

Hypothyroidism Modulates Beta Adrenergic Receptor-Adenylate Cyclase Interactions in Rat Reticulocytes

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ABSTRACT We have investigated alterations in beta adrenergic receptor binding sites of rat reticulocytes occurring in animals rendered hypothyroid by thyroidectomy. Beta adrenergic receptor interactions were assessed by measuring the number of $(-)[^3\text{H}]$ -dihydroalprenolol binding sites and the ability of an agonist to compete for occupancy of the receptors. The number of receptors was significantly reduced in cells from the hypothyroid animals. In addition, there were significant agonist-specific alterations in binding. Using computer assisted curve fitting techniques, it was found that the ability of $(-)$ -isoproterenol to stabilize a high affinity guanine nucleotide sensitive "coupled" form of the receptor was impaired. Reticulocytes from hypothyroid animals have, in addition, a reduction in the concentration of the nucleotide regulatory protein as assessed by the number of 42,000 M_r substrates for cholera toxin catalyzed ADP ribosylation. These alterations are associated with reductions in catecholamine and NaF stimulated adenylate cyclase activity. Diminished coupling of beta adrenergic receptors with other regulatory components of the adenylate cyclase system represents a mechanism by which altered thyroid states modulate beta adrenergic receptor function and beta adrenergic responsiveness of tissues.

INTRODUCTION

Thyroid hormone levels appear to modulate tissue responsiveness to beta adrenergic catecholamines,

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with increased responsiveness often observed in hyper- and decreased responsiveness in hypothyroidism (1, 2). Thyroid hormones regulate the number of beta adrenergic receptors in several tissues including rat heart (3), skeletal muscle (4), and submaxillary gland (5) as well as in turkey erythrocytes (1).

Although changes in receptor number may be partially responsible for the altered sensitivity, other changes such as alteration in agonist-receptor affinity might also be important. Radioligand binding techniques now permit the assessment not only of the number of beta adrenergic receptors but also the status of their coupling with the adenylate cyclase system. This is most effectively done by quantitative analysis of ligand binding data, using computer assisted curve modeling procedures (6). Such procedures have indicated that in beta adrenergic systems the competition curves of agonists with radiolabeled antagonists are shallow whereas antagonist competition curves are of normal steepness. This agonist specific heterogeneity of binding appears to be due to the ability of agonists, but not antagonists, to distinguish separate affinity states of the beta adrenergic receptor (R)¹ having respectively high (K_H) and low (K_L) affinity for the agonist. The high affinity state of the receptors appears to represent a "coupled" agonist stabilized complex of hormone, receptor, and nucleotide regulatory protein (N), which is an intermediate required for hormone promoted activation of the enzyme (7). Guanine nucleotides, which are required for beta adrenergic activation of adenylate cyclase, appear to mediate a transition from the high to the low affinity state of the receptor and thus shift agonist competition curves to the right. The ratio K_L/K_H for an agonist correlates with the efficacy (intrinsic activity) for activation of

¹Abbreviations used in this paper: EC_{50} , 50% displacement; $[^3\text{H}]DHA$, $[^3\text{H}]$ dihydroalprenolol; $Gpp(NH)p$, guanylyl-5'-yl-imidodiphosphate; N , nucleotide regulatory protein; R , beta adrenergic receptor; T_4 , thyroxine.

adenylate cyclase (6). The ratio K_L/K_H , reflecting the ability of an agonist to stabilize the RN complex is, therefore, a measure of the coupling of the receptor with the nucleotide regulatory protein.

The binding of agonists but not antagonists to the beta adrenergic receptor of rat reticulocyte membranes has been directly shown to induce the coupling of R with N (7). It has also been demonstrated that maturation of rat reticulocytes to erythrocytes is accompanied by a gradual uncoupling of the receptor from adenylate cyclase. This results in a loss of agonist promoted high affinity state as documented by quantitative analysis of agonist binding properties (8). This model system is now used to investigate whether decreased R-N coupling might provide a partial explanation for decreased beta adrenergic responsiveness in hypothyroidism.

