FEBS Letters 486 (2000) 208–212 FEBS 24395

Expression of β-arrestins in toxic and cold thyroid nodules

Carsten Voigt, Hans-Peter Holzapfel, Ralf Paschke*

III, Medical Department, University of Leipzig, Ph. Rosenthal Str. 27, D-04103 Leipzig, Germany

Received 17 October 2000; accepted 13 November 2000

First published online 29 November 2000

Edited by Shmuel Shaltiel

Abstract β-Arrestins mediate agonist dependent desensitization of G protein-coupled receptors. Somatic TSH receptor mutations were identified in the majority of hot thyroid nodules. When transiently overexpressed in COS 7 cells these mutations resulted in constitutive activation of the cAMP pathway. However, the in vivo mechanisms and the in vivo desensitization of these TSH receptor mutations are unknown. Moreover, constitutively activated β-adrenergic receptors are known to be constitutively desensitized. Therefore, we investigated the expression of β-arrestins in toxic thyroid nodules (TTNs) with and without somatic TSH receptor mutation and in cold thyroid nodules (CTNs) by Western blotting and ELISA. Expression of β-arrestin 2 was increased in all TTNs while β-arrestin 2 expression was decreased in CTNs compared to their corresponding surrounding tissue. The mean β-arrestin 1 expression was unchanged in the cytosol of TTNs, in membranes and cytosol of CTNs and decreased in the membranes of TTNs compared to their surrounding tissue. Transient coexpression of β-arrestins 1 or 2 with the TSH receptor in HEK 293 cells and subsequent determination of cAMP showed that in vitro both β-arrestins interact with the TSH receptor and are able to desensitize the receptor. The increased β-arrestin 2 expression in TTNs and the desensitization of the TSH receptor by β-arrestin 2 in vitro suggest that the β-arrestin 2 expression is cAMP dependent and that β-arrestin 2 very likely desensitizes the constitutively activated TSH receptor in toxic thyroid nodules.

Key words: β-Arrestin; TSH receptor; Toxic thyroid nodule; Desensitization

1. Introduction

Binding of β-arrestins to receptors phosphorylated by G protein-coupled receptor kinases (GRK's) quenches activation of G proteins and targets the receptors to clathrin-coated pits for internalization [1–8]. β-Arrestins therefore play an important role in agonist dependent desensitization of G protein-coupled receptors. β-Arrestin 1 mRNA and protein expression and its function as a negative regulator of TSH receptor stimulated cAMP has been reported for FRTL 5 cells [9,10]. TSH receptor internalization and recycling is mediated by clathrin-coated vesicles [11]. However, expression of β-arrestins and its role in TSH receptor desensitization has not been investigated in human thyroid tissues.

Somatic thyrotropin-receptor (TSHR) mutations cause a constitutive activation of the TSHR leading to chronic stimulation of the cAMP pathway in the majority of toxic thyroid nodule (TTN)s [12–15]. Less frequently constitutively activating mutations in the $G_s\alpha$-protein gene are found in TTNs [16,17]. Constitutive activation of the β_2-adrenergic receptor (β_2-AR) by mutations induces constitutive desensitization and downregulation of the receptor [18]. A similar desensitization mechanism is very likely for constitutively activating TSH receptor mutations.

The cAMP pathway is thought to be responsible for differentiation, function and growth of thyroid follicular cells [19]. Therefore chronic stimulation of cAMP is likely to explain the clinical phenotype characterized by hyperthyroidism and nodule growth. Significantly higher thyroid hormone release in TTNs than in the surrounding tissue has been reported [20]. The various TSH receptor mutations differ considerably in their functional properties, determined in in vitro assays [14,21]. However, no correlation with the phenotype could be found for somatic [22] or germline [23] TSH receptor mutations. Feedback mechanisms like β-arrestin-induced desensitization and downregulation of the receptor are possible explanations for the differences found between TSH receptor genotype and clinical phenotype.

We therefore asked the following questions: (l) Which β-arrestins interact with the TSH receptor? (2) Which β-arrestins are expressed in the human thyroid? (3) Does the expression pattern of β-arrestins in TTNs differ from normal surrounding tissue or cold thyroid nodules (CTN)?

2. Materials and methods

2.1. Tissue samples

Thyroid nodules were characterized by ultrasound and scintiscan. All TTNs showed increased technetium uptake with suppression of the surrounding tissue. None CTNs took up technetium. All preoperatively identified nodules were also identified during surgery and by histology. Somatic TSH receptor mutations in the hot nodules were previously determined by DGGE and subsequent direct sequencing of the positive bands [24]. The samples were stored in liquid nitrogen.

