Increased expression of G-protein-coupled receptor kinases 3 and 4 in hyperfunctioning thyroid nodules

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Abstract

G-protein-coupled receptor kinases (GRKs) are implicated in the pathophysiology of human diseases such as arterial hypertension, heart failure and rheumatoid arthritis. While G-protein-coupled receptor kinases 2 and 5 have been shown to be involved in the desensitization of the rat thyrotropin receptor (TSHR), their role in the pathophysiology of hyperfunctioning thyroid nodules (HTNs) is unknown. Therefore, we analyzed the expression pattern of the known GRKs in human thyroid tissue and investigated their function in the pathology of HTNs. The expression of different GRKs in human thyroid and HTNs was measured by Western blotting. The influence of GRK expression on TSHR function was analyzed by coexpression experiments in HEK 293 cells. We demonstrate that in addition to GRKs 2, 5 and 6, GRKs 3 and 4

are also expressed in the human thyroid. GRKs 2, 3, 5 and 6 are able to desensitize the TSHR *in vitro*. This GRK-induced desensitization is amplified by the additional over-expression of β -arrestin 1 or 2. We did not find any mutations in the GRKs 2, 3 and 5 from 14 HTNs without TSHR mutations and $G_s\alpha$ mutations. The expression of GRKs 3 and 4 was increased in HTNs independently from the existence of TSHR mutations or $G_s\alpha$ mutations. In conclusion, the increased expression of GRK 3 in HTNs and the ability of GRK 3 to desensitize the TSHR *in vitro*, suggest a potential role for GRK 3 as a negative feedback regulator for the constitutively activated cAMP pathway in HTNs.

Journal of Endocrinology (2004) **182**, 173–182

Introduction

G-protein-coupled receptor kinases (GRKs) and the family of arrestins have been shown to coordinately regulate the homologous desensitization of different G-protein-coupled receptors (Ferguson 2001, Kohout & Lefkowitz 2003). The multigene family of GRKs consists of 7 known members named GRKs 1 to 7 (Ferguson 2001, Kohout & Lefkowitz 2003). GRK 1, GRK 2, GRK 3 and GRK 7 are also known as rhodopsin kinase (GRK 1), β -adrenergic receptor kinase 1 (GRK 2), β -adrenergic receptor kinase 2 (GRK 3) and cone opsin kinase (GRK 7) respectively (Ferguson 2001). GRK 1 and GRK 7 are exclusively expressed in the retina where they regulate photo-transduction, while GRK 2 and GRK 3 were found to be widely expressed in different tissues (Ferguson 2001). The GRK 4 subfamily consists of GRKs 4, 5 and 6 (Ferguson 2001). GRK 4 expression is predominantly found in testis, some brain areas and to a lesser extent in rat kidney (Virlon *et al.* 1998, Ferguson 2001). GRKs 5 and 6 demonstrate a ubiquitous expression pattern (Ferguson 2001). In addition, the different GRKs are highly specific in their receptor preference (Bunemann & Hosey 1999, Ferguson 2001).

Retinal arrestin, also known as arrestin 1 or cone arrestin is exclusively expressed in the retina, where it is involved in the regulation of photo-transduction (Ferguson 2001). Arrestin 2 and 3 (β -arrestin 1 and 2) were found to be expressed in a wide range of tissues. Differences in structural homology and expression of arrestin 2 and 3 are the basis for their receptor specificity (Ferguson 2001).

GRK expression and activity has been found to be altered in several diseases. Increased GRK 2 expression and activity was found in myocytes from patients with heart failure (Ungerer *et al.* 1994) and in peripheral blood lymphocytes (PBL) of patients with arterial hypertension (Gros *et al.* 1997). Decreased GRK 2 and GRK 6 expression and activity were detected in PBL of patients with rheumatoid arthritis (Lombardi *et al.* 1999). Mutations and deletions of the GRK 1 gene have been shown to cause Oguchi disease (Yamamoto *et al.* 1997, Cideciyan *et al.* 1998). Moreover, in hypothyroid rats an increased expression of GRK 2 was found in heart and lung tissues at 60 days after birth whereas GRK 2 expression in the liver of these rats was decreased (Penela *et al.* 2001a).

