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# Activation of the $\beta_2$-adrenergic receptor promotes growth and differentiation in thyroid cells

(thyrotropin/cyclic AMP/neurotransmitters/signal transduction)

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ABSTRACT **We have introduced the $\beta_2$-adrenergic receptor into the unnatural environment of a thyroid cell to demonstrate that the activation of this receptor initiates diverse cellular programs in different cell types. The thyroid-stimulating hormone (TSH) receptor and the $\beta_2$-adrenergic receptor stimulate a common signaling pathway in distinct populations of cells. In this study, we demonstrate that the activation of the $\beta_2$-adrenergic receptor, transfected into a thyroid epithelial cell, elicits a program of growth and differentiation normally observed with TSH. In thyroid cells expressing $\beta_2$ receptors, the $\beta_2$ agonist isoproterenol activates adenylate cyclase, induces the expression of a thyroid-specific iodide carrier system, and can substitute for TSH to promote growth. Thus, in thyroid cells expressing $\beta_2$-adrenergic receptors, isoproterenol elicits the entire array of thyroid-specific functions normally activated by TSH.**

Cell–cell communication is mediated by the interaction of signaling molecules with specific membrane receptors. The cellular response to a signaling molecule is determined by the nature of the membrane receptors expressed on the cell surface, the cascade of second messenger pathways triggered by receptor activation, and the specific cellular program elicited by these second messenger systems. A single signaling molecule, such as a hormone, growth factor, or neurotransmitter, may therefore elicit distinct responses in different cell types.

Multiple receptor subtypes exist for most, if not all, neurotransmitters. The existence of multiple adrenergic receptor subtypes, each capable of interacting with different guanine nucleotide-binding proteins, provides one mechanism by which a single hormone may elicit distinct cellular responses. Adenylate cyclase activity, for example, is stimulated by the $\beta_1$- and $\beta_2$-adrenergic receptors but is inhibited by the $\alpha_2$-receptor subtype (1–3). The $\alpha_1$-receptor, however, couples with a distinct second messenger pathway to activate phospholipase C (4). In this manner, a single adrenergic ligand may interact with different receptor subtypes to elicit different responses in different cells.

In other instances, a single ligand may interact with the same receptor subtype in different cells to elicit distinct cellular responses. For example, activation of a neurotransmitter receptor on neurons may modulate the activity of ion channels, whereas activation of the same receptor on non-neuronal cells may regulate growth and differentiation (5, 6). These distinct cellular responses observed upon activation of the same receptor subtype may reflect the manner in which different cell types respond to the same set of signaling events.

We have introduced the $\beta_2$-adrenergic receptor into the unnatural environment of a thyroid cell to demonstrate that the activation of this receptor initiates diverse cellular programs in different cell types. The cell line FRTL5 is a continuous line of rat thyroid cells that is dependent upon thyroid-stimulating hormone (TSH) for both growth and differentiation (7). Activation of the TSH receptor stimulates adenylate cyclase, resulting in a rapid increase in cAMP (7, 8). The $\beta_2$-adrenergic receptor also stimulates adenylate cyclase, but this receptor subtype is not expressed in thyroid cells. We have therefore asked whether thyroid cells transfected with cDNA encoding the $\beta_2$-adrenergic receptor will undergo growth and differentiation in response to the adrenergic ligand isoproterenol. We demonstrate that in transfected cells, isoproterenol elicits the same program of thyroid-specific functions observed with TSH. Thus, the $\beta_2$-adrenergic receptor, which contributes to autonomic neurotransmission in the sympathetic nervous system, regulates growth and differentiation in the unnatural environment of a thyroid cell.

## MATERIALS AND METHODS

**Cells, Media, and Transfection.** FRTL5 cells (7) and their derivatives were maintained in a growth medium consisting of Dulbecco's modified Eagle's medium, 5% calf serum, insulin (10 $\mu$g/ml), cortisol (10 nM), transferrin (5 $\mu$g/ml), glycyl-L-histidyl-L-lysine acetate (10 $\mu$g/ml), somatostatin (10 $\mu$g/ml), and TSH (10 nM). FRTL5 cells were cotransformed with 20 $\mu$g of recombinant plasmid pKSVTF (2) and 2 $\mu$g of pSVneo (9) by the calcium phosphate transfection technique (10).