2.2. Membrane preparation

Frozen tissues were ground in liquid nitrogen, thawed and taken up in membrane preparation buffer (40 mM Tris–HCl, 250 mM sucrose, 0.1 mM DTT, 0.1 mM PMSF, pH 7.4). The homogenates were first centrifuged at $700\times g$ for 10 min at 4°C. The supernatant was further centrifuged at $60\,000\times g$ for 45 min at 4°C. The pellet (membrane fraction) was resuspended in membrane preparation buffer. The supernatant was used as cytosolic fraction. Protein concentrations were determined by measuring the absorption at 280 nm.

2.3. Cell culture and transfection

HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Karlsruhe, Germany) at 37°C in

*Corresponding author. Fax: (49)-341-9713209.
E-mail: pasr@server3.medizin.uni-leipzig.de

0014-5793/00/$20.00
PII: S0014-5793(00)02302-4

a humidified 5% CO_2 incubator. For cAMP assays, the cells were transfected using the FuGENE 6 (Roche, Mannheim, Germany) transfection reagent according to the manufacturer's instructions. In brief, 2×10^5 COS-7 cells per well were seeded into 12 well plates 24 h before cotransfection with plasmid constructs (1 μg DNA/well) containing the coding sequence of the TSHR and β-arrestin 1, β-arrestin 2 or a dominant negative β-arrestin 1 mutant (V53D) or the TSHR and the empty psvl-vector. The dominant negative β-arrestin 1 mutant (V53D) was a generous gift from Dr. J. Benovic (Thomas Jefferson University). Functional assays were performed 48 h after transfection and repeated two times.

2.4. Measurement of cAMP

For cAMP assays HEK 293 cells were washed in serum-free Dulbecco's modified Eagle's medium, followed by preincubation with the same medium containing 1 mM 3-isobutyl-1-methyl-xanthine (Sigma Chemical Co., St. Louis, MO, USA) for 20 min at 37°C in a humidified 5% CO_2 incubator. Subsequently, cells were stimulated with bTSH (10 mU/ml) for 1 h. Reactions were terminated by aspiration of the medium and addition of 0.5 ml 0.1 N HCl. Supernatants were collected and dried. The cAMP content of the cell extracts was determined with a commercial kit (Amersham Pharmacia Biotech, Braunschweig, Germany) according to the manufacturer's instructions.

2.5. Western blot analysis

The cytosolic and membrane fractions of all samples were heated at 95°C for 5 min in sample buffer [25] and electrophoresed (100 μg protein/lane) on 10% polyacrylamide gels containing 0.1% SDS using the discontinuous buffer system described by Davis [26]. Separated proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a semidry blotting system (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk in TBS/T (20 mM Tris–HCl, pH 7.6, 0.8% NaCl, 0.1% Tween 20) for 1 h at room temperature and probed overnight with mouse anti-β-arrestin 1 or 2 antibody (Transduction Laboratories, San Diego, CA, USA) (1:200 dilution) or anti-actin antibody (Sigma Chemical Co., St. Louis, MO, USA) (1:500 dilution) at 4°C in TBS/T containing 5% BSA. After washing the membranes three times for 5 min, they were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-mouse antibody (New England Biolabs, Beverly, MA, USA) diluted 1:3000 in blocking solution. Following washing with TBS/T (4×5 min), membranes were incubated with the chemiluminescence reagent (SuperSignal West Pico, Pierce, Rockford, IL, USA) for 5 min at room temperature. Immunoreactive proteins were detected with the ChemiImager 4000 (Alpha Innotech Corporation, San Leandro, USA) and quantified using the Alpha Ease 4.0 software from Alpha Innotech Corporation. The measurements were carried out in duplicates and repeated.