The thyrotropin (TSH) receptor (TSHR) regulates thyroid growth and thyroid hormone production and is known to undergo homologous desensitization (Wynford-Thomas *et al.* 1983). Although several authors have recently demonstrated an influence of GRK 2, GRK 5, GRK 6 and β -arrestin 1 on the desensitization of the rat TSHR *in vitro* (Iacovelli *et al.* 1996, Nagayama *et al.* 1996a,b), the distinct molecular mechanism of TSHR desensitization remains unclear. However, an involvement at different steps of the desensitization machinery of the TSHR has been suspected in thyroid pathologies (Kopp 1997) including differentiated thyroid carcinoma cells (Metaye *et al.* 2002).

The aim of the present study was, therefore, to characterize the expression pattern of the different GRKs in human thyroid tissue and to analyze their involvement in TSHR desensitization. We demonstrated that GRKs 2–6 are expressed in all human thyroids examined. By coexpression of the different GRKs with the TSHR in HEK 293 cells, we showed that GRKs 2, 3, 5 and 6 are able to desensitize the TSHR *in vitro*. Moreover, an over-expression of GRKs 3 and 4 could be found in hyperfunctioning thyroid nodules (HTNs) as compared with their surrounding thyroid tissue.

Materials and Methods

Tissue samples

Six HTNs (4 with TSHR mutation, 2 without TSHR mutation) and their normal surrounding tissues were used for the measurement of GRK 2, GRK 3 and GRK 4 expression. Six additional HTNs (2 with TSHR mutation, 4 without TSHR mutation) and their normal surrounding tissues were used only for the measurement of GRK 3 expression. None of the examined HTNs showed a G α mutation. The HTNs were characterized by ultrasound and scintiscan. All HTNs showed increased technetium uptake with suppression of the surrounding tissue. All preoperatively identified nodules were identified during surgery and confirmed by histology. Only samples without lymphocytic infiltration in the tumor or surrounding tissue were included in our study. At the time of surgery the patients were euthyroid. Somatic TSHR mutations in the hot nodules were previously determined by denaturing gradient gel electrophoresis and positive bands were subsequently directly sequenced (Trulzsch *et al.* 2001). Samples were stored in liquid nitrogen.

The study was approved by the local ethics committee. Written informed consent was obtained from all patients before surgery.

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 dithiothreitol (DTT), 0.1 mM PMSF, pH 7.4). The homogenates were first centrifuged at 700 *g* for 10 min at 4 °C. The supernatant was further centrifuged at 60 000 *g* for 45 min at 4 °C. The pellet (membrane fraction) was resuspended in membrane preparation buffer. The supernatant was used as the cytosolic fraction. Protein concentrations were determined by the method of Bradford (Bradford 1976).

Cell culture and transfection

HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (GibcoBRL, Karlsruhe, Germany) at 37 °C in a humidified 5% CO₂ 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 $\times$ 10⁵ HEK 293 cells were seeded onto 12-well plates 24 h before cotransfection with 1 μ g/well plasmid constructs (0.33 μ g TSHR, 0.33 μ g GRK and 0.33 μ g β -arrestin, or supplemented with empty pcDNA 3.1 vector to finally 1 μ g transfected DNA) containing the coding sequence of the TSHR and GRK 2, GRK 3, GRK 5, GRK 6 or a kinase-deficient GRK 2 mutant (K220R) and β -arrestin 1 or 2. The plasmids containing the cDNA of GRK 2, GRK 2 mutant K220R, GRK 3, GRK 5, GRK 6, β -arrestin 1 and 2 were kindly provided by J L Benovic (Duke University, Durham, USA). The coding sequence of GRK 4 α (representing the whole protein) was amplified by RT-PCR from human thyroid cDNA (primer: forward 5'-CCAGG ACATGGAGCTCGAGAAC-3', reverse 5'-TCAGCA TTGCTTGGGTTCCTTC-3') and subcloned in the pcDNA 3.1 vector. Both strands of the GRK 4 α clone were checked by sequencing. Functional assays were performed 48 h after transfection and repeated two times.

Measurement of cAMP

For cAMP assays, HEK 293 cells were washed once in serum-free Dulbecco's modified Eagle's medium, followed by a 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% CO₂ incubator. Subsequently, cells were stimulated with bovine TSH (100 mU/ml) for 1 h to get a maximal response. Reactions were terminated by aspiration of the medium and the addition of 0.5 ml 0.1 M HCl. Supernatants were collected and dried. The cAMP content of cell extracts was determined with a commercial kit (Amersham Pharmacia Biotech, Braunschweig, Germany) according to the manufacturer's instructions.

