Thyroid hormone regulation of transmembrane signalling in neonatal rat ventricular myocytes by selective alteration of the expression and coupling of G-protein α-subunits

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Thyroid hormone exerts profound effects on the activity of the hormone-sensitive adenylate cyclase system in the heart. Distinct guanine nucleotide-binding regulatory proteins (G-proteins) mediate stimulatory and inhibitory influences on adenylate cyclase activity. To examine whether the effects of thyroid hormone on adenylate cyclase involve specific changes in G-protein subunit expression, the influence of tri-iodothyronine (T_3) on the biosynthesis and activity of G-proteins in neonatal rat ventricular myocytes was determined. In myocytes challenged with T_3 for 5 days, $G_s\alpha$ levels increased by 4 ± 0.5-fold, whereas $G_i2\alpha$ levels declined by more than 80%. T_3 down-regulated $G_i2\alpha$ mRNA by 60% within 3 days, but had no effect on $G_s\alpha$ mRNA. The basis for the decline in $G_i2\alpha$ mRNA was the T_3-mediated suppression of $G_i2\alpha$ gene transcription by 80 ± 9% within 4 h. The decline in $G_i2\alpha$ mRNA in response to T_3 produced a 2-fold decrease in relative rate of synthesis of $G_i2\alpha$ but not in its half-life (46 ± 7 h). $G_s\alpha$ synthesis was not altered by T_3, but the half-life of $G_s\alpha$ increased from 50 ± 6 h in control cells to 72 ± 8 h in T_3-treated cells. In addition, T_3 provoked the translocation of $G_s\alpha$ from the cytoplasmic to the membranous compartment. Membranous $G_s\alpha$ increased from 30 ± 6% to 61 ± 7% of total cellular $G_s\alpha$, whereas cytoplasmic $G_s\alpha$ declined from 68 ± 6% to 33 ± 8% within 1 day of exposure to T_3. T_3-mediated up-regulation of $G_s\alpha$ enhanced the activation of myocardial adenylate cyclase by the stimulatory pathway whereas the down-regulation of $G_i2\alpha$ attenuated the deactivation of myocardial adenylate cyclase by the inhibitory pathway.

INTRODUCTION

Thyroid hormone produces multiple effects on muscular tissues such as the heart and skeletal muscles, metabolic tissues such as the liver and spleen, and nervous system [1]. The heart is a major target organ for tri-iodothyronine (T_3) action. Thyroid status influences the responsiveness of the heart of hormones and neurotransmitters that interact with G-protein-coupled receptors [2]. A system that is exquisitely sensitive to T_3 is the hormone-sensitive adenylate cyclase complex [3]. This system is controlled by dual pathways. The heterotrimeric G-proteins, termed G_s and G_i, mediate the actions of the stimulatory and inhibitory pathways respectively [4,5]. Each of these G-proteins is composed of a distinct α-subunit which associates with common β- and γ-subunits [5]. The β- and γ-subunits are tightly associated and are anchored into the membrane by the γ-subunit [5]. Agonist activation of receptors that are coupled to G_s leads to stimulation of adenylate cyclase and the generation of intracellular cyclic AMP. In the heart, β_1- and β_2-adrenergic receptors are coupled to G_s. Their activation results in the familiar effects of catecholamines, which include increases in heart rate, force of contraction and rhythmicity [6]. Activation of G_i-coupled receptors leads to attenuation of adenylate cyclase activity and decreased intracellular cyclic AMP levels. This inhibitory effect is dependent on the type of adenylate cyclase and may be mediated by the α-subunit of G_i or the βγ-subunits [7,8]. The α-subunits of the G_i family are composed of at least three gene products termed $G_i\alpha$, $G_i2\alpha$ and $G_i3\alpha$ [9]. In addition, eight isoforms of adenylate cyclase have been cloned, and more are likely to exist [10–12]. $G_i\alpha$ isoforms inhibit type-V and -VI adenylate cyclase but are without effect on type I [13,14]. The βγ-subunits can stimulate or inhibit adenylate cyclase in an isoform-dependent manner also [8,15,16]. βγ-subunits co-stimulate $G_s\alpha$-GTP-activated type-II and inhibit $G_s\alpha$-GTP-stimulated type-I adenylate cyclase [15,16]. The specificity of the various $G_i\alpha$ isoforms and βγ-subunits for the other species of adenylate cyclase has not been determined.

Persistent activation of either the stimulatory or inhibitory G-protein-mediated pathways results in cross-regulation of the expression of components of the other pathway and this may alter the sensitivity of adenylate cyclase to regulation by the affected pathway [17,18]. These studies revealed that the net activity of adenylate cyclase is a composite of the inputs from the stimulatory and inhibitory G-proteins [18]. Consequently, T_3 may influence the activity of adenylate cyclase by altering the levels of individual components that comprise the regulatory pathways. T_3 has been shown to influence the levels of components associated with the stimulatory and inhibitory pathways of adenylate cyclase. For example, T_3 increased the levels of myocardial β-adrenergic receptors, and this increase was associated with enhanced stimulation of adenylate cyclase activity by β-agonists [2,19,20]. A consistent effect of T_3 on G-protein subunits has been documented for $G_i2\alpha$ [21,22]. Hyperthyroidism was associated with a substantial decrease in steady-state levels of $G_i2\alpha$ in the heart and adipose tissue [21–23]. However, substantial differences exist in the effect of T_3 on G-protein subunit expression among tissues and in the biochemical basis for these changes [23].

Most of the studies that have examined the influence of T_3 on G-protein expression have focused on steady-state measurements of the protein and the mRNA of individual G-protein subunits. Thus limited information about the mechanism by which T_3 influences the expression of multiple G-protein subunits has been

Abbreviations used: T_3, 3,5,3′-tri-iodo-L-thyronine; G-protein, guanine nucleotide-binding regulatory protein; G_s, the stimulatory G-protein; G_i, the inhibitory G-protein; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; DMEM, Dulbecco's modified Eagle's medium; 1 × SSC, 150 mM NaCl/15 mM sodium citrate, pH 7.0; IBMX, 3-isobutyl-1-methylxanthine.

obtained. Moreover, a systematic correlation between changes in individual G-protein subunits and their proposed function in the signalling cascade has not been firmly established. In the present study, the effect of T_3 on steady-state levels of the various G-protein subunits was measured and the relevant biochemical mechanisms for these changes were determined. Moreover, changes in individual G-proteins were correlated with their physiological role in transmembrane signalling. These studies were conducted in primary cultures of neonatal rat ventricular myocytes because the changes in G-protein subunits in this system are comparable with those obtained in the heart *in vivo* [2].

EXPERIMENTAL

Culture of ventricular myocytes

Ventricles were isolated under aseptic conditions from 1–3-day-old Sprague–Dawley rats. They were minced and dissociated into a mixed population of muscle and non-muscle cells after six to seven 10 min periods of incubation at 37 °C with 10 ml of Hank's balanced salt solution containing 0.075% Viokase [24]. The first two batches of dissociated cells contained mostly red blood cells and debris and were discarded. The cells in the remaining supernatants were collected by centrifugation and resuspended in 30 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.5 mg of DNAase I and 10% fetal bovine serum depleted of thyroid hormones. The cells were preplated for 1 h because non-myocardial fibroblast-like cells attach to the plastic dish whereas the majority of the myocardial cells remain in suspension during this period [25]. Subsequently, the supernatant was aspirated, and the myocardial cells were adjusted to a concentration of 10^6/ml of culture medium which was composed of 68% DMEM, 17% Medium 199, 10% thyroid hormone-depleted horse serum and 5% thyroid hormone-depleted fetal bovine serum. Myocardial cells were cultured on collagen-coated plates for 24 h to allow cell attachment. The medium was replaced the next day and every 48 h thereafter. Experiments utilizing ventricular cells were initiated 48 h after culture when rhythmic contractions of more than 90% of the myocytes were evident.

Antibodies of G-protein subunits

Antisera of peptides corresponding to sequence 384–394 of rat $G_s\alpha$ (SB-07), 346–355 of rat $G_i2\alpha$ (SB-04), 345–354 of rat G_o (CM-140), 25–39 of $G_{\beta 1}$ (CM-133) and 25–39 of $G_{\beta 2}$ (CM-162) were generated for the measurement of steady-state levels and the immunoprecipitation of G-proteins [26,27]. Antisera denoted CM were kindly provided by Craig C. Malbon, SUNY at Stony Brook, NY, U.S.A.

