of T_3, binding of T_3 in calcium-free medium was significantly decreased to about two-thirds of control values (8.67±0.53 fmol/100 μg protein). Addition of 1 mM EGTA to the calcium-free medium produced no further effect (Fig. 4). In studies that examined binding of T_3 to the low affinity site, omission of calcium was also found to decrease T_3-binding. Scatchard analysis revealed this to be due to a reduction in the binding affinity, rather than the number of binding sites (Fig. 5). Thus, as would be expected, the inhibitory effect of excluding calcium became proportionately less as the concentration of T_3 was progressively increased.

Effect of L-alprenolol. Experiments to determine the effects of L-alprenolol on T_3-binding were also conducted at tracer and at relatively high concentrations of T_3, as in experiments with calcium-free medium.
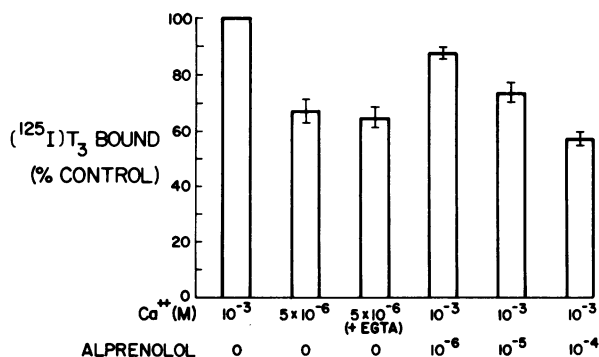

FIGURE 4 The effect of calcium depletion and of L-alprenolol on the binding of tracer concentrations of [^{125}I]T_3 by rat thymocyte plasma membranes. Plasma membranes were suspended in either standard medium (containing 1 mM Ca^{2+}) or Ca^{2+}-free medium, in the presence or absence of 1 mM EGTA. [^{125}I]T_3 (77 pM) was added, with or without various concentrations of L-alprenolol, and 5 min later binding of [^{125}I]T_3 by the plasma membranes were assessed. Results are shown as percent [^{125}I]T_3 binding in the corresponding control group. Values shown are mean±SD of those obtained in from three to six separate experiments.

In the former experiments, L-alprenolol tartrate produced a dose-related inhibition of T_3 binding (Fig. 4). Sodium tartrate (0.1 mM) had no effect (data not shown). At the higher concentrations of T_3, 10 μM L-alprenolol was also inhibitory to T_3 binding, and this effect, like that of excluding calcium, was to decrease the binding affinity, rather than the number of binding sites (Fig. 5).
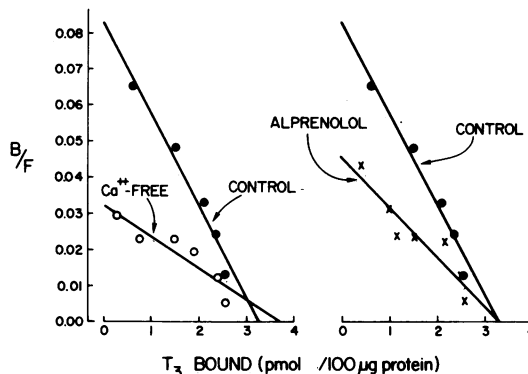
TABLE III
Inhibition of the Binding of [^{125}I]T_3 to Thymocyte Plasma Membranes by Various Thyronine Analogues

Thyronine analogue	Inhibition of [^{125}I]T_3 binding (%)	
	Analogue concentration	
	10^{-9} M	10^{-7} M
L-T_3	44.2±4.4	69.6±2.6
L-T_4	28.4±2.4	43.1±5.5
D-T_3	12.8±3.0	34.1±3.1
D-T_4	15.3±3.0	34.5±2.6
L-3,5-T_2	-----	16.7±2.8
rT_3	-----	11.8±1.9
DL-Thyronine	-----	10.9±1.8

* Plasma membranes were incubated for 5 min with [^{125}I]T_3 (77 pM), with or without two concentrations (1 nM and 0.1 μM) of various thyronine analogues, including T_3. Values represent the percent inhibition of [^{125}I]T_3-binding, relative to the binding obtained in control specimens incubated with [^{125}I]T_3 alone. Values are mean±SD of those obtained in four separate experiments. L-T_4 contained 0.5% or less of L-T_3 as a contaminant.