Fluorescence activated cell sorting (FACS) analysis

For the determination of the TSHR cell surface expression, transfected cells were incubated with anti-human

TSHR antibody (2C11, Serotec Ltd, Oxford, Oxon, UK; 10 µg/ml in PBS containing 0.1% BSA). After two washes with PBS (0.1% BSA), cells were incubated with the fluorescent conjugated F(ab')₂ rabbit anti-mouse IgG secondary antibody (Star9B, Serotec Ltd; 1:100 dilution in PBS containing 0.1% BSA) for 1 h on ice in the dark. The fluorescence of 10 000 cells per tube was assayed by a FACS Scan Cytofluorometer (Becton Dickinson, San Jose, CA, USA).

Western blot analysis

The cytosolic and membrane fractions of all samples were heated to 95 °C for 5 min in sample buffer and electrophoresed (100 µg/lane) on 10% polyacrylamide gels containing 0.1% sodium dodecylsulfate using the discontinuous buffer system described by Davis (1964). Separated proteins were electro-blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a semidry blotting system (BioRad, 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 rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against GRK 2 (1:1250), GRK 3 (1:700), GRK 4 (1:500), GRK 5 (1:500), GRK 6 (1:700) or actin (1:2000) at 4 °C in TBS/T containing 5% BSA, followed by 1 h incubation at room temperature with a horseradish peroxidase-conjugated anti-rabbit antibody (New England Biolabs, Beverly, MA, USA) and incubation with the chemiluminescence reagent (SuperSignal West Pico, Pierce, Rockford, IL, USA). Immunoreactive proteins were detected with the ChemiImager 4000 (Alpha Innotech Corporation, San Leandro, CA, USA) and quantified using the Alpha Ease 4.0 software from Alpha Innotech Corporation. The measurements were carried out in duplicate and repeated once. The intracellular β-actin was used as a control for protein loading. β-Actin was determined by stripping and reblotting the original membranes.

RNA preparation

RNA was prepared with an Atlas pure RNA isolation kit according to the manufacturer's description (Clontech, Palo Alto, CA, USA). The quality of the RNA samples was assessed by electrophoresis through denaturing agarose gels and staining with ethidium bromide. 18S and 28S RNA bands were visualized under UV illumination. The extraction yield was quantified spectrophotometrically.

Detection of GRKs by RT-PCR

The GRKs were detected in the normal surrounding tissues of HTNs. One microgram total RNA was used for cDNA synthesis. Briefly, GRK-specific primers (GRK 2:

forward 5'-CTCCGAGGGGACGTGTTCCAGAAA-3', reverse 5'-TGGCATGGGGCTTCTTCTTGA GA-3'; GRK 3: forward 5'-TGCACTACCACCTTT CACAACA-3', reverse 5'-ACATGCTGCCAGTCAA CACCT-3'; GRK 4: forward 5'-ATGGAGCTCGAGA ACATCGTG-3', reverse 5'-AGGTGCCATGTAGCC GACTGT-3'; GRK 5: forward 5'-GGCAACCCGGTG ACCAAAAA CACTT-3', reverse 5'-ACCCGGCCGC GGATCAGGTCTC-3'; GRK 6: forward 5'-GAACG GGGGCGACCTCAA-3', reverse 5'-CTAGAGGCG GGTGGGGAGCCT-3') were added to each sample (2 µmol final concentration) and heated to 70 °C for 2 min. The samples were placed on ice and a 7.5 µl master mix containing 1 µl reverse transcriptase (MMLV, Gibco-BRL), 4 µl RT buffer, 1 µl dNTPs (GibcoBRL), 0.5 µl RNase inhibitor (peqlab, Erlangen, Germany), and 1 µl DTT (GibcoBRL) were added to each sample followed by incubation at 55 °C for 1 h. Finally, the product was denatured for 5 min (94 °C) and 80 µl RNase-free water were added. The RT-PCR products were purified by polyethylene glycol precipitation and then detected by agarose gel electrophoresis.

Sequence analysis

For the GRKs 2, 3 and 4 mutation detection, 14 HTNs without TSHR mutation (including the 6 HTNs without TSHR mutations used for GRK expression analysis) were analyzed. The RT-PCR was performed with oligo-dT primers using the RT-PCR protocol described above.

