3,3',5-Triiodothyronine Administration In Vivo Modulates the Hormone-sensitive Adenylate Cyclase System of Rat Hepatocytes

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ABSTRACT The ability of 10 μM epinephrine or isoproterenol to stimulate cyclic AMP accumulation was decreased in hepatocytes isolated from hyperthyroid (triiodothyronine treated) as compared to euthyroid rats. In the presence of methylisobutylxanthine, epinephrine or isoproterenol-stimulated cyclic AMP accumulation was ~65% lower in hyperthyroid as compared with euthyroid rat hepatocytes. The ability of glucagon to stimulate a cyclic AMP response was also decreased in the hyperthyroid state, when assayed in either the absence or presence of a methyl xanthine. The character of the catecholamine-stimulated cyclic AMP response was beta adrenergic in both the hyper- and euthyroid states. No evidence for an alpha$_2$ adrenergic mediated component of catecholamine action on cyclic AMP levels was noted. Cyclic AMP phosphodiesterase activity of hepatocyte homogenates was not altered in the hyperthyroid state. Hormone-stimulated, guanine nucleotide- and fluoride-activatable adenylate cyclase activity was reduced in subcellular fractions obtained from hyperthyroid as compared with euthyroid rat hepatocytes. Beta adrenergic receptor binding was reduced ~35% and glucagon receptor binding reduced ~50% in the hyperthyroid as compared with euthyroid rat hepatocyte membrane fractions. The status of the regulatory components of adenylate cyclase were examined by in vitro treatment of subcellular fractions with cholera toxin. The ability of cholera toxin to modulate adenylate cyclase was not altered by hyperthyroidism. Cholera toxin catalyzed AD[^{32}P]ribosylation of hyperthyroid and euthyroid rat hepatocyte proteins separated electrophoretically displayed nearly identical autoradiograms. Studies of the reconstitution of adenylate cyclase activity of S49 mouse lymphoma *cyc*$^-$ mutant membranes by detergent extracts from rat hepatocyte membranes, indicated that hyperthyroidism was associated with a reduced capacity of regulatory components to confer fluoride, but not guanine nucleotide activatability to catalytic cyclase. Thyroid hormones regulate the hormone-sensitive adenylate cyclase system of rat hepatocytes at several distinct loci of the system.

INTRODUCTION

Thyroid hormones modulate the ability of catecholamine hormones to act in heart (1–4), fat (5–7), and liver (8–10). In heart and fat tissue hypothyroidism blunts, whereas hyperthyroidism potentiates, catecholamine-sensitive responses (1–7). Hyperthyroidism has been shown to potentiate the ability of catecholamines to activate rat myocardial phosphorylase *a* (1, 2). Epinephrine-stimulated cyclic AMP accumulation and lipolysis of isolated rat fat cells are likewise enhanced in those fat cells from hyperthyroid, as compared with euthyroid animals (7).

The effects of thyroid hormones on catecholamine action are quite different in liver, as compared to heart or fat tissue. Hypothyroidism dramatically potentiates, rather than blunts, the ability of beta adrenergic hormones to activate phosphorylase *a* and to stimulate cyclic AMP accumulation (8, 9). This potentiation of the phosphorylase and cyclic AMP responses noted in hypothyroid rat hepatocytes is selective for the beta adrenergic hormone stimulation and is not observed in stimulation by either alpha adrenergic agonists or glucagon (8). The basis for this enhanced beta adrenergic stimulation of cyclic AMP and phosphorylase responses of hypothyroid rat hepatocytes appears to be a two- to threefold increase in the steady-state level of beta adrenergic receptors (10).

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Information on the effects of hyperthyroidism on the regulation of hepatic cyclic AMP metabolism or glycogen phosphorylase activity by catecholamines and other hormones is lacking. We have examined the hormone-responsive adenylate cyclase system of hepatocytes from triiodothyronine-treated (hyperthyroid) as compared to control (euthyroid) rats. We find that short-term thyroid hormone administration in vivo acutely modulates the hormone-sensitive adenylate cyclase system of rat hepatocytes at several distinct loci.

METHODS

Animals. Rats (175–225 g fed female Sprague-Dawley SD strain rats from Taconic Farms, Germantown, N. Y.) were made hyperthyroid by a single injection (subcutaneous administration) of triiodothyronine (0.25 mg/kg body wt) at 48, 24, and 3 h before death. Control rats were littermates or animals of the same weight as those administered thyroid hormone. Serum triiodothyronine levels were 2.2±0.1 nmol/liter in control and 9.3±0.1 nmol/liter in triiodothyronine-treated animals. Serum thyroxine levels were 43.5±4.4 nmol/liter in control animals and reduced to <13 nmol/liter in the animals administered triiodothyronine.

Hepatocyte preparation. Hepatocytes were isolated following the procedure of Berry and Friend (11) as described previously (8). After isolation the rat hepatocytes were incubated in a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 22 mM glucose for 30 min, then washed and resuspended in fresh medium for 20 min for the experiments in plastic tubes (17 × 100 mm, model 55.468, Walter Sarstedt, Inc., Princeton, N. J.) in a total volume of 1 ml and were constantly shaken at 37°C in an orbital water bath operating at 150–200 rpm.

Cyclic AMP accumulation measurements. Hepatocytes were incubated for 20 min as described above and then exposed to a hormone or vehicle and the incubation continued for 1 min, the point of maximal cyclic AMP response. A 0.1-ml aliquot of 2 N HCl was added to terminate the cyclic AMP response. These incubation tubes were then heated in a boiling water bath for 1 min and after cooling were neutralized with NaOH. A 20-μl aliquot was taken from the tube and the cyclic AMP content assayed by a modification of the method of Gilman (12) using the cyclic AMP binding protein extracted from bovine adrenal gland. The free cyclic AMP was separated from the bound cyclic AMP by adsorption on activated charcoal, as suggested by Brown et al. (13).

Subcellular fractionation. Subcellular fractions were prepared from freshly isolated hepatocytes by a modification (10) of Neville's method (14). Hepatocytes were homogenized in 6 vol of ice-cold 1 mM $NaHCO_3$ buffer using a Willems Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) at setting 5 for 30 s and were then further homogenized by 16 strokes of a precision bore glass Potter-Elvehjem homogenizer (Arthur Thomas Co., Philadelphia, Pa.) fitted with a Teflon pestle operated on a Eberback Con-Torque unit set at maximum running speed. All steps from homogenization to preparation of other subcellular fractions were performed in an ice bath. The homogenate was centrifuged at 1,500 *g* for 10 min at 4°C to yield a supernatant that was discarded, and a pellet ("pellet-1"). This pellet was resuspended in 30 ml of 1 mM $NaHCO_3$ and homogenized again with 16 strokes of the Potter-Elvehjem homogenizer. This fraction was centrifuged at 1,500 *g* for 10 min at 4°C. The resulting supernatant was discarded and the pellet ("pellet-2") was resuspended in sufficient 69% (wt/wt) sucrose, 1 mM $NaHCO_3$ to yield precisely 44% sucrose (wt/wt). 10 ml of this mixture were placed into a 13-ml polycarbonate ultracentrifuge tube and gently overlaid with 3 ml of a 42.3% sucrose (wt/wt), 1 mM $NaHCO_3$ solution. The sucrose solutions were checked and adjusted to the indicated density by use of a Zeiss refractometer. The tubes were allowed to stand 30 min at 4°C and then centrifuged at 100,000 *g* for 2 h at 4°C in a Beckman L-65 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) fitted with an SW41-Ti rotor. After centrifugation the float ("Purified membranes") was aspirated, resuspended in 1 mM $NaHCO_3$, centrifuged at 12,000 *g* for 10 min at 4°C, and resuspended to ~2 mg of protein/ml in the same buffer. The membranes were used immediately or stored at −90°C. These membranes were stored up to 8 wk with no significant loss of adenylate cyclase activity, glucagon binding, specific [^{125}I]iodohydroxybenzylpindolol binding, cholera toxin-catalyzed ADP-ribosylation or activation of adenylate cyclase activity (10).