FIGURE 5 Scatchard plot of T_3-binding to rat thymocyte plasma membranes in the presence and absence of calcium and L-alprenolol. Plasma membranes were suspended in standard medium (1 mM Ca^{2+}), or in Ca^{2+}-free medium (5 μM Ca^{2+}). [^{125}I]T_3, together with various concentrations of nonlabeled T_3, with or without 10 μM alprenolol, was then added and 5 min later binding of [^{125}I]T_3 by the plasma membranes was measured. Values shown are mean±SD of those obtained in four separate experiments. Analysis of covariance followed by the Dunnet's test was used for comparisons between the alprenolol or Ca^{2+}-free groups and the control group.

Effect of epinephrine. Although epinephrine interacts synergistically with T_3 to increase the uptake of 2-DG in intact rat thymocytes (2), epinephrine (0.1–100 μM) had no effect on the binding of tracer concentrations of $[^{125}I]T_3$ (data not shown).

DISCUSSION

In these studies we have demonstrated the presence of saturable binding sites for T_3 and other thyroid hormones and analogues in plasma membrane preparations obtained from rat thymocytes. Saturable binding of T_3 has been observed in nuclei or mitochondria from a variety of other tissues (20–24). Nonetheless, the two T_3 binding sites that we observed in our plasma membrane preparations cannot be ascribed to contamination of membrane preparations by those subcellular elements, since both electron microscopic and marker enzyme studies indicated that our preparations were virtually devoid of mitochondria and were entirely devoid of nuclei.

Earlier studies have provided evidence of the existence of saturable binding sites for T_3 in the plasma membranes of hepatocytes and fibroblasts (25–28). However, in the case of these T_3 binding sites, there is no evidence that links them to a physiological response. Our interest in determining whether analogous T_3-binding sites are present in the plasma membrane of the rat thymocyte stemmed from our previous observations in intact thymocytes, in which substantial evidence was obtained that the prompt increase in 2-DG uptake induced by T_3 in vitro, as well as its synergistic interaction with epinephrine, is indicated at a plasma membrane level (1–5).

Not only were saturable binding sites for T_3 demonstrated, but several lines of inferential evidence were obtained linking the binding of T_3 to these sites with the biochemical response mentioned above.

First, the apparent dissociation constants of the T_3 binding sites found were in the range of concentrations at which T_3 alone was found to be effective in increasing both cellular cyclic AMP concentration and 2-DG uptake in intact thymocytes (1, 3). These concentrations are substantially higher than the assumed free T_3 concentration in vivo (5–10 pM). However, 10 pM T_3 is active in the intact cell in the presence of insulin and epinephrine (5), and, from the present binding data, it can be calculated that at this concentration of T_3 ~7.6% of the high affinity binding sites for T_3 would be occupied.

A further link between the binding sites for T_3 that we here demonstrate and physiological responses in the intact thymocyte is the fact that the relative affinities of the various thyroid hormone analogues for the T_3-binding sites in the thymocyte plasma membrane correlated quite well with their relative abilities to increase 2-DG uptake in the intact cell, as seen in earlier studies (1).

An additional line of evidence is provided by the observation that L-alprenolol inhibited the binding of T_3 to the thymocyte plasma membrane, an effect reminiscent of its ability to inhibit the increase both in the cellular cAMP concentration and the uptake of 2-DG in the intact thymocyte. Further, in the present studies, alprenolol was found to decrease the affinity, rather than the number, of T_3 binding sites. This, too, is consonant with its effects in the intact cell, which could be overcome by increasing the concentration of T_3.

A final point of similarity between the binding of T_3 to the thymocyte membrane and the effect of T_3 in the intact thymocyte is the partial or complete calcium-dependency, respectively, of these two processes. This, however, cannot be construed as evidence linking the binding of T_3 to the plasma membrane with the biochemical responses that we have described. Although omission of calcium from the media also appears to decrease the affinity of binding sites for T_3, its effects on cAMP concentration and 2-DG uptake, unlike those of alprenolol, are not overcome by higher concentrations of T_3. This suggests that these actions of T_3 require calcium at some point subsequent to the binding of T_3 to the plasma membrane. This postulate would be in accord with the demonstration by Lefkowitz and co-workers (29) that omission of calcium does not affect the binding of ACTH to the plasma membrane of an adrenal tumor cell line, but does inhibit the consequent increase in adenylate cyclase activity.

Apart from their likely relation to biochemical responses, several other properties of the T_3-binding sites in the plasma membranes of rat thymocytes seem worthy of mention. The presence of both a higher and a lower affinity binding site in the rat thymocyte plasma membrane is generally consonant with the observations of Gharbi-Chihi and Torresani (27), who found two classes of T_3 binding site in plasma membranes of rat liver and kidney, though the affinities in these membranes was somewhat lower than those we found in the thymocyte plasma membrane.