METHODS

Hypothyroidism was produced by thyroidectomy in male Sprague-Dawley rats (175–225 g) obtained from Charles River Laboratories, Newfield, N. J. Changes in thyroid hormone levels were determined 8 wk after thyroidectomy by measuring thyroxine (T_4) levels as previously described (9). Control serum T_4 ($n = 3$) was 6.9 ± 0.3 $\mu\text{g/dl}$ (mean \pm SEM) while hypothyroid serum T_4 ($n = 6$) was 1.8 ± 0.2 ($P < 0.001$).

Reticulocytosis was induced by injecting animals with 7.5, 9, and 12 mg (i.p.) of phenylhydrazine on 3 consecutive d followed by sacrifice on day 7. "Hypothyroid" reticulocytes were prepared by injecting the same quantities of phenylhydrazine 8 wk following thyroidectomy. Percent reticulocytes was determined after staining with new methylene blue. There were $88 \pm 5\%$ reticulocytes in the control group and $87 \pm 4\%$ in the hypothyroid group.

Reticulocyte membranes were prepared as previously described (8). Receptor binding assay was performed by using $(-)[^3\text{H}]\text{dihydroalprenolol}$ ($[^3\text{H}]\text{DHA}$) at concentrations of 2.0–3.5 nM in competition assays in a volume of 1.0 ml. Protein concentrations were 0.25–0.5 mg/ml. Incubations were for 10 min at 37°C with unlabeled ligands as indicated. Data are presented as specific binding, defined as binding competed for by 1 mM $(-)\text{isoproterenol}$, which was $>90\%$ of total binding.

Adenylate cyclase activity was assayed as previously described (8). Final concentrations were 32 mM Tris-HCl pH 7.65, 10 mM MgCl_2 , 0.2 mM EDTA, 0.04 mM IBMX (3-isobutyl-1-methyl-xanthine) (at 25°C), ATP 0.12 mM, and incubations were for 10 min at 37° in the presence of 0.1 mM guanosine 5'-triphosphate (GTP). Proteins were measured by the method of Lowry using bovine serum albumin as standard (10). All data not derived from computer curve fitting were expressed as mean \pm SEM and comparisons were made by an analysis of variance.

Cholera toxin catalyzed ADP-ribosylation of membranes prepared from reticulocytes obtained from euthyroid and hypothyroid rats. Preparation of reticulocyte membranes and subsequent exposure of these membranes to activated cholera toxin and $[^{32}\text{P}]\text{NAD}^+$ was carried out as previously described and validated (7, 8). Equal quantities of each of the reticulocyte membrane preparations (control and hypothyroid at ~ 30 mg/ml) were exposed for 20 min at 25°C to 50 $\mu\text{g/ml}$ of cholera toxin (preactivated for 10 min at 37° with 20 mM dithiothreitol), 5 μM $[^{32}\text{P}]\text{NAD}^+$ (5–15 Ci/mM), 750 μM GTP,

and a GTP regeneration system that included 2.8 mM phosphoenol pyruvate, 5.2 $\mu\text{g/ml}$ pyruvate kinase, and 10 $\mu\text{g/ml}$ myokinase. As a control experiment reticulocyte membranes from both euthyroid and hypothyroid rats were incubated under similar conditions in the absence of cholera toxin. At the end of the incubation period each preparation was diluted fivefold with 75 mM Tris-HCl (pH 7.5), 12.5 mM MgCl_2 , 1.5 mM EDTA and assayed for adenylate cyclase activity in the presence of GTP (0.1 mM) and NaF (10 mM). It was demonstrated that the incorporation of $[^{32}\text{P}]\text{ADP}$ ribose into the 42,000 M_r protein was linearly related to protein substrate concentration. The ^{32}P incorporation was also directly related to the extent of cholera intoxication (Fig. 1). The extent of intoxication was determined for both euthyroid and hypothyroid membranes by comparing the GTP and NaF-stimulated adenylate cyclase activity after incubation of membranes in the presence and absence of cholera toxin (7). Full (100%) intoxication in the rat reticulocyte system (7, 8) means that adenylate cyclase activity in the presence of GTP is equivalent to that observed in the presence of NaF. The extent of intoxication was $92 \pm 7\%$ for euthyroid and $82 \pm 8\%$ for hypothyroid membranes in six experiments.