Immunoblot analysis of G-protein subunits

Myocytes were routinely prepared from the ventricles of a large number of neonatal rats (> 60 per batch). They were cultured in T_3-deficient medium for 2 days to allow recovery and the restoration of rhythmic contractions in more than 90% of the myocytes. Thereafter, they were exposed to diluent or 10 nM T_3, and then harvested 1–5 days later. They were detached non-enzymically [28], and pelleted by centrifugation at 1000 $g_{av.}$ for 2 min. The cell pellet was resuspended for 10 min in 50 vol. of ice-cold lysis buffer composed of 20 mM Hepes, pH 7.2, 2 mM $MgCl_2$, 1 mM EDTA and the protease inhibitors leupeptin (10 μg/ml), aprotinin (10 μg/ml) and phenylmethanesulphonyl fluoride (0.1 mM). The cells were then broken open by 40 strokes of a Dounce homogenizer fitted with a type-A pestle. The homogenate was centrifuged at 2000 $g_{av.}$ for 5 min at 2 °C in a JS-13 rotor, and the supernatant was collected and centrifuged at 80000 $g_{av.}$ in an SW-42 rotor for 20 min at 2 °C. The pellet, which was composed of crude membranes, was resuspended in 50 mM Tris/HCl, pH 7.4, containing 10 mM $MgCl_2$ and protease inhibitors. It was frozen at −80 °C until use. Myocyte membranes were solubilized in 0.125 M Tris/HCl, pH 6.8, containing 4% SDS, 5% sucrose and 20 mM dithiothreitol and incubated at 37 °C for 30 min, followed by alkylation with excess 2-iodoacetamide. The solubilized proteins were subjected to electrophoresis on 11% polyacrylamide gels containing 0.1% SDS and the separated proteins transferred electrophoretically to nitrocellulose. The nitrocellulose blot was incubated in 10% albumin in PBS for 30 min at room temperature, rinsed with water, and incubated with rabbit antiserum directed against the various G-protein subunits. The sera were diluted in 0.3% (v/v) Tween 20/PBS, and the blot was incubated for 2 h at 37 °C. The blot was washed and incubated with goat anti-rabbit antibody conjugated to calf alkaline phosphatase and 10^6 c.p.m./ml ^{125}I-labelled goat anti-rabbit IgG to localize the immunoreactive bands by the chromogenic phosphatase precipitate and to quantify the immunoreactive bands by autoradiography [29].

Quantitative Immunoblotting of $G_s\alpha$ subunit

$G_s\alpha$ was purified from BL21-DE3 strain of *Escherichia coli* that was transformed with plasmid pQE-60 containing cDNA encoding the 45 kDa form of bovine $G_s\alpha$ (kindly provided by Alfred Gilman, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). Expression of recombinant $G_s\alpha$ from its cDNA was induced by the addition of isopropyl β-D-thiogalactopyranoside, and the protein was purified essentially as described by Graziano et al. [30]. The cytosolic fraction of myocytes was concentrated by the addition of trichloroacetic acid to a final concentration of 10% before electrophoresis. In these experiments, 5–100 ng of $G_s\alpha$ was electrophoresed side-by-side with the protein samples and the gels were processed as described in the preceding section.

Extraction of RNA and Northern-blot analysis

RNA was extracted using the guanidinium isothiocyanate and ethanol precipitation method [31]. The integrity of the RNA was assessed by electrophoresis on 3% formaldehyde/1.2% agarose gel. The RNA was electroblotted from the gel to Nylon membranes (Nytran; Schleicher and Schuell) overnight in 25 mM sodium phosphate, pH 6.5. The blot was prehybridized in solution containing 50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0), 5% SDS, 2 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA) and 250 μg/ml sheared salmon sperm DNA at 42 °C for 6 h. After prehybridization, the blot was incubated in prehybridization solution containing [^{32}P]dCTP-radiolabelled *Eco*RI cDNA of the various G-proteins (2 × 10^6 c.p.m./ml) for 16 h at 42 °C. The blot was washed twice for 5 min in 2 × SSC/0.5% SDS at 25 °C and three times for 15 min in 0.1 × SSC/0.5% SDS at 54 °C, covered with Saran wrap, and subjected to autoradiography. To determine minor differences in RNA transfer between the lanes, the blot was then stripped of radioactivity as recommended by the manufacturer and rehybridized with a ^{32}P-radiolabelled *Pst*I fragment of α-tubulin cDNA in plasmid pT_1 [32]. α-Tubulin mRNA levels in cardiac myocytes are not subjected to regulation by T_3 [33]. The cDNA probes for G-proteins were derived as follows: the $G_s\alpha$ probe was a 1.85 kb *Eco*RI fragment of G2 cDNA, which encodes the larger form of $G_s\alpha$; the $G_i2\alpha$ probe

was a 2.3 kb *Eco*RI fragment of G18 cDNA; and the $G_i3\alpha$ probe was a 3.5 kb *Eco*RI fragment of G16 cDNA [9]. The G-protein cDNA and avian α-tubulin cDNA in pT_1 plasmid were provided by Randall R. Reed and Donald W. Cleveland (Howard Hughes Medical Institute, The Johns Hopkins University Medical Centre, Baltimore, MD, U.S.A.) respectively.

Receptor mRNA stability

The half-life of $G_i2\alpha$ mRNA was determined by the method of Rodgers et al. [34], as previously reported [26]. Myocytes were exposed to T_3 or vehicle for 2 days, and then actinomycin D (5 μg/ml) was added; RNA was prepared over the next 24 h. The levels of $G_i2\alpha$ mRNA in total cellular RNA were quantified by Northern-blot analysis as described in the preceding section.

Preparation of myocardial nuclei and nuclear run-on assay

Nuclei were isolated from cultured myocytes as described [35]. To each 100 mm dish was added 1 ml of buffer I (10 mM Tris/HCl, pH 8.0, 10 mM NaCl, 2.5 mM $MgCl_2$ and 5 mM dithiothreitol) for 10 min. Then 1 ml of buffer I containing 0.6 M sucrose and 0.6% Triton X-100 was added, and the cells were scraped into a Dounce homogenizer. The cells were homogenized by six strokes of a type-A pestle and layered over buffer I containing 0.6 M sucrose. Nuclei were collected after centrifugation at 2000 $g_{av.}$ for 10 min. The nuclei were resuspended in glycerol buffer (50 mM Tris/HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA) for immediate assay [36]. Frozen nuclei were found to be unsuitable for the nuclear run-on assays. To detect nascent transcripts, 2×10^7–3×10^7 nuclei in 200 μl were added to 200 μl of a reaction buffer composed of 10 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, unlabelled GTP, ATP and CTP, and 10 μl of [α-^{32}P]UTP (800 Ci/mmol; New England Nuclear). Newly transcribed labelled RNA was extracted and then incubated for 36 h at 65 °C with plasmid DNA immobilized on nitrocellulose [37]. After hybridization, each sample was washed twice with 2 × SSC for 60 min at 65 °C. The samples were then treated with RNAase A for 30 min at 37 °C followed by a wash with 2 × SSC at 37 °C for 60 min. The filters were dried and subjected to autoradiography for 5 days with an intensifying screen. Relative changes in transcription were assessed by scanning densitometry of the autoradiogram. The $G_i2\alpha$ plasmid was linearized by *Eco*RI and the pT_1 plasmid was linearized by *Pst*I. Then 10 μg of linearized $G_i2\alpha$ and 5 μg of pT_1 were added to each slot.

Metabolic labelling of myocytes

To determine the relative rates of synthesis of $G_s\alpha$ and $G_i2\alpha$, cells were cultured in vehicle or 10 nM T_3 for 4 days to allow a sufficient period of time for the action of T_3 on $G_s\alpha$ and $G_i2\alpha$ mRNAs to take effect. The cells were washed twice with PBS and recultured in methionine-free DMEM containing 10% horse serum and 5% fetal calf serum. Both sera were depleted of thyroid hormone and then dialysed extensively against PBS. To each 100 mm plate were added 300 μCi of [^{35}S]methionine and either vehicle or 10 nM T_3 for 1–8 h. Cells were harvested at the times indicated in Figure 4, washed twice with PBS, and then used as a source for the preparation of crude membranes. To determine the relative rates of degradation of $G_s\alpha$ and $G_i2\alpha$, myocytes were cultured in T_3-depleted serum for 3 days. The culture medium was replaced by a methionine-free medium as described above. To each 100 mm dish was added 400 μCi of [^{35}S]methionine for 8 h to achieve maximal incorporation of the label into the G-proteins. At the end of the incubation, the cells were washed free of radiolabelled methionine and recultured in media supplemented with 1 mM unlabelled methionine and either vehicle or 10 nM T_3. Membranes were prepared at the end of the metabolic pulse and 1, 2 and 3 days after the chase with unlabelled methionine.

Immunoprecipitation of $G_s\alpha$ and $G_i2\alpha$

The protein concentration in [^{35}S]methionine-labelled membranes was assayed by the method of Bradford [38] and in each experiment 100 μg of protein was used. To each batch of membranes were added 10 μg of benzamidine and 15 μg of bacitracin, and the volumes of the samples were adjusted to 75 μl. Membrane proteins were dissolved by boiling in 6% SDS for 2 min. Dithiothreitol (10 mM) was added, and the solubilized proteins were incubated at 37 °C for 30 min, followed by alkylation with excess *N*-ethylmaleimide. Then 1.2 ml of IPA buffer without detergents (20 mM Tris/HCl, pH 8.0, 150 mM NaCl and 5 mM EDTA) was added, followed by Triton X-100 to a final concentration of 10%. To minimize non-specific immunoprecipitation, each sample was incubated for 2 h at room temperature with 0.1 ml of non-immune rabbit IgG preadsorbed to Protein A–Sepharose. The samples were centrifuged at 14000 × $g_{av.}$ for 2 min, and the supernatant was transferred to another tube containing 50 μl of G-protein IgG preadsorbed to Protein A–Sepharose. These samples were incubated overnight at 4 °C in a tumbling shaker. The Protein A–Sepharose beads were isolated by centrifugation, washed four times in IPA buffer containing 1% Triton X-100 and 0.2% SDS, and subjected to electrophoresis on 11% polyacrylamide gels [17,18]. The gels were stained and subjected to fluorography and autoradiography. ^{35}S incorporated into the desired bands was quantified by liquid-scintillation counting. The autoradiograms were scanned with a laser densitometer to calculate the absorbance of each band.

