GRK2 and β-Arrestin1 as Negative Regulators of Thyrotropin Receptor-Stimulated Response

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Arrestins are regulatory proteins for a number of G-coupled receptors. The binding of arrestin to receptor phosphorylated by G protein-coupled receptor kinase (GRK) quenches the activation of the G protein, thus resulting in receptor homologous desensitization. We have previously shown that the levels of β-arrestin1 are regulated by intracellular cAMP and proposed that this may represent one homeostatic mechanism with which to regulate some cellular responses. To test this hypothesis, we focused on the TSH receptor using a rat thyroid cell line, FRTL5. We found that β-arrestin1 is the only detectable isoform of arrestin expressed in FRTL5 and that its expression is regulated by TSH. To investigate the possible role of GRK2/β-arrestin1 machinery in the mechanism of TSH receptor homologous desensitization, we used a cotransfection approach. The TSH-induced cAMP accumulation in COS7 cells transfected with TSH receptor was reduced by 35–45% when cotransfected with GRK2 and/or β-arrestin1, indicating that the TSH receptor can be regulated by a GRK/arrestin mechanism. This raised the hypothesis that TSH increases the levels of β-arrestin1, which in turn could regulate the TSH stimulation. To test this point a FRTL5-derived cell line overexpressing β-arrestin1 was generated. In these cells the TSH-stimulated cAMP accumulation and, more importantly, the mitogenic activity were substantially blunted. Our results show that TSH receptor-stimulated cAMP accumulation and cell proliferation can be controlled by a GRK2/β-arrestin1 mechanism. (Molecular Endocrinology 10: 1138–1146, 1996)

INTRODUCTION

The G protein-coupled receptor family includes many receptors that bind a large array of different molecules, ranging from photons, neurotransmitters, and neuropeptides, to autacoid substances, hormones, and immunomodulators acting through different intracellular second messengers. For a number of G protein-coupled receptors a rapid, drastic, and reversible loss of responsiveness has been shown to occur upon exposure to agonists (homologous desensitization). Two types of proteins have been shown to play a major role in determining homologous desensitization: G protein-coupled receptor kinases (GRKs) (Ref. 1 for recent review), which phosphorylate agonist-occupied G protein-coupled receptors, and their functional cofactors arrestins (2, 3). The multigene family of GRKs consists so far of six members named GRK 1 to 6. Three of these kinases are also known as rhodopsin kinase, β-adrenergic receptor kinase (βARK)1, and βARK2 and correspond to GRK1, GRK2, and GRK3, respectively. Among the arrestin/β-arrestin gene family, retinal-arrestin and cone-arrestin are essentially expressed in the retina where they regulate phototransduction, while a wide tissue distribution has been reported for the other two members, β-arrestin1 and β-arrestin2. Arrestins share 90% overall similarity between species although the C terminus and the N terminus have only 45% and 60% of similarity, respectively. We have documented by molecular cloning the existence of two isoforms for both β-arrestin1 (named β-arrestin1A and 1B) and arrestin (named arrestin A and B) generated by a similar process of alternative splicing (4). More recently other splice variants were reported by other laboratories (3).

Both GRK and arrestin can be regulated within the cells. We have previously shown that the gene expression of GRK2 can be up-regulated by chronic activation of protein kinase C (PKC) either via receptor agonist or direct activation by phorbol ester (phorbol-12-myristate-13-acetate) (5). On the other hand, GRK2 can be covalently modified through phosphorylation by acutely activated PKC, resulting in increased GRK2 activity (6). Both ways of increasing cellular GRK2 functionality resulted in expected elevation in potency of receptor homologous desensitization. While activation of PKC up-regulated GRK2, chronic elevation of cAMP increased β-arrestin1 gene expression probably via a protein kinase A (PKA)-mediated mechanism

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(4). As β-arrestin1 functions by a 1:1 stoichiometric mechanism of receptor desensitization, an increase in cellular content of β-arrestin1 would result in more efficient homologous desensitization (7).

In the present investigation we studied the regulation of β-arrestin1 by TSH in a rat thyroid cell line FRTL5 (8). We also investigated whether GRK2 and β-arrestin1 could, in turn, regulate TSH-stimulated cAMP accumulation and cell proliferation. Our results show that TSH receptor-stimulated cAMP accumulation and cell proliferation can be controlled by a GRK2/β-arrestin1 mechanism.