**cAMP Assay.** Thyroid cells seeded in 24-well plates were grown to a density of $\approx 10^5$ cells per well in TSH-containing medium and were then incubated for 4 days in growth medium without TSH. Wells were then washed with 1 ml of Hanks' balanced salts solution (HBSS) and exposed to 1 ml of HBSS containing either 20 $\mu$M forskolin, 10 $\mu$M isoproterenol, or 10 nM TSH, in the presence of 10 $\mu$M 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterases. The HBSS was then removed and 1 ml of ethanol was added to each well. After 2 hr of incubation at room temperature, the ethanol was removed and dried, and the levels of cAMP were determined with a radioimmunoassay kit (NEN; catalogue no. NEK-033). The protein content of the samples was determined by Coomassie blue G250 binding (11).

**Iodide Uptake Assay.** *Nylon disc replica assay.* Cells were seeded at a density of 1000 cells per 10-cm dish and were grown to colonies of about 100 cells. Nylon discs were placed over the cells and a single layer of glass beads was poured over the discs to hold them flat against the bottom of the plate. Cell colonies were grown in contact with the nylon

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Abbreviations: TSH, thyroid-stimulating hormone; HBSS, Hanks' balanced salts solution.

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discs for 2 days. The plates and filters were incubated for 4 days in growth medium lacking TSH and were then exposed for 48 hr to growth medium supplemented with either 10 nM TSH or 1 $\mu$M isoproterenol. The nylon discs were then removed, washed once in HBSS, and incubated for 45 min at 37°C in HBSS containing $Na^{125}I$ (27 Ci/mg, NEN; 1 Ci = 37 GBq) at 1 $\mu$Ci/ml. The nylon discs were then washed twice in ice-cold HBSS and autoradiographed at −80°C for 12 hr.

*Quantitation of iodide uptake.* Cells were seeded in 24-well plates as in the cAMP assay, deprived of TSH for 4 days, and incubated for 48 hr in growth medium supplemented with either 10 nM TSH or various concentrations of isoproterenol. The wells were washed once with HBSS and incubated for 45 min with 1 ml of HBSS containing 1 $\mu$Ci of $Na^{125}I$. The wells were then washed twice with ice-cold HBSS. One milliliter of 95% ethanol was added for 15 min at room temperature and then removed for measurement of radioactivity in a $\gamma$ counter.

**Incorporation of [$^3$H]Thymidine.** Cells were seeded in 24-well plates as described for the quantitative iodide uptake assay. After 4 days of TSH deprivation, cells were incubated for 48 hr in growth medium supplemented with either TSH or isoproterenol and containing 100 $\mu$M [$^3$H]thymidine (5 $\mu$Ci/ml). The wells were washed twice with ice-cold HBSS and 1 ml of 95% ethanol was added for 15 min at room temperature. After removal of the ethanol, the fixed cells were dissolved in 1 ml of 0.3 M NaOH for 1 hr at room temperature. This solution was neutralized with 100 $\mu$l of 1.5 M HCl and 1.1 ml of cold 10% (wt/vol) trichloroacetic acid was added for 15 min on ice. The precipitate was collected on glass-fiber filters, which then were washed five times with cold 5% trichloroacetic acid followed by 95% ethanol. Filters were dried for liquid scintillation counting.

## RESULTS

We have used gene transfer to generate a rat thyroid epithelial cell line expressing the human $\beta_2$-adrenergic receptor. An expression vector, pKSVTF (2), containing the cDNA encoding the human $\beta_2$-adrenergic receptor, was introduced into the FRTL5 thyroid cell line by cotransformation with the bacterial neomycin phosphotransferase gene. Colonies resistant to the neomycin derivative G418 were expanded and a clone expressing high levels of $\beta_2$-receptor mRNA ($HB_2X_3$) was isolated. We then examined the functional consequences of activation of the $\beta_2$-adrenergic receptor in this transformed thyroid cell line.