Assay of adenylate cyclase

Myocyte membranes were prepared as outlined earlier, except that phenylmethanesulphonyl fluoride was omitted. They (100 μg of protein) were incubated at 30 °C in a final volume of 0.1 ml containing 50 mM Tris/HCl, pH 7.4, 1 mM $MgCl_2$, 10 mM phosphocreatine, 1 mM cyclic AMP, 2 mM 2-mercaptoethanol, 1 mg/ml BSA, 0.4 mM EGTA, 2 mg/ml creatine kinase, 0.2 mM ATP containing 2 μCi of [α-^{32}P]ATP, and the indicated amounts of guanine nucleotides or other reagents. The assay was initiated by the addition of membranes and terminated after 10 min [22]. The cyclic AMP that formed was isolated and quantified by the procedure of Salomon et al. [39]. Assays were routinely performed in triplicate.

Cyclic AMP accumulation in ventricular myocytes

Myocytes were cultured on 35 mm dishes in the presence or absence of 10 nM T_3 for 5 days. On the day of the experiment, the medium was aspirated and replaced with 68% DMEM and 17% Medium 199 with or without T_3. The cells were returned to the incubator and used 1 h later. This procedure is necessary to restore the basal levels of cyclic AMP in these cells. Cells were pretreated with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) at a concentration of 0.5 mM for 15 min and then exposed to 10 μM forskolin and the desired concentrations of carbachol and endothelin-1 for 5 min at 30 °C. The medium was aspirated, 1 ml of 1 M HCl was added, and the plates were quickly frozen on solid CO_2. The contents of the plates were thawed by heating at 95 °C for 5 min, and the cell extract was scraped off, clarified by centrifugation and

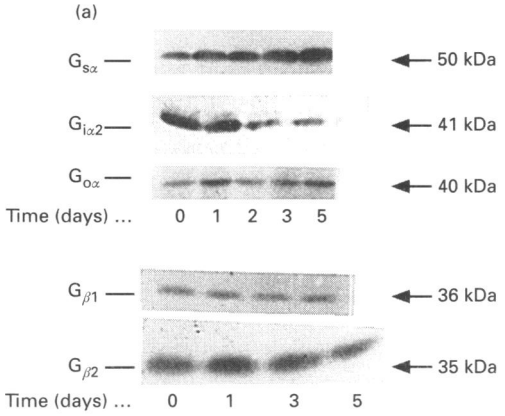
lyophilized. The lyophilized pellet was rehydrated with 20 mM sodium phosphate, pH 6.0, and cyclic AMP levels were determined as described [40]. Protein levels were determined on all the samples by the method of Bradford [38].

Presentation of data

Unless noted otherwise, data are expressed as means ± S.E.M. for separate experiments. Rates of $G_s\alpha$ and $G_i2\alpha$ synthesis are presented as mean values of separate experiments. Statistical significance was determined using Student's *t* test.

RESULTS

Membranes prepared from ventricular myocytes were probed by immunoblotting with the $G_s\alpha$ antiserum to identify the size of the $G_s\alpha$ subunit in these cells. The $G_s\alpha$ that was expressed in the neonate was the 52 kDa form (Figure 1). The predominant form of $G_s\alpha$ in adult myocardium, however, is the 45 kDa form [21]. A single gene encodes both these forms. The gene is alternatively spliced to generate four mRNAs that encode for either the 45 kDa form or the 52 kDa form, which contains an additional 15 amino acids [41]. Quantitative immunoblotting of myocyte membranes revealed that the levels of $G_s\alpha$ in the absence of T_3 were 1.2 ± 0.3 ng/μg of membrane protein. T_3 increased the absolute amounts of $G_s\alpha$ to 2.2 ± 0.4 ng/μg within 1 day and to 2.5 ± 0.5 ng/μg within 2 days. Therefore T_3 increased $G_s\alpha$ immunoreactivity in myocyte membranes about 2-fold (2.0 ± 0.4; $n = 3$; Figure 1) within 1 day. The levels of $G_s\alpha$ continued to increase in response to T_3 and by the third day, they were elevated to 3.7 ± 0.6 ng/μg and by 5 days (the latest time point examined), they were still elevated by about 4-fold to 4.7 ± 0.6 ng/μg of membrane protein. The effect of T_3 on $G_i2\alpha$ levels was investigated using the antiserum (SB-04) directed to the C-terminal decapeptide of $G_i1,2\alpha$ [9,26]. The myocardium does not express $G_i1\alpha$ mRNA [9], therefore, SB-04 detected $G_i2\alpha$ specifically in this tissue. Immunoblot analysis of myocyte membranes with the SB-04 antiserum revealed a single immunostained protein of molecular mass 41 kDa corresponding to $G_i2\alpha$ (Figure 1). Exposure of myocytes to 10 nM T_3 produced a gradual but marked decrease in the relative immunoreactive levels of $G_i2\alpha$. Within 2 days of continuous T_3 exposure, $G_i2\alpha$ levels declined by about 50% (45 ± 3% of control; $n = 3$), and these levels dimin-



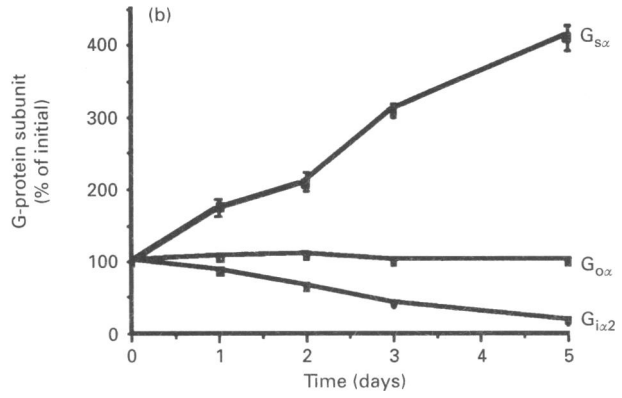


Figure 1 Relative changes in concentrations of G-protein subunits in membranes prepared from control and T_3-treated myocytes

(**a**) Myocytes were incubated with 10 nM T_3 or buffer for 0–5 days, and then the membranes were prepared as described in the Experimental section. Samples (100 μg) were separated by electrophoresis on 11% polyacrylamide gels and transferred electrophoretically to nitrocellulose. The blots were incubated with the specific G-protein subunit antiserum, and bound antibody was detected with ^{125}I-labelled goat anti-rabbit IgG and phosphatase-labelled goat anti-rabbit IgG. The sera were diluted as follows: anti-$G_s\alpha$, anti-$G_i1,2\alpha$ and anti-$G_o\alpha$, 1:200 dilution; anti-$G_{\beta1}$ and anti-$G_{\beta2}$, 1:100 dilution. (**b**) The intensity of the bands in the autoradiograms was measured by scanning densitometry and the desired bands were excised and counted with a γ-counter. G-protein levels are expressed as a percentage of the corresponding value obtained from membranes of myocytes cultured for the same period of time in the absence of T_3. Data are means ± S.E.M. of three membrane preparations that were processed separately.

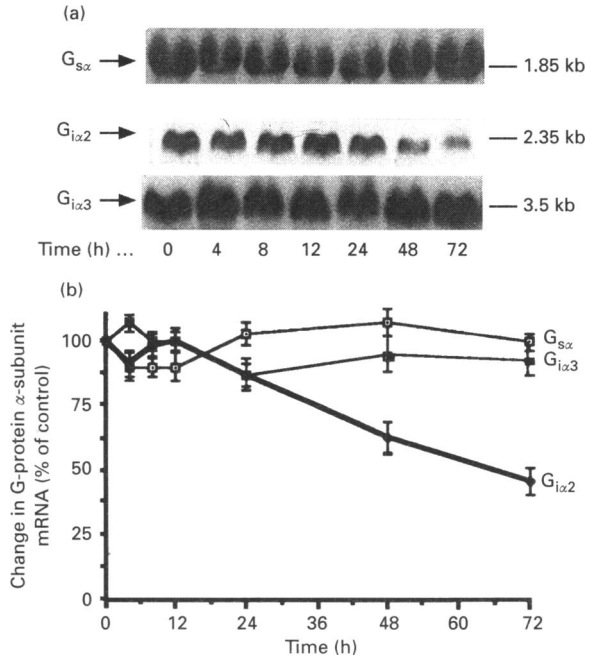


Figure 2 Relative changes in G-protein-subunit mRNA levels in myocytes exposed to diluent or T_3

(**a**) Total cellular RNA was isolated from myocytes exposed to buffer or 10 nM T_3 for 0–72 h. The RNA (50 μg) was subjected to Northern-blot analysis using radiolabelled cDNA probes specific for mRNA encoding $G_s\alpha$, $G_i2\alpha$ and $G_i3\alpha$. Then the blots were subjected to autoradiography for 6 h for $G_s\alpha$ and 24 h for $G_i2\alpha$ and $G_i3\alpha$. (**b**) Autoradiographic intensities of each individual band were determined by scanning densitometry. The blot was stripped from the first probe and rehybridized with α-tubulin cDNA to correct for minor differences in the transfer of the RNA. The intensity of each G-protein band was corrected for transfer efficiency and expressed as a percentage of the values obtained from RNA prepared from myocytes cultured in the absence of thyroid hormone. Data are presented as means ± S.E.M. of four separate determinations.