Polyacrylamide gel electrophoresis. The membranes, after incubation in the presence and absence of cholera toxin, were washed five times in a buffer containing 75 mM Tris-HCl (pH 7.5), 2 mM EDTA/Trasyolol (5 U/ml) before being dissolved in gel sample buffer (8). Polyacrylamide gel electrophoresis was carried out (8) with equal amounts of protein (control and hypothyroid membrane preparation) placed on each gel lane. $[^{32}\text{P}]\text{ADP}$ -ribosylated proteins were detected by autoradiography for 1–3 d using Kodak XR-5 film (Eastman-Kodak Co., Rochester, N. Y.) in the presence or absence of intensifying screens. $[^{32}\text{P}]\text{ADP}$ -ribosylated proteins were quantitated by slicing each gel lane into 1.5–2-mm slices and then allowing the slices to incubate overnight in 5 ml of scintillation fluid containing Soluene-350 (Packard Instrument Co. Inc., Downers Grove, Ill.) before counting in a Packard Tricarb liquid scintillation spectrometer. The $[^{32}\text{P}]\text{ADP}$ ribose counts peak representing the 42,000 M_r protein (1–2 slices in width) were corrected to counts per minute per milligram protein and compared on a percent basis. In the six experiments a mean of 0.67 ± 0.06 mg protein was placed in each gel lane. However, in each individual experiment there was never a protein difference of >0.03 mg between gel lanes.

Data analysis. Competitive binding assay data were analyzed by a nonlinear least squares curve fitting procedure based on the law of mass action and applied to complex ligand-receptor systems. The method provided estimates of the affinity of ligands for different states of the receptors and for the proportion of these states (percent R_H and percent R_L). Fitted estimates are provided for the data, assuming either one or two affinity states. Statistical analysis comparing "goodness of fit" between one and two affinity state models was determined as previously described (6) and the more complex model was retained only when it significantly improved the fit.

Statistical analysis used in comparing curves and individual parameters was previously described (6). Each curve was formed by averaging individual experiments (control = 3, hypothyroid = 4) and each of the mean curves was initially analyzed independently allowing each parameter estimate to reach its optimum value. Then, the curves were analyzed simultaneously and parameters were constrained to a common value. If the effect of sharing parameters was not to significantly worsen the goodness of fit, then the parameters were considered indistinguishable. Significance was defined as $P < 0.05$.

