Hypothyroidism is associated with an enhanced sensitivity of rat fat cells to the inhibitory action of adenosine and adenosine agonists. The sensitivity of the forskolin-stimulated cyclic AMP response of rat fat cells to the adenosine agonist N6-phenylisopropyladenosine is amplified 3-fold by hypothyroidism. Forskolin-stimulated adenylate cyclase activity is more sensitive to inhibition by this adenosine agonist in membranes of fat cells isolated from hypothyroid as compared to euthyroid rats. Hypothyroidism does not significantly alter the number or affinity of binding sites for N6-cyclohexyl[3H]adenosine or N6-phenylisopropyladenosine in membranes of rat fat cells. GTP-induced inhibition of forskolin-stimulated adenylate cyclase was markedly enhanced in the hypothyroid state, suggesting an alteration in the inhibitory regulatory component (N6)-mediated control of adenylate cyclase. Incubating membranes with [α-32P]NAD and preactivated pertussis toxin results in the radiolabeling of two peptides with M, = 40,000 and 41,000 as visualized in autoradiograms of polyacrylamide gels run in sodium dodecyl sulfate. The amount of label incorporated by pertussis toxin into these two peptides (putative subunits of N6) per mg of protein of membrane is increased 2–3-fold in the hypothyroid state. The amount of the stimulatory regulatory component, N, in fat cell membranes is not altered by hypothyroidism (Malbon, C. C., Graziano, M. P., and Johnson, G. L. (1984) J. Biol. Chem. 259, 3254–3260). The amplified response of hypothryroid rat fat cells to the inhibitory action of adenosine appears to reflect a specific increase in the activity and abundance of N,.

Thyroid hormones exert a permissive effect on the sensitivity of many tissues to stimulation by hormones such as catecholamines and insulin. β-Adrenergic catecholamine action in heart (McNeil and Brody, 1968), liver (Malbon et al., 1978b; Malbon and Greenberg, 1982), and fat tissue (Debons and Schwartz, 1961), for example, is regulated by thyroid hormones. Insulin action in fat cells and skeletal muscle is also modulated by thyroid status (Czech et al., 1980). The biochemical mechanisms responsible for this permissive effect of thyroid hormones on the actions of a variety of other hormones is not fully understood. A large body of information has accumulated on the effects of thyroid hormones on β-adrenergic hormone action, and it is clear that thyroid hormones can regulate receptor number and function (for review, see Stiles et al., 1984). Thyroid hormones also appear to be capable of regulating other components of the β-adrenergic receptor-coupled adenylate cyclase system, including the stimulatory regulatory component, N, (Stiles et al., 1981; Malbon and Greenberg, 1982), and the catalytic moiety (Malbon and Greenberg, 1982).

In addition to the stimulatory pathway, mammalian cells also display a receptor-mediated inhibitory control of adenylate cyclase. Stimulation of α2-adrenergic receptors (Steer and Wood, 1979; Aktories et al., 1979; Sabol and Nirenberg, 1979; Jakobs, 1979) and cholinergic muscarinic receptors (Hazenki and Ui, 1981), for example, has been shown to reduce intracellular cyclic AMP in a variety of tissues and cell types. Little information is available concerning the influence of thyroid status on receptor-mediated inhibition of adenylate cyclase.

The rat fat cell appears to be an ideal model system for study of the possible interactions between thyroid hormone status and receptor-mediated inhibitory control of adenylate cyclase. Adenosine and its purine-modified analogs such as N6-phenylisopropyladenosine (PIA) or N6-cyclohexyladenosine (CHA) are potent inhibitors of cyclic AMP accumulation and lipolysis of rat fat cells (Fain, 1973; Fain and Wieser, 1975; Trost and Stock, 1977). GTP regulates adenylate cyclase in a biphasic manner; low concentrations of GTP acting via N, activate the cyclase, while higher concentrations acting via an inhibitory regulatory component, N, inhibit the activity of adenylate cyclase (Yamamura et al., 1977; Cooper et al., 1979). Fat cells from the hypothyroid rat display enhanced sensitivity to the inhibitory actions of adenosine with regard to both lipolysis (Ohisalo and Stouffer, 1979) and cyclic AMP accumulation (Malbon and Graziano, 1983) stimulated by β-adrenergic agonists. In the present paper, we investigate the influence of hypothyroidism on receptor-mediated inhibition of adenylate cyclase by adenosine in rat fat cells. Part of this work has been presented in abstract form (Rapiejko et al., 1984).