The parental cell line, FRTL5, requires TSH for both growth and differentiation. If FRTL5 cells are cultured in the absence of TSH, cells stop dividing, extinguish differentiated thyroid functions, and remain quiescent for long periods of time. Upon exposure to TSH, cAMP levels rapidly increase. By 24 hr, a functional iodide carrier is expressed and the cells accumulate high levels of iodide (8). After 48 hr with TSH, the cells resume a normal division cycle. The resumption of growth and differentiation is thought to be a consequence of the earlier activation of adenylate cyclase (8, 12).

In initial experiments, we examined whether activation of the $\beta_2$-adrenergic receptor in the transformed line, $HB_2X_3$, could elevate the level of cAMP. FRTL5 and $HB_2X_3$ cells, when grown in the absence of TSH and isoproterenol, exhibit low, resting levels of cAMP. Exposure of either cell line to TSH or forskolin, a diterpene that directly stimulates adenylate cyclase, resulted in a 30-fold increase in cAMP (Fig. 1). The adrenergic agonist isoproterenol elicited a 25-fold increase in cAMP in the transformed $HB_2X_3$ cells but had no effect on the level of cAMP in parental FRTL5 cells (Fig. 1). Thus, activation of either the TSH receptor or the $\beta_2$-adrenergic receptor in transformed thyroid cells leads to a striking increase in adenylate cyclase activity.

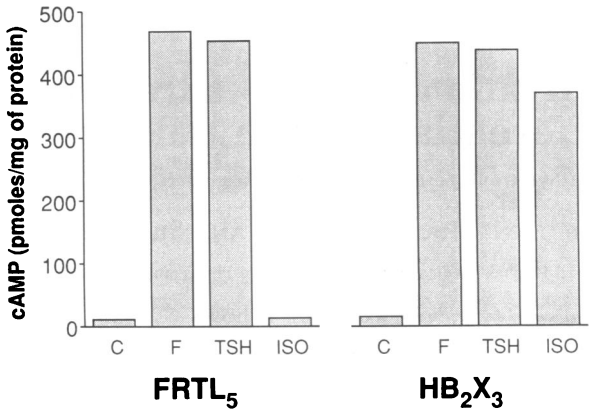


FIG. 1. cAMP levels are elevated by TSH and isoproterenol in $HB_2X_3$ cells. Cells were seeded in 24-well plates at $\approx 10^5$ cells per well. Cells were deprived of TSH for 4 days and exposed for 30 min to control medium (HBSS with 10 $\mu$M 3-isobutyl-1-methylxanthine) alone (C) or supplemented with 20 $\mu$M forskolin (F), 10 $\mu$M isoproterenol (ISO), or 10 nM TSH. cAMP levels were determined and are expressed in pmol per mg of protein. Two independent experiments were carried out; each value was determined in duplicate from two separate wells, and results differed by <10%.

Activation of the TSH receptor and the subsequent increase in cAMP is essential for the growth of FRTL5 cells. We therefore asked whether isoproterenol can serve as a growth factor to promote the growth of $HB_2X_3$ cells in the absence of TSH. When FRTL5 or $HB_2X_3$ cells are seeded in the presence of TSH and then deprived of TSH, the cells cease division and become quiescent. Such quiescent cells were exposed to either TSH or isoproterenol. The addition of TSH to either FRTL5 or $HB_2X_3$ cells resulted in a resumption of cell division within 48 hr, and the colonies then expanded (Fig. 2). Isoproterenol, however, restored division in $HB_2X_3$ cells but had no effect on the growth of the parental FRTL5 line. The rate of growth of $HB_2X_3$ in medium with isoproterenol was about half that observed with TSH. We also measured an increase in the incorporation of [$^3$H]thymidine in $HB_2X_3$ cells after exposure to either isoproterenol or TSH (Fig. 3). The response to isoproterenol was maximal at 1 $\mu$M and the $EC_{50}$ was about 0.1 $\mu$M, a value consistent with the $EC_{50}$ of the $\beta_2$-adrenergic receptor in other cell lines (13). In parental cells, incorporation of [$^3$H]thymidine was only observed following exposure to TSH.