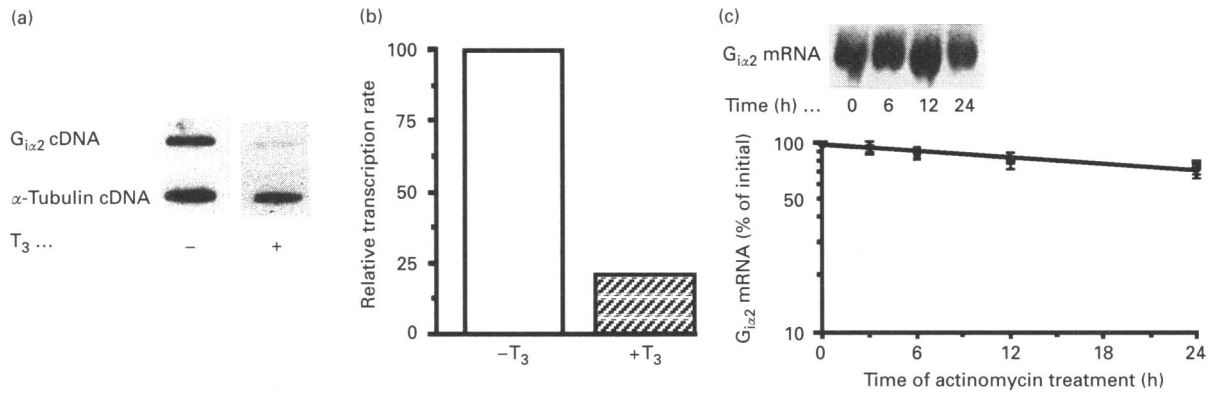


Figure 3 Reduction of the relative transcriptional rate of the $G_i2\alpha$ gene by T_3 in ventricular myocytes without $G_i2\alpha$ transcript stability being affected

(**a**) Nuclei were prepared from ventricular myocytes cultured in the absence or presence of 100 nM T_3 for 4 h as described in the Experimental section. Transcript elongation in the presence of [^{32}P]UTP was allowed to proceed for 30 min at 30 °C, and then RNA was extracted. Equal amounts of ^{32}P-labelled RNA from control and T_3-treated nuclei (4×10^6–6×10^6 c.p.m.) were hybridized to nitrocellulose blots containing 10 μg of either $G_i2\alpha$ or 5 μg of α-tubulin cDNA that were linearized with *Eco*RI and *Pst*I respectively. A typical autoradiogram is shown that was obtained after 3 days of exposure. (**b**) The autoradiogram was scanned, and the absorbance of the $G_i2\alpha$ band was divided by the absorbance of the corresponding α-tubulin cDNA to correct for hybridization efficiency. Then the absorbance of $G_i2\alpha$ in the presence of T_3 was divided by that in its absence to obtain the percentage inhibition in the relative transcriptional rate of the $G_i2\alpha$ gene in T_3-treated nuclei. The data indicate that exposing the cells to T_3 for 4 h reduced the relative transcriptional rate by 80 ± 9% ($n = 3$). (**c**) Myocytes were cultured in the absence (□) or presence (■) (symbols overlap) of T_3 for 1 day, actinomycin D (5 μg/ml) was added, and total cellular RNA was isolated at the times indicated on the abscissa. RNA (50 μg) was subjected to Northern-blot analysis using ^{32}P-labelled $G_i2\alpha$ cDNA. The autoradiogram is a 24 h exposure with one intensifying screen. The percentage of $G_i2\alpha$ mRNA remaining after each actinomycin D time period was calculated and plotted on a semilogarithmic scale versus the time period of actinomycin D treatment. The half-life of $G_i2\alpha$ mRNA (48 ± 7 h) was deduced from the 50% value that was calculated by extrapolation. These data are means ± S.E.M. of four separate experiments.

ished by more than 80% within 5 days (19 ± 4% of control; $n = 4$). The steady-state concentrations of $G_o\alpha$ (39 kDa) in membranes of untreated cells and cells challenged with T_3 were unchanged (Figure 1). The levels of G_β subunits in myocyte membranes were also measured. The relative immunoreactive concentrations of $G_{\beta 1}$ (molecular mass 36 kDa) were significantly lower than $G_{\beta 2}$ (molecular mass 35 kDa). This may be due to either the lower abundance of $G_{\beta 1}$ protein in ventricular myocytes or the weaker immunoreactivity of $G_{\beta 1}$ with the antiserum used in this study, particularly as several other polypeptides in the membranes reacted with the $G_{\beta 1}$ antiserum (results not shown). Nevertheless, the band corresponding to $G_{\beta 1}$ was sufficiently distinct to permit accurate assessment of the effect of T_3 on this protein. These studies revealed that T_3 had no appreciable effect on $G_{\beta 1}$ and $G_{\beta 2}$ levels in myocyte membranes.

To characterize further the mechanism underlying the diverse effects of T_3 on $G_s\alpha$ and $G_i2\alpha$ levels in ventricular myocytes, the effect of thyroid status on G-protein α-subunit mRNA was determined using Northern-blot analysis (Figure 2). In cells cultured in the absence or presence of 10 nM T_3, total cellular RNA levels per dish were comparable. The $G_s\alpha$ probe hybridized to a 1.85 kb message, which was the most abundant among the G-protein mRNAs. In cells exposed to T_3 for 0–72 h, $G_s\alpha$ mRNA levels were not affected by this hormone. Hybridization with the $G_i2\alpha$ probe detected a message of 2.35 kb. The levels of $G_i2\alpha$ mRNA in myocytes treated with T_3 were not altered during the first day of exposure. Thereafter they declined gradually, and by 72 h were less than 40% of those in myocytes cultured throughout this period in T_3-depleted serum. The effect of T_3 on $G_i3\alpha$ mRNA was also determined because the study of Levine et al. [21] suggested that $G_i3\alpha$ mRNA levels in adult rat ventricles were influenced by thyroid status. In this system, $G_i3\alpha$ mRNA levels were not influenced by T_3, and the effect of T_3 on $G_i3\alpha$ was not explored further.

Steady-state levels of most mRNAs are regulated by rate of transcription of the encoding gene and rate of degradation of the mRNA [42]. To explore the basis for the delayed yet marked effect of T_3 on $G_i2\alpha$ mRNA, the effect of T_3 on the rate of transcription of the $G_i2\alpha$ gene and on the half-life of $G_i2\alpha$ mRNA were determined (Figure 3). Many of the physiological effects of T_3 are mediated transcriptionally via the ligand-activated nuclear T_3 receptor [43,44]. Therefore the effect of T_3 on the relative rate of transcription of the $G_i2\alpha$ gene was determined by means of nuclear run-on transcription assays in nuclei prepared from myocytes exposed to T_3 or vehicle for 4 h. In nuclei prepared from cells treated with T_3, the rate of transcription of the $G_i2\alpha$ gene decreased by 80% relative to that in vehicle-treated cells (Figure 3b). The effect of T_3 on $G_i2\alpha$ mRNA stability was determined next (Figure 3c). The decay of $G_i2\alpha$ mRNA in myocytes was slow, with a half-life of approx. 48 ± 7 h. This value was determined by extrapolating the percentage of $G_i2\alpha$ remaining after 24 h of continuous exposure of myocytes to actinomycin D and does not take into account changes in the rate of decay that might occur in later time frames. Moreover, the myocytes ceased to contract about 12 h after continuous exposure to actinomycin D. Treatment of myocytes with 10 nM T_3 yielded a similar rate of decay for $G_i2\alpha$ mRNA, indicating that T_3 had no detectable effect on $G_i2\alpha$ mRNA stability (Figure 3c).

To obtain a more complete understanding of the regulation by T_3 of $G_s\alpha$ and $G_i2\alpha$ expression, the relative rates of synthesis and degradation of these proteins were evaluated (Figures 4 and 5). The rate of $G_s\alpha$ synthesis in both vehicle- and T_3-treated cells plateaued after 4 h of labelling, indicating that no significant changes in relative rates of synthesis of $G_s\alpha$ occurred between control cells and cells treated with T_3. In contrast, the rate of $G_i2\alpha$ synthesis in cells exposed to vehicle was faster than in cells exposed to T_3. Maximal rates of $G_i2\alpha$ synthesis in cells cultured in T_3-depleted serum were attained within 4 h ($t_{\frac{1}{2}} = 1$ h; $n = 3$), whereas the maximal rate of synthesis in T_3-treated cells was achieved in 8 h ($t_{\frac{1}{2}} = 2$ h; $n = 3$). These data agree with those describing the effect of T_3 on steady-state levels of the mRNA of

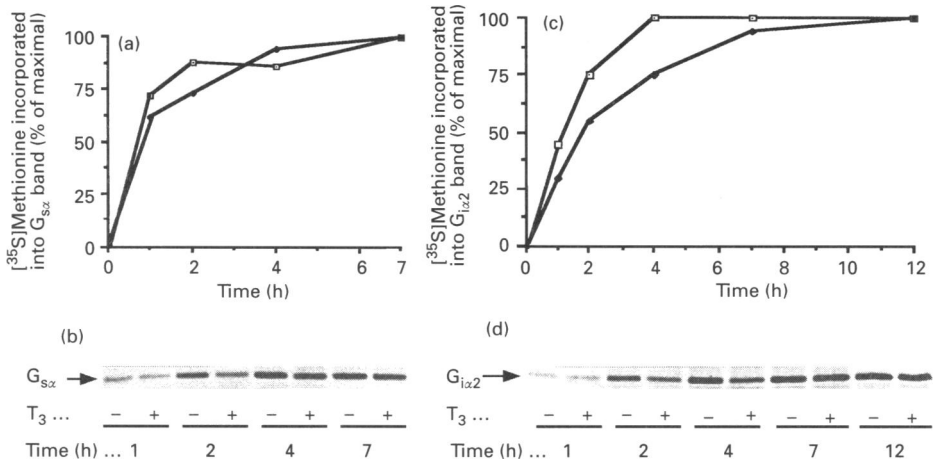


Figure 4 Effect of exposure of ventricular myocytes to T_3 on the relative rates of synthesis of $G_s\alpha$ (a, b) and $G_i2\alpha$ (c, d)

Myocytes were cultured in culture medium supplemented with vehicle (□) or 10 nM T_3 (■) for 4 days. They were then incubated with [^{35}S]methionine (300 μCi/100 mm dish) for 1, 2, 4, 7 or 12 h. At each of these time points, the metabolically labelled cells were harvested, and membrane fractions were prepared. Immunoprecipitation of metabolically labelled $G_i2\alpha$ and $G_s\alpha$ was performed using 100 μg of membrane protein from each sample. The immunoprecipitants were subjected to SDS/PAGE and fluorography. The gels were exposed to X-ray film, and autoradiograms of immunoprecipitations were generated after 8 days ($G_s\alpha$) and 12 days ($G_i2\alpha$) of exposure (**b** and **d** respectively). Next, the gels were subjected to phosphorimage analysis for 2 days ($G_s\alpha$) and 3 days ($G_i2\alpha$). Finally, the metabolically labelled species identified by fluorography were excised and the amount of label was quantified by phosphorimage analysis and liquid-scintillation spectrometry. The percentile of ^{35}S incorporated into $G_s\alpha$ and $G_i2\alpha$ at each time point was calculated by phosphorimage analysis and liquid-scintillation spectrometry and the means of these determinations are presented in (**a**) and (**c**).