**EXPERIMENTAL PROCEDURES**

Animals—Fed, female Sprague-Dawley (SD strain) rats weighing 150–175 g were used in these studies. Rats were rendered hypothyroid by maintenance on an iodine-deficient diet and drinking water containing 150 g were used in these studies. Rats were rendered hypothyroid by maintenance on an iodine-deficient diet and drinking water containing...
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Cyclic AMP accumulation of fat cells is stimulated by the plant diterpene forskolin, and this response is sensitive to inhibition by the adenosine analog PIA (Fig. 1). Hypothyroidism is associated with a 3-fold increase in the sensitivity of the fat cells to inhibition by PIA. Half-maximal inhibition of forskolin-stimulated cyclic AMP accumulation by rat fat cell membranes is achieved at about 0.01 μM PIA. One micromolar PIA inhibits the adenylate cyclase response of fat cell membranes from euthyroid rats about 50%. Half-maximal inhibition of the forskolin response of hypothyroid rat fat cell membranes is achieved at about 0.01 μM PIA. One micromolar PIA inhibits the adenylate cyclase response by more than 75% in the hypothyroid state. Inhibition of forskolin-stimulated adenylate cyclase activity of rat fat cell membranes was maximal at 1 μM PIA. Increasing the concentration of PIA from 1 to 10 μM fails to produce any additional inhibition of the forskolin-stimulated adenylate cyclase response of membranes.

RESULTS

Sensitivity of Fat Cells to the Inhibitory Actions of PIA—Fat cells from the hypothyroid rat display enhanced sensitivity to the actions of adenosine (Malbon and Graziano, 1983). Cyclic AMP accumulation of fat cells is stimulated by the plant diterpene forskolin, and this response is sensitive to inhibition by the adenosine analog PIA (Fig. 1). Hypothyroidism is associated with a 3-fold increase in the sensitivity of the fat cells to inhibition by PIA. Half-maximal inhibition of forskolin-stimulated cyclic AMP accumulation occurs at 6 ± 1 nM PIA in fat cells from euthyroid and hypothyroid rats, respectively (n = 5, p < 0.05 for the difference). This differential sensitivity of the cyclic AMP response to inhibition by PIA results into a 10–50-fold increase in the sensitivity of the lipolytic response of the cells from the hypothyroid animal to inhibition by PIA (Ohsialo and Stouffer, 1979).

Adenylate Cyclase Inhibition by PIA—The effect of PIA on forskolin-stimulated adenylate cyclase activity was investigated. Expression of PIA inhibition of forskolin-stimulated adenylate cyclase was found to require several modifications of the standard assay medium. Dose-dependent inhibition of forskolin-stimulated adenylate cyclase by PIA required the following modifications: the elimination of 1-methyl-3-isobutylxanthine, an adenosine antagonist, the substitution of Ro 20-1724 as a phosphodiesterase inhibitor, the inclusion of 0.1 M NaCl (Londos et al., 1981), the inclusion of adenosine deaminase to eliminate competing endogenous adenosine, the use of an ATP preparation that is free of adenosine contamination, the inclusion of an inhibitory concentration of GTP (1 μM) and low MgCl2 (Londos et al., 1978), the use of submaximal concentrations of the activator forskolin, and the use of freshly prepared fat cell membranes (data not shown).

Under these conditions, PIA inhibits forskolin-stimulated adenylate cyclase activity of fat cell membranes (Fig. 2). One micromolar PIA inhibits the adenylate cyclase response of fat cell membranes from euthyroid rats about 50%. Half-maximal inhibition of the forskolin response of hypothyroid rat fat cell membranes is achieved at about 0.01 μM PIA. One micromolar PIA inhibits the adenylate cyclase response by more than 75% in the hypothyroid state. Inhibition of forskolin-stimulated adenylate cyclase activity of rat fat cell membranes was maximal at 1 μM PIA. Increasing the concentration of PIA from 1 to 10 μM fails to produce any additional inhibition of the forskolin-stimulated adenylate cyclase response of membranes.