2.6. ELISA

98-Well plates were coated overnight at 4°C with 0.5–2.0 μg sample protein per well diluted in 100 μl coating buffer (15 mM Na_2CO_3, 0.35 m $NaHCO_3$, 3 mM NaN_3, 0.1 mM PMSF). After blocking of unspecific protein bindings (100 μl/well TBS/T containing 5% non-fat dry milk, pH 7.6) for 1 h at room temperature and three washes (5 min in 150 μl TBS/T per well) the plates were incubated with a 1:250 dilution of a β-arrestin 2 antibody (Transduction Laboratories, San Diego, CA, USA) in TBS containing 0.05% Tween 20 (100 μl/well) for 90 min at room temperature. Subsequently, plates were washed three times with TBS/T and incubated for 1 h at room temperature in a 1:2500 dilution of a horseradish peroxidase-conjugated anti-mouse antibody (New England Biolabs, Beverly, MA, USA) in TBS containing 0.05% Tween 20. After final washes in TBS/T (3×5 min, 150 μl/well), the reaction was developed using a substrate system containing *o*-phenyldiamine (0.6 mg/ml) and H_2O_2 (30%, 0.5 μl/ml) in phosphate-citrate buffer (0.15 M, pH 5.0). The enzymatic reaction was stopped after 30 min at 37°C with 3 M HCl (100 μl/well) and the samples were measured at 492 nm using a microplatereader. All measurements were carried out twice.

2.7. Data analysis

The distribution of β-arrestins was expressed as percent of the sum of β-arrestins in nodular and in surrounding tissue, β-arrestin expression is given as means ± S.E.M. of duplicate measurements. cAMP values of cotransfection experiments are given as means ± S.E.M. of one representative experiment, carried out in duplicates. Statistical analysis was carried out by Student's *t*-test.

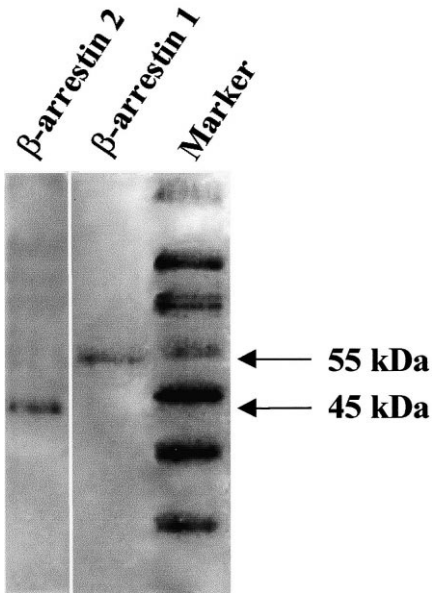

Fig. 1. Expression of β-arrestin 1 (55 kDa) and β-arrestin 2 (45 kDa) in normal thyroid tissue. The tissue homogenates were electrophoresed on SDS–polyacrylamide gels (100 μg protein per lane), transferred to nitrocellulose membranes and probed with monoclonal mouse antibodies followed by HRP-linked second antibody and chemiluminescence imaging.

3. Results

Both, β-arrestin 1 as well as β-arrestin 2 were detectable by Western blotting in all investigated thyroid tissues (Fig. 1). For better quantification of β-arrestin 2 we designed a very sensitive ELISA. The specificity of the β-arrestin 2 detection in the ELISA was also tested by Western blot for some samples. The Western blot showed the same relations of β-arrestin 2 expression between nodular and surrounding tissue as the ELISA (data not shown). For quantification of β-arrestin 1 we used Western blotting followed by chemiluminescence imaging, because none of the available β-arrestin 1 antibodies used in the ELISA was able to detect β-arrestin 1. All quan-

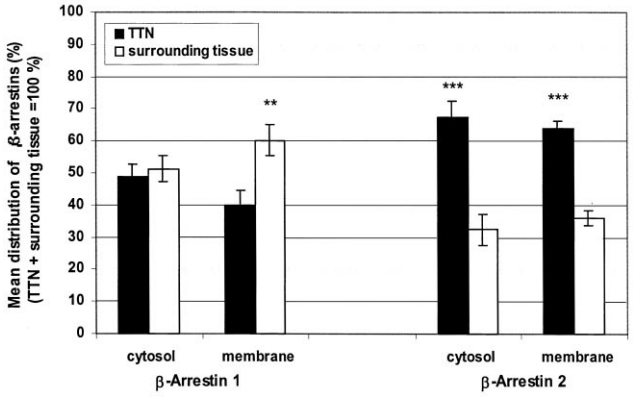

Fig. 2. Mean distribution of β-arrestins in TTN. β-Arrestin 1 was measured as duplicates in 12 TTNs and quantified by Western blotting followed by chemiluminescence imaging. β-Arrestin 2 was measured as duplicates in 12 TTNs and quantified by ELISA. (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