Adenylate cyclase assay. Adenylate cyclase activity was assayed in a final volume of 0.1 ml containing 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM creatine phosphate, 100 U/ml creatine phosphokinase, 0.5 mg/ml bovine serum albumin, 1 mM cyclic AMP, 0.1 mM ascorbic acid, 0.5 mM [α-^{32}P]ATP (35–50 cpm/pmol), subcellular fractions (50–150 μg protein), and hormones, guanine nucleotides, or sodium fluoride as indicated. The low specific activity and relatively small response of the liver homogenate adenylate cyclase to catecholamine stimulation necessitated higher amounts of protein (0.5–1.2 mg) for some assays. The results were verified with assays using lower amounts of protein (100–200 μg) and higher specific activity ATP. The assays were initiated by addition of the enzyme preparation to reaction tubes and were performed in duplicate or triplicate at 30° for 10 min. The reaction was terminated and the [^{32}P]cyclic AMP produced was quantitated by the method of Salomon et al. (15).

Cyclic AMP phosphodiesterase assay. Cyclic AMP phosphodiesterase activity was measured by a modification of the method of Thompson and Appleman (16). The final volume of the assay mixture was 0.1 ml containing 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.125 or 1.025 μM [^{3}H]cyclic AMP (60,000 cpm), and the enzyme preparation (~1 mg of homogenate fraction). The incubation period was 7 min in length and was terminated by boiling for 1 min. A 0.1-ml aliquot of snake venom (1 mg/ml, *Ophiophagus hannah*, from Sigma Chemical Co., St. Louis, Mo.) was added and the mixture then incubated at 37°C for 10 min. The labeled adenosine thus formed was separated from labeled cyclic AMP by the addition of 1.0 ml of 1:3 (wt/wt) slurry of washed Dowex 1-X8 (200–400 mesh) containing 25 mg/100 ml of adenosine and inosine. Under the conditions used ~15% of total cyclic AMP was converted to AMP.

Cholera toxin activation of adenylate cyclase. Initial studies on the effects of cholera toxin on the liver cyclase were conducted in standard adenylate cyclase assay medium (consisting of 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM creatine phosphate, 100 U/ml creatine kinase, 0.5 mg/ml bovine serum albumin, 1 mM cyclic AMP, 0.1 mM ascorbic acid, 0.5 mM ATP containing 1 mM NAD^+ and 10 μg/ml of cholera toxin (preactivated with 20 mM dithiothreitol at 30°C for 10 min) and rat hepatocyte membranes. The mixture was incubated for 10 min at 30°C, then diluted 20-fold with ice-cold 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$,

pelleted, resuspended in fresh cyclase assay medium and immediately assayed for adenylate cyclase assay activity over a 10-min incubation at 30°C using the Salomon et al. (15) procedure. Under these conditions toxin treatment increased basal adenylate cyclase activity of purified membranes 40%, GTP (100 μM)-stimulated activity by 10%. In contrast, toxin treatment of hepatocyte whole homogenate preparations (under identical conditions) increased basal adenylate cyclase activity ~120%, GTP-stimulated activity ~100%, and decreased fluoride-stimulated activity 30–50%. We therefore adopted the rat hepatocyte whole homogenate fraction for the study of cholera toxin action.

Our ultimate aim was to use cholera toxin and this in vitro broken-cell system to AD[^{32}P]ribosylate and identify the adenylate cyclase regulatory proteins of the rat hepatocyte. Moss and Vaughan (17) reported that high phosphate or acetate buffers facilitated cholera toxin-catalyzed ADP-ribosylation of arginine methyl esters, so we reasoned that it might be useful to examine the ability of cholera toxin to activate hepatocyte cyclase in these two buffers. A high-phosphate buffer (250 mM KH_2PO_4, pH7) containing 100 μM ATP, 100 μM GTP, 20 mM arginine-HCl, 20 mM thymidine, and 1 mM NAD^+ supported cholera toxin (100 μg/ml) activation of hepatocyte adenylate cyclase in a fashion similar to that noted with cyclase assay medium. This buffer has been recently shown to reduce nonspecific AD[^{32}P]ribosylation of S49 (18) and human erythrocyte (19) membranes. We have confirmed these observations in this liver system (data not shown).

Using the high phosphate buffer and [^{32}P]NAD^+ we have examined cholera toxin-catalyzed ADP-ribosylation of hepatocyte plasma membrane proteins. The toxin treatment was performed in 250 mM KH_2PO_4, pH 7.0, 0.1 mM ATP, 0.1 mM GTP, 20 mM arginine-HCl, 20 mM thymidine, 10 μM [^{32}P]NAD+ (10–20 Ci/mmol), with and without varying amounts of cholera toxin (preactivated with dithiothreitol [20]) or a dummy solution and 0.1 mg of membrane protein for 10 min at 30°C. At the end of the incubation, the mixture was diluted 20-fold in ice-cold 250 mM KH_2PO_4 buffer, centrifuged, and the resultant pellet solubilized in 1% sodium dodecyl sulfate (SDS) containing 5% 2-mercaptoethanol and boiled for 3 min. This aliquot was electrophoresed on one-dimensional SDS-polyacrylamide slab gels (10% acrylamide) according to the method of Laemmli (21). The gels were then stained and fixed (20 min in 0.2% Coomassie Blue, 50% acetic acid, 10% ethanol) and destained (10% acetic acid, 10% ethanol). The gels were dried and the autoradiography performed on Kodak X-Omat R film (Eastman Kodak Co., Rochester, N. Y.) for 2–3 d.

The reconstitution of guanine nucleotide- and fluoride-sensitive adenylate cyclase of S49 mouse lymphoma *cyc*$^-$ mutant membranes was performed by a modification of the original method of Ross and Gilman (22) as described by Kaslow et al. (19).

Glucagon binding. ^{125}I-Glucagon binding to hepatocyte membranes was determined using the assay described by Lin et al. (23).

Beta adrenergic receptor binding. Binding of [^{125}I]-iodohydroxybenzylpindolol to the subcellular fractions was performed according to a modification (10) of the method of Wolfe et al. (24). Binding assays were routinely performed in triplicate at 37°C for 40 min in a final volume of 0.2 ml containing 50 mM potassium phosphate, pH 7.5, 4 mM $MgSO_4$, 0.01–4 nM [^{125}I]iodohydroxybenzylpindolol, 70–100 μg of the purified membranes fraction (0.3 mg of the pellet-1 fraction, or 1–1.2 mg of homogenate protein), and with or without the indicated beta adrenergic agonists or antagonists. At the end of the incubation period 50 ml of 20 mM KH_2PO_4, pH 7.5 at 37°C, 1 mM $MgSO_4$ buffer was added to each tube and the contents of the tube filtered under vacuum onto a Whatman GFC filter (Whatman, Inc., Clifton, N. J.) (2.4 cm). The filter was washed with 15 ml of the same buffer to remove nonspecific binding. Specific binding was defined as the radioligand binding which was inhibited in the presence of 0.3 μM (−)-propranolol (24). This concentration of (−)-propranolol was approximately two orders of magnitude higher than its dissociation constant (K_d), a condition where 99% of the beta adrenergic receptors would be occupied by this ligand (10, 24). Nonspecific binding [binding retained in the presence of 0.3 μM (−)-propranolol] was 30–40% of bound radioligand for fractions from euthyroid or hypothyroid rat hepatocytes alike.