FIG. 1. Inhibition of forskolin-stimulated cyclic AMP accumulation by PIA in rat fat cells: effects of hypothyroidism. Rat fat cells from euthyroid and hypothyroid rats were isolated and incubated in Krebs-Ringer phosphate buffer containing 4% BSA in the presence of forskolin (50 μM), adenosine deaminase (1.0 μg/ml), and increasing concentrations of PIA. After a 2-min period, the incubation was terminated and the cyclic AMP content of the cells was determined. The data represent the mean value ± S.E. from five separate experiments performed on separate occasions. Basal and forskolin-stimulated cyclic AMP accumulation was 0.5 ± 0.1 and 11.0 ± 1.5 pmol/10⁶ cells in euthyroid and 0.7 ± 0.1 and 11.2 ± 0.8 pmol/10⁶ cells in hypothyroid rat fat cells.
protein in euthyroid and hypothyroid rat preparations, re-
inhibited by CHA and display Ib0 values of 1-10 nM for CHA
lipolysis and cyclic AMP accumulation of rat fat cells are
oligand binding to adenosine receptors of fat cell membranes
adenosine action in rat fat cells. Catecholamine-stimulated
yladenosine, like PIA, is a potent, full agonist with respect to
from hypothyroid rats. The adenosine analog N'-cyclo-
logs on lipolysis and cyclic AMP accumulation in fat cells
enhanced inhibitory effects of purine-modified adenosine an-
ong on lipolysis and cyclic AMP accumulation in fat cells
hypothyroid rats. The adenosine analog N⁶-cyclohex-
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lipolysis and cyclic AMP accumulation of rat fat cells are
hibited by CHA and display Ib0 values of 1-10 nM for CHA
(Trost and Stock, 1977). Tritiated CHA was employed to
assay receptor status in this system.
The binding of [³H]CHA to fat cell membranes is rapid,
reversible, and temperature-dependent (data not shown). Spe-
cific binding is saturated at approximately 100 nM ligand and
is half-maximal at about 10 nM [³H]CHA (data not shown).
Specific [³H]CHA binding to fat cell membranes is sensitive
to competition by PIA (Fig. 3). Half-maximal inhibition of
[³H]CHA binding is achieved at approximately 10 nM PIA.
These data on CHA binding are in good agreement with the
status of adenosine receptors was probed in an effort to ascertain the basis for the
enhanced sensitivity of adenylate cyclase to PIA in
hypothyroidism. The binding of [³H]CHA to fat cell
membranes of euthyroid and hypothyroid rats is examined. At saturating concen-
trations of [³H]CHA, fat cell membranes bind 0.20 ± 0.03 and
0.17 ± 0.02 (n = 5) pmol of radioligand/mg of membrane
protein in euthyroid and hypothyroid rat preparations, re-
spectively (Fig. 4). Analysis of these binding data by the
method of Scatchard (1949) provides a Kd for the interaction of
[³H]CHA with its binding site of 3 and 5 nM in euthyroid
and hypothyroid rat fat cell membranes, respectively (Fig. 4, inset). These data indicate that there is no major alteration
in the amount of receptor sites for purine-modified adenosine
agonists in fat cell membranes from the hypothyroid rat and
that the affinity of these receptor sites for [³H]CHA is slightly
lower in the hypothyroid case. These changes cannot account
for the enhanced sensitivity of adenylate cyclase to PIA
inhibition observed in hypothyroidism.

Fat Cell N₁-mediated Actions in Hypothyroidism

Fig. 2. Inhibition of forskolin-stimulated adenylate cyclase activity by PIA in membranes isolated from rat fat cells: effects of hypothyroidism. Membranes freshly prepared from fat
cells isolated from euthyroid and hypothyroid rats were assayed for
adenylate cyclase activity in the presence of forskolin (10 μM) and
increasing concentrations of PIA. Forskolin-stimulated adenylate
cyclase activity was 340 ± 23 and 290 ± 20 pmol/min/mg of membrane
protein in the euthyroid and hypothyroid preparations, respectively. The
data represent mean values ± S.E. from five separate experiments
for each.