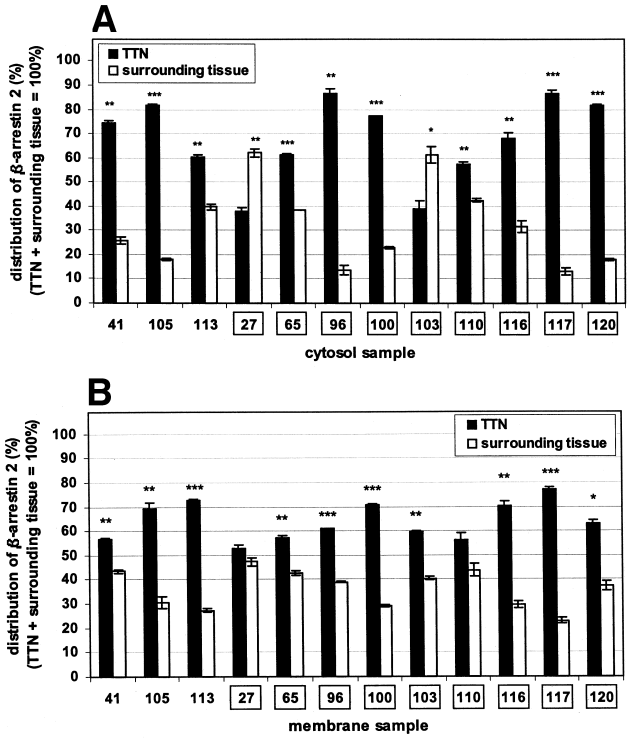

Fig. 3. Distribution of cytosolic (A) and membrane-bound (B) β-arrestin 2 in TTNs quantified by ELISA. Data are given as means ± S.E.M. of duplicate measurements (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; boxed numbers: samples with TSH receptor mutation).

tifications of β-arrestins were reproducible. There were no differences in the actin expression between the nodular and surrounding tissue samples (data not shown). As purified β-arrestins are not commercially available the total amount of β-arrestins could not be determined. The distribution of β-arrestins was expressed as percent of the sum of β-arrestins in nodular and in surrounding tissue. The sum of each β-arrestin in nodular and in surrounding tissue represents 100%.

3.1. Expression of β-arrestins in TTNs

In the membrane fraction of 12 investigated TTNs we found higher amounts (63.9 ± 2.3%; $P < 0.001$; Fig. 2) of β-arrestin 2 in the TTNs than in the surrounding tissues. Except for two of the 12 TTNs the differences were significant for all

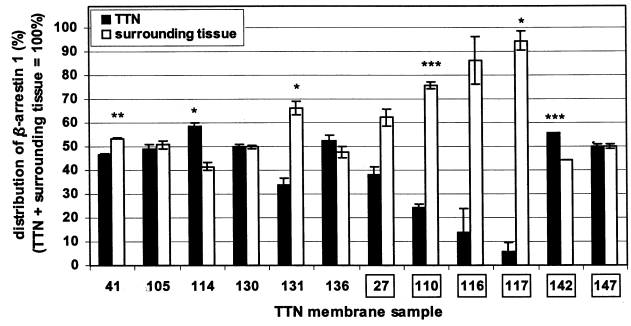

Fig. 4. Distribution of membrane-bound β-arrestin 1 in TTN quantified by Western blotting followed by chemiluminescence imaging. Data are given as means ± S.E.M. of duplicate measurements (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; boxed numbers: samples with TSH receptor mutation).

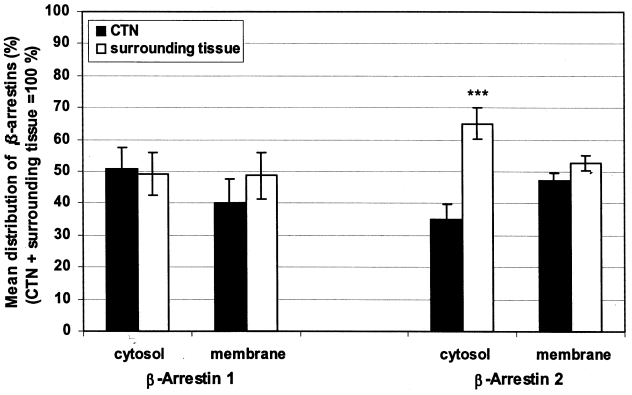

Fig. 5. Mean distribution of β-arrestins in CTN. β-Arrestin 1 was measured as duplicates in nine CTNs and quantified by Western blotting followed by chemiluminescence imaging. β-Arrestin 2 was measured as duplicates in nine CTNs and quantified by ELISA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

