Post-transcriptional induction of β_1-adrenergic receptor by retinoic acid, but not triiodothyronine, in C6 glioma cells expressing thyroid hormone receptors

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Thyroid hormone (triiodothyronine; T_3) has been shown to control the expression of β_1-adrenergic receptors (β_1-AR) in cardiac myocytes, but not in C6 glioma cells. This cell specificity has been attributed to low expression of T_3 receptors and high expression of the *c-erbA*α_2 splice variant that interferes with the action of T_3. To check this hypothesis we have expressed the *c-erbA*/thyroid hormone receptor (TR) α_1 gene in C6 glioma cells and investigated their response to thyroid hormone. Cells expressing TRα_1, but not wild-type cells, were responsive to T_3 as shown by increased expression of mitochrondrial hydroxymethylglutaryl CoA synthase after T_3 exposure. However, T_3 had no effect on β_1-AR gene expression in either set of cells. The β_1-AR mRNA concentrations were, however, altered by retinoic acid (RA) treatment. Retinoic acid caused a rapid up-regulation of β_1-AR mRNA levels that was blocked by cycloheximide. Retinoic acid did not increase the β_1-AR gene transcription rate in run-on experiments. These results indicate an indirect post-transcriptional effect of RA. Control of β_1-AR expression in C6 cells is also exerted at the translational level, because there was no correlation between mRNA and protein induction, as determined by radioligand binding studies. We conclude that lack of responsiveness of the β_1-AR gene in C6 cells to T_3 is not due to high expression of *c-erbA*α_2 but to undefined cell-specific factors.

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The β_1-adrenergic receptor (β_1-AR) is a member of the family of G-protein-coupled catecholamine (adrenaline and noradrenaline) receptors. Upon hormone binding, β_1-AR stimulates the enzyme adenylate cyclase leading to the generation of cAMP. Both the β_1 and β_2 adrenergic receptors are expressed in the brain, where their density seems to be regulated by thyroid hormone (1–3). Thyroid hormone also increases β-adrenergic receptors in the heart muscle and isolated cardiac myocytes (4, 5), in which it specifically activates β_1-AR gene transcription (6). In contrast to cardiac myocytes, glial C6 cells have been shown to be unresponsive to thyroid hormone in terms of β_1-AR induction (6). This fact has been attributed to the high expression of the *c-erbA*α_2 form, which results from differential splicing of the TR/*c-erbA*α gene and encodes a protein that is unable to bind thyroid hormone but inhibits transcriptional activation by T_3 (7, 8). In addition, C6 cells express low levels of T_3 receptors: levels of the RNAs coding for the active receptors TR/*c-erbA*α_1 and TR/*c-erbA*β_1 are undetectable or much lower than those of *c-erbA*α_2 RNA in Northern analyses. For these reasons, we decided to express in C6 cells an exogenous TR/*c-erbA*α_1 gene to investigate whether the lack of β_1-AR inducibility by T_3 in these cells could be due to the pattern of expression of the different erbA forms or, alternatively, to an intrinsic inability of β_1-AR to respond to T_3 treatment.

On the other hand, the vitamin A metabolite all-*trans*-retinoic acid (RA), considered as a modulator of differentiation of a number of cell types in higher organisms, causes a threefold increase in the number of β-adrenergic receptors in mouse teratocarcinoma F9 cells (9). Recently, RA has also been reported to induce the expression in C6 glioma cells of proteolipid protein (PLP), the major protein constituent of myelin (10, 11). These data prompted us also to investigate possible actions of RA on β_1-AR expression in these cells, which are a commonly used system to study hormonal effects on glial cells. C6 glioma cells express high and low levels, respectively, of β_1-adrenergic and β_2-adrenergic receptor RNA and protein (6, 12). The RNA levels for

both receptors are down-regulated by their agonist isoproterenol and also by contact inhibition and by protein kinase C activators such as phorbol esters (12, 13). We report here the effects of RA and T_3 on the expressions of the β_1-AR gene in C6 glioma cells, and discuss them in relation to their reported regulatory actions in different cell types and on other genes.