Adenosine Receptor Status—The status of adenosine recep-
tors was probed in an effort to ascertain the basis for the
enhanced inhibitory effects of purine-modified adenosine an-
logs on lipolysis and cyclic AMP accumulation in fat cells
from hypothyroid rats. The adenosine analog N⁶-cyclohex-
yladenosine, like PIA, is a potent, full agonist with respect to
adenosine action in rat fat cells. Catecholamine-stimulated
lipolysis and cyclic AMP accumulation of rat fat cells are
hibited by CHA and display Ib0 values of 1-10 nM for CHA
(Trost and Stock, 1977). Tritiated CHA was employed to
assay receptor status in this system.
The binding of [³H]CHA to fat cell membranes is rapid,
reversible, and temperature-dependent (data not shown). Spe-
cific binding is saturated at approximately 100 nM ligand and
is half-maximal at about 10 nM [³H]CHA (data not shown).
Specific [³H]CHA binding to fat cell membranes is sensitive
to competition by PIA (Fig. 3). Half-maximal inhibition of
[³H]CHA binding is achieved at approximately 10 nM PIA.
These data on CHA binding are in good agreement with the
values for the Ib0 with respect to both inhibition of catechol-
amine-stimulated lipolysis and cyclic AMP accumulation in
fat cells (Trost and Stock, 1977) and the inhibition of radi-
oligand binding to adenosine receptors of fat cell membranes
The binding of [³H]CHA to fat cell membranes of euthyroid
and hypothyroid rats was examined. At saturating concen-
trations of [³H]CHA, fat cell membranes bind 0.20 ± 0.03 and
0.17 ± 0.02 (n = 5) pmol of radioligand/mg of membrane
protein in euthyroid and hypothyroid rat preparations, re-
spectively (Fig. 4). Analysis of these binding data by the
method of Scatchard (1949) provides a Kd for the interaction of
[³H]CHA with its binding site of 3 and 5 nM in euthyroid
and hypothyroid rat fat cell membranes, respectively (Fig. 4, inset). These data indicate that there is no major alteration
in the amount of receptor sites for purine-modified adenosine
agonists in fat cell membranes from the hypothyroid rat and
that the affinity of these receptor sites for [³H]CHA is slightly
lower in the hypothyroid case. These changes cannot account
for the enhanced sensitivity of adenylate cyclase to PIA
inhibition observed in hypothyroidism.

Adenylyl Cyclase Inhibition by GTP—GTP-dependent in-
hibition of forskolin-stimulated adenylate cyclase was next
investigated. In agreement with previous studies (Cryer et al.,
1969; Harwood et al., 1973; Yamamura et al., 1977), GTP at
concentrations above 0.1 μM inhibits adenylate cyclase activ-
ity of fat cell membranes (Fig. 5). Inhibition of forskolin-

Fig. 3. Competitive inhibition of N⁶-cyclohexyl[³H]adenosine binding to fat cell membranes by N⁶-phenylisopropyl-
adenosine. Fat cell membranes were incubated with 10 nM N⁶-cyclo-
hexyl[³H]adenosine and the indicated concentrations of N⁶-phenyl-
isopropyladenosine as described under “Experimental Procedures.”
The data represent mean values ± S.E. from four separate membrane
preparations performed on as many occasions.

Fig. 4. Binding of N⁶-cyclohexyl[³H]adenosine to membranes prepared from fat cells isolated from euthyroid and
hypothyroid rats. Membranes freshly prepared from euthyroid and
hypothyroid rat fat cells were incubated at 22 °C for 20 min with
increasing concentrations of radioligand. Specific binding was assayed
as described under “Experimental Procedures.” Inset, Scatchard anal-
ysis of the binding data. In each experiment, membranes were pre-
pared concurrently from three to five euthyroid and hypothyroid rats
and assayed immediately for radioligand binding. The data presented
represent mean values ± S.E. from five separate experiments
performed on as many occasions.
stimulated adenylate cyclase is maximal at 10 \mu M GTP under these assay conditions (data not shown). Fat cell membranes of both euthyroid and hypothyroid rats display inhibition of forskolin-stimulated adenylate cyclase by GTP (Fig. 5). The inhibition by GTP is more profound in the hypothyroid state. One micromolar GTP inhibits forskolin-stimulated adenylate cyclase activity about 25% in euthyroid and nearly 70% in hypothyroid rat fat cell membranes. These data indicate that the inhibitory actions of GTP, like that of PIA, are enhanced in the hypothyroid state and likely reflect a change in an element in the system common to both pathways of inhibition.