We next asked whether isoproterenol elicits the same set of differentiation events induced by TSH. To this end, we

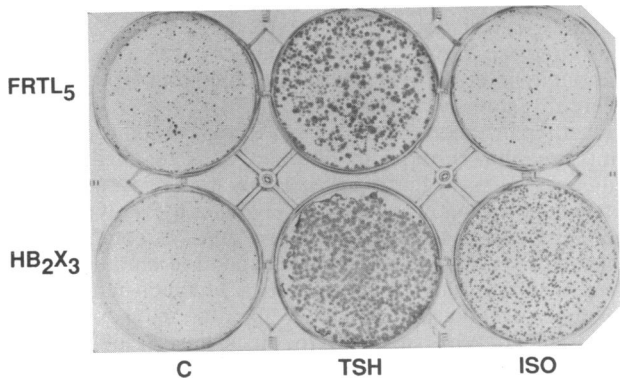


FIG. 2. Isoproterenol and TSH promote the growth of $HB_2X_3$ cells. FRTL5 and $HB_2X_3$ cells were seeded at low density in 6-well plates (500 cells per well) and allowed to grow for 15 days in growth medium without TSH (C) or supplemented with either 10 nM TSH or 1 $\mu$M isoproterenol (ISO). Finally, cells were fixed in methanol and stained with Giemsa reagent.

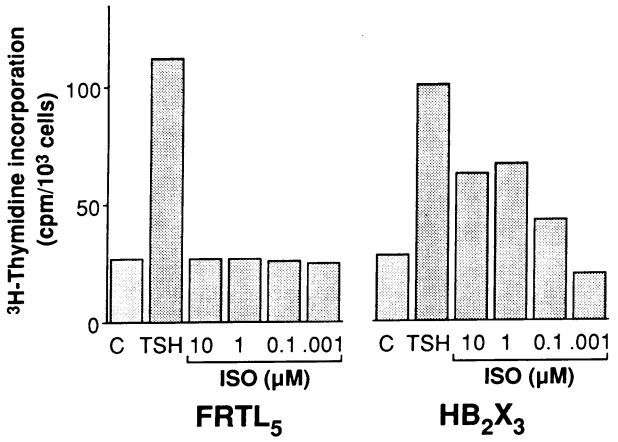


FIG. 3. Isoproterenol and TSH increase [$^3$H]thymidine incorporation in $HB_2X_3$ cells. FRTL5 and $HB_2X_3$ cells were seeded in 24-well plates at about $10^5$ cells per well. They were deprived of TSH for 4 days and exposed for 48 hr to growth medium without TSH (C) or supplemented with 10 nM TSH or various concentrations of isoproterenol (ISO, 0.001–10 μM). [$^3$H]Thymidine incorporation was determined and is expressed in cpm per 1000 cells. Two independent experiments were carried out; each value was determined in duplicate, and results differed by <15%.

used a colony assay to examine the expression of the iodide carrier in parental and transformed thyroid cells (S.O., unpublished data). FRTL5 and $HB_2X_3$ cells were grown as colonies on nylon filters in the presence of either TSH or isoproterenol. Filters were then incubated with [$^{125}$I]iodide, washed, and exposed to film for 12 hr. Colonies capable of concentrating [$^{125}$I]iodide from the medium generated a strong localized signal upon radiographic exposure. This assay demonstrated that both the parental and the transformed cells accumulated iodide in the presence of TSH (Fig. 4). Isoproterenol, however, induced the expression of the iodide carrier only in transformed cells expressing the $\beta_2$ receptor. Iodide uptake was not observed in control cells cultured in the absence of TSH or isoproterenol.