Protein was determined by method of Lowry et al. (25).

Most reagents were obtained from sources listed previously (10). (−)Isoproterenol, (−)epinephrine, (−)norepinephrine (bitartrate salts), neutral alumina, imidazole, bovine serum albumin (fraction V), and cyclic AMP were purchased from Sigma Chemical Co. 1-Methyl-3-isobutyl xanthine was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. The (+)- and (−)-stereoisomers of propranolol were gifts from Ayerst Laboratories, New York. Crude collagenase type II (lot 45A145) was purchased from Worthington Biochemicals (Freehold, N. J.); creatine phosphate, creatine phosphokinase, and ATP from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; [^{3}H]cyclic AMP (35 Ci/mmol), [^{32}P]NAD^+ (5–10 Ci/mmol), [^{125}I]iodohydroxybenzylpindolol (2,200 Ci/mmol), and Aquasol-2 from New England Nuclear, Boston, Mass.; [α-^{32}P]ATP (5–10 Ci/mmol) from Amersham Corp., Arlington Heights, Ill.; and Dowex AG-50 W-X4 (200–400 mesh) cation-exchange resin from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Since we had previously demonstrated that the ability of epinephrine, isoproterenol, or glucagon to stimulate glycogen phosphorylase activity was blunted in hepatocytes isolated from triiodothyronine-treated as compared with control rats (26), it was of interest to investigate the influence of hyperthyroidism on the ability of these glycogenolytic hormones to modulate intracellular cyclic AMP levels (Fig. 1). In the absence of a methyl xanthine, the cyclic AMP response of the hepatocytes stimulated by epinephrine or isoproterenol was relatively small, as previously noted (8, 27). The cyclic AMP response of the hyperthyroid rat hepatocytes to either isoproterenol or epinephrine was not significantly different from the response in the euthyroid state except at the highest concentration of either hormone where a reduced response in the hyperthyroid rat hepatocytes was evident. In the presence of 50 μM methylisobutylxanthine the cyclic AMP responses were potentiated and the blunted maximal response of the hyperthyroid as compared to euthyroid rat hepatocytes to the stimulation by epinephrine or isoproterenol was highlighted (Fig. 1). Under these conditions, the stimulation of cyclic AMP accumulation by 10 μM epinephrine or isoproterenol was 50%

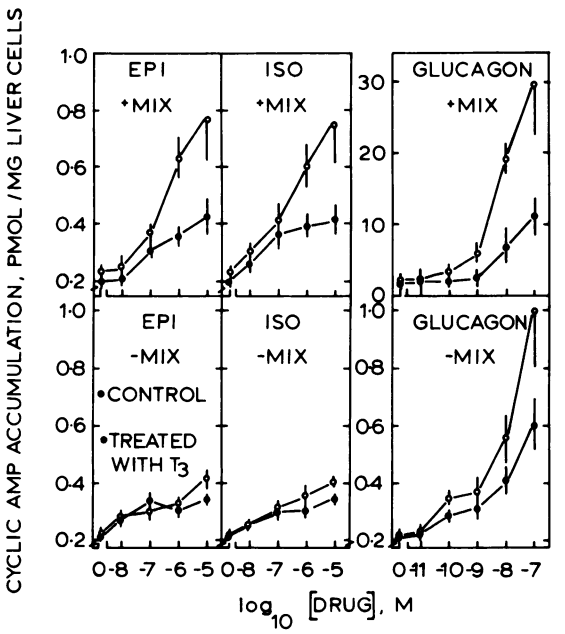

FIGURE 1 The stimulation of cyclic AMP accumulation by epinephrine, isoproterenol, or glucagon in hepatocytes isolated from control and triiodothyronine-treated rats. Rat hepatocytes (50 mg cell wet wt/tube) were isolated from euthyroid (control) and hyperthyroid (triiodothyronine-treated) rats, incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 22 mM glucose for 30 min, then resuspended in fresh medium for another 20 min for the experiments. The indicated agents were added 1 min prior to the end of the 20-min incubation and were added in the absence (−MIX) or presence (+MIX) of 50 μM methylisobutylxanthine. At the end of a 1-min incubation with these indicated agents the incubation was terminated and the cyclic AMP accumulation assayed as described. The values provided are mean values±SEM of data derived from at least four separate experiments performed on separate occasions for both control and triiodothyronine-treated rat hepatocytes. Epinephrine (EPI), isoproterenol (ISO), and glucagon were present in the incubation at the final concentrations indicated. Please note different scale for glucagon stimulation in the presence of the methyl xanthine.

less in hepatocytes from the triiodothyronine-treated as compared with control rats. Glucagon-stimulated cyclic AMP accumulation was also blunted in the hyperthyroid rat hepatocytes (Fig. 1). This impaired response to glucagon in the hyperthyroid state was apparent even when the methyl xanthine was omitted from the incubation (Fig. 1). Short-term (48 h) administration of triiodothyronine to rats in vivo clearly impairs the ability of catecholamines or glucagon to stimulate cyclic AMP accumulation in hepatocytes isolated from these animals.

The abilities of the potent alpha adrenergic antagonist prazosin and beta adrenergic antagonist propranolol to block catecholamine-stimulated cyclic AMP accumulation was examined in order to probe the nature of the altered adrenergic response noted in the hepatocytes obtained from the triiodothyronine-treated rats (Table I). Catecholamine-stimulated cyclic AMP accumulation of control rat hepatocytes displayed a beta adrenergic character that was effectively blocked by propranolol but not by prazosin. The inclusion of methylisobutylxanthine in the incubation did not alter the character of the response. The relatively poor cyclic AMP response of the hyperthyroid rat hepatocytes necessitated that the blocking studies be performed in the presence of the methyl xanthine. Under these conditions, catecholamine-stimulated cyclic AMP accumulation by hyperthyroid rat hepatocytes was effectively blocked by propranolol indicating a beta adrenergic nature. Interestingly, prazosin also partially blocked the response of the hyperthyroid rat hepatocytes to epinephrine or isoproterenol. The basis for this observation is unclear.

The blunted cyclic AMP response of the hyperthyroid rat hepatocytes to hormonal stimulation could reflect an increase in cyclic AMP phosphodiesterase activity, a decrease in the number or affinity of hormone receptors coupled to the cyclase, a decrease in the efficiency of transmembrane signaling between the receptors and the cyclase, or some complex modulation of the cyclase system involving several of these loci. Because a linkage between thyroid hormones and cyclic AMP phosphodiesterase activity has been reported in fat cells (28) the role of phosphodiesterase in the hyperthyroid rat hepatocyte was investigated. The low Michaelis constant (K_M) cyclic AMP phosphodiesterase activities of homogenate fractions, assayed at 0.125 μM substrate, were unaltered by triiodothyronine treatment being 4.1±0.3 and 4.0±0.2 pmol/min per mg protein for control and hyperthyroid rat hepatocytes, respectively. When measured at 1.025 μM substrate, phosphodiesterase activities for control and hyperthyroid were again the same, 19.8±2.1 and 21.0±1.2 pmol/min per mg protein, respectively.