Materials and methods

Culture techniques

The C6 glioma cell line was grown in RPMI medium containing 10% horse serum and 5% fetal calf serum (Gibco, UK). Cells were maintained at 37°C in a 5% CO_2 atmosphere. The TR/c-*erbA*α_1 and v-*erbA*-expressing cells were generated by using recombinant retroviruses encoding the chicken TR/c-*erbA*α_1 or v-*erbA* genes described previously (14). Expression of active TR proteins was assayed by T_3-binding assays (not shown). These analyses showed that the concentration of TRs increased around tenfold in TR/c-*erbA*α_1 cells. The concentrations of T_3 (25×10^{-9} mol/l) and RA (10^{-6} mol/l) used gave maximal biological responses in these cells.

Isolation and analysis by RNA

Poly(A)$^+$ RNA was purified as described elsewhere (15), electrophoresed on a 1.2% agarose gel containing 2.2 mol/l formaldehyde and blotted onto Gene Screen membranes according to standard protocols (16). The cDNA probes were labeled by the random priming method (17). Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mmol/l sodium phosphate buffer (pH 7.2) and 1 mmol/l EDTA (18). Filters were washed twice for 30 min each in 1% SDS and 40 mmol/l sodium phosphate buffer (pH 7.2) at 65°C. Sizes of respective mRNAs were calculated using an RNA ladder as a marker (BRL, Bethesda). Membranes were exposed to Kodak X-OMAT AR films. Autoradiograms were analyzed using a La Cie scanner connected to a Macintosh IIci computer using Adobe Photoshop™ 2.0 and NIH Image programs. All the experiments were done in triplicate unless stated otherwise. Statistical comparison between means was done by Student's *t*-test.

Run-on assay

Nuclear run-on reactions were performed using [α-^{32}P]UTP (Amersham) and 2×10^7 nuclei (9). Labeled RNA was purified by phenolchloroform extraction after the addition of 3 volumes of guanidine isothiocyanate solution (20) and isopropanol precipitation. Unincorporated label was removed by centrifugation through Sephadex G-50 spin columns. The probes were 5 μg of linearized plasmid DNA immobilized on nylon filters after denaturation in NaOH. Hybridizations were performed in 0.2 mol/l sodium phosphate buffer (pH 7.2), 1 mmol/l EDTA, 7% SDS and 45% formamide containing 250 μg/ml *E. coli* tRNA as a carrier, at 42°C for 3 days. Following hybridization, the filters were washed in 40 mmol/l sodium phosphate buffer (pH 7.2) containing 1% SDS at 37°C.

Binding assays

Radioligand binding studies were conducted using intact cells. Control or RA-treated cells were detached and washed three times with RPMI + 20 mmol/l HEPES (pH 7.4) medium. Resuspended cells were incubated (0.5×10^6 cells/assay) for 1 h at 37°C in the same medium in the presence of 2 nmol/l [^{3}H]dihydroalprenolol (Amersham), and binding reactions were terminated by rapid filtration over GF/C glass-fiber filters (Whatman). Total cellular β-adrenergic receptor was defined as the amount of radioligand binding inhibited by 50 μmol/l (±) propranolol; similar results were obtained by displacing with the specific β_1-adrenergic receptor antagonist ICI 89,406 (a generous gift from ICI Pharmaceuticals). All experiments were carried out in quadruplicate (SEM < 10%).

Results

Lack of effect of thyroid hormone on β_1-AR mRNA levels in C6 cells

As shown in Fig. 1, C6 cells were found to express the β_1-AR gene. Addition of RA but not T_3 induced β_1-AR mRNA by about a factor of 3, as measured by densitometric analysis of autoradiographs after correction by expression of the cyclophilin gene used as control. The combination of RA plus T_3 did not change the response to RA. The presence of cycloheximide in the culture medium blocked the effect of RA, suggesting an indirect effect through the synthesis of other RA-induced proteins. In order to clarify conclusively whether the unresponsiveness to thyroid hormone of the β_1-AR gene in C6 cells is the consequence of the very low expression of T_3 receptors and high expression of the c-*erbA*α_2 isoform, as suggested (6), we analyzed the pattern of expression of endogenous TR/c-*erbA* by Northern blotting. Results obtained were analogous to those reported previously: higher expression of c-*erbA*α_2 RNA (2.6 kb) than of TR/c-*erbA*α_1 RNA (5.0 kb) (Fig. 2). We then decided to express in these cells an exogenous (chicken) TR/c-*erbA*α_1 cDNA and study the effect of T_3 administration. For comparison, the mutant viral v-*erbA* gene, whose product is unable to bind hormone, was also expressed in parallel. Expression of both genes in C6 cells was carried out by using retroviral vectors available in our laboratory (see Materials and methods). First, their respective expression in infected cultures after G418 selection was analyzed. Figure 2 clearly shows the high levels of the respective retroviral mRNAs detected using the hormone-binding domain of the