The cDNA of GRKs 2, 3 and 5 were amplified by PCR using the following primers: GRK 2: forward 5'-GCCCCAAGATGGCGGACCTGGA-3', reverse 5'-TCAGAGGCCGTTGGCA CTGCCG-3'; GRK 3: forward 5'-CAACATGGCGGACCTGGAGGC-3', reverse 5'-TGTCAGAGGCCGTTGCTGTTC-3'; GRK 5: forward 5'-CGACTGTCAATGGAGCTGGA-3', reverse 5'-GCCGAACTAGCTGCTTCCG-3'. The PCR was performed including 3 min of initial denaturation at 95 °C followed by 30 cycles with 30 s denaturation at 95 °C, 60 s annealing at 60 °C and 90 s elongation at 72 °C.

Additionally, one of each type of PCR corresponding to each target, was migrated on agarose gel electrophoresis and showed a unique band of the expected size. Both strands of the PCR products were directly sequenced using the following primer pairs: GRK 2: forward 5'-GCCCCGAGCCCAGCATCC-3', reverse 5'-TGGCAA AACCGTGTGAACTTATCG-3'; GRK 3: forward 5'-TCACAGCCCTTTCAGACAACATA-3', reverse 5'-ACAGTCCTTCCCCAGAGCGTAATC-3'; GRK 5: forward 5'-GAG TCCATGGCCCTCAATGAG-3', reverse 5'-TCCTTGGTCTCGTAGGCATAG-3'.

There are four splice variants of GRK 4 (Premont *et al.* 1996) and two of GRK 6 (Firsov & Elalouf 1997). Our own unpublished observations have shown an additional five splice variants of GRK 4 by RT-PCR using specific

Figure 1 Expression of GRKs in human thyroid tissue. (A) Detection of GRK mRNA in normal surrounding tissue of a hyperfunctioning thyroid nodule by RT-PCR using GRK specific primers as described in Materials and Methods. The white triangles mark the 500 bp bands. (B) Detection of GRK proteins in normal surrounding tissue of a hyperfunctioning thyroid nodule by Western blot. One hundred micrograms of the cytosolic fraction were run on a 10% polyacrylamide gel (PAGE) followed by electro-blotting onto a nitrocellulose membrane. The expression of the GRKs was determined by antibodies for GRKs 2, 3, 4, 5 and 6 followed by chemiluminescence imaging. The corresponding bands for the different GRKs are marked by arrows. The first lane of each GRK blot shows the molecular weight marker visualized by secondary antibodies and chemiluminescence imaging.

primers (manuscript in preparation). Therefore, it was not possible to screen for GRK 4 or GRK 6 mutations.

Data analysis

The distribution of the GRKs protein expression was expressed as fold GRK expression in the nodular tissue compared with the surrounding tissue. GRK expression is given as the mean $\pm$ S.E.M. of two independent experiments with duplicate measurements. cAMP values of cotransfection experiments are given as the mean $\pm$ S.E.M. of one representative experiment out of four experiments carried out in duplicate. Student's *t*-test was performed to test for statistically significant differences in GRK expression or cAMP accumulation.

Results

Expression of GRKs in human thyroid tissue

Since GRKs 1 and 7 are exclusively expressed in the retina where they regulate photo-transduction (Ferguson 2001), we only examined the expression of GRKs 2, 3, 4, 5 and 6 in the thyroid. All of the examined GRKs could be detected by RT-PCR and by Western blotting of non-nodular normal thyroid tissue (Fig. 1). Additional bands on the Western blot in the case of GRK 5 and GRK 6 represent unspecific binding of the GRK antibodies used since they could not be displaced by specific blocking peptides (data not shown).

In contrast to previous findings, we could detect GRK 3 and GRK 4 in human thyroid tissue. GRK 3 from

Figure 2 GRK expression in hyperfunctioning thyroid nodules in comparison to the surrounding tissue. Mean GRK expression in hyperfunctioning thyroid nodules (fold (nodule:st)) quantified by Western blotting followed by chemiluminescence imaging. GRKs 2 and 4 were measured as duplicates in 6 HTNs and GRK 3 was measured as duplicates in 12 HTNs in 2 independent experiments. Data are given as means $\pm$ S.E.M. of duplicate measurements; * $P < 0.05$; ** $P < 0.01$. ST/st, surrounding tissue.

human thyroid was found to have the identical molecular weight as described for other tissues. Multiple fragments of different sizes were detected by RT-PCR and Western blotting for GRK 4 (Fig. 1). Nine different splice variants of the GRK 4 could be identified by RT-PCRs (unpublished observations). However, because of the loss of the antibody consensus sequence after splicing they are mostly not identified by the antibody used.