Status of N, and M — Treating fat cells with pertussis toxin attenuates the ability of inhibitory hormones to regulate cyclic AMP accumulation and lipolysis (Moreno et al., 1983; Olansky et al., 1983). Pertussis toxin catalyzes the ADP-ribosylation of a membrane protein that both binds guanine nucleotides and regulates adenylate cyclase (Katada and Ui, 1982a, 1982b). Incubating rat fat cell membranes with activated pertussis toxin and [\alpha-\text{32P}]NAD⁺ results in the incorporation of radiolabel into two peptides, M₁ = 40,000 and 41,000, that are distinct from the peptides radiolabeled in the presence of activated cholera toxin and [\alpha-\text{32P}]NAD⁺ (Fig. 6). Cholera toxin catalyzes the ADP-ribosylation of M₁ = 42,000 and 46,000/48,000 peptides in rat fat cell membranes, as previously noted (Malbon and Gill, 1979; Malbon et al., 1984a).

Bacterial toxin-catalyzed radiolabeling by [\text{32P}]NAD⁺ of peptides of fat cell membranes from euthyroid and hypothyroid rats was examined (Fig. 6). The electrophoretic mobilities of the peptides radiolabeled by [\text{32P}]NAD⁺ in the presence of either pertussis toxin (lanes 3 and 4) or cholera toxin (lanes 5 and 6) are the same in fat cell membranes from euthyroid and hypothyroid rats. Pertussis toxin catalyzes radiolabeling of M₁ = 40,000 and 41,000 peptides (lanes 3 and 4). Cholera toxin catalyzes radiolabeling of M₁ = 42,000, 46,000/48,000 peptides (5, 6). Most striking are the relative amounts of [\text{32P}] label incorporated into fat cell membranes from euthyroid (lane 3) as compared to hypothyroid (lane 4) rats by pertussis toxin. Whereas the amount of label incorporated into the M₁ = 42,000 and 46,000/48,000 peptides (subunits of N₁) was equivalent in membranes from euthyroid and hypothyroid rat fat cell membranes (63 and 50 fmol of [\text{32P}]/mg of protein, respectively), the amount of label incorporated into fat cell membrane M₁ = 40,000/41,000 peptides by pertussis toxin was 2.5-fold greater in membranes from the hypothyroid as compared to euthyroid rats (32 and 13 fmol/mg of protein, respectively).

Labeling of M₁, 40,000/41,000 peptides of fat cell membranes was examined in the presence of pertussis toxin (100 \mu g/ml) and various concentrations of [\text{32P}]NAD⁺. The final concentration of NAD⁺ was obtained by addition of unlabeled NAD⁺ to 0.1 \mu M [\text{32P}]NAD⁺ (20 \mu Ci/lane). The autoradiogram of the labeled products separated on polyacrylamide gels reveals that at 0.1, 1.0, and 10 \mu M NAD⁺, the amount of label incorporated into fat cell membranes is greater in hypothyroid as compared to euthyroid preparations (Fig. 7). The amount of phosphate incorporated per mg of protein is 13, 75, and 570 fmol in the euthyroid and 21, 177, and 820 fmol in the hypothyroid at 0.1, 1.0, and 10 \mu M NAD⁺, respectively.

Pertussis toxin-catalyzed labeling of the M₁ = 40,000 and 41,000 peptides of fat cell membranes in the presence of a fixed concentration of [\text{32P}]NAD⁺ (10 \mu M) and increasing concentrations of the activated toxin was investigated also. Under standard incubation conditions, the amount of label incorporated into both the M₁ = 40,000 and 41,000 peptides...
increases as the concentration of pertussis toxin is increased from 0.1 to 3 mg/ml, the highest concentration of toxin employed in these studies (data not shown). The amount of label incorporated into these two substrates in the presence of [32P]NAD+ and 1 or 3 mg/ml pertussis toxin is 65 and 45% greater, respectively, in the fat cell membranes prepared from hypothyroid as compared to euthyroid rats (data not shown).

The ability of pertussis toxin in attenuate the inhibitory action of PIA on forskolin-stimulated cyclic AMP accumulation of rat fat cells from euthyroid and hypothyroid rats was explored. Incubating fat cells from the euthyroid rat with pertussis toxin (0.1 µg/ml) for 3 h nearly abolishes the inhibitory action of PIA (Fig. 8). Fat cells from the hypothyroid rat display a greater sensitivity to the inhibitory action of PIA, and this enhanced response to PIA is only slightly attenuated following incubation with 0.1 µg/ml pertussis toxin (Fig. 8).