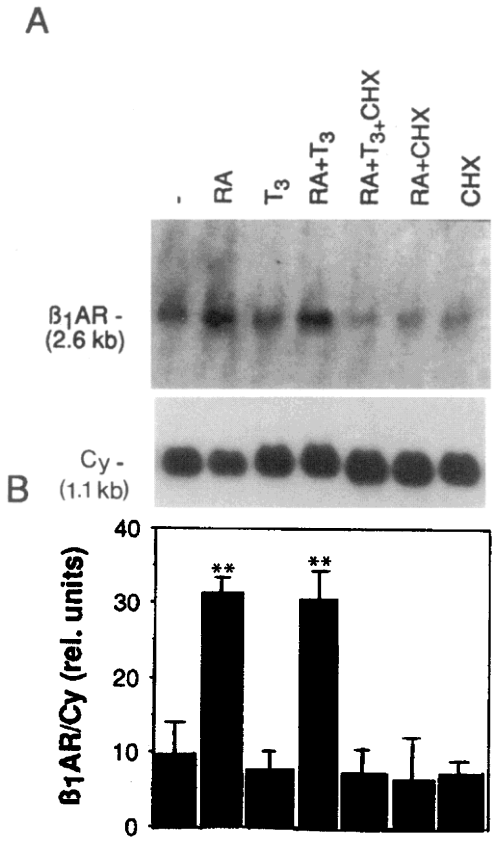

Fig. 1. Retinoic acid, but not T_3, increases β_1-adrenergic receptor mRNA levels in glioma C6 cells. (A) Poly(A)$^+$ RNA (10 μg) from C6 cells treated for 6 h with 10^{-6} mol/l retinoic acid (RA), 25×10^{-9} mol/l T_3 or both together in the presence or absence of 8 μg/ml cycloheximide (CHX) were sequentially hybridized with β_1-adrenergic receptor (β_1-AR) and cyclophilin (Cy) probes. The autoradiograms were exposed 18 h (β_1-AR) and 6 h (Cy), respectively. The size of the β_1-AR mRNA is indicated. (B) Densitometric determinations of the ratio of expression of β_1-AR mRNA versus Cy mRNA obtained as described in the text. Results are the mean ± SD of four sets of data.

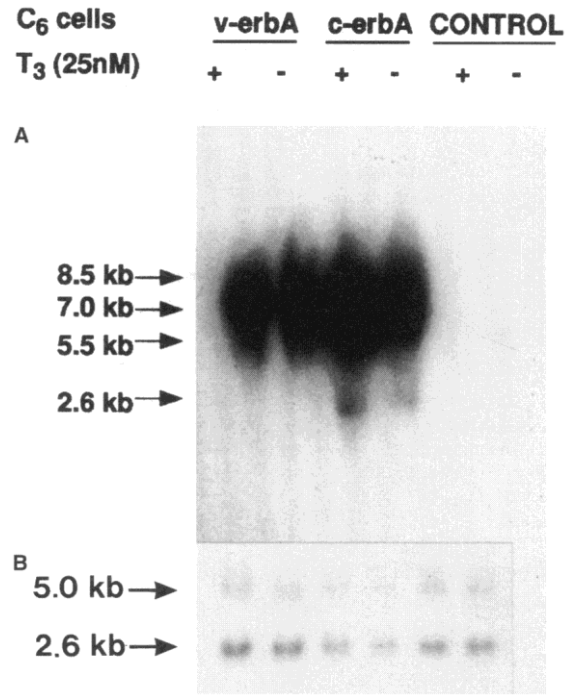

Fig. 2. Northern analysis of exogenous (chicken) TR/c-*erbA*α_1 or v-*erbA* gene expression in C6 cells. Normal C6 cells were infected with recombinant retroviruses encoding the (chicken) TR/c-*erbA*α_1 or the v-*erbA* genes (see text); 10 μg of poly(A)$^+$ RNA from C6 cells infected or uninfected, and treated or not with 25×10^{-9} mol/l T_3 for 2 days, were hybridized first with a specific probe corresponding to the hormone binding domain of the chicken TR/c-*erbA*α_1 cDNA (A) and then with a rat-specific probe corresponding to the same domain (B). The autoradiograms were exposed for 5 days.