The hormone-sensitive adenylate cyclase system of the hyperthyroid rat hepatocyte was investigated next, in an attempt to identify the basis for the impaired cyclic AMP response, which apparently did not involve a change in the phosphodiesterase activity. Hepatocytes isolated from control and triiodothyronine-treated rats were homogenized and the hormone-sensitive adenylate cyclase activity measured in the whole homogenate, a crude membrane (pellet-1), and a purified membrane fraction. Hepatocyte yield and the amount of total protein per hepatocyte preparation were not affected by this triiodothyronine treatment (26). Isoproterenol failed to stimulate adenylate cyclase activity in hyperthyroid rat hepatocyte fractions (Table II). No stimulation was noted in hyperthyroid rat hepa-

TABLE I

Alpha and Beta Adrenergic Antagonist Effects on Hormonal Stimulation of Cyclic AMP Accumulation in Hepatocytes from Control and Triiodothyronine-treated Rats

	Source of hepatocytes		
	Control rat		Triiodothyronine-treated rat
Incubation with 50 μM MIX	−	+	+
	Change from basal cyclic AMP accumulation, pmol/mg of liver cells		
Agent			
Epinephrine, 10 μM	+0.25	+0.42	+0.31
+ Prazosin, 10 μM	+0.23	+0.33*	+0.14*
+ Propranolol, 10 μM	+0.04*	+0.02*	+0.01*
Isoproterenol, 10 μM	+0.27	+0.35	+0.23
+ Prazosin, 10 μM	+0.31	+0.32	+0.18
+ Propranolol, 10 μM	+0.04*	−0.06*	−0.04*
n	(4)	(3)	(3)

Hepatocytes were isolated from control rats and rats administered 25 μg of triiodothyronine/100 g body wt at 48, 24, and 3 h before hepatocyte preparation. The cells were incubated without and with 50 μM methylisobutylxanthine (MIX) and no agent or the hormones indicated and the cyclic AMP accumulation assessed as described in Methods. The data are mean values of triplicates from "*n*" number of separate hepatocyte preparations. Basal cyclic AMP values were 0.27±0.01, 0.33±0.07, and 0.19±0.02 pmol/mg of cells in control without MIX, control with MIX, and triiodothyronine-treated groups. * $P < 0.05$ in comparison with change obtained in the absence of any blocking agent in response to the indicated agent.

tocyte homogenates at even 100 μM isoproterenol (data not shown). The ability of glucagon to stimulate adenylate cyclase activity was diminished ~40% in the hyperthyroid state (Table II). Maximal stimulation of the cyclase by glucagon was 50% less in purified membranes from hyperthyroid as compared to euthyroid rat hepatocytes (Fig. 2). The concentration of glucagon required to achieve half-maximal stimulation of purified membrane adenylate cyclase activity was higher for the hyperthyroid (28±4 nM, $n = 5$) as compared to euthyroid (7±1 nM, $n = 5$) rat hepatocyte preparations. These data implicate the hormone-sensitive adenylate cyclase system as the primary site of the impaired hormonal response of the hyperthyroid rat hepatocyte.

The possibility that alterations in hormone receptor status may be an important component of the impaired responses of the hyperthyroid rat hepatocyte was investigated via radioligand binding analysis. Beta adrenergic receptors were studied using the potent antagonist [^{125}I]iodohydroxybenzylpindolol, as previously described (10). A 30% reduction in the amount of specific [^{125}I]iodohydroxybenzylpindolol binding per milligram of protein was noted in purified membranes obtained from triiodothyronine-treated (B_{max} = 27±2 fmol/mg protein, $n = 4$) as compared with control (B_{max} = 37±4 fmol/mg protein, $n = 4$) rat hepatocytes (Fig. 3). Similar reductions in the amount of specific [^{125}I]iodohydroxybenzylpindolol binding per milligram of protein were noted in the whole homogenate and low-speed pellet fractions (crude membranes) obtained from the triiodothyronine-treated as compared to control rat hepatocytes (data not shown). A significant increase in the K_d for specific [^{125}I]iodohydroxybenzylpindolol binding to hepatocyte membranes was noted in the hyperthyroid (K_d = 0.32 ±0.03 nM, $n = 4$) as compared to the euthyroid (K_d≐0.15±0.02 nM, $n = 4$) states (Fig. 3). Nonspecific binding was the same for both control and thyroid hormone-treated rat hepatocyte preparations (data not shown).

Specific ^{125}I-glucagon binding to particulate membrane fractions was ~50% less in the hyperthyroid as compared in euthyroid rat hepatocyte fractions measured at 0.9, 1.9, or 10.9 nM radioligand (Table III). Scatchard analysis of steady-state binding data generated in this system yields curvilinear plots (data not shown) and thereby precludes definition of K_d or B_{max} values.

The amount of fluoride-stimulatable adenylate cyclase activity was measured in hyperthyroid and euthyroid rat hepatocytes to provide an index of the cat-

TABLE II

Hormonal Stimulation of Adenylate Cyclase Activity of Subcellular Fractions of Hepatocytes Isolated from Control Rats and Rats Administered Triiodothyronine

Fraction	Agent	Adenylate cyclase activity	
		Control rats	T_3-treated rats
		pmol/10 min/mg of protein	
Homogenate	None	20±4	27±6
	Isoproterenol	35±4*	26±4
	Glucagon	137±20*	102±11*
Pellet-1	None	30±4	21±3
	Isoproterenol	37±5	26±3
	Glucagon	134±24*	71±16*‡
Purified membranes	None	68±9	64±9
	Isoproterenol	68±3	66±9
	Glucagon	158±19*	99±12*‡

The concentrations of each agent are as follows: isoproterenol, 10 μM; glucagon, 10 μM.

Adenylate cyclase activity was assayed in subcellular fractions prepared from hepatocytes isolated from control rats and rats administered 25 μg of triiodothyronine/100 g body wt at 48, 24, and 3 h prior to the cell isolation. Details of the cell isolation, subcellular fractionation, and adenylate cyclase assay appear in Methods. The data are mean values±SEM from four to seven separate hepatocyte preparations for control and triiodothyronine (T_3) treated rats alike.

* Differs significantly ($P < 0.05$) from unstimulated value.

‡ Differs significantly ($P < 0.05$) from control rat adenylate cyclase activity value.

alytic activity of the cyclase and an indication as to whether the hyperthyroid state might be associated with a change in this parameter. Total fluoride-stimulated adenylate cyclase activity per hepatocyte homogenate was 30% less for the triiodothyronine-treated as compared to euthyroid rat hepatocytes, whereas total protein content was not affected (Table IV). Fluoride-stimulated activity was 52–55% less in the pellet-1 and purified membrane fractions of the hyperthyroid as compared to euthyroid rat hepatocytes. Although the protein yield of the particulate membrane preparations was reduced in the hyperthyroid rat hepatocyte fractions, it was of insufficient magnitude to explain the loss of catalytic cyclase activity (Table IV).