TTNs (Fig. 3B). In the cytosol of these TTNs we also found higher amounts (67.6 ± 4.9%; $P < 0.001$; Fig. 2) of β-arrestin 2 in the nodules than in the surrounding tissues. Only two of 12 samples showed an inverse expression pattern for cytosolic β-arrestin 2. The differences were significant for all TTNs (Fig. 3A). In contrast to the relatively homogeneous results obtained for β-arrestin 2 there was a lower amount of β-arrestin 1 in the membrane fraction of six TTNs, no different β-arrestin 1 expression in the membrane fraction of four TTNs and a significantly higher β-arrestin 1 expression in the membrane fraction of two TTNs (Fig. 4). The mean distribution of β-arrestin 1 in the membrane fraction of all 12 investigated TTNs showed a significantly decreased level (39.8 ± 4.9%; $P < 0.01$; Fig. 2) compared to the surrounding tissue. The β-arrestin 1 expression in the cytosol of 12 investigated TTNs was not different from the surrounding tissue (Fig. 2).

3.2. Expression of β-arrestins in CTNs

In contrast to the homogeneous expression of β-arrestin 2 in TTNs there was no predominant expression pattern for β-arrestins 1 or 2 in CTNs. In the cytosol of six out of nine investigated CTNs we detected significantly lower (35.0 ± 6.4%; $P < 0.001$; Fig. 5) amounts of β-arrestin 2 in the nodules compared to the surrounding tissues whereas no difference was found in the cytosol of one CTN and an inverse pattern was observed in the cytosol of two CTNs (Fig. 6). The mean level of β-arrestin 2 in the membrane fraction of

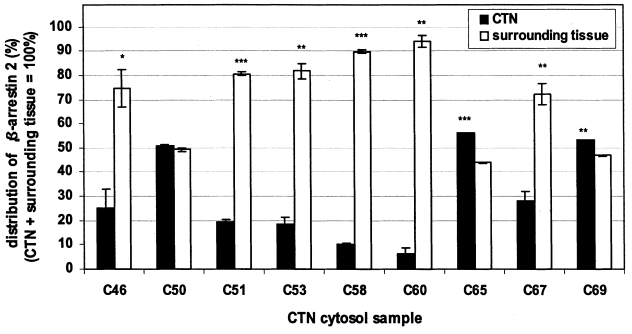

Fig. 6. Distribution of cytosolic β-arrestin 2 in CTN quantified by ELISA. Data are given as means ± S.E.M. of duplicate measurements (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

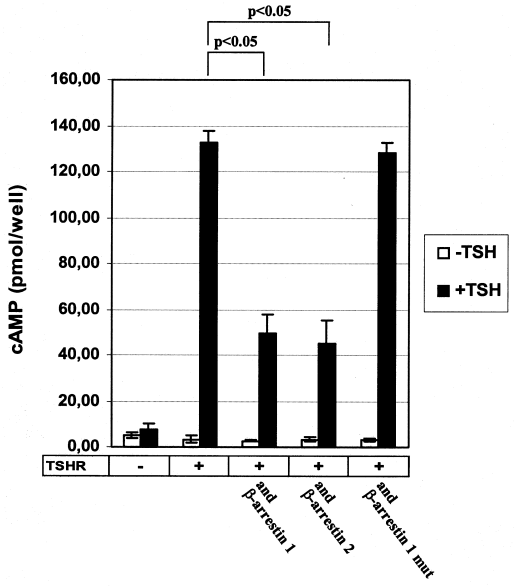

Fig. 7. Basal and TSH-stimulated (10 mU/ml, 1 h) cAMP accumulation in HEK 293 cells cotransfected with the TSH receptor and β-arrestin constructs. Data are given as means ± S.E.M. of one representative experiment, carried out in duplicate.

CTNs was not different from the surrounding tissue (Fig. 5). Moreover, the mean β-arrestin 1 level in membranes or cytosol of nine CTNs was not different from the surrounding tissue (Fig. 5).

The expression data were compared to the thyroid size, clonality and mutations of the TSH receptor of each sample, but no coherence could be found (data not shown).

3.3. Cotransfection experiments

TSH stimulated (10 mU/ml, 1 h) cAMP accumulation in HEK 293 cells, cotransfected with the TSH receptor and β-arrestin 1 or 2 was significantly decreased to 37.2% ± 6.4 ($P < 0.05$) for β-arrestin 1 and to 33.9 ± 7.5% ($P < 0.05$) for β-arrestin 2 compared to HEK 293 cells transfected only with the TSH receptor (100%) (Fig. 7). cAMP was unchanged after TSH stimulation in COS 7 cells cotransfected with the TSH receptor and a dominant negative β-arrestin 1 mutant (V53D) compared to COS 7 cells transfected only with the TSH receptor. All cotransfection experiments were reproducible.