chicken TR/c-*erbA*α_1 cDNA as probe, which recognizes all four mRNA species: chicken and rat TR/c-*erbA*α_1, rat c-*erbA*α_2 and v-*erbA* (14) (see also this ref. for a detailed description of the expected genomic and subgenomic retroviral RNAs). This result confirmed that the infection with the recombinant retroviruses was successful, and that each cell type expressed the expected *erbA*-encoding mRNAs. Next, expression of functional thyroid receptors was assessed by binding assays using [^{125}I]T_3. The C6 cells expressing TR/c-*erbA*α_1 showed fivefold higher levels of TRs (17,918 cpm bound [^{125}I]T_3) than normal or v-*erbA*-expressing cells (3660 and 4492 cpm bound [^{125}I]T_3 respectively).

The susceptibility of the generated C6 cells types to β_1-AR mRNA induction by T_3 was studied by Northern analysis. Results shown in Fig. 3 indicate that thyroid hormone failed to increase β_1-AR mRNA levels in cells expressing TR/c-*erbA*α_1 as well as in uninfected or v-*erbA*-expressing cells. This result indicates that the low level of endogenous TR is not the reason for the T_3 unresponsiveness of C6 cells. Figure 3 also shows that RA induced a similar increase (around threefold, as measured by densitometric scanning and correcting by cyclophilin expression) in β_1-AR mRNA levels in both TR/c-*erbA*α_1- and v-*erbA*-expressing cells than that observed in control C6 cells.

To prove that the exogenously expressed TRc-*erbA*α_1 gene in C6 cells was biologically active and to discard any possible aberrant character or uncharacterized mutation of the C6 cells that might make them totally T_3-resistant, we tested the hormonal inducibility of the mitochondrial hydroxymethylglutaryl CoA synthase (HMGCoA synthase) gene, which is T_3-inducible in rat liver (21). As seen in Fig. 4, TR/c-*erbA*α_1 C6 cells but not uninfected or v-*erbA* cells responded to thyroid hormone, increasing the HMGCoA synthase RNA levels in a statistically significant fashion ($p < 0.05$).

Retinoic acid increases β_1-AR mRNA levels by a post-transcriptional mechanism

In view of the upregulation of β_1-AR mRNA levels following RA addition, we investigated whether this

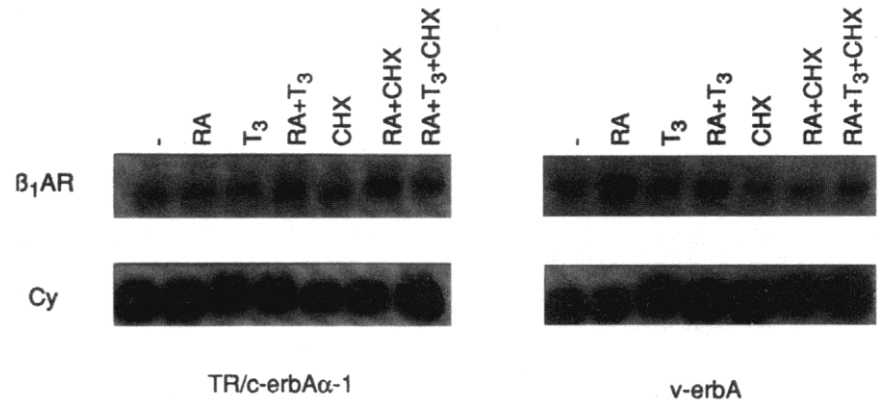

Fig. 3. β_1-Adrenergic receptor induction by retinoic acid (RA) and T_3 in TR/c-*erbA*α_1- or v-*erbA*-expressing C6 cells. Poly(A)$^+$ RNA (10 μg) from C6 cells untreated or treated for 6 h with 10^{-6} mol/l RA or 25×10^{-9} mol/l T_3 were sequentially hybridized with the β_1-adrenergic receptor (β_1-AR) and cyclophilin (Cy) probes. The autoradiograms were exposed for 18 h for β_1-AR and 6 h for Cy. Results are the mean ± SD of three sets of data.

could be the result of an effect on the transcription rate of the gene. To test this possibility, run-on assays were performed using nuclei from untreated cells or cells treated with RA for 30 or 120 min. Retinoic acid β receptor (RAR-β) gene was used as a positive control of RA regulation, and cyclophilin as an unchanged gene. As shown in Fig. 5, the effect of RA on RAR-β transcription was significant, with $p < 0.01$ (asterisks) both at 30 min and at 120 min after addition to the culture medium. In contrast, no changes in transcription were observed with the β_1-AR probe. Together with the cycloheximide sensitivity data, this finding suggests that the observed effect of RA can be ascribed to a post-transcriptional stabilization of β_1-AR mRNAs.