Expression of GRK 3 and GRK 4 is increased in HTNs

HTNs are characterized by a constitutive activation of the cAMP pathway, most frequently caused by TSHR mutations (57%) and less frequently caused by $G_s\alpha$ mutations (3%) (Trulzsch *et al.* 2001). No correlation with the phenotype of HTNs could be found for the different functional properties of somatic (Arturi *et al.* 1998) or germline (Fuhrer *et al.* 1999) TSHR mutations. Defects in feedback mechanisms such as GRK-induced desensitization of the TSHR receptor are possible explanations for these differences. Therefore, we hypothesized that GRK levels are increased in HTNs due to the constitutively activated cAMP. We measured the protein expression of GRKs 2, 3 and 4 in HTNs and compared them with the corresponding

surrounding non-nodular tissue. As the antibodies used for GRKs 5 and 6 produced too many unspecific bands in the Western blots of most of the samples examined, GRKs 5 and 6 have not been quantified. In order to exclude differences in protein levels between membrane bound and cytosolic GRKs, we isolated both fractions and measured the specific GRKs by Western blotting. There were no differences in the β -actin control protein between the nodular and surrounding tissues.

The mean distribution of GRK 2 in the HTNs did not show any significant differences compared with the surrounding tissues either in the cytosol or in the membrane fraction (Fig. 2).

GRK 3 expression was increased in the cytosol in 10 out of 12 investigated HTNs compared with the cytosol of their surrounding tissues. Two HTNs showed unchanged GRK 3 expression in the cytosol. There was increased expression of GRK 3 in the membrane fraction in 8 out of 12 HTNs. Four HTNs showed unchanged GRK 3 expression in the membrane fraction. The mean GRK 3 expression in the HTNs was significantly higher in the cytosol (1.74-fold; $P < 0.001$; Fig. 2) and in the membrane fraction (1.49-fold; $P < 0.001$; Fig. 2) as compared with the surrounding tissue.

Figure 3 TSH receptor (TSHR) desensitization by coexpression of GRKs and/or β -arrestins in HEK 293 cells. The cells were cotransfected with TSHR, GRKs and/or β -arrestins as indicated. Forty-eight hours after transfection, the cells were stimulated with 100 mU TSH/ml for 1 h, and the cAMP accumulation was measured as described in Materials and Methods. cAMP accumulation is shown in pmol/well. Data are given as the mean $\pm$ S.E.M. of one representative experiment, carried out in duplicate. The experiment was repeated four times. * $P < 0.05$, ** $P < 0.01$. GRK 2 m, GRK 2 mutant.

We observed increased GRK 4 expression in the cytosol of 4 out of 6 HTNs compared with their surrounding tissues. Two HTNs showed unchanged GRK 4 expression in the cytosol. In the membrane fraction of HTNs we detected increased expression of GRK 4 in 5 out of 6 HTNs compared with their surrounding tissues. One HTN showed unchanged GRK 4 expression in the membrane fraction. The mean GRK 4 expression in the HTNs was significantly increased in the cytosol (1.52-fold; $P < 0.05$; Fig. 2) and in the membrane fraction (1.38-fold; $P < 0.05$; Fig. 2) compared with the surrounding tissue.

There were no correlations between the expression of GRKs 2, 3 or 4 and the clinical phenotype of the HTNs (including histology, size of the nodule, age of the patient, TSH, free thyroid hormones) or the molecular properties (including clonality of the tumor, TSHR mutations) (Krohn *et al.* 1999, Trulzsch *et al.* 2001). There were no differences in the expression of GRKs 2, 3 or 4 between HTNs with or without TSHR mutations.

In vitro effect of increased GRK levels on TSHR signal transduction

The involvement of GRKs 2, 3, 4 α , 5 and 6 over-expression on TSHR signaling was investigated by

cotransfection experiments of these GRKs with human TSHR in HEK 293 cells. The over-expression of GRKs in HEK 293 cells was confirmed by cotransfection of HEK 293 cells with the TSHR and the GRK containing vector or the empty vector followed by Western blot. A transfection efficiency of 40–50% was measured by FACS analysis. The TSHR cell surface expression was not affected by coexpression with the GRKs (data not shown).