In an effort to explain the inability of pertussis toxin treatment to abolish the inhibitory action of PIA on forskolin-stimulated cyclic AMP accumulation in fat cells from hypothyroid rats, we prepared membranes from these cells, incubated these membranes with [32P]NAD+ and pertussis toxin, and identified the peptides that were radiolabeled. Pertussis toxin-catalyzed labeling by [32P]NAD+ is again confined to the Mr, = 40,000/41,000 peptides and is greater in fat cell membranes of hypothyroid as compared to euthyroid rats (Fig. 9). Prior treatment of fat cells from euthyroid rats with pertussis toxin in vitro virtually eliminates the subsequent labeling of the Mr, = 40,000/41,000 peptides by the toxin in the presence of [32P]NAD+ (compare lanes 8 and 12). These data agree well with the loss of the inhibitory response of these fat cells to PIA (Fig. 8). Membranes from pertussis toxin-treated fat cells of the hypothyroid rat, in contrast, still display a considerable amount of labeling of the Mr, = 40,000/41,000 peptides upon a second incubation with pertussis toxin and [32P]NAD+ (compare Fig. 9, lanes 2 and 6). These peptides available for pertussis toxin-catalyzed labeling by [32P]NAD+ appear to represent residual substrates that are transucing the marked inhibitory response to PIA observed in these cells (Fig. 8). The ability of pertussis toxin treatment to attenuate
The properties of \(^{3}H\)CHA binding to fat cell membranes agree well with those expected for the sites by which PIA and CHA act on the hormone-sensitive adenylate cyclase system. Studies of the binding of \(^{3}H\)CHA to membranes of euthyroid and hypothyroid rat fat cells showed no significant change in either receptor number or affinity that could explain the increase sensitivity to PIA in the hypothyroid state. The observation that GTP inhibition of forskolin-stimulated adenylate cyclase, which is a 4-day exposure. The amount of \(^{32}P\) label incorporated into fat cell membrane \(M_i = 40,000/41,000\) peptides is as follows: lane 2, 1400; lane 6, 440; lane 8, 1010; lane 12, 80.

found in fat cell membranes obtained from hypothyroid as compared to euthyroid rats. These data provided compelling evidence that hypothyroidism is associated with an amplified inhibitory control of adenylate cyclase by adenosine in the rat fat cell.

An alteration in either receptor number or affinity may explain the enhanced response of the cells to inhibition by PIA. The properties of \(^{3}H\)CHA binding to fat cell membranes agree well with those expected for the sites by which purine-modified analogs of adenosine (like PIA and CHA) act upon the adenylate cyclase system. Studies of the binding of \(^{3}H\)CHA to membranes of euthyroid and hypothyroid rat fat cells provide, however, no insight into the biochemical basis of the enhanced sensitivity to PIA in hypothyroidism. The total number of binding sites was less, not more, in hypothyroidism, and the affinity of these receptors was slightly reduced, not enhanced, in the hypothyroid state. Study of the binding of 1,3-diethyl-8-[\(^{3}H\)]phenylxanthine, and adenosine antagonist, to fat cell membranes also revealed no significant change in either receptor number or affinity that could explain the increase sensitivity to PIA in the hypothyroid state.

The observation that GTP inhibition of forskolin-stimulated adenylate cyclase was greater in hypothyroid than in euthyroid rat fat cells provided a key insight. Hypothyroidism appeared to be exerting an influence on element(s) of the cyclase system that are common to PIA- and GTP-induced inhibition of forskolin-stimulated adenylate cyclase. Pertussis toxin-catalyzed ADP-ribosylation by \(^{32}P\)NAD\(^{+}\) was employed to probe the status of inhibitory regulatory component of adenylate cyclase (Ni). Murayama and Ui (1983) reported that pertussis toxin treatment of rat fat cells abolishes those activities that are transduced via Ni and that incubating fat cell membranes with pertussis toxin in the presence of \(^{32}P\)NAD\(^{+}\) results in the radiolabeling of a \(M_i = 41,000\) peptide subunit of Ni. In the present report, two peptides \(M_i = 40,000/41,000\) were radiolabeled in fat cell membranes incubated with purified, activated pertussis toxin and \(^{32}P\)NAD\(^{+}\) (Figs. 6, 7, and 9). Membranes prepared by homogenization (present study) or hypotonic lysis (as described by Murayama and Ui, 1983) and labeled in a variety of buffer systems demonstrated without exception two substrates \(M_i = 40,000/41,000\) for pertussis toxin-catalyzed labeling. Partial proteolytic peptide maps indicate that these two substrates for pertussis toxin-catalyzed ADP-ribosylation are homologous, but non identical (Malbon et al., 1984b). Definition of the basis for these apparent differences in the number of pertussis toxin substrates of rat fat cell membranes will require additional study.