4. Discussion

Up to now the role of β-arrestins for the signaling of the human TSH receptor in thyroid tissue has not been investigated. β-arrestin 1 and 2 are the known members of the β-arrestin family and are expressed in a broad range of human tissues. In this study we found β-arrestin 1 and 2 expression in human thyroid tissue. Expression of β-arrestin 1 but not β-arrestin 2 in FRTL 5 cells has recently been reported [9,10]. In addition a thyroid specific β-arrestin 2 cDNA with a high homology to β-arrestin 2 has been cloned [27]. However, the functional impact of the β-arrestin expression in human thyroid tissue remains to be clarified. The desensitizing effect of β-arrestins on G protein-coupled receptor signaling has been demonstrated in many studies [28–34]. Overexpression of β-arrestin 1 in stably transfected Chinese hamster ovary cell lines enhanced the desensitization of β_2-AR [33]. Moreover, a partially purified preparation of β-arrestin 1 inhibited the ability of β-adrenergic receptor kinase (β-ARK)-phosphorylated β_2-AR to activate G_s by $>75\%$ [3], indicating that β-arrestin 1 works in concert with β-ARK to induce agonist specific desensitization of the β_2-AR. Furthermore, β-arrestin binding to β_2-AR not only terminates receptor-G protein coupling followed by a decrease of cAMP. It also initiates a second wave of signal transduction in which the desensitized receptor acts as a structural component of a mitogenic signaling complex [6].

TSH stimulation of FRTL 5 cells increased the level of cytosolic β-arrestin 1 and, in turn, the elevated levels of β-arrestin 1 attenuated TSH-induced cAMP accumulation and increased TSH receptor desensitization [9]. In contrast Nagayama et al. [10] failed to demonstrate a TSH dependent mechanism of β-arrestin 1 regulation in FRTL 5 cells.

Evidence for a functional importance of β-arrestins for TSH receptor signaling is also implied by experiments with the lutropin/choriogonadotropin receptor which displays a high homology to the TSH receptor. The desensitization of lutropin/choriogonadotropin receptor-stimulated adenylyl cyclase activity in porcine ovarian follicular membranes is mediated by β-arrestin 1 [30]. Furthermore, cotransfection of human kidney 293 cells with the lutropin/choriogonadotropin receptor and GRK2 or β-arrestin 1 or β-arrestin 2 enhances internalization and increases downregulation of the receptor [28,32]. Moreover, the folitropin receptor in human kidney 293 cells [29,31] and in mouse Ltk cells [34] shows enhanced agonist-induced internalization after cotransfection with β-arrestin 1 or 2.

Overexpression of β-arrestin 1 in COS 7 cells or in FRTL 5 cells decreased TSH receptor dependent cAMP production [9,10] probably by an increased desensitization of the TSH receptor.

We found that both β-arrestins are able to desensitize the TSH receptor in vitro in a coexpression system in HEK 293 cells (Fig. 7). In contrast no decrease of TSH-stimulated cAMP could be detected in HEK 293 cells cotransfected with the TSH receptor and a dominant negative β-arrestin 1 mutant compared to HEK 293 cells transfected only with the TSH receptor. Our results confirm previous data indicating a β-arrestin 1 dependent desensitization mechanism for the TSH receptor [9,10] and extend the possibilities for TSH receptor desensitization to β-arrestin 2.

The cytosol contains free β-arrestin and the membrane fraction receptor-bound β-arrestin [6,35]. The amount of free (cytosolic) β-arrestin reflects the entire β-arrestin pool [7]. The TSH receptor is desensitized by β-arrestin binding [9]. Therefore, the amount of receptor-bound β-arrestin which is found in the membrane fraction reflects the β-arrestin fraction which is involved in receptor desensitization (inactivation). Therefore, we separated the cytosolic and the membrane fraction for each sample.