The transfected GRKs exerted different effects on TSHR function. TSHR coexpression with GRK 2 significantly reduced the TSH-stimulated cAMP response to 37.0% ($P < 0.01$; Fig. 3) of the level obtained when expressing the TSHR alone. GRK 3 leads to a significant reduction in the TSH-stimulated cAMP accumulation to 51.6% ($P < 0.05$) of the level obtained when expressing the TSHR alone. Coexpression of the TSHR with GRK 5 or GRK 6 also significantly reduced the TSH-stimulated cAMP signaling to 47.9% (GRK 5, $P < 0.05$) or 38.9% (GRK 6, $P < 0.01$).

In contrast to the results obtained for GRKs 2, 3, 5 and 6, no significant effect on cAMP accumulation was detected for the coexpression of the TSHR with the kinase-deficient GRK 2 mutant, K220R, or the GRK 4 α (Fig. 3).

Modulation of the GRK effects by coexpression of β -arrestins

In addition to the functional effects seen for GRKs, cAMP signaling of the TSHR is influenced by the expression and function of β -arrestins. Because of the known over-expression of β -arrestin 2 in HTNs (Voigt *et al.* 2000), we investigated the influence of over-expressed GRKs together with β -arrestin 1 or 2 on the TSHR-induced cAMP signaling in the HEK 293 cell system. In cotransfection experiments with the TSHR and β -arrestin 1 or 2, the cAMP accumulation was significantly reduced to 37.2% (β -arrestin 1; $P < 0.05$; Fig. 3) or to 33.9% (β -arrestin 2; $P < 0.05$; Fig. 3) compared with transfection of the TSHR and the empty pcDNA vector (previously described in Voigt *et al.* 2000).

The triple transfection with TSHR, GRK 2 and β -arrestin 1 or 2 further decreased the TSH-induced cAMP accumulation to 32.9% ($P < 0.05$) or 34.2% ($P < 0.05$) respectively of the TSH-stimulated cAMP accumulation obtained without β -arrestins (Fig. 3). Similar results were obtained for GRK 3 when cotransfected with the TSHR and either β -arrestin 1 (39.2%; $P < 0.05$) or β -arrestin 2 (30.6; $P < 0.05$). Triple transfections with the TSHR and GRK 5 and either β -arrestin 1 or β -arrestin 2 reduced the TSH-stimulated cAMP accumulation to only 69.5% or 53.1% ($P < 0.05$) respectively of the level obtained without β -arrestins. Triple transfections with the TSHR and GRK 6 and either β -arrestin 1 or β -arrestin 2 reduced the TSH-stimulated cAMP accumulation to 51.7% ($P < 0.05$) or 48.4% ($P < 0.05$) respectively of the level obtained without β -arrestins. The triple transfections with the TSHR and GRK 4 α and either β -arrestin 1 or β -arrestin 2 reduced the TSH-stimulated cAMP accumulation only to the level obtained for cotransfection with the TSHR and β -arrestins. A similarly effect could be found for the kinase-deficient GRK 2 mutant, K220R.

Sequence analysis of GRKs 2, 3 and 5 cDNA from HTNs

Mutations in the TSHR gene, which lead to a constitutive activation of the cAMP system, are the most frequent molecular causes of HTNs (Trulzsch *et al.* 2001). Mutations in the GRK 1 and arrestin 1 genes, which both influence the signaling of the rhodopsin receptor, are molecular causes of Oguchi disease, a special form of stationary night blindness (Yamamoto *et al.* 1997, Cideciyan *et al.* 1998). Accordingly, mutations in the genes of GRKs could be underlying causes of HTNs.

Therefore, samples of 14 TSHR mutation negative HTNs (including the 6 samples without TSHR mutation used for expression analyses) were analyzed for mutations in the cDNAs of GRKs 2, 3 and 5 by RT-PCR and direct sequencing of the PCR products. Seven out of the fourteen samples showed a monoclonal pattern without evidence of somatic mutations determined by

X-chromosomal inactivation (Krohn *et al.* 1998), while the clonality of the other seven samples from male patients could not be determined. No mutations in the cDNA of GRKs 2, 3 and 5 were found in any of the 14 samples examined. The mutational screens of the cDNA of GRKs 4 and 6 have not been performed due to the existence of multiple splice variants (Fig. 1).