RESULTS

The Expression of β-Arrestin1 in FRTL5 Is Regulated by TSH

The present study sought to investigate the regulation of arrestins in FRTL5 cells and its possible functional implications. FRTL5 is a continuous line of rat thyroid cells whose growth and differentiation are regulated by a mixture of six hormones (6H) (8). Among those, TSH is the most important regulator of these functions, acting through the increase of cAMP levels. First, we determined which isoform of arrestins is expressed in these cells. The monoclonal antibody F4C1 raised against the epitope DGVVLVD (4), which is conserved in all the known mammalian isoforms of arrestins including rat arrestin and β-arrestin1 and 2 (3), was used. Only one band was detected with an apparent M_r of ~50,000, corresponding to β-arrestin1 from transfected COS7, which was used as standard (Fig. 1A). No immunoreactivity for β-arrestin2 was observed in FRTL5. Northern blot analysis using probes derived from human β-arrestin1 and rat β-arrestin2 confirmed these results. A strong mRNA signal for β-arrestin1 was detected, consisting as described previously (4) of two bands of ~7.5 and ~3.0 kb, which are presently interpreted as different products of mRNA processing (4). The 7.5-kb species was the most abundant and was the one considered when comparing different levels of expression. In parallel Northern blot experiments, β-arrestin2 mRNA was barely detectable, if at all (not shown). These results indicate that β-arrestin1 is the only detectable isoform of arrestin in FRTL5, and therefore we investigated the regulation and function of this isoform. GRK2 was also abundantly expressed in FRTL5 as documented by Northern and Western blots (Fig. 1B). The expression of GRK2 mRNA in FRTL5 was compared with that in human mononuclear leukocytes (MNL), which is the preferential site of expression of this kinase (6). While in human MNL there was a major mRNA species of 4 kb in size with a second minor band of 2.4 kb, in FRTL5 the two species were expressed at similar levels (Fig. 2B), as usually observed in rat tissues and cells (M. Sallese and A. De Blasi, unpublished observations).

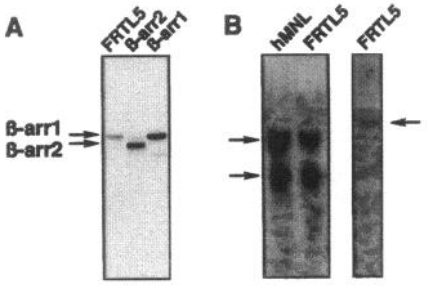

Fig. 1. Expression of β-Arrestin1 and GRK2 in FRTL5

A, β-Arrestin1 is the only isoform of arrestins detectable in FRTL5. Forty micrograms of cytosolic proteins from FRTL5 cells (FRTL5) and COS7 cells transfected with β-arrestin2 (β-arr2) or β-arrestin1 (β-arr1) were prepared, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. β-Arrestin1 and 2 immunoreactivity was detected by the F4C1 monoclonal antibody, followed by alkaline phosphatase-coupled second antibody. Data represent three separate experiments. The positions of β-arrestin1 and β-arrestin2 bands are indicated by *arrows*. B, Expression of GRK2 in FRTL5. On the *left*, blots of total mRNA (20 μg) from human MNL (h. MNL) and FRTL5 were hybridized with a probe for GRK2. The two mRNA species are indicated by *arrows*. On the *right*, GRK2 immunoreactivity (*arrow*) in cytosolic proteins (100 μg) from FRTL5.

We studied the mRNA expression of β-arrestin1 in FRTL5 following starvation, to determine whether it is hormone-controlled (Fig. 2). The expression of β-arrestin1 decreased upon starvation from 6 to 24–48 h, at which times the mRNA levels were 40–50% of the control values. Similar changes were observed at the protein level by Western blot (Fig. 2C). This change proved to be selective as the expression of GRK2 mRNA was not affected in the same cells (not shown). When cells starved for 12 h were exposed to TSH (1 nM) for an additional 12 or 36 h, the β-arrestin1 mRNA expression was increased to 4.4- and 3.0-fold the parallel control values, respectively (Fig. 3). A similar increase (3.8-fold) was documented at the protein levels by Western blot analysis (Fig. 3). Exposure of starved cells to complete 6H medium increased the β-arrestin1 mRNA (not shown) and protein (Fig. 3) expression to the same extent as TSH treatment. The mRNA levels of β-arrestin1 were decreased when FRTL5 were cultured in their complete growth medium (which includes 5% serum) lacking TSH only (Fig. 4). This effect was similar to the effect of starvation (Fig. 4), supporting the idea that β-arrestin1 expression is regulated by TSH.

Regulation of TSH-Stimulated cAMP Response by GRK/β-Arrestin-Desensitizing Mechanism

Binding of TSH to its own receptor reduces the cAMP response to a subsequent stimulation with the hormone (9). This rapid and agonist-dependent regulation is similar to the phenomenon of homologous desensitization observed for several G-coupled receptors. The molecular mechanisms of TSH receptor desensi-

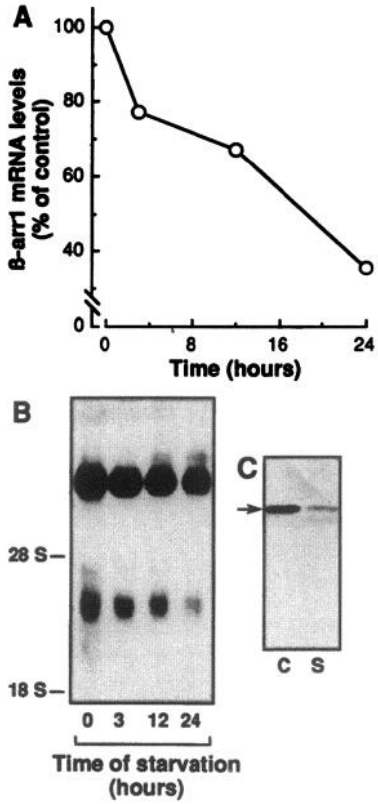

Fig. 2. The Expression of β-Arrestin1 in FRTL5 Cells Is Decreased Following Starvation

FRTL5 cells (70% confluent) were washed twice with HBSS and kept in the starvation medium for the indicated times. At the end of the incubation the cells were lysed in guanidinium isothiocyanate for Northern blot, or cytosolic proteins were prepared for immunoblots. A, Messenger RNA expression, quantified by densitometric analysis of autoradiographic films, is expressed as percentage of the control (time 0). Data are means of two separate experiments. B, Blots of total mRNA (20 μg) were hybridized with a probe for β-arrestin1. Washed filters were exposed at −80 C for 72 h. C, β-Arrestin1 immunoreactivity (*arrow*) in FRTL5 after 24 h of starvation (S) compared with the time 0 control (C).