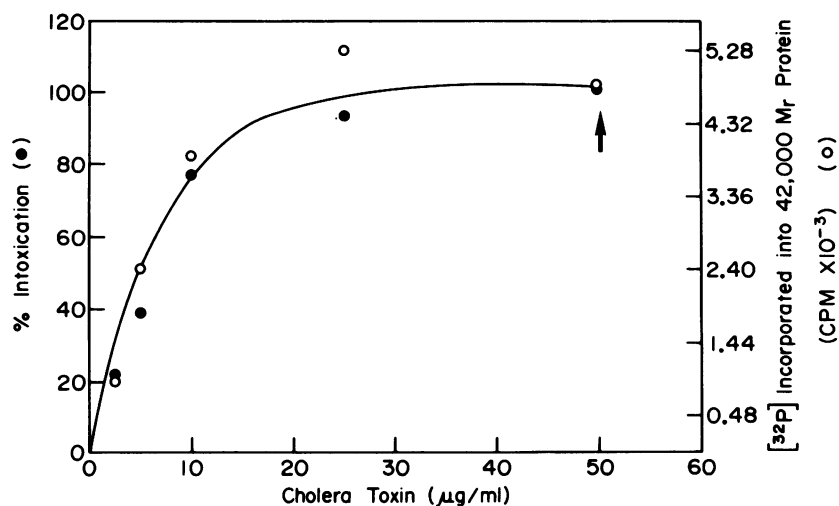


FIGURE 1 The concentration-dependent effects of cholera toxin on adenylate cyclase activity and [^{32}P]ADP ribose incorporation into the 42,000- M_r protein. Intoxication was performed as described in Methods. 100% intoxication of the enzyme adenylate cyclase in this system means that GTP (0.1 mM)-stimulated activity is equivalent to the activity elicited by NaF (10 mM). The ^{32}P incorporated into the 42,000 M_r protein was measured by cutting the gel lane into slices and counting the appropriate region (as determined by a marker protein) in a scintillation counter (Methods). Arrow indicates concentration of cholera toxin used in each experiment. Results are the means of duplicate determinations.

The dissociation constant (K_D) and maximum binding capacity for the antagonist [^3H]DHA were determined by performing saturation experiments over a range of 0.5–10 nM. The data were analyzed according to the same generalized model and using a one-state fit. The EC_{50} (50% displacement) and the steepness factors of competition curves were determined by fitting the data to a four parameter logistic equation (11).

RESULTS

The reticulocytes from hypothyroid animals were found to have significantly fewer beta adrenergic receptors 625 ± 42 fmol/mg ($n = 3$) than did control reticulocytes $1,351 \pm 215$ fmol/mg ($n = 5$) ($P < 0.03$). Similar decreases in receptor number have been observed in other tissues (1). There was no change in the K_D for [^3H]DHA (control 1.2 ± 0.9 nM and hypothyroid 1.0 ± 0.5 nM).

Isoproterenol competition curves always modeled better to a two state fit (K_H and K_L) than a one state fit while isoproterenol curves in the presence of guanylyl-5'-yl-imidodiphosphate (Gpp(NH)p) were always adequately described by a one state model (K_L) which was not improved by a two state fit. The K_L for these two curves (i.e., with and without Gpp(NH)p) were not significantly different, consistent with the interconvertibility of these states.

An important finding was that the isoproterenol competition curve for the hypothyroid reticulocyte was shifted to the right as shown in Fig. 2 with a significant increase in the EC_{50} but no change in the

slope factor (Table I). The isoproterenol plus Gpp(NH)p curve demonstrated no significant change in either EC_{50} or the slope factor.

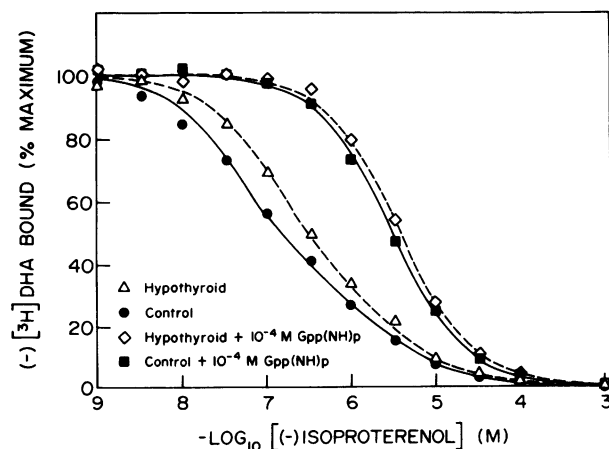


FIGURE 2 Computerized curve fitting of binding data from displacement of [^3H]DHA by (-)-isoproterenol in reticulocytes from control and hypothyroid rats in the presence and absence of Gpp(NH)p. The reticulocyte membranes were incubated with 2.0–3.5 nM [^3H]DHA in competition with the indicated concentration of (-)-isoproterenol in the presence and absence of 0.1 mM Gpp(NH)p. The data points represent the mean of three experiments (control) or four experiments (hypothyroid) each performed in duplicate. Binding in the absence of competitor was ~900 fmol/mg in the control membranes. Data were normalized for purposes of graphic representation.