The ability of guanine nucleotide triphosphates and fluoride to stimulate adenylate cyclase activity in homogenate and purified membrane fractions obtained from control and triiodothyronine-treated rats is shown in Table V. Stimulation of adenylate cyclase by GTP or the guanine nucleotide analogue guanylylimidodiphosphate (GppNHp) was blunted 30–50% in the hepatocyte fraction obtained from the hyperthyroid as compared to euthyroid rat. These data suggest that not only the activation by hormones such as isoproterenol or glucagon but also that the level of activity measured in the presence of either fluoride or GTP are reduced in the hyperthyroid rat hepatocyte. Thus, the reduced hormonal response may reflect alterations in the hormone receptor number, and in either the capacity of the cyclase to utilize a guanine nucleotide triphosphate, or in the amount of catalytic cyclase itself.

It was important to distinguish between a possible reduction in the amount of catalytic cyclase and a reduced amount of adenylate cyclase regulatory proteins necessary for the activation of catalytic cyclase by GTP or fluoride. The regulatory proteins that confer guanine nucleotide, fluoride, and hormone-sensitivity to adenylate cyclase systems replete with hormone receptors and catalytic cyclase are the target for the action of cholera toxin on adenylate cyclase (29–31). The A_1-subunit of cholera toxin specifically catalyzes ADP-ribosylation of these regulatory proteins at the expense of NAD^+ and irreversibly activates the cyclase activity (cf. 32). The incubation of hepatocyte homogenate with cholera toxin and NAD^+ (1 mM) increased basal and GTP-stimulated adenylate cyclase activity while inhibiting fluoride-activatable activity in a dose-dependent manner (data not shown). Under these conditions, cholera toxin produced maximal effects on the cyclase activity at 30 μg/ml. The kinetics of these effects of the in vitro cholera toxin treatment were examined and found to be rapid, achieving near maximal

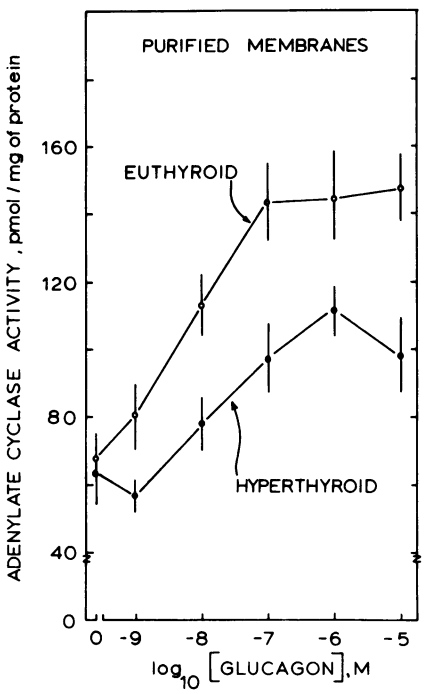

FIGURE 2 Glucagon-stimulated adenylate cyclase activity of purified membranes of hepatocytes isolated from control (euthyroid) and treated (hyperthyroid) rats. See the legend to Table IV for details of these experiments. The data shown are mean values±SEM of five separate hepatocyte membrane preparations for euthyroid and hyperthyroid rat hepatocytes alike. The adenylate cyclase activity was determined over a 10-min incubation period.

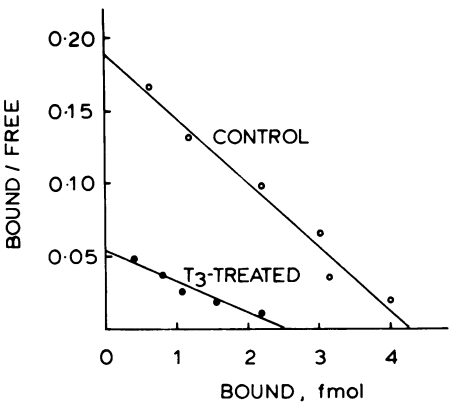

FIGURE 3 Scatchard analysis of specific [^{125}I]iodohydroxybenzylpindolol binding to purified membranes prepared from euthyroid (control) or hyperthyroid (T_3-treated) rat hepatocytes. Details of the cell isolation, membrane preparation, and radioligand binding assay appear in Methods. Each point is the mean of triplicate determinations from a single experiment, representative of at least three different hepatocyte preparations from control and thyroid hormone-treated rats alike. Purified membranes were diluted to obtain 0.1 mg of membrane/binding assay for membranes derived from both control and T_3-treated rat hepatocytes in this particular experiment.

levels within 10 min at 30°C under the conditions specified (data not shown).

Having demonstrated the ability of cholera toxin to influence the adenylate cyclase system of the rat hepatocyte, we then investigated the effects of toxin treatment on homogenates prepared from hyperthyroid as compared to euthyroid rat hepatocytes (Table VI). Cholera toxin increased basal and GTP-stimulated adenylate cyclase activity in hyperthyroid rat hepatocyte preparations, but did not produce the same level of activity noted in the euthyroid rat preparations. Similarly, the difference in fluoride-stimulated activity between the hyperthyroid and euthyroid conditions persisted following the toxin treatment although both were inhibited by the action of cholera toxin (Table VI).

When [^{32}P]NAD^+ was used in the in vitro cholera toxin treatment, autoradiography of the products separated on SDS-polyacrylamide gels by electrophoresis disclosed the toxin-catalyzed AD[^{32}P]ribosylated components (putative regulatory proteins of adenylate cyclase). In the presence of [^{32}P]NAD^+ and no cholera toxin, little radioactivity was incorporated into either purified membranes (lane A of Fig. 4) or homogenate (lane C of Fig. 4) hepatocyte proteins. Cholera toxin treatment of purified membranes from hepatocytes performed with labeled NAD^+ resulted in the incorporation of label into several distinct bands on SDS-polyacrylamide gels which were visualized in the autoradiogram (lane B, Fig. 4). The predominant labeling was noted at 41.6K daltons (M_r). A minor band of labeling was evident at 44.4K daltons (lane B, Fig. 4), but the appearance of this band in autoradiograms from toxin-treated purified membranes was quite variable being nearly absent in some preparations.

The amount of toxin-specific labeling per milligram protein was nearly doubled when the reaction was performed with whole homogenate as compared with purified plasma membrane fractions (data not shown). In contrast to the single major toxin-specific band of ADP-ribosylation noted with plasma membrane fractions, three heavily labeled bands were evident in autoradiograms of the ADP-ribosylated products from a homogenate fraction (lane D, Fig. 4). The apparent molecular weights (M_r) of these toxin targets as determined under the denaturing, reducing conditions of the electrophoresis were 41.6, 44.4, and 45.9K daltons (average of eight determinations). The 41.6K dalton target is the predominant band when using the homogenate fraction in the toxin-catalyzed reaction. The dose-response with respect to toxin for the cholera toxin-catalyzed ADP-ribosylation of hepatocyte membrane proteins (using the whole homogenate fractions in the reaction) is shown in Fig. 4, lanes E–L. As was

TABLE III

^{125}I-Glucagon Binding to Pellet-1 and Purified Membranes of Hepatocytes Obtained from Control Rats and Rats Administered Triiodothyronine

Fraction	Source	^{125}I-Glucagon binding, pmol/mg of protein		
		Radioligand concentration, nM		
		0.9	1.9	10.9
Pellet-1	Control	0.29±0.03	0.43±0.04	0.87±0.06
	T_3-treated	0.16±0.02*	0.22±0.03*	0.57±0.08*
Purified membranes	Control	1.00±0.30	1.40±0.45	2.25±0.74
	T_3-treated	0.43±0.09*	0.70±0.11*	1.00±0.18*

Hepatocytes were isolated from control rats and rats administered 25 μg of triiodothyronine/100 g body wt at 48, 24, and 3 h prior to cell isolation. After cell isolation, pellet-1 and purified membrane fractions were prepared as described in Methods. ^{125}I-Glucagon binding was assayed according to the method of Lin et al. (23). The data are expressed as mean values±SEM from three separate experiments each assayed in triplicate using three different hepatocyte preparations for control and triiodothyronine (T_3) treated rats alike.