Most striking was the increased amount of \(^{32}P\) label incorporated into fat cell membrane \(M_i = 40,000/41,000\) peptides of the hypothyroid rat following incubation with pertussis toxin and \(^{32}P\)NAD\(^{+}\). Hypothyroidism was associated with a 1.7-2.5-fold increase in the amount of label in these peptides, whereas the amount of \(^{32}P\) label incorporated into the \(M_i = 42,000/46,000/48,000\) peptides by cholera toxin and \(^{32}P\)NAD\(^{+}\) was not significantly altered. If it is assumed that bacterial toxin-catalyzed ADP-ribosylation of membranes by \(^{32}P\)NAD\(^{+}\) is an index of the relative abundance of N\(_i\) and N\(_{s}\) then these data suggest that hypothyroidism is associated with a substantial increase in the amount of N\(_i\) and little change in N\(_{s}\). Assays of N\(_i\) via the reconstitution of the hormone-sensitive adenylate cyclase of S49 cys\(^{-}\) mutant cell membranes were shown to reflect the relative amounts of label incorporated into N\(_i\) via cholera toxin-catalyzed ADP-ribosylation (Malbon et al., 1984a). The study of PIA- and GTP-induced inhibition of adenylate cyclase of fat cell membranes in the hypothyroid as compared to euthyroid preparation also appears to reflect the amount of label incorporated into N\(_i\) via pertussis toxin-catalyzed ADP-ribosylation, \(i.e\) increased N\(_i\) activity and increased amounts of label incorporated into \(M_i = 40,000/41,000\) peptides by pertussis toxin in the hypothyroid state. The inability of the lower concentrations of pertussis toxin to abolish the N\(_i\)-mediated inhibition of PIA (Fig. 8) or subsequent labeling of N\(_i\) peptides by activated pertussis toxin and \(^{32}P\)NAD\(^{+}\) (Fig. 9) in hypothyroid, but not euthyroid, rat fat cells also suggests an increase in the amount of N\(_i\) in the hypothyroid state.

The rat fat cell clearly provides an interesting model for the study of the interactions between thyroid hormones and the hormone-sensitive adenylate cyclase system. Both receptor-mediated inhibition (via R\(_i\)) and stimulation (via R\(_s\)) of adenylate cyclase transduced through N\(_i\) and N\(_{s}\), respectively, operate in these cells. Adenosine analogs like PIA or CHA inhibit, while hormones such as \(\beta\)-adrenergic agonists and glucagon stimulate cyclic AMP accumulation and lipolysis in rat fat cells. In the hypothyroid state, both pathways are modified, the stimulatory pathway is dampened (Malbon et al., 1984a), while the inhibitory pathway is enhanced (Ohisalo and Stouffer, 1979, present study). Since N\(_i\) and N\(_{s}\) share common subunits (Bokoch et al., 1984; Codina et al., 1984), changes in the relative amount of one of these components

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2 C. C. Malbon, and T. J. Mangano, manuscript in preparation.
would be expected to alter the complexion of both responses. This is indeed the case in the hypothyroid fat cell, since stimulation of adenylate cyclase by a number of hormones is impaired (Correze et al., 1974) even though a receptor defect has only been identified for the β-adrenergic receptor (Malbon et al., 1984a). The increase in N2 appears to explain, in part, the attenuated responsiveness of these cells to both β-agonists and other stimulatory hormones and provides an explanation for the increased sensitivity of the N1-mediated pathway. It is likely that thyroid hormones and other agents that exert a permissive influence on hormone action may also express their actions via alterations in the status of N1 and N2.

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