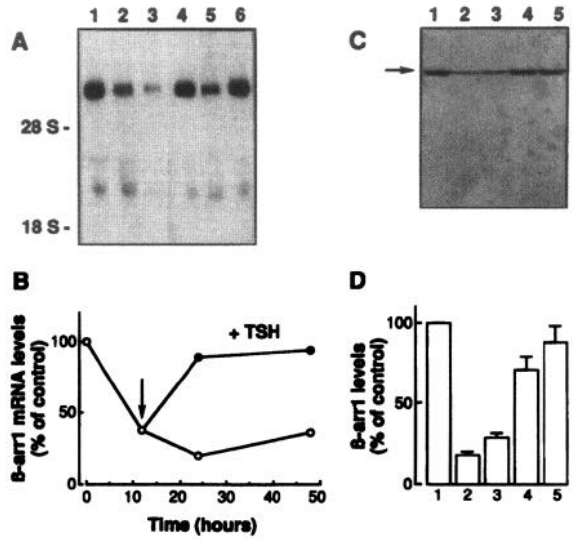

Fig. 3. TSH Restores the Decrease of β-Arrestin1 Expression by Starvation

A, Blots of total mRNA (20 μg) from FRTL5 cells cultured under different conditions were hybridized with a probe for β-arrestin1. FRTL5 cells (70% confluent, time 0) (lane 1) were kept in the starvation medium for 12 h (lane 2) and then exposed for an additional 12 h (lanes 3 and 4) or 36 h (lanes 5 and 6) to the starvation medium or 1 nM TSH, respectively. B, FRTL5 cells (70% confluent, time 0) were kept in the starvation medium for 12 h and then exposed for an additional 12 or 36 h to the starvation medium (*open circles*) or 1 nM TSH (+TSH, *closed circles*). The *arrow* indicates the time when TSH was added. Messenger RNA expression, quantified by densitometric analysis of autoradiographic films, is expressed as a percentage of the control (time 0). Data are means of two separate experiments. C, β-Arrestin1 immunoreactivity (*arrow*) on Western blot using cytosolic proteins (40 μg) from FRTL5 cultured under different conditions. FRTL5 cells, grown until 70% confluent (time 0, lane 1), were kept in the starvation medium for 12 h (lane 2) and then exposed for an additional 12 h to the starvation medium (lane 3), 1 nM TSH (lane 4), or 6H medium (lane 5). D, Densitometric analysis of β-arrestin1 immunoblots as in panel C. Data are the means ± SE of three experiments.

tization, which involves a decreased coupling of the receptor with Gs, are unknown, and a possible role for GRK/arrestin was suggested (10). To test this possibility, we measured the TSH-stimulated cAMP accumulation in COS7 cells transfected with the TSH receptor alone or in combination (cotransfection) with GRK2 and/or β-arrestin1 (Fig. 5). COS7 cells do not express the TSH receptor (our data not shown and Ref. 11). When transfected with the rat TSH receptor cDNA, these cells showed a TSH-dependent increase of intracellular cAMP (up to 13-fold the basal levels) indicating that the receptor is expressed and properly coupled to Gs and adenylyl cyclase (Fig. 5). In cells cotransfected with cDNA of GRK2 and/or β-arrestin1, the TSH-stimulated cAMP accumulation was significantly reduced by 26–35% and 35–46% after 15 min and 30 min of TSH (10 nM), respectively. The cAMP response was reduced also after 10 min of TSH treatment, but this effect reached statistical significance ($P < 0.05$) only for cells cotransfected with both GRK2 and β-arrestin1 (Fig. 5A). Forskolin-stimulated (30 min) cAMP accumulation was not significantly different between COS7 transfected with TSH receptor alone (17.8 ± 1.8 pmol/well) and cells cotransfected with GRK2 (14.5 ± 2.2), β-arrestin1 (19.9 ± 3.2) and GRK2 plus β-arrestin1 (13.9 ± 0.7). The level of expression of TSH receptor was the same in COS7 transfected with TSH receptor cDNA or cotransfected with β-arrestin1 and/or GRK2, as documented by immunoblot (Fig. 5B). The overexpression of GRK2 and β-arrestin1 was confirmed by immunoblot using specific antibodies (Fig. 5B). These blots also documented the presence of endogenous GRK2 and β-arrestin1 in COS7 cells not transfected with these genes.

The use of an alternative protocol of homologous desensitization further confirmed these results. In

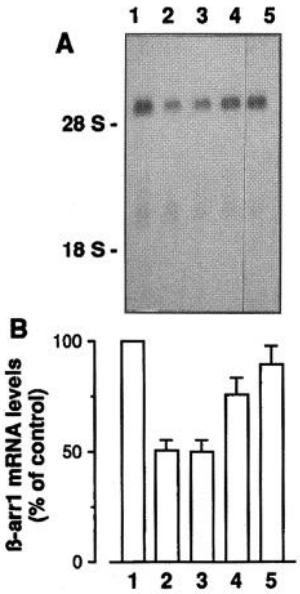

Fig. 4. The Expression of β-Arrestin1 in FRTL5 Cells Is Regulated by TSH

A, Blots of total mRNA (20 μg) from FRTL5 cells cultured under different conditions were hybridized with a probe for β-arrestin1. FRTL5 cells (70% confluent, lane 1) were exposed for 24 h to starvation medium (lane 2), complete growth medium + serum lacking TSH (5H, lane 3), 6H medium (lane 4), and 1 nM TSH in starvation medium (lane 5). B, Messenger RNA expression, quantified by densitometric analysis of autoradiographic films, is expressed as a percentage of the control (time 0, column 1). Treatments are as in panel A. Data are means ± SE of three separate experiments.