These colony assays measuring iodide accumulation were complemented by more quantitative studies of iodide accumulation (Fig. 5). Cells were treated with either TSH or isoproterenol for 48 hr and then exposed to [$^{125}$I]iodide for 45 min and iodide accumulation was measured in a γ counter. TSH induced about a 30-fold increase in iodide accumulation in both FRTL5 and $HB_2X_3$ cells. Isoproterenol (1 μM)

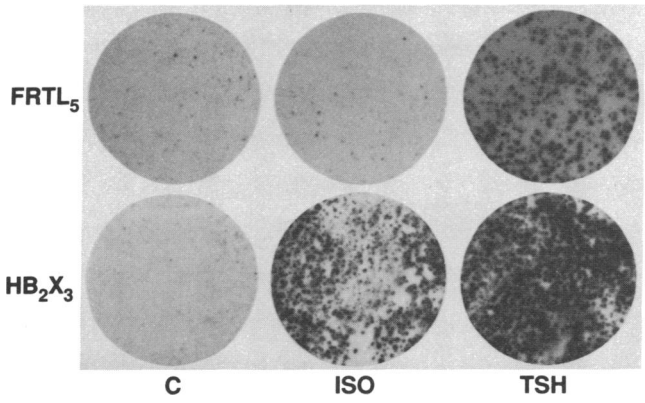


FIG. 4. TSH and isoproterenol induce iodide uptake in colonies of $HB_2X_3$ cells. Colonies of FRTL5 and $HB_2X_3$ cells were grown on nylon discs. TSH was withdrawn for 4 days and the cells were finally exposed for 48 hr to growth medium without TSH (C), or supplemented with 10 nM TSH or 1 μM isoproterenol (ISO). Filters were incubated with [$^{125}$I]iodide, washed, and autoradiographed.

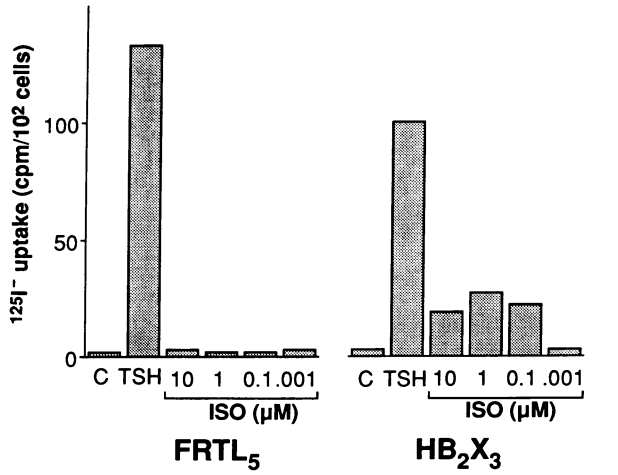


FIG. 5. Iodide uptake in response to TSH and isoproterenol in FRTL5 and $HB_2X_3$ cells. Cells were seeded in 24-well plates at a density of about $10^5$ per well. Cells were deprived of TSH for 4 days and then exposed for 48 hr to growth medium without TSH (C) or supplemented with 10 nM TSH or various concentrations of isoproterenol (ISO, 0.001–10 μM). $^{125}$I uptake was determined and is expressed in cpm per 100 cells. Five independent experiments were carried out; each value was determined in duplicate, and results differed by 15%.

resulted in a 9-fold increase in the idodide content of $HB_2X_3$ cells but had no effect on iodide uptake in the parental cells. Thus, in cells expressing the $\beta_2$-adrenergic receptor, isoproterenol can serve as a growth factor as well as an inducer of a thyroid-specific function normally induced by TSH.