TABLE I
Effects of Hypothyroidism on (-)Isoproterenol Binding
in the Rat Reticulocyte Using Computer-derived
Parameters from Displacement
Curves with [³H]DHA

	Control (n = 3)	Hypo- thyroidism (n = 4)	Signifi- cance
			<i>P</i>
Slope factor without Gpp(NH)p	0.63±0.02	0.70±0.02	NS
Slope factor with Gpp(NH)p	0.94±0.03	1.01±0.04	NS
EC ₅₀ (nM) without Gpp(NH)p	180±20	380±30	<0.001
EC ₅₀ (nM) with Gpp(NH)p	2,800±200	3,700±400	NS
K _H (nM)	26±2	63±5	<0.001
K _L (nM)	1,380±40	1,730±50	<0.001
K _L /K _H	53±8	28±4	<0.001
R _H %	71±5	66±7	NS

For each set of displacement curves (Fig. 1) the computer programs provided estimates of the EC₅₀ (50% displacement), the slope factors, percentage of total receptors in the high affinity state (R_H%), the high (K_H) and low (K_L) affinity state dissociation constants. The results are expressed as mean ± SEE (standard error of the estimate). The statistical significance was determined as described in Methods. *n*, the number of individual experiments.

The K_H and K_L of the isoproterenol competition curves were both altered significantly in the hypothyroid cells but the fold change in K_H (2.4-fold) was much greater than that in the K_L (1.2-fold). There was a concomitant significant decrease in the K_L/K_H ratio while no significant change was seen in the percent R_H (Table I).

In several other situations where K_L/K_H of an agonist for beta adrenergic receptors is reduced, such as after desensitization or with partial agonists, the biological correlate is a decreased ability of agonists to stimulate adenylate cyclase (6). Accordingly, we investigated the status of the adenylate cyclase system in the reticulocyte membranes. As can be seen from the representative experiment in Fig. 3, there was a 50% fall in maximum isoproterenol (0.1 mM) and F⁻ (10 mM)-stimulated enzyme activity in cells from the hypothyroid animals. In five experiments basal activity did not change significantly between control 5.7±1.4 vs. hypothyroid 3.1±0.9 pmol/min per mg (*P* = 0.28) cells. By contrast, maximum isoproterenol-stimulated activity 51.9±5.7 vs. 20.3±3.8 pmol/min per mg (*P* < 0.002), and fluoride-stimulated activity 56.0±5.7 vs. 25.2±2.6 pmol/min per mg (*P* < 0.001) were significantly reduced. Although the EC₅₀ for isoproterenol stimulation was not significantly altered 1.1±0.7 μM (control) vs.

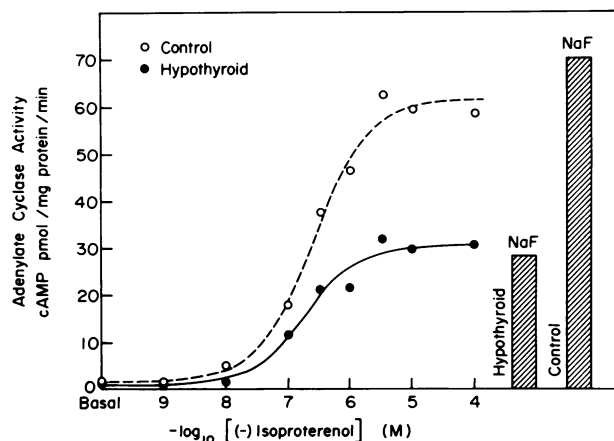


FIGURE 3 Isoproterenol and NaF stimulation of rat reticulocyte adenylate cyclase. The reticulocyte membranes were incubated with the indicated concentrations of isoproterenol or NaF (10 mM) for 10 min at 37° in the presence of 0.1 mM GTP and a standard adenylate cyclase assay mixture (Methods). The data points represent the mean of duplicate determinations from a representative experiment (*n* = 5).

0.7±0.6 μM (hypothyroid) (*P* = 0.71), the day-to-day variability in this parameter was substantial and small changes may not have been evident.