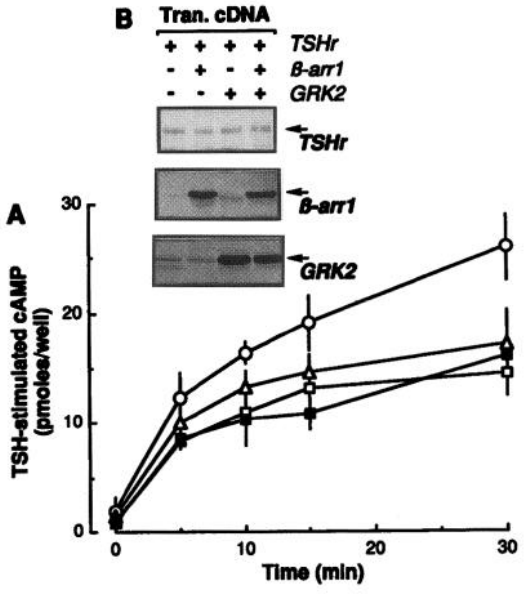

Fig. 5. TSH-Stimulated cAMP Accumulation Is Reduced by GRK2 and/or β-Arrestin1 Overexpression

A, Time course of cAMP accumulation stimulated by 10 nM TSH in COS7 cells transiently transfected with the TSH receptor alone (*circles*) or cotransfected with β-arrestin1 (*triangles*), GRK2 (*open squares*), and the two together (*closed squares*). Data are means ± SEM of three separate experiments each performed in duplicate. For β-arrestin1-cotransfected cells the cAMP accumulation was significantly reduced at 15 min ($P < 0.05$) and 30 min ($P < 0.05$) compared with cells transfected with TSH receptor alone; for GRK2-cotransfected cells the reduction was statistically significant at 15 min ($P < 0.01$) and 30 min ($P < 0.01$); for cells cotransfected with β-arrestin1 plus GRK2 the reduction was statistically significant at 10 min ($P < 0.05$), 15 min ($P < 0.01$), and 30 min ($P < 0.05$). B, TSH receptor (TSHr), β-arrestin1 (β-arr1), and GRK2 immunoreactivity (*arrows*) on Western blot from COS7 cells transfected with TSH receptor (TSHr) and cotransfected as indicated. Tran. cDNA, Transfected cDNA.

COS7 cells transfected with TSH receptor alone, the TSH-induced cAMP accumulation was reduced to 86% and 80% of control values after preexposure to 10 nM TSH for 5 and 10 min, respectively. This indicates the occurrence of homologous desensitization. The desensitization was enhanced by coexpression of TSH receptor plus GRK2 and/or β-arrestin1. After preexposure to TSH for 5 or 10 min, the subsequent TSH-induced cAMP accumulation was reduced, respectively, to 65% and 56% in COS7 cotransfected with β-arrestin1, to 59% and 49% in COS7 cotransfected with GRK2, and to 66% and 49% in COS7 cotransfected with β-arrestin1 plus GRK2 (n = 2).

Regulation of TSH-Stimulated cAMP Accumulation and Proliferative Response in FRTL5 Overexpressing β-Arrestin1

The experiments described so far document that the levels of β-arrestin1 in FRTL5 cells are regulated by TSH and that a GRK/arrestin mechanism can regulate the TSH receptor. This raised the hypothesis that TSH increases the levels of β-arrestin1, which, in turn, could regulate the TSH-induced intracellular responses including cAMP stimulation and cell proliferation. To test this hypothesis, we have generated FRTL5-derived cell lines, which were permanently transfected with β-arrestin1 (L5β-arr). FRTL5 transfected in parallel with the empty vector (L5pBJI) were used as control. The overexpression of β-arrestin1 was documented by immunoblot (Fig. 6A). Northern blot analysis of transfected cells showed that the bands at ~7.5 and ~3.0 kb, corresponding to the endogenous β-arrestin1 mRNA, were similar in L5pBJI and L5β-arr. An abundant new species of specific mRNA, generated by tranfection, was observed in L5β-arr (Fig. 6B). The mRNA levels of TSH receptor were comparable in L5β-arr and in control cells, as demonstrated by Northern blot analysis (Fig. 6B). In L5β-arr the TSH-stimulated cAMP accumulation was reduced by 38–42% as compared with L5pBJI (Fig. 7). Forskolin-stimulated cAMP accumulation was similar in L5pBJI and L5β-arr. After exposure to 10 μM forskolin for 30 min, intracellular cAMP levels were 6.7 and 10.1 pmol/well for L5pBJI and L5β-arr, respectively, and after 100 μM forskolin were 20 and 17 pmol/well for L5pBJI and L5β-arr, respectively. As an alternative