Binding of T_3 to the thymocyte plasma membrane was very prompt, reaching a maximum at 5 min and declining thereafter. Progressive loss of binding activity with increasing incubation time did not depend on whether T_3 was present during preincubations prior to the addition of $[^{125}I]T_3$. Therefore, it evidently was not due to what has been termed desensitization, which results from occupancy of the binding site by its ligand. We would suggest, instead, that the loss of T_3 binding activity with time results from turnover or degradation of the receptor, since plasma membrane proteins are

known to undergo rapid turnover (30); alternatively, it may reflect a change in the conformation of the membrane that leads to a loss of available binding sites.

Finally, one may consider the mechanisms by which alprenolol and calcium inhibit the binding of T_3. The inhibitory effects of alprenolol could result from either a direct competition with T_3 for a common binding site or an effect on the T_3-binding site secondary to binding of alprenolol to its own binding site elsewhere on the plasma membrane. It is unlikely that the effect of L-alprenolol can be ascribed to a nonspecific detergent-like effect on the membrane, since, in the intact thymocyte, comparable concentrations of D-alprenolol do not affect cell viability or the accumulation of 2-DG, which functions are highly sensitive to perturbation of the plasma membrane (31). With respect to the inhibitory effect of calcium, it is well known that plasma membranes have specific binding sites for calcium (32–34) and that calcium increases the binding of particular ligands to the plasma membranes of several tissues (35, 36), but it is not known whether the binding of calcium initiates the latter responses.

ACKNOWLEDGMENTS

REFERENCES

1. Segal, J., and S. H. Ingbar. 1979. Stimulation by triiodothyronine of the in vitro uptake of sugars by rat thymocytes. *J. Clin. Invest.* **63:** 507–515.
2. Segal, J., and S. H. Ingbar. 1980. Stimulation of 2-deoxy-D-glucose uptake in rat thymocytes in vitro by physiological concentrations of triiodothyronine, insulin, or epinephrine. *Endocrinology.* **107:** 1354–1358.
3. Segal, J., and S. H. Ingbar. 1981. Studies on the mechanism by which 3,5,3'-triiodothyronine stimulates 2-deoxyglucose uptake in rat thymocytes in vitro. Role of calcium and adenosine 3':5'-monophosphate. *J. Clin. Invest.* **68:** 103–110.
4. Segal, J., and S. H. Ingbar. 1982. Enhancement of adenylate cyclase activity by 3,5,3'-triiodothyronine (T_3) in thymocyte plasma membranes. *Clin. Res.* **30:** 276A (Abstr.).
5. Segal, J., and S. H. Ingbar. 1980. Direct and synergistic interactions of 3,5,3'-triiodothyronine and the adrenergic system in stimulating sugar transport by rat thymocytes. *J. Clin. Invest.* **65:** 958–966.
6. Minneman, K. P., L. R. Hegstrand, and P. Molinoff. 1979. Simultaneous determination of beta-1 and beta-2 adrenergic receptors in tissues containing both receptor subtypes. *Mol. Pharmacol.* **16:** 34–46.
7. Stemerman, M B., and R. Ross. 1972. Experimental arteriosclerosis. I. Fibrous plaque formation in primates, an electron microscope study. *J. Exp. Med.* **136:** 769–789.
8. Burton, K. 1966. Determination of DNA concentration with diphenylamine. *Methods Enzymol.* **12:** 163–166.
9. Bosmann, H. B., and C. Z. Pike. 1971. Membrane marker