Impaired formation of the high affinity state of the receptor as well as diminished catecholamine- and fluoride-sensitive adenylate cyclase are all consistent with an alteration of the nucleotide regulatory protein. Accordingly, in six additional experiments we quantitated the concentration of 42,000 *M_r* substrates for cholera toxin catalyzed ADP-ribosylation in the membranes, since this technique appears to label the major subunit of the regulatory protein (7, 8). Results of a representative experiment are shown in Fig. 4 as assessed by polyacrylamide gel electrophoresis. The apparent molecular size of the cholera toxin substrate (~42,000 *M_r*) was not different in hypothyroid vs. control reticulocyte membranes. However, the quantity was diminished by 40% in the experiment shown. In this series of six experiments each performed with a separate set of animals, we measured and compared isoproterenol (0.1 mM) and NaF (10 mM)-stimulated adenylate cyclase activity and the quantity of ³²P radioactivity incorporated into the 42,000 *M_r* protein. We found that in the hypothyroid membranes the isoproterenol-stimulated activity was decreased by 44±12%, the NaF-stimulated activity was decreased by 36±11% and the ³²P radioactivity incorporated was decreased by 40±10% compared to control membranes. The percent decrease in isoproterenol and F⁻-stimulated activity is in good agreement with the percent decrease in ³²P incorporation into the 42,000 *M_r* protein. This seems reasonable since both isoproterenol- and fluoride-stimulated activities are

DISCUSSION

The present study suggests that thyroid hormone modulates beta adrenergic function in several ways and indicates that simple enumeration of receptor number is not sufficient to document these interrelationships. We have documented several alterations in the beta adrenergic receptor-adenylate cyclase system of rat reticulocyte membranes derived from hypothyroid animals. These include the following: (a) the number of beta adrenergic receptors is decreased. (b) There is a highly significant decrease in the ability of agonists to stabilize a high affinity state of the beta adrenergic receptor. (c) Maximal isoproterenol and NaF-stimulated adenylylase are decreased. (d) The amount of ADP-ribosylated cholera toxin substrates (N) is decreased.

The alteration in high affinity state formation is apparent as a twofold decrease in the ratio of the dissociation constants of the agonist for the high and low affinity states of the receptor (K_L/K_H). This is mainly due to a twofold change in K_H . Because the high affinity state of the receptor appears to consist of a complex of HR and N (7), these results suggest alterations in the agonist promoted R-N interaction. This could be due to changes occurring at several sites. Our data demonstrate that the concentrations of both receptor and N protein are reduced in membranes from hypothyroid animals. Thus, the impaired ability to form this complex in the hypothyroid membranes may be a consequence of the decreased concentration of both of these components. In addition to the decrease in receptor number, this impaired R-N interaction may well contribute to the decreased sensitivity to catecholamines in hypothyroidism. The changes observed in agonist affinity for the beta adrenergic receptors contrast with the lack of change in antagonist [3 H]DHA affinity in these cells. Because antagonists do not induce a high affinity nucleotide-sensitive form of the receptor, this result seems reasonable.

Our data do not exclude other possible factors that might contribute to the altered catecholamine sensitivity of the adenylylase, such as structural changes in R or N (apparent molecular weight is unchanged) or some change in the membrane environment in which R and N interact. We also cannot exclude changes in the catalytic unit at this point but these would not be expected to perturb agonist binding.

Since N is involved in mediating the stimulatory effects of both catecholamines and F^- on adenylylase (12), the alteration observed in N could explain the decreased K_L/K_H in the binding assay as well as the decreased isoproterenol- and F^- -stimulated adenylylase activity.