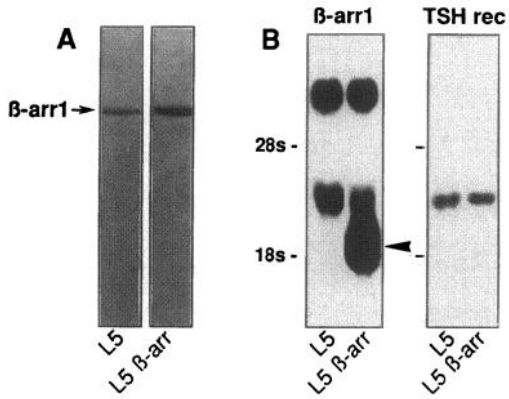

Fig. 6. Characterization of a FRTL5 Cell Line Permanently Overexpressing β-Arrestin1

A, β-Arrestin1 immunoreactivity (*arrow*) on Western blot using cytosolic proteins (40 μg) from L5pBJI (L5) and L5β-arr. B, Blots of total mRNA (20 μg) from L5pBJI (L5) and L5β-arr (L5 β-arr) were hybridized with a probe for β-arrestin1 (β-arr1) or for the TSH receptor (TSH rec). The new species of β-arrestin1 mRNA generated by the transfection is indicated by the *arrow*.

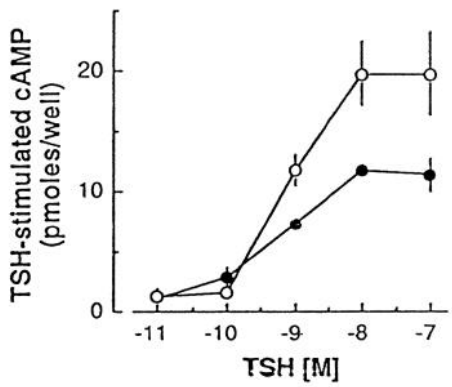

Fig. 7. TSH-Stimulated cAMP Accumulation Is Reduced in L5β-arr

L5pBJI (*open circles*) and L5β-arr (*closed circles*) were incubated for 30 min with the indicated concentrations of TSH. Data are means ± SEM of three separate experiments each performed in duplicate. The cAMP accumulation induced by 1, 10, and 100 nM TSH was significantly reduced in L5β-arr ($P < 0.01$).

protocol of homologous desensitization, cells were preexposed to TSH and extensively washed, and then TSH-induced cAMP accumulation was measured. After preexposure to TSH (10 nM for 15 min), subsequent TSH-induced cAMP accumulation was reduced by 25% in FRTL5 and by 40% in L5β-arr, indicating an enhanced homologous desensitization in cells overexpressing β-arrestin1 (n = 2).

[^{3}H]Thymidine uptake experiments were used to study the cell proliferation in clones overexpressing β-arrestin1. A dramatic reduction of mitogenic response induced by different doses of TSH was found in L5β-arr as compared with L5pBJI (Fig. 8).

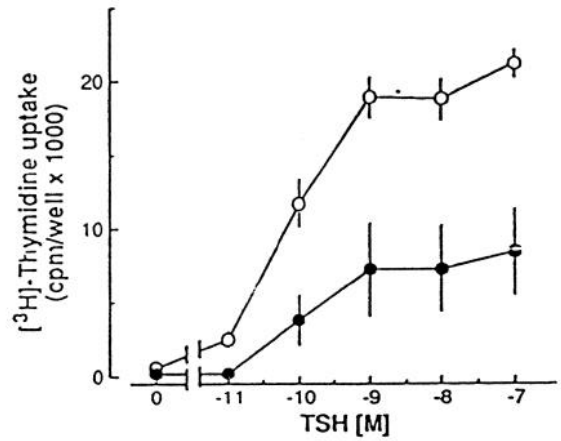

Fig. 8. TSH-Stimulated [^{3}H]Thymidine Incorporation Is Reduced in L5β-arr

L5pBJI (*open circles*) and L5β-arr (*closed circles*) were exposed to the indicated concentrations of TSH, and [^{3}H]thymidine incorporation was measured. Data are means ± SEM of six separate experiments each performed in triplicate. The [^{3}H]thymidine incorporation was significantly reduced for all the doses of TSH in L5β-arr compared with L5pBJI ($P < 0.01$).

The experiments reported so far were performed in FRTL5 overexpressing the long isoform of β-arrestin1 (β-arrestin1A) (4). Since an additional splice variant of β-arrestin1 (named β-arrestin1B), which differed from β-arrestin1A in that it lacked an 8-amino acid sequence near the C-terminus, was recently identified in our laboratory, a FRTL5 cell line was generated permanently transfected with this isoform (L5β-arrB). Overexpression of β-arrestin1B in these cells was confirmed by Western and Northern blot analysis (not shown). In parallel experiments, cAMP accumulation stimulated by 10 nM TSH was significantly reduced in L5β-arr (8.52 ± 0.64 pmol/well, $P < 0.001$) and L5β-arrB (10.6 ± 0.42, $P < 0.05$) as compared with L5pBJI (13.0 ± 0.64, n = 6). Similar to what was observed in L5β-arr, the proliferative response stimulated by different doses of TSH, as measured in [^{3}H]thymidine uptake experiments, was drastically reduced also in cells overexpressing β-arrestin1B (Fig. 9). [^{3}H]Thymidine uptake basal levels were similar in L5pBJI (392 ± 35 cpm/well, n = 18), L5β-arr (355 ± 38, n = 18), and L5β-arrB (292 ± 32, n = 15).