β_1-Adrenergic receptors in RA-treated C6 cells

To investigate whether the observed increase in β_1-AR mRNA induced by RA correlates with a higher number of β_1-ARs, radioligand binding studies were performed. β_1-Adrenergic receptor protein expression was assessed by using the lipophylic antagonist

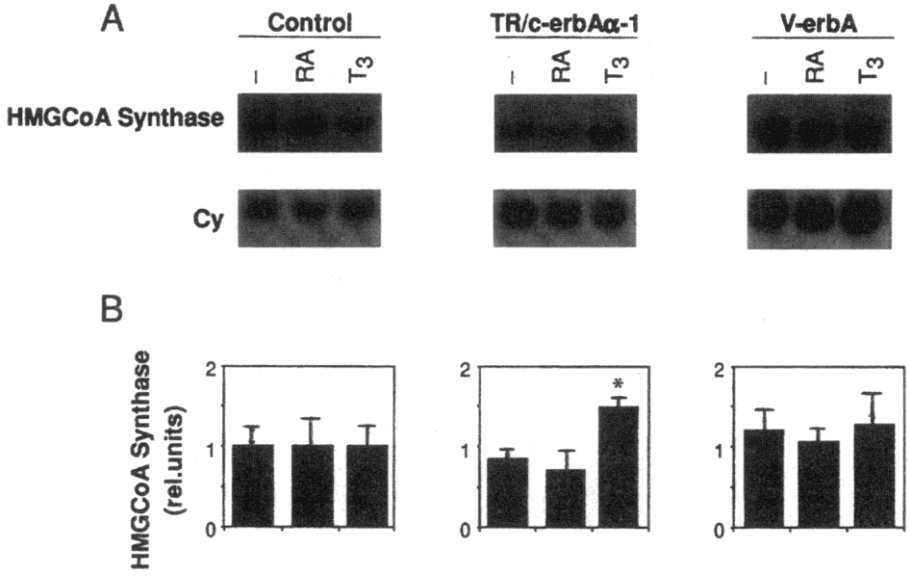

Fig. 4. Thyroid hormone induction of the mitochondrial hydroxymethylglutaryl CoA (HMGCoA) synthase gene in normal or infected C6 cells. (A) Poly(A)$^+$ RNA (10 μg) from uninfected, TR/c-*erbA*α_1 or v-*erbA* expressing C6 cells untreated or treated for 6 h with 10^{-6} mol/l retinoic acid (RA) or 25×10^{-9} mol/l T_3 or both were sequentially hybridized with HMGCoA synthase and cyclophilin (Cy) probes. The autoradiograms were exposed for 48 h for the HMGCoA synthase and 6 h for Cy. (B) Densitometric determinations of the ratio of expression of HMGCoA synthase mRNA versus Cy mRNA. Results are the mean ± SD of three sets of data. The effect of T_3 on HMGCoA synthase was significant (*$p < 0.05$) only in TR/c-*erbA*α_1 cells.