Based on the previous findings in FRTL 5 cells [9] we expected an increased expression of β-arrestin 1 in TTNs with mutation-induced TSH receptor activation. However, unchanged levels of β-arrestin 1 in the cytosol of TTNs (Fig. 2) and decreased levels of β-arrestin 1 in the membrane fraction of TTNs compared to the normal surrounding tissue were detected (Figs. 2 and 4). These findings most likely indicate an unchanged total pool of β-arrestin 1 whereas the internalization and degradation of the β-arrestin 1-TSH receptor com-

plex seems to be increased. This assumption is supported by the significant decrease of cAMP accumulation in TSH-stimulated HEK 293 cells cotransfected with the TSH receptor and β-arrestin 1. Similar results have been reported for constitutively activating rhodopsin mutants [36]. The phosphorylation of these constitutively activating rhodopsin mutants by rhodopsin kinase and the subsequent arrestin binding apparently led to sequestration of the entire arrestin pool.

Higher levels of β-arrestin 2 in the membrane fraction of all screened TTNs compared to the surrounding tissue (Figs. 2 and 3b) together with increased expression of β-arrestin 2 in the cytosol of 10 out of 12 screened TTNs compared to the surrounding tissue (Figs. 2 and 3a) suggest a increased TSH receptor desensitization by β-arrestin 2 in these toxic nodules. Chronic stimulation of the cAMP-pathway is very likely in TTNs, especially those with constitutively activating TSH receptor mutations. Therefore, the increased β-arrestin 2 expression in the toxic nodules is most likely induced by the constitutive activation of the cAMP pathway. As the cAMP production in the thyroid is mainly induced by the TSH receptor, β-arrestin 2 is most likely part of a feedback mechanism which desensitizes the TSH receptor. The higher levels of β-arrestin 2 compared to β-arrestin 1 in the membrane fraction of TTNs are most likely due to the predominant role of β-arrestin 2 in TSH receptor desensitization.

The β-arrestin 1 level in the cytosol and the membrane fraction of CTNs was not different from the normal surrounding tissue (Fig. 5) most likely indicating a normal β-arrestin 1 expression and TSH receptor desensitization in these nodules and the surrounding tissue. Moreover, we found a decreased expression of β-arrestin 2 in the cytosol of CTNs (Figs. 5 and 6) compared to the surrounding tissue. Unchanged levels of β-arrestin 2 in membrane fractions of CTNs (Fig. 5) compared to surrounding tissues indicate an equal desensitization of the TSH receptor in cold nodules and corresponding surrounding tissues by β-arrestin 2. As the cAMP-pathway is most likely not constitutively activated in CTNs in contrast to TTNs the decreased β-arrestin 2 in the cytosol of CTNs further supports the hypothesis that the main signaling pathways in cold and TTNs are different. In addition, the feedback mechanism which desensitizes the TSH receptor is apparently not activated in CTNs.

In summary, our findings demonstrate the expression of both, β-arrestin 1 and 2 in the human thyroid. In vitro both β-arrestins are capable to interact with the TSH receptor and to desensitize the receptor after stimulation. However, in hot nodules β-arrestin 2 seems to be the predominant cAMP dependent regulator of the TSH receptor activity.

Acknowledgements: This study was supported by Grants from the Deutsche Forschungsgemeinschaft (DFG Pa423/10-1) and the Interdisciplinary Center for Clinical Research at the University of Leipzig (IZKF B14). We thank Dr. J. Benovic for the β-arrestin 1 (V53D) mutant cDNA.