* $P < 0.05$ with respect to difference from control glucagon binding values.

noted in the pigeon erythrocyte (30), fat cell (33), and S49 (31) cell membranes, the level of AD[^{32}P]ribose incorporated per milligram of liver membrane protein increased linearly with toxin concentration without apparent clear-cut saturation.

Successful labeling of cholera toxin targets in intact cells via prelabeling the intact cells with ^{32}P-inorganic phosphate has not been reported. To establish whether or not the toxin targets that are ADP-ribosylated in the in vitro broken-cell assay are targets related to cholera toxin action in vivo, we examined the effects of incubation of intact hepatocytes with cholera toxin at 37° for 4 h on the subsequent ability of cholera toxin to AD[^{32}P]ribosylate hepatocyte membrane proteins in the broken cell system (Fig. 5). The AD[^{32}P]ribosylation of the 41.6, 44.4, and 45.9K dalton targets by cholera toxin was blocked in the fractions obtained from hepatocytes exposed to 10 μg/ml of holocholera toxin for 4 h. (Compare lane F representing cells preincubated without toxin to lane C, Fig. 5 representing cells preincubated with cholera toxin prior to homogenization and in vitro toxin-catalyzed AD[^{32}P]ribosylation.) These data suggest that these targets are sites for toxin action in the intact cells.

TABLE IV

Adenylate Cyclase Activities of Subcellular Fractions of Hepatocytes from Control Rats and Rats Treated with Triiodothyronine

Fraction	Source			
	Control rats		Triiodothyronine-treated rats	
	Protein	NaF-stimulated adenylate cyclase activity	Protein	NaF-stimulated adenylate cyclase activity
	mg	*pmol/10 min*	*mg*	*pmol/10 min*
Homogenate	989±80	272.4±9.2 × 10^3	1,080±130	205.3±5.1 × 10^3*
Pellet-1	429±40	114.4±3.9 × 10^3	351±30	54.0±9.9 × 10^3*
Purified membranes	14±2	9.3±1.3 × 10^3	10±2	3.7±0.2 × 10^3‡

Hepatocytes were isolated from control rats and rats administered 25 μg of triiodothyronine/100 g body wt at 48, 24, and 3 h before cell isolation. Protein was assayed according to Lowry et al. (25) and adenylate cyclase was assayed in the presence of 10 mM NaF using the whole homogenate, pellet-1 and purified membrane fractions prepared from the isolated hepatocytes. The data are mean values±SEM from three separate hepatocyte preparations for control and triiodothyronine-treated rats alike.

* $P < 0.05$ with respect to control activity value.

‡ $P < 0.01$ with respect to control activity value.

TABLE V

Guanine Nucleotide and Fluoride Stimulation of Adenylate Cyclase Activity of Subcellular Fractions of Hepatocytes from Control Rats and Rats Administered Triiodothyronine

Fraction	Agent	Adenylate cyclase activity	
		Control rats	T_3-treated rats
		pmol/10 min/mg of protein	
Homogenate	None	20±4	27±6
	GTP, 1 μM	43±5	30±4
	GTP, 100 μM	51±4	32±3
	GppNHp, 1 μM	77±2	55±3
	GppNHP, 100 μM	101±3	91±2
	NaF, 10 mM	223±18	154±10
Purified membranes	None	68±9	64±9
	GTP, 1 μM	192±33	122±15
	GTP, 100 μM	200±37	114±13
	GppNHp, 1 μM	377±59	232±44
	GppNHp, 100 μM	434±76	242±37
	NaF, 10 mM	436±48	227±29

Details of these experiments are described in the legend to Table II. The data are mean values±SEM from four to seven separate hepatocyte preparations for control and triiodothyronine-treated rats alike.

TABLE VI

Effects of Cholera Toxin Treatment on the Adenylate Cyclase Activity of Whole Homogenates Prepared from Hyperthyroid as Compared with Euthyroid Rat Hepatocytes

Pretreatment	Euthyroid		Hyperthyroid	
	−CT	+CT	−CT	+CT
	Adenylate cyclase activity, pmol/10 min/mg of protein			
Agent				
None	26±1	71±4	22±1	56±4*
GTP, 0.1 mM	47±3	173±5	33±3*	149±1*
naF, 10 mM	161±2	112±8	123±5*	79±2*

Aliquots of whole homogenates (0.7–1.0 mg of protein) were incubated in a final volume of 0.1 ml containing 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM creatine phosphate, 100 U/ml creatine phosphokinase, 0.05 mg bovine serum albumin, 1 mM cyclic AMP, 0.1 mM ascorbic acid, 0.5 mM ATP, 1 mM NAD^+, with and without 10 μg of cholera toxin that was previously activated with 0.5% SDS and 20 mM dithiothreitol (24). This mixture was incubated for 20 min at 30°C and the incubation stopped by a 20-fold dilution with ice-cold 1 mM $NaHCO_3$ buffer. The contents were then centrifuged at 20,000 *g* for 15 min at 4°C and the resultant pellet resuspended to a protein concentration of 1 mg/ml with 1 mM $NaHCO_3$ buffer. Adenylate cyclase activity was immediately assayed according to Salomon et al. (15) in the absence or presence of 0.1 mM GTP or 10 mM NaF for a 10-min period at 30°C. The data are expressed as the mean values±SEM from three separate hepatocyte homogenate preparations for euthyroid and hyperthyroid alike. Cholera toxin is abbreviated CT.

* $P < 0.05$ for difference with respect to euthyroid adenylate cyclase values.

It was important to eliminate the possibility that these multiple targets might be generated during the homogenization or in the subsequent steps of the procedure. It was found that the labeling pattern noted in Fig. 5 was not affected when the final membrane pellets were solubilized in SDS/mercaptoethanol and electrophoresed with no boiling, or 1-3 min of boiling. The inclusion of the proteolytic enzyme inhibitor phenylmethanesulfonyl fluoride (0.1 mM) in either the cell homogenization step, or in the toxin-labeling reaction, or both, did not alter this pattern (compare lane H to lane J, Fig. 5). Aprotinin and tosyl-L-lysine chloromethyl ketone were similarly without effect on this pattern (data not shown). Exposing liver membranes which had been incubated with [^{32}P]NAD^+ and cholera toxin to mild proteolysis with trypsin or α-chymotrypsin did not cause the 44.4 or 45.9K dalton band to migrate at the 41.6K dalton region or to any single 30–40K dalton region (data not shown). These data support our hypothesis that there are multiple toxin-specific AD[^{32}P]ribosylated targets in rat hepatocyte membranes that exist in vivo as discrete 45.9, 44.4, and 41.6K dalton entities and that these membrane components may be toxin targets involved in the activation of the cyclase by cholera toxin (presumably representing liver adenylate cyclase regulatory proteins).