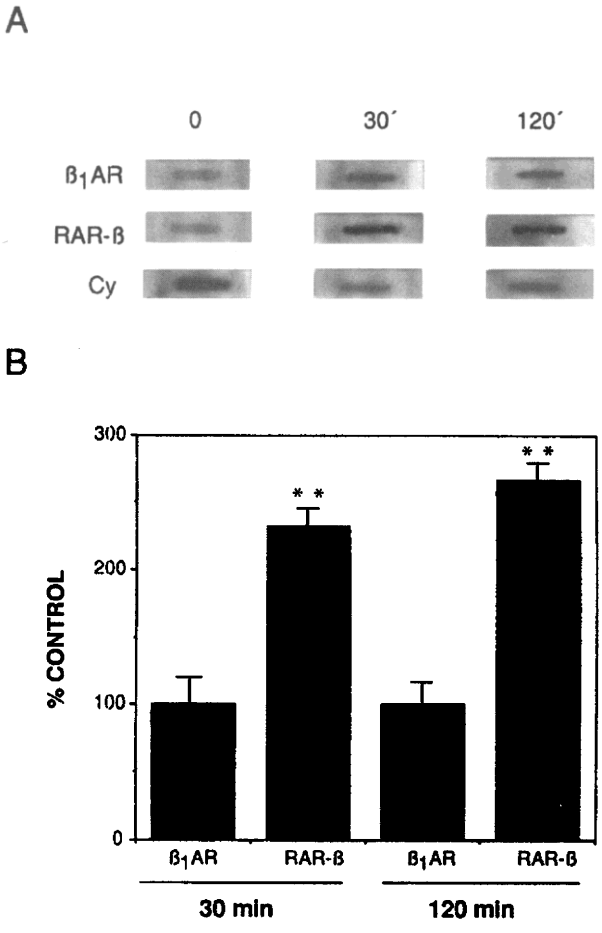

Fig. 5. Transcription rate of the β_1-adrenergic receptor (β_1-AR) gene in C6 cells. (A) Twenty million nuclei from C6 cells treated or not with 10^{-6} mol/l retinoic acid (RA) for 30 or 120 min were used to perform nuclear run-on assays. The probes were 5 μg of linearized plasmid DNA of β_1-AR gene, retinoic acid β receptor (RAR-β) gene as an RA-induced gene and cyclophilin (Cy) as control gene. The autoradiograms were exposed for 12 days. (B) Densitometric scanning was performed as described in the text for Northern analysis. Determinations of the ratio of transcription of β_1-AR versus Cy and RAR-β versus Cy are shown. Results are the mean ± SD of three sets of data. **Significant differences versus untreated samples, $p < 0.01$.

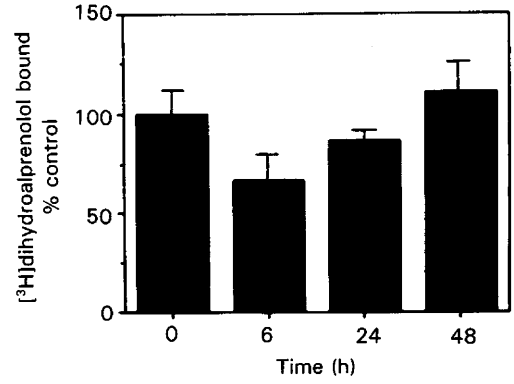

Fig. 6. Effect of retinoic acid (RA) treatment on β-adrenergic receptor number in C6 glioma cells. Cells were incubated with 1 μmol/l RA for the indicated periods of time, and whole-cell β_1-adrenergic receptor number was determined by radioligand binding using [^{3}H]dihydroalprenolol as described in the text. The figure shows the mean ± SD of three independent experiments performed in quadruplicate. The number of β-adrenergic receptors in control, untreated cells (165 ± 19 fmol/10^6, mean = ± SEM of three sets of data) was taken as 100%. No statistically significant effect could be demonstrated at any time after RA addition.

[^{3}H]dihydroalprenolol; no statistically significant increase in the total number of cellular receptors was found in RA-treated cells, and even a slight decrease was observed at short times of treatment (Fig. 6). These results indicate that the regulation of β_1-AR expression is a complex process that takes place at both mRNA and protein levels.

Discussion

The expression of relatively high levels of β_1-ARs in neurons and glia is well documented. Despite the importance of adrenergic function, very little is known about the agents controlling the expression of these receptors. An influence of the thyroid status on the number of different receptors, including β-adrenergic (1–3), α-adrenergic (22) and other receptors (23), in the central nervous system (CNS) has been reported. Hypothyroidism induced by propylthiouracil (PTU) treatment causes a reduced expression of β-ARs in the developing rat brain (24). Correspondingly, primary cultures of astrocytes from hypothyroid animals display a similar reduction in receptors (24). Another recent study suggests a permissive effect of thyroid hormone because PTU treatment resulted in a decrease in the number of β-receptor binding sites but, however, no increase was observed following T_3 administration (25). The results described in this paper show that thyroid hormone has no effect on the expression of β-ARs in C6 cells. This discrepancy may be due to an intrinsic T_3 unresponsiveness of the C6 cells used, or to the requirement of the concerted action of other factors. Supporting the hypothesis of a cell-type specific effect, thyroid hormone has been shown to upregulate the β_1-AR gene at the transcriptional level in cultured cardiac myocytes. In contrast, in the same study thyroid hormone had no effect on C6 cells (6). It was suggested that the high expression of the c-*erbA*α_2 isoform could interfere with the action of low levels of active TRs in these cells. However, we show here that over-expression of TR/c-*erbA*α_1 by retrovirally mediated gene transfer, which makes these cells sensitive to T_3, has no effect on the response of β-ARs. On the other hand, the use of a long-term cultured transformed cell line such as C6 may contribute to the differences observed. In line with this, several groups have described differences in the response of C6 cells to β-adrenergic activation, depending on the passage (26).