All the results with permanently transfected FRTL5 cells were confirmed using at least two independent clones for each group.

DISCUSSION

Arrestins are regulatory proteins that control the second messenger generation stimulated by a number of G-coupled receptors. The binding of arrestin to receptor phosphorylated by GRK quenches the activation of the G protein, thus resulting in receptor-homologous

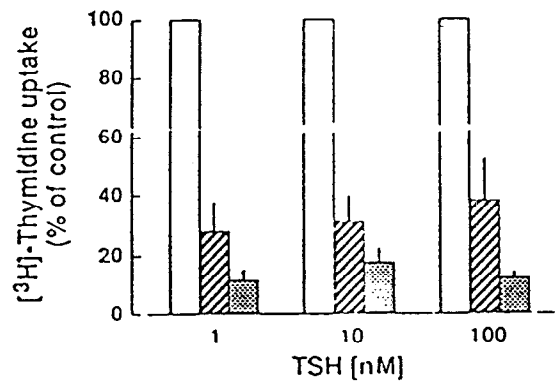

Fig. 9. Similar Effect of Two β-Arrestin1 Isoforms Overexpression on TSH-Stimulated [^{3}H]Thymidine Incorporation

L5pBJI (*open bars*) L5β-arr (*hatched bars*), and L5β-arrB (*stippled bars*) were exposed to the indicated concentrations of TSH, and [^{3}H]thymidine incorporation was measured. Data are means ± SEM of three separate experiments each performed in triplicate. The [^{3}H]thymidine incorporation was significantly reduced for all the doses of TSH in L5β-arr and L5β-arrB compared with L5pBJI ($P < 0.001$). Differences between L5β-arr and L5β-arrB were not statistically significant.

desensitization (2, 3). We have previously shown that the levels of β-arrestin1 are regulated by intracellular cAMP and proposed that this may represent one homeostatic mechanism by which to regulate cellular response to G-coupled receptor-mediated stimulation (4). To test this hypothesis, among the G-coupled receptors, we focused on the TSH receptor using as a cellular model a continuous line of rat thyroid cells, FRTL5, in which, as in normal thyroid gland, growth and differentiation are regulated by TSH through the increase of intracellular cAMP (8). Using this approach we documented a reciprocal relationship between TSH-induced cellular response and β-arrestin1: sustained exposure of FRTL5 to TSH increased the expression of β-arrestin1 and, in turn, elevated levels of β-arrestin1 attenuated the TSH-induced cAMP accumulation and mitogenic response.

β-Arrestin1 is the only detectable isoform of arrestins expressed in FRTL5, as demonstrated using antibodies raised against an epitope common to all the mammalian arrestins identified so far. In these cells, the level of β-arrestin1 mRNA and protein is regulated by TSH with a time course similar to the increase of β-arrestin1 previously observed in human endothelial cell and smooth muscle cells in which intracellular cAMP was raised by activation of receptor (by iloprost), Gs (by cholera toxin), or adenylyl cyclase (by forskolin) (4). Since okadaic acid, a potent and specific inhibitor of phosphatases 1 and 2A, substantially potentiated the effect of cAMP, it was suggested that this effect could be mediated by PKA (4). In an attempt to obtain more direct evidence for a possible role of PKA on β-arrestin1 expression on FRTL5, we used the specific cell permeant inhibitor of PKA H89. Unfortunately, this treatment had dramatic effects on FRTL5 shape and proliferation, indicating that this agent at the doses used (1–3 μM) was toxic for FRTL5. The use of such PKA inhibitors on alternative cell models will be considered for further studies.

Previous studies have shown that the TSH receptor is desensitized in a rapid and agonist-dependent manner (homologous desensitization) (9). The molecular mechanism of this process, which includes uncoupling of the receptor from Gs, has not been determined. To investigate a possible role of GRK2/β-arrestin1 machinery in the mechanism of TSH receptor-homologous desensitization, we measured the TSH-stimulated intracellular cAMP in COS7 cells transfected with the TSH receptor alone or cotransfected with GRK2 and/or β-arrestin1. If the receptor is under the control of these regulatory proteins, the stimulation of intracellular second messengers is expected to be quenched in cells cotransfected with GRK2 and/or β-arrestin1. The cotransfection approach has been previously used to demonstrate a regulatory role of GRK and/or arrestin for several receptor systems including β1-adrenergic (12), α1-adrenergic (13), and D1A-dopamine receptors (14). The TSH-induced cAMP accumulation in cells transfected with TSH receptor was reduced by 35–45% when cotransfected with GRK2 and/or β-arrestin1 cDNAs. This observation strongly indicates that the TSH receptor can be regulated by the GRK2/β-arrestin1 system and provides the first evidence that GRK2 and β-arrestin1 are involved in the process of TSH-receptor homologous desensitization. The use of an alternative protocol of homologous desensitization (*i.e.* pretreatment of transfected cells with TSH and rechallenge with the same hormone after extensive washing) further confirmed this idea. The possibility of the TSH receptor to be regulated by GRK/arrestin is supported by the analysis of the amino acid sequence of the TSH receptor (11) showing that the long cytosolic tail of this receptor contains a number of serines and threonines that could be phosphorylation substrates for GRKs.