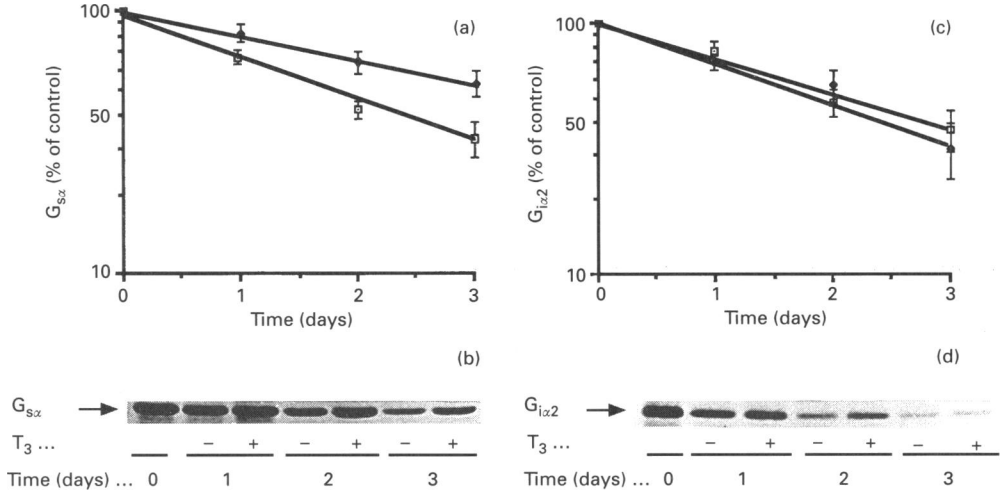


Figure 5 Effect of exposure of ventricular myocytes to T_3 on the relative rates of degradation of $G_s\alpha$ (a, b) and $G_i2\alpha$ (c, d)

Myocytes were cultured in culture medium supplemented with vehicle (□) or 10 nM T_3 (●) for 3 days. They were then incubated with [^{35}S]methionine (400 μCi/100 mm dish) for 8 h. At the end of the labelling period, cells were harvested from five dishes and membrane fractions were prepared as described in the experimental section. The remaining cells were washed with PBS, and recultured in media containing 1 mM unlabelled methionine. Then vehicle (control) or 10 nM T_3 was added. At 1, 2 and 3 days later, cells were harvested, and membrane fractions were prepared from each group of cells. Immunoprecipitation of metabolically labelled $G_i2\alpha$ and $G_s\alpha$ was performed using 100 μg of membrane protein from each sample. The immunoprecipitants were subjected to SDS/PAGE and fluorography (**b** and **d**). The gels were exposed to X-ray film, and autoradiograms of immunoprecipitations were generated after 7 days ($G_s\alpha$) and 11 days ($G_i2\alpha$) of exposure. Metabolically labelled species identified by fluorography were excised and the amount of label was quantified by phosphorimage analysis and liquid-scintillation spectrometry (**a** and **c**).

these G-proteins, in that reduced $G_i2\alpha$ mRNA resulted in a lower rate of synthesis of the $G_i2\alpha$ protein.

The relative rates of degradation of $G_s\alpha$ and $G_i2\alpha$ were explored to complete our understanding of the influence of T_3 on the biosynthetic pathway of these G-proteins (Figure 5). The half-life of $G_s\alpha$ in myocytes cultured in T_3-depleted serum was 50 ± 6 h, whereas that of $G_s\alpha$ in myocytes exposed to T_3 was 72 ± 8 h ($n=4$). The half-life of $G_i2\alpha$ was 46 ± 7 h ($n=4$) in vehicle- and T_3-treated myocytes.

Membranous $G_s\alpha$ concentrations are regulated at the levels of synthesis and redistribution between the cytoplasmic and membranous compartments [45–47]. Redistribution has been implicated as a major pathway for regulating the activity of $G_s\alpha$ [46,47]. The influence of T_3 on the redistribution of $G_s\alpha$ between

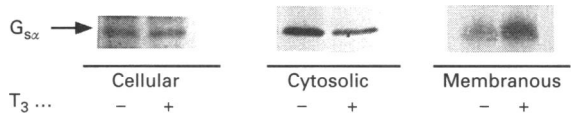


Figure 6 Effect of thyroid hormone on the short-term distribution of $G_s\alpha$ in ventricular myocytes: analysis by quantitative immunoblotting

Myocytes were incubated with 10 nM T_3 or buffer for 1 day. Total cellular proteins were prepared by adding 1 ml of Laemmli solution to a culture dish. Supernatants from the 2000 $g_{av.}$ spin (cytosol) or crude membranes were prepared as outlined in the Experimental section. Some 40 μg from each fraction was subjected to electrophoresis along with 5–100 ng of $G_s\alpha$. The separated proteins were transferred to nitrocellulose and the immunoreactive $G_s\alpha$ was visualized as described in the legend of Figure 1. The autoradiogram for cellular proteins is a 5-day exposure with one intensifying screen, whereas those for cytosol and membranes correspond to 1 day and 0.5 day respectively. The amount of ^{125}I incorporated into the known amounts of $G_s\alpha$ and into the samples was determined by γ-counting. From these data the absolute amounts of $G_s\alpha$ were calculated. Total cellular proteins prepared from myocytes that were exposed to buffer or 10 nM T_3 contained 0.17 ± 0.03 and 0.16 ± 0.04 ng of $G_s\alpha/\mu g$ of protein respectively. Cytosolic proteins from myocytes cultured in the absence of T_3 contained 0.9 ± 0.05 ng of $G_s\alpha/\mu g$ and those that were prepared from myocytes exposed to T_3 for 1 day contained 0.52 ± 0.06 ng of $G_s\alpha/\mu g$. The amount of $G_s\alpha$ was 1.2 ± 0.06 ng/μg in membranes prepared from myocytes cultured in the absence of T_3 and 2.2 ± 0.07 ng/μg in membranes prepared from myocytes exposed to T_3 for 1 day. The data are means ($\pm$S.E.M.) of two separate determinations.

Table 1 Effect of T_3 on the distribution of $G_s\alpha$ within ventricular myocytes

Myocytes were cultured on 100 mm dishes for 2 days, then incubated with 10 nM T_3 or buffer for 1 day. The various cell fractions were prepared as described in the Experimental section. Actual amounts of protein were determined and 40 μg of protein from each condition in the presence or absence of T_3 were electrophoresed separately with 5–100 ng of $G_s\alpha$ protein. The amount of $G_s\alpha$ per lane (each lane contained 40 μg of protein) was determined as outlined in the legend of Figure 6, and the amount of $G_s\alpha$ in ng/dish for each parameter was calculated. The protein data for the cytosol and membranes were derived from ten dishes. *Significant effect of T_3 ($P < 0.05$; $n = 2$).

Condition	Cell fraction	Total protein (μg/dish)	$G_s\alpha$ (ng/lane)	$G_s\alpha$ (ng/dish)
$-T_3$	Whole cells	3540 ± 142	6.8 ± 1.2	601 ± 106
$+T_3$	Whole cells	3608 ± 130	6.4 ± 1.6	577 ± 144
$-T_3$	Cytosol	459 ± 38	36 ± 2	413 ± 60
$+T_3$	Cytosol	408 ± 35	21 ± 2.4*	214 ± 42*
$-T_3$	Membranes	148 ± 10	48 ± 2.5	178 ± 13
$+T_3$	Membranes	159 ± 14	88 ± 3*	350 ± 19*

cytoplasm and membrane was determined by quantitative immunoblotting of total cellular, cytoplasmic and membrane fractions of myocytes (Figure 6). In these experiments we determined the effect of T_3 on the short-term distribution of immunoreactive $G_s\alpha$ because T_3 up-regulated membranous $G_s\alpha$ levels by 2-fold within 1 day (Figure 1a). T_3 did not alter the amount of total cellular $G_s\alpha$, instead it shifted it from the cytoplasm to the membrane (Figure 6 and Table 1). Cytosolic $G_s\alpha$ decreased from 0.9 ± 0.05 ng/μg of protein to 0.52 ± 0.06 ng/μg, 1 day after exposure of the myocytes to T_3. Thus cytoplasmic $G_s\alpha$ decreased from 68 ± 6 to 37 ± 8% of total cellular $G_s\alpha$ in response to T_3 (Table 1). In the same time frame, membranous $G_s\alpha$ increased from 30 ± 6% to 61 ± 7% (Table 1).