Discussion

Expression of GRKs 2, 5 and 6 has previously been demonstrated in rat and human thyroid tissue and in FRTL 5 cells (Iacovelli *et al.* 1996, Nagayama *et al.* 1996a,b, Metaye *et al.* 2002). In addition to GRKs 2, 5 and 6, we identified GRKs 3 and 4 in the human thyroid using RT-PCR and Western blot (Fig. 1). GRK 3 is known to be expressed in a broad range of tissues, whereas GRK 4 expression has mostly been limited to the testis and to a lesser amount in the kidney (Ferguson 2001). The previous lack of detection of GRKs 3 and 4 in thyroid tissue was most likely caused by species-specific differences, by the use of less specific antibodies or by the use of degenerated primers, which decrease the specificity of the RT-PCR. Moreover, Iacovelli *et al.* (1996) demonstrated TSHR desensitization by GRK 2 and β -arrestin 1. Receptor and G-protein selectivity has been demonstrated for GRK 3 (Bunemann & Hosey 1999, Penn *et al.* 2000, Ferguson 2001). GRK 4 has been shown to desensitize the follitropin (FSH) receptor (Lazari *et al.* 1999) and the luteinizing hormone/chorionic gonadotropin (LH/CG) receptor (Premont *et al.* 1996). Increased GRK 2 activity and decreased GRK 5 expression have been reported for differentiated human thyroid carcinomas (Metaye *et al.* 2002). A GRK 6-dependent desensitization of the TSH-stimulated cAMP pathway has been shown in FRTL 5 and COS 7 cells (Nagayama *et al.* 1996 a,b).

Since the cAMP system is constitutively activated in hyperfunctioning thyroid nodules, an increased expression and/or activity of the specific GRKs, which desensitize these pathways in HTNs, is therefore very likely. In line with this reasoning, an increased phosphodiesterase (PDE) activity has been found in HTNs with TSH receptor or $G_s\alpha$ protein mutations, indicating a stimulated feedback mechanism in order to reduce the constitutively activated cAMP production in these HTNs (Persani *et al.* 2000).

We found an unchanged expression of GRK 2 in HTNs compared with their surrounding tissues (Fig. 2). However, since a GRK 2-dependent TSHR desensitization was demonstrated in hyperfunctioning thyroid nodules (Iacovelli *et al.* 1996), an increased GRK 2 expression is to be expected as part of the TSHR downregulation mechanism. This apparent contradiction can most likely be explained by the previously reported increased β -arrestin 2 expression in HTNs (Voigt *et al.* 2000) which is known to support the rapid degradation of GRK 2

(Penela *et al.* 2001b). Therefore, the normal GRK 2 expression in HTNs is very likely the result of the high β -arrestin 2 expression in HTNs followed by increased GRK 2 degradation.

Furthermore, we found increased expression of GRK 3 and GRK 4 in HTNs compared with their surrounding tissues (Fig. 2). Since the rapid degradation by β -arrestin 2 was only described for GRK 2 (Penela *et al.* 2001b), the increased expression of GRK 3 and GRK 4 is very likely the result of an activated TSHR desensitization mechanism induced by the chronic stimulation of the cAMP pathway in HTNs. Since there was no difference in the expression pattern of GRKs 2, 3 and 4 between HTNs with and without TSH receptor mutations, the level of GRK expression is most likely not related to this molecular cause of HTNs.

In cotransfection experiments in HEK 293 cells with the TSHR and GRKs 2, 3, 5 or 6 we found decreased cAMP accumulations after TSH stimulation (Fig. 3). This finding supports the previously described TSHR desensitization by GRKs 2, 5 and 6 (Iacovelli *et al.* 1996, Nagayama *et al.* 1996a,b, Metaye *et al.* 2002) and further indicates the ability of GRK 3 to desensitize the TSHR. Since GRK 4 could desensitize the FSH and LH/CG receptors (Premont *et al.* 1996, Lazari *et al.* 1999), a possible involvement of GRK 4 in the desensitization of the TSHR seems very likely, given the high structural homology of TSH receptor, FSH receptor and LH/CG receptor. However, no TSHR desensitization was detected for GRK 4 α (representing the whole protein) in the HEK 293 cell system (Fig. 3). This finding is most likely due to the use of a heterologous cell system like HEK 293 that could produce results which may differ from thyroid cells because of artificial additional intracellular protein interactions or disruption of normal protein interactions (e.g. with G-protein subunits, regulators of G-protein signaling (RGS) proteins, etc.). However, since our results for GRKs 2 and 5 are in agreement with previous data (Iacovelli *et al.* 1996, Nagayama *et al.* 1996 a,b) obtained with FRTL 5 cells over-expressing GRKs 2, 5 and β -arrestin 1, the influence of the heterologous cell expression system on our results is most likely very small. Furthermore, the GRK 4 α splice variant which is used for cotransfection experiments, is most likely not able to desensitize the TSHR. Other GRK 4 splice variants may possibly be able to desensitize the TSHR. Therefore, further investigations should clarify which GRK 4 splice variants interact with the TSHR.