References

[1] Lohse, M.J., Krasel, C., Winstel, R. and Mayor, F.J. (1996) Kidney Int. 49, 1047–1052.
[2] Bennett, N. and Sitaramayya, A. (1988) Biochemistry 27, 1710–1715.
[3] Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G. and Lefkowitz, R.J. (1990) Science 248, 1547–1550.
[4] Lohse, M.J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J.P., Caron, M.G. and Lefkowitz, R.J. (1992) J. Biol. Chem. 267, 8558–8564.
[5] Wilden, U., Hall, S.W. and Kuhn, H. (1986) Proc. Natl. Acad. Sci. USA 83, 1174–1178.
[6] Luttrell, L.M., Ferguson, S.S., Daaka, Y., Miller, W.E., Maudsley, S., Della, R.G., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G. and Lefkowitz, R.J. (1999) Science 283, 655–661.
[7] Ferguson, S.S., Barak, L.S., Zhang, J. and Caron, M.G. (1996) Can. J. Physiol. Pharmacol. 74, 1095–1110.
[8] Goodman, O.B.J., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R.B., Gagnon, A.W., Keen, J.H. and Benovic, J.L. (1996) Nature 383, 447–450.
[9] Iacovelli, L., Franchetti, R., Masini, M. and De Blasi, A. (1996) Mol. Endocrinol. 10, 1138–1146.
[10] Nagayama, Y., Tanaka, K., Namba, H., Yamashita, S. and Niwa, M. (1996) Thyroid 6, 627–631.
[11] Baratti-Elbaz, C., Ghinea, N., Lahuna, O., Loosfelt, H., Pichon, C. and Milgrom, E. (1999) Mol. Endocrinol. 13, 1751–1765.
[12] Parma, J., Duprez, L., Van Sande, J., Cochaux, P., Gervy, C., Mockel, J., Dumont, J. and Vassart, G. (1993) Nature 365, 649–651.
[13] Parma, J., Van Sande, J., Swillens, S., Tonacchera, M., Dumont, J. and Vassart, G. (1995) Mol. Endocrinol. 9, 725–733.
[14] Van Sande, J., Parma, J., Tonacchera, M., Swillens, S., Dumont, J. and Vassart, G. (1995) J. Clin. Endocrinol. Metab. 80, 2577–2585.
[15] Fuhrer, D., Holzapfel, H.P., Wonerow, P., Scherbaum, W.A. and Paschke, R. (1997) J. Clin. Endocrinol. Metab. 82, 3885–3891.
[16] Lyons, J., Landis, C.A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q.Y., Clark, O.H., Kawasaki, E. and Bourne, H.R. (1990) Science 249, 655–659.
[17] Paschke, R. and Ludgate, M. (1997) N. Engl. J. Med. 337, 1675–1681.
[18] Pei, G., Samama, P., Lohse, M., Wang, M., Codina, J. and Lefkowitz, R.J. (1994) Proc. Natl. Acad. Sci. USA 91, 2699–2702.
[19] Roger, P.P., Christophe, D., Dumont, J.E. and Pirson, I. (1997) Eur. J. Endocrinol. 137, 579–598.
[20] Poertl, S., Kirner, J., Mann, K. and Hoermann, R. (1996) Exp. Clin. Endocrinol. Diabetes 104 (Suppl. 4), 39–40.
[21] Wonerow, P., Stötzner, H., Chey, S. and Paschke, R. (1999) J. Endocrinol. Invest. (Abstr.) 22, 17–17.
[22] Arturi, F., Capula, C., Chiefari, E., Filetti, S. and Russo, D. (1998) Exp. Clin. Endocrinol. Diabetes 106, 234–236.
[23] Fuhrer, D., Mix, M., Wonerow, P., Richter, I., Willgerodt, H. and Paschke, R. (1999) Thyroid 9, 757–761.
[24] Trülzsch, B., Krohn, K., Wonerow, P. and Paschke, R. (1999) Biotechniques 27, 266–268.
[25] Laemmli, U.K. (1970) Nature 227, 680–685.
[26] Davis, B.J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
[27] Rapoport, B., Kaufman, K.D. and Chazenbalk, G.D. (1992) Mol. Cell. Endocrinol. 84, R39–R43.
[28] Lazari, M.F., Bertrand, J.E., Nakamura, K., Liu, X., Krupnick, J.G., Benovic, J.L. and Ascoli, M. (1998) J. Biol. Chem. 273, 18316–18324.
[29] Lazari, M.F., Liu, X., Nakamura, K., Benovic, J.L. and Ascoli, M. (1999) Mol. Endocrinol. 13, 866–878.
[30] Mukherjee, S., Palczewski, K., Gurevich, V., Benovic, J.L., Banga, J.P. and Hunzicker-Dunn, M. (1999) Proc. Natl. Acad. Sci. USA 96, 493–498.
[31] Nakamura, K., Krupnick, J.G., Benovic, J.L. and Ascoli, M. (1998) J. Biol. Chem. 273, 24346–24354.
[32] Nakamura, K., Lazari, M.F., Li, S., Korgaonkar, C. and Ascoli, M. (1999) Mol. Endocrinol. 13, 1295–1304.
[33] Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M.G., Lefkowitz, R.J. and Lohse, M.J. (1993) J. Biol. Chem. 268, 3201–3208.
[34] Troispoux, C., Guillou, F., Elalouf, J.M., Firsov, D., Iacovelli, L., De Blasi, A., Combarnous, Y. and Reiter, E. (1999) Mol. Endocrinol. 13, 1599–1614.
[35] von Zastrow, M. and Kobilka, B.K. (1992) J. Biol. Chem. 267, 3530–3538.
[36] Rim, J. and Oprian, D.D. (1995) Biochemistry 34, 11938–11945.