To determine whether the biochemical effects of T_3 on $G_s\alpha$ and $G_i2\alpha$ levels in myocytes were correlated with corresponding changes in the physiological functions attributed to these G-proteins, functional assays involving measurement of adenylate

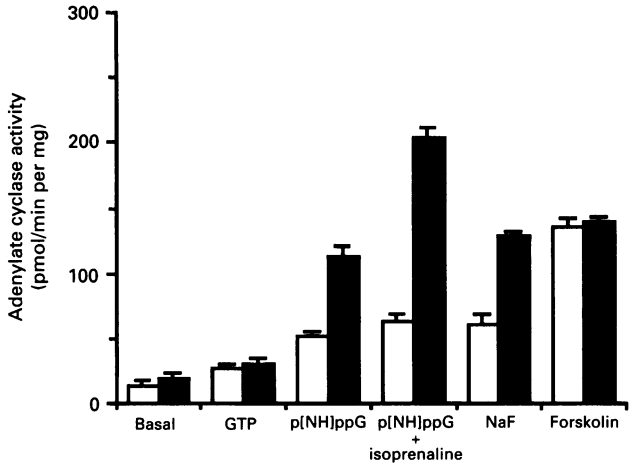


Figure 7 Enhanced guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG) and NaF stimulation of adenylate cyclase after continuous exposure of myocardial ventricular myocytes to T_3

Ventricular myocytes were incubated without (□) or with 10 nM T_3 (■) for 5 days. The cells were harvested and the membranes prepared as described in the Experimental section. Adenylate cyclase response to no added activator (basal), GTP (100 μM), p[NH]ppG (1 μM), p[NH]ppG and isoprenaline (100 μM), NaF (10 mM) or forskolin (10 μM) was measured in 100 μg of membranes in each assay. Data are presented as means $\pm$ S.E.M. of four separate experiments in which each assay was performed in triplicate.

cyclase activity in myocardial membranes and cyclic AMP accumulation in myocytes were performed (Figure 7). Basal and GTP-stimulated adenylate cyclase activities in membranes from cells cultured in the absence or presence of 10 nM T_3 for 5 days were comparable, even though the levels of $G_s\alpha$ were elevated 4-fold in T_3-treated cells. The effects of agents that directly activate G_s, such as p[NH]ppG and NaF, were measured next. p[NH]ppG and NaF caused a 2–3-fold increase in adenylate cyclase activity over that caused by GTP in membranes prepared from myocytes cultured in the absence of T_3. These stimulated activities correlated with the elevated levels of $G_s\alpha$ observed in myocytes pre-exposed to T_3 for at least 3 days, when the concentration of Mg^{2+} in the adenylate cyclase assay was equal to or lower than 1 mM. Stimulation of adenylate cyclase by p[NH]ppG and NaF in membranes prepared from myocytes cultured in the absence or presence of T_3 for 1–2 days was independent of T_3 status (results not shown). As shown in Figure 1, the immunoreactive levels of $G_s\alpha$ in membranes of myocytes cultured in the presence of T_3 for 2 days were 2-fold higher than in those prepared from myocytes cultured in T_3-depleted media, but this change in $G_s\alpha$ did not translate into an enhancement in p[NH]ppG or NaF-mediated stimulation of myocardial adenylate cyclase except after 3 or more days of continuous exposure of the myocytes to T_3. Thus the experiments detailed in Figure 7 were conducted using membranes prepared from myocytes that were exposed to T_3 for at least 4 days (mean 5 ± 0.5). To explore the role of Mg^{2+}, stimulation of myocardial adenylate cyclase by p[NH]ppG and NaF was conducted in 1 mM and 6 mM Mg^{2+}. At 1 mM Mg^{2+}, p[NH]ppG and NaF caused a 2-fold increase in adenylate cyclase activity in T_3-treated membranes compared with its effect on membranes prepared from myocytes cultured in T_3-depleted media (Figure 7). At high Mg^{2+} concentrations such as 6 mM, adenylate cyclase activities in response to p[NH]ppG and NaF in membranes from myocytes cultured in the absence of T_3 did not differ significantly from those of membranes from myocytes

Table 2 Inhibition of the effects of endothelin-1 and carbachol on forskolin-stimulated cyclic AMP accumulation in ventricular myocytes by pertussis toxin

Myocytes were cultured on 35 mm dishes for 2 days and then incubated with or without pertussis toxin (100 ng/ml) for 16 h. They were then incubated for 15 min with 0.5 mM IBMX and then forskolin (10 μM) was added for 5 min. Actual cyclic AMP content (pmol/dish) for forskolin treatment (100% value) was 95 ± 8 and 160 ± 12 in the absence and presence of pertussis toxin respectively. The effects of endothelin-1 (20 nM) and carbachol (100 μM) on the response to forskolin are shown. *Significant effect of pertussis toxin treatment, compared with matched control ($P < 0.001$; $n = 4$).

	Maximal stimulation (%)		
	Forskolin	Forskolin + carbachol	Forskolin + endothelin-1
Control	100	52 ± 7	79 ± 6
Pertussis toxin	100	98 ± 4*	95 ± 6*

exposed to T_3 for 5 days. At 6 mM Mg^{2+}, the activity of adenylate cyclase in pmol/min per mg in membranes prepared from myocytes cultured in the presence or absence of 10 nM T_3 for 5 days was as follows: basal, 50 ± 6; 100 μM GTP, 78 ± 12; 1 μM p[NH]ppG, 200 ± 32; 10 mM NaF, 289 ± 41. Stimulation of adenylate cyclase by the diterpene, forskolin, which stimulates the cyclase, produced a 4–6-fold increase in adenylate cyclase activity that was independent of T_3 status. The fold induction by forskolin in the presence of 20 mM Mn^{2+}, which is believed to provide information on cyclase activity in the absence of G-protein regulation [48], was independent of T_3 status. The activity of adenylate cyclase in membranes exposed to 10 μM forskolin in high Mn^{2+} was 337 ± 16 pmol/min per mg of protein in the absence of T_3 and 329 ± 18 pmol/min per mg in membranes from myocytes exposed to T_3 for 5 days. These data reveal that the activation of adenylate cyclase by forskolin was not influenced by T_3 status, suggesting that the amount of effector enzyme was unchanged.

The functional consequences produced by altered $G_i2\alpha$ levels were determined by investigating carbachol- and endothelin-1-dependent inhibition of forskolin-stimulated adenylate cyclase. Carbachol interacts with M2 muscarinic receptors in the ventricle to inhibit β-agonist- and forskolin-stimulated adenylate cyclase in a pertussis-toxin-sensitive manner [49]. This effect is transduced by $G_i2\alpha$ because carbachol-mediated inhibition of the catalyst was lost in membranes prepared from ventricles of transgenic mice in which $G_i2\alpha$ gene expression was knocked out by homologous recombination [50]. Endothelin-1, a 21-amino acid peptide, is secreted by the vascular endothelium and has effects in several organs including the heart, where it produces positive inotropic and chronotropic responses [51]. Endothelin-1 stimulates phosphatidylinositol turnover and reduces cyclic AMP accumulation in cardiac myocytes by interacting with the endothelin$_A$ (endothelin-1-selective) receptors on the cell surface of these cells [52,53]. The effect of endothelin-1 on adenylate

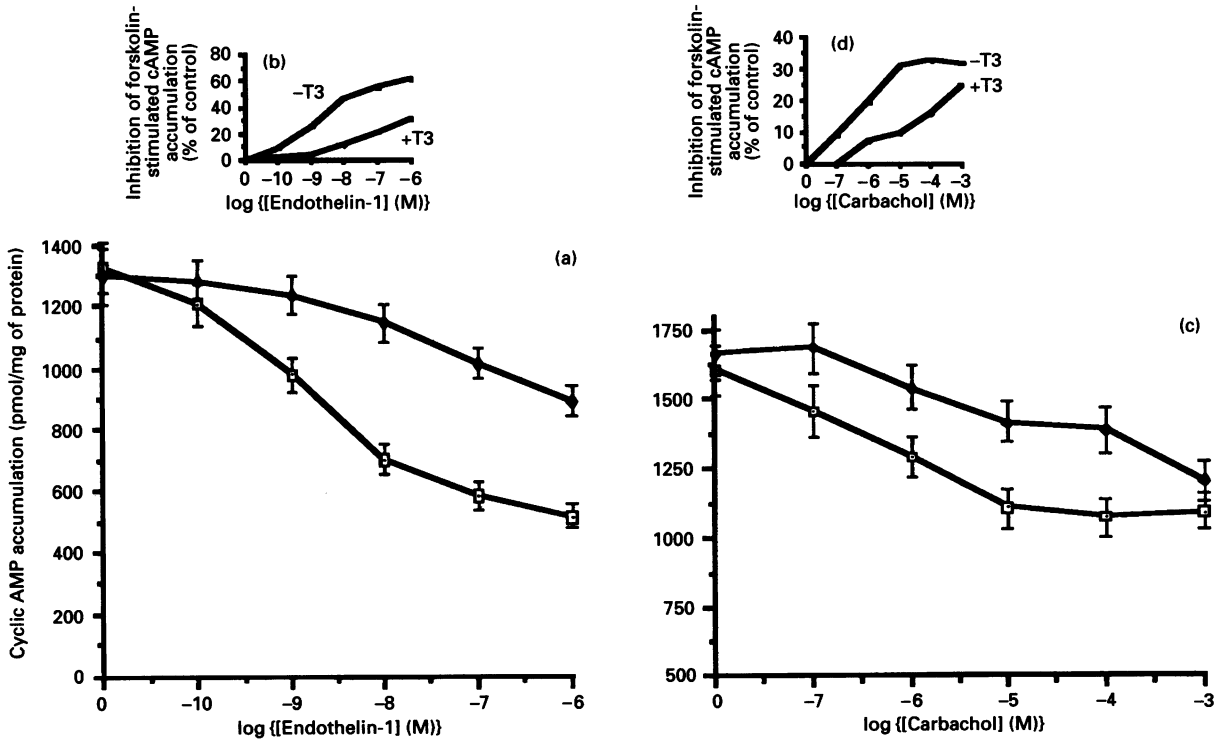


Figure 8 Reduction of forskolin effect on cyclic AMP accumulation by carbachol and endothelin-1