In addition to the previously reported TSHR desensitization by β -arrestin 1 or 2 (Voigt *et al.* 2000), we have shown that the β -arrestins together with GRKs 2, 3, 5 and 6 induce a stronger TSHR desensitization than with β -arrestins alone (Fig. 3). This finding supports the hypothesis that the TSHR desensitization by β -arrestins is amplified by phosphorylation of the receptor by these GRKs.

While more than 57% of the HTNs are caused by constitutively activating TSHR mutations and $G_s\alpha$ -protein mutations (Paschke & Ludgate 1997, Trulzsch *et al.* 2001, Krohn & Paschke 2002) the molecular etiology of nodular growth in the remaining HTNs is currently not known. Because of the similar clinical phenotype of hyperfunctioning thyroid nodules, with or without TSHR mutations, mutations in other genes influencing the cAMP pathway (e.g. GRKs) are likely.

Point mutations and deletions in the coding region of GRK 1 are a frequent molecular cause of Oguchi disease, a special form of stationary night blindness (Yamamoto *et al.* 1997, Cideciyan *et al.* 1998). Cardio-selective knockout mice heterozygous for the GRK 2 gene showed an enhanced catecholamine responsiveness due to decreased β -adrenergic receptor desensitization (Koch *et al.* 1996, Rockman *et al.* 1998). However, by direct sequencing of the cDNA of 14 HTNs without TSHR and $G_s\alpha$ mutations, no mutations could be identified in GRKs 2, 3 or 5. These data provide evidence that mutations in the coding regions of GRKs 2, 3 or 5 are unlikely to be a molecular cause of HTNs. However, mutations in the GRK 4 or GRK 6 gene could still be a cause of HTNs.

The expression and function of GRKs is also regulated through the phosphorylation by other kinases (protein kinase (PK) A, PKC, c-Src, MAP kinase) or the interaction with G-protein subunits, lipids (e.g. PIP₂), anchoring proteins (caveolin, clathrin) and calcium-binding proteins (e.g. calmodulin) (reviewed in Penela *et al.* 2003). Furthermore, β -arrestins, another part of the TSHR desensitization mechanism, are found to interact as scaffold proteins with G-protein-coupled receptors and MAP kinases to induce mitogenic pathways (McDonald *et al.* 2000, Luttrell *et al.* 2001). Defects in these mechanisms can also be considered as possible causes of HTNs.

In conclusion, GRKs 2, 3, 4, 5 and 6 are expressed in human thyroid tissue. GRKs 2, 3, 5 and 6 are able to desensitize the human TSHR in transfected HEK 293 cells. GRKs 2, 3, 5 and 6 are able to amplify the β -arrestin-induced TSHR desensitization. Somatic mutations in the genes of GRKs 2, 3 or 5 are very likely not a cause of HTNs without TSHR or $G_s\alpha$ mutations. The increased expression of GRK 3, the ability of GRK 3 to desensitize the TSHR *in vitro* and the previously described increased expression of β -arrestin 2 in HTNs (Voigt *et al.* 2000), may contribute to the desensitization of the TSHR as a negative feedback for the constitutively activated cAMP pathway in HTNs.

Acknowledgements

We thank Dr J L Benovic for providing the GRK 2, GRK 2 K220R mutant, GRKs 3, 5 and 6 and β -arrestin 1 and 2 constructs.

Funding

This work was supported by BMBF Interdisciplinary Center for Clinical Research at the University of Leipzig (01 KS 9504, project B14) and a grant from the Deutsche Forschungsgemeinschaft (DFG Ho1764/4-1).

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Received 15 January 2004

Accepted 30 March 2004

Made available online as an

Accepted Preprint 7 April 2004