## DISCUSSION

Receptors for specific signaling molecules are often selectively expressed on subpopulations of cells, whereas the second messenger systems they activate are expressed on a far broader range of cell types. The TSH receptor and the $\beta_2$-adrenergic receptor activate a common signaling pathway on distinct populations of cells. In this study, we demonstrate that activation of the $\beta_2$-adrenergic receptor, in the unnatural environment of a thyroid epithelial cell, elicits a program of growth and differentiation normally observed with TSH. In thyroid cells expressing $\beta_2$ receptors, the $\beta_2$ agonist isoproterenol activates adenylate cyclase, resulting in a 25-fold increase in cAMP. Isoproterenol also induces the expression of a thyroid-specific iodide carrier system and can substitute for TSH to promote growth. Thus, in thyroid cells expressing $\beta_2$-adrenergic receptors, isoproterenol elicits the entire array of thyroid-specific functions normally activated by TSH.

The ability of both TSH and a $\beta_2$ agonist to initiate the identical program of growth and differentiation in transfected thyroid cells is likely to result from the ability of these two ligands to activate adenylate cyclase and elevate cAMP levels. This mechanism is supported by the observation that cholera toxin and forskolin, which stimulate adenylate cyclase, mimic the action of TSH in thryoid cells. $\beta_2$-Adrenergic agonists, which mediate a diverse set of functions in the peripheral nervous system by activation of adenylate cyclase, serve to elicit growth and differentiation via the same mechanism in thyroid cells. Thus, the distinct phenotypic consequences of $\beta_2$-receptor activation in different cell types may reflect different ways in which individual cells are programed to respond to the same set of signaling events.

Although the responses to TSH and isoproterenol appear qualitatively similar, TSH is a more effective inducer of both the iodide uptake mechanisms and cell growth. This differential response may reflect the fact that long-term stimulation

may result in desensitization of the $\beta_2$-adrenergic receptor. Alternatively, the TSH receptor, which clearly activates adenylate cyclase, may couple to other signaling pathways not activated by $\beta_2$ agonists. Finally, although both the TSH and the $\beta_2$-adrenergic receptor activate adenylate cyclase, it is conceivable that they exert this function by coupling with different members of the family of stimulatory guanine nucleotide-binding proteins.

The function of the $\beta_2$-adrenergic receptor has recently been studied in the mouse adrenal cortical cell line Y1 (14). Steroid synthesis and secretion are stimulated in this cell line by transient rises in cAMP elicited by corticotropin. In Y1 cells transfected with cDNA encoding the $\beta_2$ receptors, isoproterenol mimics the action of corticotropin. Thus, in both adrenal cortical cells and thyroid cells, activation of the $\beta_2$-adrenergic receptor elicits changes in the growth and differentiation normally observed with the cell-specific growth factors TSH and corticotropin. In addition, DNA encoding the serotonin 5HT1c receptor, a neurotransmitter receptor restricted to cells of the nervous sytem, functions as an oncogene when expressed in NIH mouse 3T3 cells (D. Julius, T. J. Livelli, Thomas M. Jessell, and R.A., unpublished data). Moreover, serotonin, as well as the peptide neurotransmitters, stimulates DNA synthesis and cell division in multiple cell types in culture (5, 15, 16). Thus, the functional distinction between a neurotransmitter receptor and a growth factor receptor (or even an oncogene product) may depend critically on the cellular environment.

The observation that activation of the $\beta_2$-adrenergic receptor in thyroid cells elicits a series of events that are easily detectable and often selectable provides a genetic system to examine the function of receptors that couple with adenylate cyclase. At present, the function of these receptors and receptor mutants is usually analyzed by direct biochemical measurements of adenylate cyclase activity. We have devised two simple visual assays that permit the analysis of receptor function at the level of individual colonies: iodide uptake and cell growth. These indicators of receptor function should facilitate the selection of receptor mutants *in vivo*, as well as the analysis of mutants constructed *in vitro*, and therefore facilitate somatic cell genetic analysis of neurotransmitter receptors. Finally, these assays in concert with gene transfer techniques may ultimately permit the cloning of other receptors coupled to adenylate cyclase.

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