To investigate the possible functional effect of the reciprocal regulation between TSH receptor and β-arrestin, FRTL5 cells were stably transfected with β-arrestin1, generating cell lines (L5β-arr) that constantly express elevated levels of this protein. The TSH-stimulated cAMP accumulation in L5β-arr was significantly reduced as compared with FRTL5 transfected with the empty vector and, more importantly, the mitogenic activity stimulated by TSH was substantially blunted in FRTL5 overexpressing β-arrestin1. Similar results were obtained with both splice variants of β-arrestin (A and B) previously identified in our laboratory (4). This represents the first indication that both splice variants of β-arrestin1 are functionally active.

From our data, the transduction pathway triggered by TSH receptor stimulation and leading to cell proliferation appears to be regulated by a homeostatic mechanism between TSH stimulation and β-arrestin1 activity in thyroid: TSH, which induces differentiation

and mitogenic response, also increases the expression of β-arrestin1, which in turn controls cellular responsiveness to TSH itself. Other mechanisms controlling TSH stimulation of thyroid cells are documented. For example, prolonged exposure to TSH induces down-regulation of the TSH receptor by decreasing its mRNA levels. This mechanism, which involves cAMP and inducible cAMP early repressor, has been recently elucidated (10).

Every protein able to activate the TSH-stimulated transduction cascade could acquire oncogenic properties, if permanently activated by somatic mutations or by other genetic alterations (15). Point mutations resulting in constitutive activation of the TSH receptor have been found in a number of hyperfunctional thyroid adenomas (16). Another study reported that 25% of hyperfunctioning adenomas were bearing activating mutations of Gsα (15). Conversely, the tumorigenic phenotype could also appear due to loss of function of proteins that control the proliferative response. While the oncogenic mutations are often dominant, the tumor suppressor genes act in a recessive manner, and the genes of both alleles must be lost in order for the tumor to grow. This is the case in thyroid anaplastic carcinomas, in which a high prevalence of inactivating point mutations of p53 has been described. The appearance of the oncogenic phenotype could be linked to somatic chromosome deletions that cause the loss of regulatory proteins, mapping in the deleted region. The deletion of some regions of the 11q13 chromosome has been specifically observed in thyroid follicular adenomas but not in papillary carcinomas, suggesting the presence of tumor suppressor genes in that region that have not yet been identified (17). Importantly, we have shown that the two genes coding for GRK2 and β-arrestin1 both map to 11q13 (18). Thus this finding and the present study, demonstrating that the TSH-induced proliferative response is regulated by GRK2/β-arrestin1 mechanism, raise the possibility for these genes to be oncosuppressors in certain thyroid tumors.

While this manuscript was under revision, a paper was published describing the involvement of GRK5 in homologous desensitization of TSH receptor (19). The main conclusion of this paper is that TSH receptor is regulated by a GRK-mediated mechanism, and this is in agreement with our findings. These workers showed that TSH receptor-mediated cAMP accumulation is reduced following overexpression of GRK5 or GRK6. Using an RT-PCR-based approach, they also suggested that GRK5 is the predominant GRK expressed in thyroid and failed to observe any expression of GRK2 and GRK3 (19). In the present investigation we documented the expression of GRK2 in the rat thyroid FRTL5 cell line by both Northern and Western blot (Fig. 1B). The mRNA expression was quite substantial, as compared with human MNL, which represent the preferential site of expression of this kinase (6). We believe that the different approach used (RT-PCR *vs.* Northern blot) to determine mRNA expression can account for these apparently divergent results.

MATERIALS AND METHODS

Cell Culture and Treatments

FRTL5 cells were cultured as previously described (20). Briefly, FRTL5 cells were maintained in Coon's modified F12 medium supplemented with 5% calf serum, 20 mM glutamine, and a mixture of six hormones (TSH, insulin, transferrin, somatostatin, cortisol, and glycyl-L-hystidyl-L-lysine acetate) (6H medium). COS7 were grown in DMEM supplemented with 10% FCS. The cells were grown at 37 C in a humified atmosphere of 5% CO_2 and 95% air. To study the effect of TSH on the expression of GRK2 and β-arrestin1, FRTL5 cells, grown in 100-mm petri dishes until 70% confluent, were washed twice with HBSS and kept in the starvation medium (Coon's modified F12 plus 0.3% BSA and 20 mM glutamine) for 12 h and then exposed to starvation medium, 6H medium, or 1 nM TSH, which is the concentration of TSH in the 6H medium, for the indicated times. The different media were replaced every 12 h during the treatment to avoid the accumulation of any possible autocrine factor. After any time of starvation, FRTL5 viability was higher than 90%. At the end of the incubation, the cells were lysed in guanidinium isothiocyanate for total mRNA preparation, or proteins were prepared for immunoblots.