The results of our studies of cholera toxin-catalyzed AD[^{32}P]ribosylation of homogenate proteins obtained from hyperthyroid as compared to euthyroid rats are displayed in Fig. 6. The autoradiograms for hyperthyroid and euthyroid rat hepatocyte homogenates were virtually identical. Scanning densitometry of these autoradiograms failed to identify any significant differences in either the pattern or relative abundance of the toxin-specific ADP-ribosylated proteins in the hyperthyroid as compared with euthyroid rat hepatocyte preparations. The toxin-catalyzed ADP-ribosylation of these rat hepatocyte targets (presumed to be regulatory proteins of adenylate cyclase) appears to be unaltered by the hyperthyroidism induced in these experiments.

The ability of detergent extracts of hepatocyte membranes obtained from euthyroid and hyperthyroid rats to reconstitute the adenylate cyclase of S49 mouse lymphoma *cyc*$^-$ mutant membranes [lacking both functional adenylate cyclase regulatory proteins (34) and toxin-specific labeling products (31)] was examined to provide an estimate of the functional status

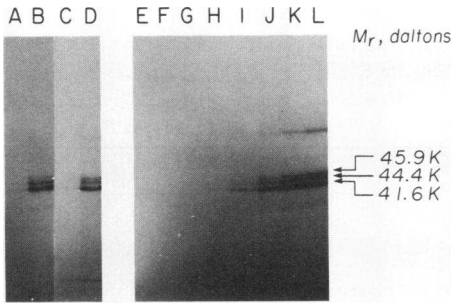

FIGURE 4 Autoradiograph of SDS-polyacrylamide gel electrophoresis of cholera toxin-catalyzed AD[^{32}P]ribosylated products of hepatocyte plasma membrane and whole homogenate subcellular fractions. Cholera toxin-catalyzed ADP-ribosylation of hepatocyte purified membranes (lanes A and B) or hepatocyte homogenate (lanes C–L) was examined in 250 mM KH_2PO_4, pH 7.0, 0.1 mM ATP, 0.1 mM GTP, 20 mM arginine-HCl, 20 mM thymidine, 10 μM [^{32}P]NAD^+ (10–20 Ci/mmol), in the absence (lanes A, C, and E) or presence of 10 μg/ml (lanes B and D), or 0.1 (lane F), 0.33 (lane G), 1.0 (lane H), 3.3 (lane I), 10 (lane J), 33 (lane K), or 75 (lane L) μg/ml of cholera toxin preactivated with 20 mM dithiothreitol (34). Incubation of the hepatocyte subcellular fractions and toxin was performed at 30° for 10 min. At the end of the incubation the mixture was diluted 20-fold with cold 250 mM KH_2PO_4, pH 7.0, buffer, centrifuged, and the resulting pellet solubilized in 1% SDS containing 5% 2-mercaptoethanol and boiled for 3 min. The SDS-polyacrylamide electrophoresis, staining, destaining, and autoradiography are described in Methods.

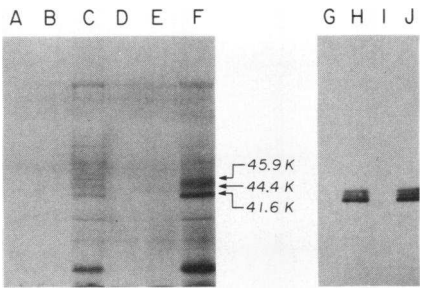

FIGURE 5 Autoradiograph of SDS-polyacrylamide gel electrophoresis of cholera toxin-catalyzed AD[^{32}P]ribosylation products of hepatocyte homogenates derived from cells previously incubated with and without cholera toxin. Hepatocytes were isolated and divided into two equal portions, one portion incubated with cholera toxin the other incubated without the toxin. The incubations were performed in Krebs-Ringer bicarbonate buffer containing 22 mM glucose for 4 h at 37°C with constant gassing with 95% O_2/5% CO_2. At the end of this incubation the cells were washed twice with fresh buffer, resuspended in 1 mM $NaHCO_3$ buffer, homogenized, and an aliquot of this homogenate used for cholera toxin-catalyzed AD[^{32}P]ribosylation as described in the legend to Fig. 4. Toxin-catalyzed labeling of homogenates from cells incubated with the holotoxin for 4 h is displayed in lanes A–C, whereas that of homogenates from cells not incubated with the toxin appears in lanes D–F. The concentration of preactivated cholera toxin in the broken-cell assay was 0 (lanes A and D), 0.3 (lanes B and E), or 10 (lanes C and F) μg/ml. The effects of the protease inhibitor phenylmethylsulfonyl fluoride on cholera toxin-catalyzed AD[^{32}P]-ribosylation of hepatocyte homogenate proteins (lanes G–J). Hepatocytes were isolated and then homogenized and ADP-ribosylated in the absence (lanes G and H) or presence (lanes I and J) of 0.1 mM phenylmethylsulfonyl fluoride. The in vitro intoxication reaction was performed in the absence (lanes G and I) or presence (lanes H and J) of 10 μg/ml of preactivated cholera toxin as described in the legend to Fig. 4. This autoradiogram is of SDS-polyacrylamide gel electrophoresis of the AD[^{32}P]ribosylated products of the reaction.

of the regulatory proteins for adenylate cyclase. Lubrol extracts prepared from hepatocyte membranes were heated at 37°C for 10 min to inactivate catalytic cyclase activity of the extracts and then mixed with *cyc*$^-$ membranes and the adenylate cyclase activity of this mixture assayed in the presence of GppNHp or fluoride. The heated extracts contained virtually no catalytic adenylate cyclase activity (Table VII). The S49 mouse lymphome *cyc*$^-$ membranes, deficient in endogenous regulatory proteins, displayed no adenylate cyclase activity in the presence of the activators GppNHp or fluoride. The reconstituted mixture, however, displayed both guanine nucleotide- and fluoride-stimulatable adenylate cyclase activity (Table VII). Lubrol extracts of hyperthyroid rat hepatocyte membranes reconstituted to the euthyroid level of GppNHp-stimulated cyclase activity but to only 80% of the euthyroid level of fluoride-stimulated activity. These data indicate that Lubrol extracts of hyperthyroid rat hepatocyte membranes possess functional adenylate cyclase regulatory proteins that can reconstitute guanine nucleotide- and fluoride-stimulatable adenylate cyclase activity of S49 *cyc*$^-$ mutant membranes, but that only the guanine nucleotide response and not that for fluoride attains the levels displayed by extracts from the euthyroid counterpart.

DISCUSSION

Our studies demonstrate that acute hyperthyroidism induced by short-term administration of triiodothyronine in vivo is associated with an impaired ability of beta adrenergic hormones and glucagon to stimulate hepatic cyclic AMP accumulation. Hyperthyroidism has been shown to exert the opposite influence of beta adrenergic hormone action in fat cells (7) and heart (3, 4), enhancing rather than blunting the responsiveness of these tissues to stimulation by beta adrenergic agonists.