Together, these results indicate that much care should be taken when extrapolating the data obtained in C6, and probably other cell lines, to the in vivo situation, and that C6 cells may not be an appropriate system in which to study thyroid hormone effects in the brain.

On the other hand, our results indirectly demonstrate that RA has a probable post-transcriptional effect on the β_1-AR gene, leading to an increased accumulation of its mRNA. In agreement with the possibility of post-transcriptional regulation by RA, we do not detect changes in the transcriptional rate of the β_1-AR gene in run-on assays, and the presence of neither high levels of TR/c-*erbA*α_1 nor of v-*erbA* protein are able to block the increase in β_1-AR mRNA. These proteins have been described to inhibit transcriptional effects of RA in other cell systems (27, 28). As shown in Fig. 3, cycloheximide does not block RA induction of β_1-AR mRNA in TR/c-*erbA*α_1-expressing cells. Expression of TR/c-*erbA*α_1 might regulate, recruit or stabilize a protein(s) required for RA action, which could be a co-activator or perhaps the RAR protein. Alternatively, it may substitute in the nucleus for a short-lived, cycloheximide sensitivity protein required for RA action. The lack of a transcriptional effect by RA is further supported by run-on experiments. As in the case of the PLP gene in the same C6 cells (9) and of other genes in different cell lines (29–31), RA may affect β_1-AR mRNA stability, affecting its half-life. Recent data suggest that the stability and turn-over of the β_1-AR mRNA is a highly regulated process, and that different agents, including protein kinase A, insulin or dexamethasone, may modulate it (32). It is tempting to speculate that RA may interact at this level with other extracellular messengers in order to modulate β_1-AR expression. Interestingly, a novel action of RA has been described recently (33). Retinoic acid can regulate gene expression by a mechanism independent of the transcriptional rate or the stabilization of cytoplasmic mRNAs. Thus, RA regulates alkaline phosphatase expression by stabilizing the nascent RNA chains in the nucleus (33). The regulation of RNA processing at the level of this very early post-transcriptional step is another possible mechanism of RA action on the β_1-AR gene in C6 cells.

The lack of correlation between the increase in mRNA levels and the number of total active receptors indicates a complex regulation. Translational or post-translational regulation may contribute to receptor function. Muscle nicotinic acetylcholine receptor number (nAChR) is regulated at both levels (34, 35). Similarly to our observations, recent data show that alterations in nAChR mRNA levels induced by NGF in PC12 cells are not paralleled by changes at the protein level (36). The elevated receptor protein could also be kept in a non-functional state by modulation of its phosphorylation or stability, or by redistribution of internal pools in the case of an excess of pre-existing receptors. Examples of these types of control have been described elsewhere (37, 38). Testing these possibilities will require additional studies, including the search for other regulatory factors probably not present in C6 cells. An additional example of this phenomenon is the up-regulation by thyroid hormone of the expression of its own TR/c-*erbA*β_1 receptor gene in rat cerebral hemisphere astrocyte cultures (39). Here, again, thyroid hormone specifically increases TR/c-*erbA*β_1 mRNA levels without changing hormone binding capacity.

Based on a series of data and considerations, including its endogenous presence and the expression of different RA binding proteins and receptors, RA has also been proposed to play a crucial role in CNS development and regulation (40, 41). Emphasizing their importance, β – adrenergic receptors have been implicated in the modulation of the synthesis and release of growth factors in glial cells (42, 43). In line with our findings, RA has been reported to increase β_1-AR mRNA and protein in mouse teratocarcinoma F9 cells (9). Whether this represents a real discrepancy with our data on the glial C6 cells due to cell-specific RA effects or simply differences due to culture conditions as discussed above remains to be clarified. Examples of cell-specific gene regulatory action of RA are known: RA induced RAR-β_2 expression in lung and liver and in F9 cells but not in several pituitary cell lines (44, 45).

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