Myocytes were cultured on 35 mm dishes and exposed to diluent or T_3 for 5 days. Myocytes were incubated for 15 min with 0.5 mM IBMX and then forskolin with endothelin-1 (**a** and **b**) or forskolin with carbachol (**c** and **d**) were added for 5 min to appraise cyclic AMP production. (**a**) Concentration-dependence of cyclic AMP accumulation on endothelin-1 in the absence (□) or presence (■) of T_3. (**b**) Percentage inhibition of cyclic AMP accumulation by endothelin-1 in myocytes exposed to buffer or T_3 for 5 days. (**c**). Concentration-dependence of cyclic AMP accumulation on carbachol in the absence (□) or presence (■) of T_3. (**d**) Percentage inhibition of cyclic AMP accumulation by carbachol in myocytes exposed to buffer or T_3 for 5 days.

cyclase activity in adult rat ventricular myocytes was inhibited by pertussis toxin, suggesting the involvement of G_i in this pathway [52–54]. The effect of pertussis toxin on carbachol- and endothelin-1-mediated inhibition of forskolin-stimulated increase in cyclic AMP accumulation was reproduced in the neonatal rat ventricular myocyte system (Table 2). Endothelin-1 was more effective and more potent than carbachol in inhibiting forskolin-mediated cyclic AMP accumulation. The inhibitory effect of carbachol and endothelin-1 on forskolin-stimulated cyclic AMP levels was blunted by prior treatment of the cells with pertussis toxin, suggesting the involvement of G_i- or G_o-related G-proteins in this effect.

The effect of T_3 status on the inhibition of cyclic AMP accumulation by endothelin-1 and carbachol in neonatal rat ventricular myocytes was examined. Myocytes were cultured in the absence or presence of 10 nM T_3 for 5 days. They were then preincubated for 15 min with 0.5 mM IBMX, followed by the addition of 10 μM forskolin and either carbachol or endothelin-1 at the concentrations outlined in Figure 8. In myocytes cultured in T_3-depleted medium, carbachol inhibited the effect of forskolin by 20–25% with an IC_{50} of 0.3 μM. In myocytes cultured in T_3-supplemented medium, the maximal inhibition caused by carbachol was slightly reduced, and the IC_{50} value was 10 μM. Endothelin-1 reduced the effect of forskolin on cyclic AMP accumulation in myocytes cultured in T_3-deficient media by 55–60% with an IC_{50} of 1–2 nM. In myocytes exposed to T_3, the potency and effectiveness of endothelin-1 in reducing the effect of forskolin were significantly reduced. Endothelin-1 maximally inhibited the forskolin effect by 25–30% only and the effective IC_{50} was above 20 nM.

DISCUSSION

Signal transduction via G-protein-linked pathways is a dynamic process, with each of the primary components (receptor, G-protein and effector) representing a potential target for regulation in normal and pathophysiological states. Altered thyroid status is one example where a single hormone exerts a profound influence on the various components of this pathway [3]. At the receptor level, hyperthyroidism increases the number of β_1-adrenergic receptors in the heart but not in the liver or adipose tissue [2]. T_3 also alters the functional responsiveness of these receptors by regulating their interaction with G-proteins. In the heart, levels of $G_i2\alpha$ are consistently down-regulated in hyperthyroidism and up-regulated in hypothyroidism [21,23]. However, the influence of T_3 status on the expression of the other subunits of G-proteins has not been found to be uniform. In one study, altered thyroid states were found to be associated with changes in the levels of $G_i3\alpha$, $G_{\beta 1}$ and $G_{\beta 2}$ in the myocardium [21]; in another, G_β levels were unchanged, but G_o and $G_s\alpha$ levels were affected [23]. Despite its marked effect in the heart, T_3 exerted no appreciable changes in G-protein subunit expression in the rat cortex, liver and vas deferens, reinforcing the notion that the actions of T_3 are tissue-specific and that the heart is one of the major targets [23]. To gain a better understanding of the basis of these changes in G-protein subunit expression in the heart, the influence of T_3 on G-protein subunit biosynthesis in cultured ventricular myocytes was examined. These cells maintain many of the parameters associated with functioning myocardial cells, and their exposure to T_3 can be readily regulated [55]. Immunoblot analysis of membranes prepared from myocytes reveals that the major influence of T_3 is exerted on $G_s\alpha$ and $G_i2\alpha$ subunits. A T_3-mediated decrease in $G_i2\alpha$ levels has been a common finding in most studies on the effect of thyroid status on myocardial and adipose-tissue G-proteins [21–23]. $G_i2\alpha$ appears to be the major target for the action of T_3 in ventricular myocytes. The levels of $G_i2\alpha$ are severely reduced in response to T_3 in this system, in agreement with previous determinations in the heart and adipose tissue [21–23].

T_3-mediated down-regulation of membranous $G_i2\alpha$ is preceded by a decline in $G_i2\alpha$ mRNA, which occurs several days after continuous exposure of the myocytes to T_3. I previously determined, by means of nuclear run-on assays, that T_3 up-regulates myocardial β_1-adrenergic receptor mRNA by transcriptional activation of the gene [37]. Using this technique, I determined that T_3 markedly suppresses the transcription of the $G_i2\alpha$ gene in ventricular myocytes. The transcriptional effects of T_3 are mediated by its binding to nuclear T_3 receptors, which bind to genes containing T_3-responsive elements [44]. Nuclear T_3 receptors belong to the c-*erbA* family of transcription factors, and mRNA encoded by the c-*erbA* α and β genes is detected in ventricular myocytes [37]. However, the location of the putative T_3 response element(s) within the promoter region of the $G_i2\alpha$ gene is still unknown.

Although $G_i2\alpha$ transcription was suppressed within 4 h, $G_i2\alpha$ mRNA levels were not diminished except after 48 h, suggesting that $G_i2\alpha$ mRNA may turn over very slowly. Indeed, $G_i2\alpha$ mRNA is very stable in ventricular myocytes. Therefore the rate of decay of the already synthesized $G_i2\alpha$ controls the time at which a detectable change in this parameter becomes evident. Further support for this tenet is provided in the study of Loganzo and Fletcher [56], which revealed that the $t_{\frac{1}{2}}$ values of $G_i2\alpha$ and $G_s\alpha$ mRNA in Sertoli cells are 44 and 51 h respectively.

The next series of experiments focused on whether the down-regulation of $G_i2\alpha$ mRNA in response to T_3 was correlated with comparable changes in the biosynthesis of $G_i2\alpha$. These studies are important in the light of the mismatch between $G_i2\alpha$ mRNA and protein levels in other cells. For example, persistent inhibition of the adenylate cyclase signalling pathway by the adenosine receptor agonist N^6-(2-phenylisopropyl)adenosine increased $G_i2\alpha$ mRNA levels in hamster vas deferens DDT_1MF-2 smooth-muscle cells [18]. However, $G_i2\alpha$ protein concentration decreased by 50% in spite of the augmented $G_i2\alpha$ mRNA because degradation of $G_i2\alpha$ increased under these conditions. In neonatal rat ventricular myocytes, T_3-mediated down-regulation of $G_i2\alpha$ mRNA produces a net decrease in the rate of synthesis of $G_i2\alpha$ without altering its half-life. The data also revealed that the values of $G_i2\alpha$ mRNA and protein are about equal (48 h). On the basis of these data, I propose that the loss of membranous $G_i2\alpha$ is the result of the primary effect of T_3, which is transcriptional silencing of the $G_i2\alpha$ gene. Consequently, the synthesis of $G_i2\alpha$ mRNA is diminished by more than 80% and the already synthesized $G_i2\alpha$ mRNA is degraded at a rate proportional to its $t_{\frac{1}{2}}$ [57]. In those instances in which half-lives of the mRNA and protein are equal, the decay of the protein can be modelled by a single-compartment model system [58]. On the basis of this model, suppression of $G_i2\alpha$ transcription by more than 80% causes a 50% loss in $G_i2\alpha$ mRNA at a period significantly longer than its $t_{\frac{1}{2}}$, as revealed in the data of Figure 2. Similarly, the kinetics of degradation of the $G_i2\alpha$ protein should be slow because of the relative stability of the already synthesized $G_i2\alpha$. Therefore, as illustrated in Figure 1, the decrease in levels of membrane $G_i2\alpha$ developed slowly during the few days after transcriptional silencing.