Alpha$_1$ and alpha$_2$ adrenergic receptors have been identified in the rat liver (35, 36). The alpha$_1$ receptors mediate the alpha adrenergic activation of glycogen phosphorylase (35, 36). The actions mediated by alpha$_2$-receptors have not been elucidated in the rat hepatocyte, but in a variety of tissues activation of alpha$_2$-receptors results in an inhibition of the cyclic

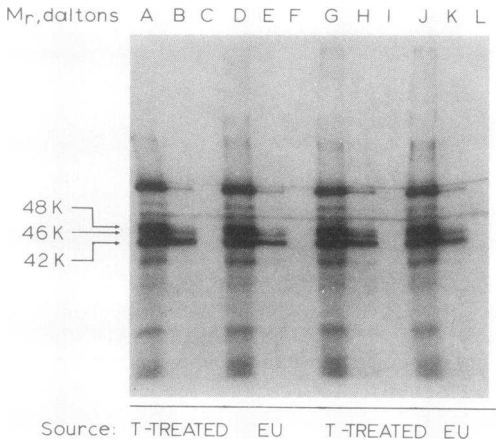

FIGURE 6 Cholera toxin-catalyzed [^{32}P]ADP-ribosylation of proteins of hepatocyte homogenates from control as compared with hyperthyroid rats. Hepatocytes were isolated from control animals or animals administered 25 μg of triiodothyronine/100 g body wt at 48, 24, and 3 h prior to cell isolation. Whole homogenate fractions were prepared and cholera toxin catalyzed ADP-ribosylation was performed using [^{32}P]NAD$^+$. The details of the intoxication, SDS-polyacrylamide gel electrophoresis, and autoradiography appear in the legend to Fig. 4. Equivalent amounts of homogenate protein were used and the amount of protein loaded onto the gels was 0.1 mg in all lanes. Hepatocyte homogenates obtained from T_3-treated (lanes A–C, G–I), and control (lanes D–F, J–L) rats were treated with no toxin (lanes C, F, I, L), 5.0 μg/ml of activated toxin (lanes B, E, H, K), or 50 μg/ml of activated toxin (lanes A, D, G, J). This autoradiogram is representative of three such experiments, each performed with different rat hepatocyte membrane preparations.

TABLE VII

Reconstitution of S49 Mouse Lymphoma cyc$^-$ Membrane Adenylate Cyclase with Lubrol Extracts of Hepatocyte Membranes Obtained from Control and Triiodothyronine-treated Rats

	Source of Lubrol extract			
	Control rat hepatocyte		Triiodothyronine-treated rat hepatocyte	
S49 *cyc*$^-$ membranes	−	+	−	+
	Adenylate cyclase activity, pmol/20 min/ml			
Activator*				
Gpp(NH)p, 0.1 mM	0	26.4±2.3	0.6	24.2±0.3
NaF, 10 mM	0	37.6±0.2	0	31.0±1.0‡

Plasma membranes of hepatocytes prepared from control and triiodothyronine-treated rats were extracted with Lubrol 12A9, and the extracts heated at 37°C for 10 min to inactive catalytic adenylate cyclase activity of these extracts (24). The extracts (0.44–0.53 mg protein/ml) were mixed without or with S49 mouse lymphoma *cyc*$^-$ membranes (0.14 mg protein/ml) in adenylate cyclase assay medium (minus [^{32}P]ATP) for 20 min at 30°C. At the end of this incubation [^{32}P]ATP was added and adenylate cyclase activity measured after a 20-min period at 30°C. The data represent the mean values±SEM of data from three separate membrane preparations for each group.

* Present in the assay from initial incubation through adenylate cyclase assay period.

‡ Difference from control euthyroid rat preparation values significant ($P < 0.001$).

AMP response (cf. 37). Jard et al. (38) recently reported the conditions under which epinephrine inhibited adenylate cyclase activity of rat liver membranes via alpha$_2$ adrenergic receptors. The possibility that the blunted cyclic AMP response of hyperthyroid rat hepatocytes to epinephrine might reflect, in part, an enhanced alpha$_2$ adrenergic action was investigated via blocking studies. 10 μM prazosin, a concentration sufficient to block both the alpha$_1$ and alpha$_2$ adrenergic receptors in the liver (36), did not rectify the blunted cyclic AMP response of hyperthyroid rat hepatocytes to epinephrine stimulation.

Cyclic AMP phosphodiesterase, reported to be a site of thyroid hormone action rat fat cells (6) and liver (39), is unaltered in hepatocytes from either hyperthyroid (present study) or hypothyroid (10) rats.

Thyroid hormones regulate beta adrenergic receptor status in rat heart (40–42), skeletal muscle (42), submaxillary gland (44), and turkey erythrocytes (45). Previously, we demonstrated that the beta adrenergic responsiveness and the number of beta adrenergic receptors were increased in hepatocytes from hypothyroid rats (8, 10). In the present study hyperthyroidism was found to be associated with a reduction in hepatic beta adrenergic responsiveness, receptor affinity, and receptor number.

The ability of glucagon to stimulate hepatocyte cyclic AMP levels or to stimulate particulate membrane adenylate cyclase is decreased by hyperthyroidism. Glucagon binding data suggest that these blunted responses may, in part, be a reflection of a reduction in glucagon receptors noted in the hyperthyroid state. Sperling et al. (46) reported that thyroxine administration in vivo did not alter the amount of glucagon binding of whole liver membranes. In contrast to the observed normal cyclic AMP response of hypothyroid rat hepatocytes to glucagon stimulation reported by our laboratory (8) and confirmed by others (47), Sperling et al. (46) reported a reduction in both glucagon-stimulated adenylate cyclase activity and glucagon receptors in liver membranes from thyroidectomized rats. The basis for these discrepant data is not obvious.

The regulatory component of adenylate cyclase that confers guanine nucleotide and fluoride sensitivity to the catalytic moiety of the cyclase is the target for cholera toxin action and is ADP-ribosylated by the toxin (cf. 48). Cholera toxin-catalyzed AD[^{32}P]ribosylation of rat hepatocyte homogenates identified

three toxin-specific targets, M_r = 41.6, 44.4, and 45.9 kilodaltons (present study). Hyperthyroidism did not alter the pattern or level of these toxin-specific targets for ADP-ribosylation, suggesting that acute hyperthyroidism is not associated with changes in these subunits of the regulatory component of adenylate cyclase. This conclusion was further supported by the finding that Lubrol extracts of purified membranes of control and hyperthyroid rats reconstituted equivalent levels of guanine nucleotide-sensitive adenylate cyclase activity of S49 *cyc*$^-$ mouse lymphoma membranes. These data suggest that the reduced ability of guanine nucleotides to stimulate the adenylate cyclase of hyperthyroid rat hepatocytes is probably a reflection of an alteration in the catalytic moiety and not the regulatory component transducing guanine nucleotide action. Fluoride-sensitive adenylate cyclase activity of the *cyc*$^-$ membranes was significantly lower in the reconstituted system using Lubrol extracts from hyperthyroid as compared with control animals. The impaired response of the hyperthyroid rat hepatocyte cyclase to fluoride stimulation would appear to reflect both the reduction in catalytic cyclase discussed above, and an alteration that involves the ability of the regulatory component to transduce fluoride action to the catalytic cyclase.

Thyroid hormones have been shown to influence hormone receptor status in a number of tissues, including as shown in this study, the rat hepatocyte. It is clear from the present study that thyroid hormones may regulate the hormone-sensitive adenylate cyclase system at a number of additional loci such as the regulatory component or catalytic component of the cyclase. The physiological impact of these thyroid hormone-induced alterations on the short-term regulation of hepatic metabolism by hormones is currently being investigated.

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