10. Hatefi, Y. 1978. Regulation of complex II and isolation of succinate dehydrogenase. *Methods Enzymol.* **53:** 27–35.
11. Wallach, D. F. H., and V. B. Kamat. 1966. Preparation of plasma-membrane fragments from mouse ascites tumor cells. *Methods Enzymol.* **8:** 164–169.
12. Baudhuin, P. 1974. Isolation of rat liver peroxisomes. *Methods Enzymol.* **81:** 356–368.
13. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* **8:** 115–118.
14. Borges, M., Z. Eisenstein, A. G. Burger, and S. H. Ingbar. 1981. Immunosequestration: A new technique for studying peripheral iodothyronine metabolism in vitro. *Endocrinology.* **108:** 1665–1671.
15. Zar, J. H. 1974. Multiple comparison. *In* Biostatistical Analysis. Prentice-Hall, Inc, Englewood Cliffs, NJ. 151–162.
16. Van Blitterswijk, W. J., P. Emmelot, and C. A. Feltkamp. 1973. Studies on plasma membranes. xix. Isolation of and characterization of a plasma membrane fraction from calf thymocytes. *Biochim. Biophys. Acta.* **298:** 577–592.
17. Kornfeld, R., and C. Siemers. 1974. Large scale isolation and characterization of calf thymocyte plasma membranes. *J. Biol. Chem.* **249:** 1295–1301.
18. Kabir, S. 1978. The isolation and characterization of plasma membranes from calf thymocytes. *Prep. Biochem.* **8:** 155–169.
19. Schmidt-Ullrich, R., E. Ferber, H. Kniifermann, H. Fischer, and D. F. H. Wallach. 1974. Analysis of the proteins in thymocyte plasma membrane and smooth endoplasmic reticulum by sodium deodecylsulfate-gel electrophoresis. *Biochim. Biophys. Acta.* **332:** 175–191.
20. Oppenheimer, J. H. 1979. Thyroid hormone action at the cellular level. *Science (Wash., DC).* **203:** 971–979.
21. Samuels, H. H., and J. S. Tsai. 1973. Thyroid hormone action in cell culture: demonstration of nuclear receptors in intact cells and isolated nuclei. *Proc. Natl. Acad. Sci. USA* **70:** 3488–3492.
22. Spindler, B. J., K. M. McLeod, J. Ring, and J. D. Baxter. 1975. Thyroid hormone receptors. Binding characteristics and lack of hormonal dependency for nuclear localization. *J. Biol. Chem.* **250:** 4113–4119.
23. Sterling, K. 1979. Thyroid hormone action at the cell level. *N. Engl. J. Med.* **300:** 173–177.
24. Sterling, K., P. O. Milch, M. A. Brenner, and J. H. Lazarus. 1980. Thyroid hormone action: the mitochondrial pathway. *Science (Wash., DC).* **197:** 996–999.
25. Pliam, N. B., and I. D. Goldfine. 1977. High affinity thyroid hormone binding sites on purified rat liver plasma membranes. *Biochem. Biophys. Res. Commun.* **79:** 166–172.
26. Gharbi, J., and J. Torresani. 1979. High affinity thyroxine binding to purified rat liver plasma membranes. *Biochem. Biophys. Res. Commun.* **88:** 170–177.
27. Gharbi-Chihi, J., and Y. Torresani. 1981. Thyroid hormone binding to plasma membrane preparations: studies in different thyroid states and tissues. *J. Endocrinol. Invest.* **4:** 177–183.
28. Maxfield, F. R., M. C. Willingham, I. Pastan, P. Dragsten, and S. Y. Cheng. 1981. Binding and mobility of the cell surface receptors for 3,3',5-triiodo-L-thyronine. *Science (Wash., DC).* **211:** 63–65.
29. Lefkowitz, R. J., Y. Ross, and I. Pastan. 1970. Effects of

enzymes: isolation, purification and properties of 5'-nucleotidase from rat cerebellum. *Biochim. Biophys. Acta.* **227:** 402–412.

calcium on ACTH stimulation of the adrenal: separation of hormone binding from adenyl cyclase activation. *Nature (Lond.).* **228:** 864–866.

30. Doljanski, F., and M. Kapeller. 1976. Cell surface shedding—the phenomenon and its possible significance. *J. Theor. Biol.* **62:** 253–270.

31. Segal, J., and S. H. Ingbar. 1981. A postulated mechanism for the coordinate effects of A23187 on calcium uptake and cell viability. *Biochim. Biophys. Acta.* **684:** 7–11.

32. McDonald, J. M., D. E. Bruns, and L. Jarett. 1976. Calcium binding to adipocyte plasma membrane. *J. Biol. Chem.* **251:** 5345–5351.

33. Tolone, G., L. Bonasera, F. Vitale, and G. M. Pontieri. 1980. Calcium binding to mastocyte plasma membrane. *Int. Arch. Allergy Appl. Immunol.* **63:** 236–239.

34. Mikkelsen, R. B., and D. L. Freerksen. 1980. Calcium binding to plasma membranes from normal and SV40 transformed hamster lymphocytes. *J. Receptor Res.* **1:** 239–260.

35. Desai, K. S., B. Zinman, G. Steiner, and C. H. Hollenberg. 1978. Effect of calcium on [^{125}I] insulin binding to rat adipocytes. *Can. J. Biochem.* **56:** 843–848.

36. Williams, L. T., D. Mullikin, and R. J. Lefkowitz. 1978. Magnesium dependence of agonist binding to adenylate cyclase-coupled hormone receptors. *J. Biol. Chem.* **253:** 2984–2989.