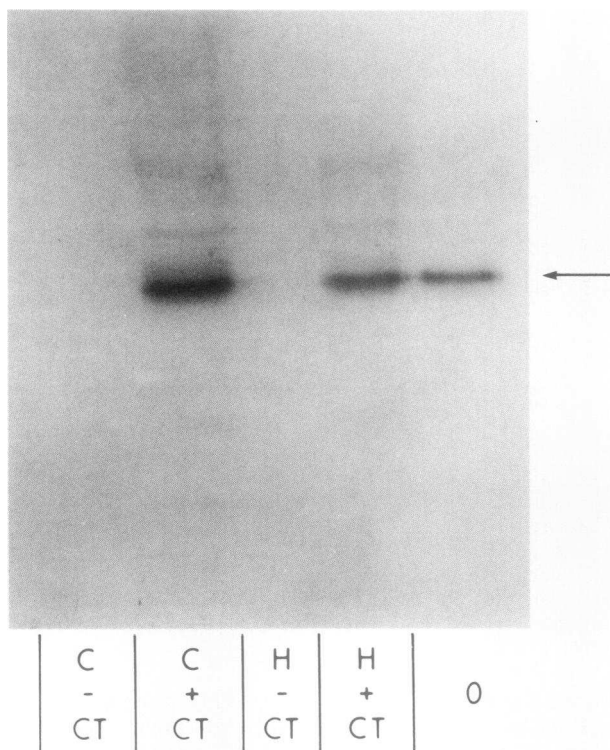


FIGURE 4 Autoradiogram of [32 P]ADP-ribosylated membranes fractionated by SDS-polyacrylamide gel electrophoresis. Reticulocyte membranes from euthyroid and hypothyroid animals were prepared and underwent [32 P]ADP-ribosylation and electrophoresis as described in Methods. 0, I 125 -ovalbumin, provides a mobility marker for a $M_r = 43,000$ protein. H, hypothyroid membrane preparation incubated with 5 μ M [32 P]NAD $^+$ in the presence (H + CT) or absence (H - CT) of cholera toxin (50 μ g/ml). C, euthyroid membranes prepared in the same manner and incubated in the presence (C + CT) or absence of (C - CT) cholera toxin. The amount of protein placed in the control lane was 0.77 mg and in hypothyroid lane was 0.78 mg.

dependent on the presence of the N protein for stimulation to occur (12).

It can be observed that there is also a reduction in the intensity of two higher molecular weight bands in the hypothyroid sample in the experiment shown in Fig. 4. In fact these two were the only bands whose labeling was decreased other than the N protein in this series of experiments. However the labeling of these two additional proteins was quite variable and low (<5% of the specifically incorporated 32 P) and their function is unknown. Since thyroid hormones are known to produce many of their effects by modulating the rate of synthesis of a variety of proteins it is not surprising that a few proteins other than the beta adrenergic receptor and N protein might also be affected by the hypothyroid state.

Our findings in this hypothyroid model are quite similar to those found by Brodde et al. (2) in a rat atrial model. They found decreased numbers of beta adrenergic receptors and a significant decrease in maximal catecholamine- and NaF-stimulated adenylate cyclase in atrial membranes from hypothyroid rats. They, however, did not assess agonist binding or the quantity of cholera toxin substrates.

In contrast, Malbon (13, 15) has found very different results in membranes from fat cells of hypothyroid rats. In this system there is no change in beta adrenergic receptor number (15), and a decrease in catecholamine but not NaF-stimulated adenylate cyclase is observed (14). In addition, agonist binding is apparently altered as manifested by a rightward shift in an agonist competition curve with [³H]DHA and a complete loss in the ability of guanine nucleotides to affect agonist binding. Cholera toxin substrates appear to be slightly increased (20–30%) in fat cell membranes from hypothyroid animals (14). Because the number of receptors and N protein as well as maximal adenylate cyclase activity are all undiminished in the hypothyroid fat system, it becomes necessary to postulate alterations in as yet undisclosed components of the system to explain the altered sensitivity. In contrast, in the reticulocyte system, alterations in known components of the system (R and N) may explain the altered sensitivity. As noted above, the alterations observed in rat heart (2) strikingly resemble these in reticulocyte system.

This study demonstrates the hormonal regulation of two components of the adenylate cyclase system (R and N) and this regulation of N highlights the fact that simple enumeration of R is insufficient for full evaluation of beta adrenergic receptor interactions in pathophysiological conditions. Modification of beta adrenergic receptor – N site interactions may be a novel mechanism of thyroid hormone control of catecholamine responsiveness. Such coupling changes can be quantitatively assessed by careful analysis of agonist binding properties as well as quantification of receptor number and N protein.

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