In addition to its suppression of $G_i2\alpha$ expression, T_3 caused a significant increase in membranous $G_s\alpha$. A 2-fold increase in $G_s\alpha$ levels occurred within 1 day and culminated in a 4-fold increase after 5 days of T_3 exposure (Figure 1). Quantitative immunoblotting revealed that more than 60% of total cellular $G_s\alpha$ in ventricular myocytes is cytosolic, in agreement with the data of

Roth et al. [59] concerning the distribution of $G_s\alpha$ in the rat atrium. The magnitude of the T_3-mediated redistribution of $G_s\alpha$ from the cytosolic to the membranous compartment is large enough to provide a rationale for the short-term up-regulation of membranous $G_s\alpha$ (Table 1). T_3-provoked palmitoylation of $G_s\alpha$, for example, provides an explanation for the T_3-induced shift of $G_s\alpha$ to the membrane [45–47]. In addition to eliciting the redistribution of $G_s\alpha$, T_3 increased the stability of membranous $G_s\alpha$. T_3-mediated stabilization of $G_s\alpha$ can bring about a net increase in membranous $G_s\alpha$ in about two half-lives ($\sim$ 100 h), which coincide with the onset of the second phase of $G_s\alpha$ up-regulation. Therefore a combination of redistribution and membrane-stabilization mechanisms operating in tandem provide an explanation for the up-regulation of membranous $G_s\alpha$ by T_3 in early and late time frames.

The redistribution of $G_s\alpha$ that occurred shortly after exposure of the myocytes to T_3 did not result in a comparable increase in membranous $G_s\alpha$ activity. p[NH]ppG and NaF-mediated stimulation of adenylate cyclase activity in membranes prepared from myocytes exposed to T_3 or diluent were comparable for the first 2 days. Therefore, in agreement with the results of Roth et al. [59], it appears that $G_s\alpha$ which redistributes from the cytoplasm to the membrane is non-functional. The present data reveal that several days of continuous exposure to T_3 are needed to attain higher adenylate cyclase activities in T_3-treated than vehicle-treated myocytes. The reason for this is unclear, but may be related to the half-life of $G_s\alpha$. Thus, since the $t_{\frac{1}{2}}$ of $G_s\alpha$ is long, a significant amount of time is required for the $G_s\alpha$ synthesized *de novo* to accumulate in the appropriate membranous compartment before a net increase in its activity is detected. In addition to the temporal effects of T_3 on the functional expression of $G_s\alpha$, T_3-mediated increase in the levels of functional $G_s\alpha$ are not associated with enhanced responsiveness of adenylate cyclase to stimulation by p[NH]ppG and NaF except when the free Mg^{2+} concentration is low. High concentrations of Mg^{2+} promote the dissociation of G-protein oligomers and the formation of an activated state of G_α [60]. At modest concentrations of Mg^{2+} (< 1 mM), however, GDP stabilizes the oligomer G-protein and dissociates from it extremely slowly [61]. In the experiments described here, the role of Mg^{2+} is unclear, but low Mg^{2+} decreases the rate of activation of $G_s\alpha$ by stable analogues of GTP [62]. Thus, in low Mg^{2+}, particularly when the concentration of $G_s\alpha$ is limiting, the catalytic activation of adenylate cyclase by p[NH]ppG and NaF appears to be proportional to the concentration of $G_s\alpha$ in the membrane.

Hormonal inhibition of adenylate cyclase is mediated via activation of G_i by inhibitory receptors. The inhibition of adenylate cyclase activity by $G_i\alpha$ appears to be dependent on both the type of adenylate cyclase and the activator [13]. The heart contains type-V and -VI adenylate cyclase, which are inhibited directly by the $G_i\alpha$-GTP subunit [12,13,63]. The manner in which $G_i\alpha$ is activated is particularly important. It can be activated directly by high concentrations of p[NH]ppG or by the inhibitory receptor pathway. The use of p[NH]ppG to activate G_i unavoidably activates G_s because the latter is activated at lower concentrations of p[NH]ppG than G_i [49]. The study of Levine et al. [21] was the first to report that levels of G_i in ventricular membranes prepared from hyperthyroid rats were lower than those prepared from euthyroid rats. However, when p[NH]ppG-mediated inhibition of forskolin-stimulated adenylate cyclase in euthyroid membranes was compared with that in hyperthyroid membranes, the results were indistinguishable. Therefore, to compare the activity of G_i in myocytes cultured in the presence or absence of T_3, G_i was activated by the receptor-mediated pathway to avoid co-activating G_s. In the atrium, M2 muscarinic receptors exert a profound inhibitory input on adenylate cyclase that is stimulated directly by forskolin or via the β-agonist receptor pathway [64]. Moreover, M2 muscarinic receptors inhibit type-VI adenylate cyclase in cells stably expressing this isoform [12]. This response in the ventricle, however, is weak, and the maximum inhibition of adenylate cyclase by the M2 muscarinic agonist carbachol is about 20–30 %. Therefore demonstrating a statistically significant difference between carbachol-mediated inhibition of forskolin-stimulated cyclic AMP accumulation in vehicle- and T_3-treated myocytes will be difficult because the statistical error in the cyclic AMP assay is comparable with the magnitude of carbachol inhibition. Another potent inhibitor of myocardial adenylate cyclase is endothelin-1 [52]. A key feature of endothelin-1-mediated inhibition is its potency and efficacy. Endothelin-1 reduces forskolin- or isoprenaline-stimulated cyclic AMP accumulation by 60 % with an IC_{50} of 1–2 nM [52]. It inhibits the protein kinase A-dependent chloride current in ventricular myocytes via the endothelin$_A$ (endothelin-1-selective) receptor [54]. Like M2 agonists, endothelin-1 inhibits the cyclase in a pertussis-toxin-sensitive manner [52] (Table 2). The effect of T_3 on endothelin-1-mediated inhibition of forskolin-stimulated cyclic AMP accumulation was tested because the effect of forskolin on this parameter is independent of T_3 status [21] (Figure 7). These experiments revealed that the endothelin-1 response in the T_3-exposed myocyte is desensitized. The efficacy (i.e. maximal inhibition) and potency (IC_{50}) of endothelin-1 are both reduced in myocytes exposed to T_3 for at least 5 days. T_3-mediated desensitization of endothelin-1 response increased the IC_{50} of endothelin-1 by 25-fold. I speculate that the shift in the IC_{50} of endothelin-1 is more related to a diminished interaction between $G_i2\alpha$ and the endothelin$_A$ receptor than to a potential loss in membranous endothelin$_A$ receptors because the affinity of a receptor for its agonist is related to its functional coupling to the G-protein and is independent of the receptor density [65]. Endothelin-mediated inhibition of the myocardial chloride channel lengthens the action potential and protects the heart muscle undergoing ischaemia against shortening of the action potential caused by catecholamines [53,54]. In the present report, these findings are extended to show that T_3 may exacerbate the effects of catecholamines on ischemia because it desensitizes the endothelin-signalling pathway and increases the responsiveness of the catecholamine pathway. In addition, it is shown that the pertussis-toxin-sensitive G-protein that imparts endothelin-1-mediated inhibition of adenylate cyclase is most probably $G_i2\alpha$.

A hallmark of hyperthyroidism is the increased sensitivity of the hormone-sensitive adenylate cyclase pathway to stimulation by β-agonists [1–3]. Continuous exposure of the myocytes to T_3 leads to an enhancement of the stimulatory pathway and desensitization of the inhibitory pathway. In addition to the well-documented increase in myocardial β-adrenergic receptors, T_3-promoted increase in $G_s\alpha$ is another factor contributing to increased sensitivity of the stimulatory pathway. These effects of T_3 are tissue-specific and occur mostly in the heart and adipose tissue [2]. In addition to its effect on the stimulatory pathway, T_3 causes desensitization of the inhibitory pathway which, in turn, increases the responsiveness of the stimulatory pathway to agonists. These effects are displayed in Figure 9. The increased sensitivity of myocardial adenylate cyclase to β-agonists is promoted, in part, by at least three different mechanisms: the increase in $G_s\alpha$, increase in the complement of β-adrenergic receptors and decrease in $G_i2\alpha$. Thus it appears that T_3 selectively elevates the levels of receptors and G-protein subunits positively coupled to stimulation of myocardial adenylate cyclase while markedly decreasing the levels of G-protein oligomers that inhibit

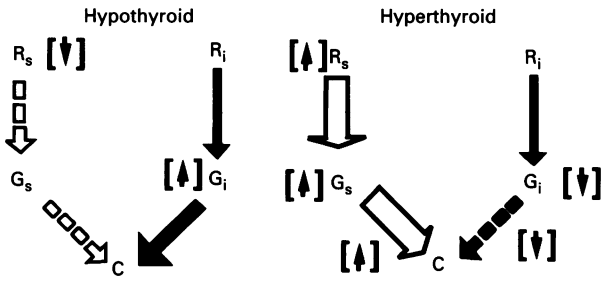


Figure 9 Regulation of the ventricular myocyte hormone-sensitive adenylate cyclase system by thyroid hormones

Stimulation of adenylate cyclase (C) by activation of stimulatory cell-surface receptors R_s (typified by myocardial β-adrenergic receptors) is mediated via the G-protein G_s. White arrows depict flow of stimulatory signal from receptor to catalytic unit. Inhibition of myocardial adenylate cyclase by activation of inhibitory cell-surface receptor R_i (typified by myocardial M2 muscarinic or endothelin-1 receptors) is mediated via the G-protein G_i. Black arrows depict flow of inhibitory signal from receptor to effector unit. Alteration of thyroid states yields a state of thyroid hormone deficiency, 'hypothyroid', and a short-term thyroid hormone excess, 'hyperthyroid'. Broken arrows depict interruptions in the flow of signals from receptors to catalyst. Changes in the abundance in altered thyroid state are indicated ([↓] [↑]).

myocardial adenylate cyclase. These multiple but selective stoichiometric changes produced by T_3 in the repertoire of this signalling pathway reveal new insights into the role of this hormone in the integration of information in signalling networks.

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