Cell Transfection

The full-length cDNAs of rat TSH receptor, kindly provided by L. D. Kohn (11), human β-arrestin2, kindly provided by B. Rapoport (21), and human β-arrestin1 (4) and GRK2 (22), previously cloned in our laboratory, were used for cotransfection experiments. COS7 cells were transiently transfected as previously described (22) with minor modifications. In cotransfection experiments, 25 μg total cDNA/150-mm petri dish were transfected, and the amount of cDNA was kept constant using the empty pBJI vector. The day after transfection, the cells were trypsinized and seeded in 24-well plates for cAMP assay at a density of 10^5 cells per well (see above) and in 100-mm petri dishes for cytosol and membrane preparation and Western blot analysis. FRTL5 cells were permanently transfected with β-arrestin1 cDNA using the calcium-phosphate precipitation method (23). FRTL5 cells were seeded at a density of approximately 8×10^5 cells per 100-mm petri dish and after 48 h were transfected with 40 μg β-arrestin1 cDNA or with the same amount of the pBJI empty vector in serum-free medium. After about 4 h and 30 min, the transfection medium was washed out, and the glycerol shock was performed for 2 min. Forty eight hours after transfection, cells were plated in medium containing 400 μg/ml of G 418. Colonies of resistant cells appeared after about 3 weeks and were picked for expansion 2 weeks later. Cells were always grown under antibiotic selective pressure.

Northern Blot Analysis

Northern blot analysis was performed as previously described (4). Total RNA (20 μg) isolated by the guanidinium isothiocyanate/cesium chloride method was fractioned on a 1% agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane. Northern blot analysis of GRK2 and TSH receptor were performed using the random-primed cDNA fragments as probes. For β-arrestin1 an antisense single-stranded cDNA (bp 684–855), generated as extensively described (4), was used as a probe. RNA blots were hybridized, washed, and subjected to autoradiography at −80 C.

Western Blot Analysis

The monoclonal antibody F4C1 (4), raised against the highly conserved epitope DGVVLVD, was kindly provided by K. Palczewski and L. A. Donoso. The TSH receptor antibody was kindly donated by L. Kohn (11). GRK2-specific antibody was prepared as described (5). Cytosolic proteins (40 μg) or membrane proteins (for TSH receptor immunoblot, 100 μg) prepared as described (4) were suspended in Laemmli buffer and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes with a tank transfer system. Efficiency of transfer was verified by Ponceau red staining of the blots and Coomassie blue staining of gels after transfer. β-Arrestin1 and 2, TSH receptor, and GRK2 were detected with their specific antibody diluted in polyethylene glycol buffer containing 5% FCS and developed with alkaline phosphatase-conjugated donkey anti-rabbit IgG (TSH receptor and GRK 2) and alkaline phosphatase-conjugated goat anti-mouse IgG (β-arrestin 1) and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. For the TSH receptor the major band detected was at 95–100 kDa, which represents the functional cell surface form of the receptor (11). The blots were scanned four times with LKB Ultroscan XL laser densitometer (LKB Instruments, Rockville, MD).

Cyclic AMP Assay

The intracellular content of cAMP in FRTL5- and in COS7-transfected cells was determined by a method previously described with minor modifications (20). Briefly, FRTL5 cells were grown in 96-well plates to confluency, then starved from hormones and serum, and kept for 48 h in Coon's modified F12 medium plus 0.3% BSA and 20 mM glutamine. These procedures are necessary to measure TSH-stimulated cAMP response (8). COS7 cells were seeded in 24-well plates at the density of 10^5 cells per well the day after transfection. Cells were washed twice with prewarmed HBSS and added with HBSS containing 0.4% BSA, 10 mM HEPES, and 0.5 mM 3-isobutyl-1-methylxanthine, pH 7.3 (cAMP incubation buffer). Incubations were continued at 37 C for 30 min for the dose-response experiments. The reaction was stopped by aspirating the cAMP incubation medium and adding ice-cold ethanol. The intracellular cAMP content was measured using a commercial RIA. Data are expressed as picomoles of cAMP/well. Experiments were performed in duplicate for COS7-transfected cells and in triplicate for FRTL5-transfected cells.

Thymidine Uptake

DNA synthesis in FRTL5 and in L5β-arr was evaluated by the method of [^{3}H]thymidine uptake as described previously (20).

Statistical Analysis

All experiments are presented as the average of duplicate or triplicate determinations repeated at least two times. Statistical analysis was carried out either by Student's *t* test or Dunnett ANOVA.

Materials

Gene Screen Plus membranes were from New England Nuclear (Boston, MA). ^{32}P-labeled deoxynucleotide triphosphates and the random priming kit were purchased from Amersham Corp (Arlington Heights, IL); culture media, FCS, newborn calf serum, HBSS, guanidine isothiocyanate, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium were from GIBCO BRL (Gaithersburg, MD); forskolin from Calbiochem (La Jolla, CA); glutamine and penicillin/streptomycin from Seromed (Berlin, Germany); Tris, SDS, bromophenol blue, acrylamide, and bisacrylamide from Bio-Rad Laboratories (Richmond, CA); cAMP assay kit and [^{3}H]thymidine from Amersham; alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphatase-conjugated goat anti-mouse IgG from Pierce Chemical Co. (Rockford, IL); G418 from Fluka Chemie AG (Buchs, Switzerland). All the other materials were obtained from Sigma Chemical Co. (St. Louis, MO).

Acknowledgments

This paper is dedicated to the memory of Professor Alfredo Leonardi. We thank D. Corda for FRTL5 cells, L. D. Kohn for rat TSH receptor cDNA and antibodies, V. Tassi for support, and R. Bertazzi and S. Menna for expert assistance in preparation of the figures.

Received March 8, 1996. Re-revision received July 1, 1996. Accepted July 2, 1996.

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This work was supported by the Associazione Italiana per la Ricerca sul Cancro and by the Italian National Research Council (Convenzione C.N.R.-Consorzio Mario Negri Sud).

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