Effect of mutations in the β_1-thyroid hormone receptor on the inhibition of T_3 binding by desethylamiodarone

H.C. van Beeren[a,*], O. Bakker[a], V.K.K. Chatterjee[b], W.M. Wiersinga[a]

[a] *Department of Endocrinology, Academic Medical Centre F5-171, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands*
[b] *Department of Medicine, University of Cambridge, Level 5 Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, UK*

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Abstract Desethylamiodarone (DEA) acts as a competitive inhibitor of triiodothyronine (T_3) binding to the α_1-thyroid hormone receptor ($TR\alpha_1$) but as a non-competitive inhibitor with respect to $TR\beta_1$. To gain insight into the position of the binding site of desethylamiodarone on $TR\beta_1$ we investigated the naturally occurring mutants Y321C, R429Q, P453A, P453T and the artificial mutants L421R and E457A in the ligand binding domain of human $TR\beta_1$. The IC_{50} values (in μM) of DEA for P453A (50 ± 11) and P453T (55 ± 16) mutant $TR\beta_1$ are not different from that for the wild type $TR\beta_1$ (56 ± 15), but the IC_{50} values of R429Q (32 ± 7; $P < 0.001$) and E457A (17 ± 3; $P < 0.001$) are significantly lower than of the wild type. Scatchard plots and Langmuir analyses indicate a non-competitive nature of the inhibition by DEA of T_3 binding to all four mutant $TR\beta_1$s tested. Mutants P453A and P453T do not influence overall electrostatic potential, and also do not influence the affinity for DEA compared to wild type. Mutant E457A causes a change from a negatively charged amino acid to a hydrophobic amino acid, enhancing the affinity for DEA. Mutant R429Q, located in helix 11, causes an electrostatic potential change from positive to uncharged, also resulting in greater affinity for DEA. We therefore postulate that amino acids R429 and E457 are at or close to the binding site for DEA, and that DEA does not bind in the T_3 binding pocket itself, in line with the non-competitive nature of the inhibition of T_3 binding to $TR\beta_1$ by DEA.

Key words: Desethylamiodarone; Mutant β_1-thyroid hormone receptor; Inhibition of triiodothyronine binding

1. Introduction

Amiodarone, an iodinated benzofuran derivative, is a potent antiarrhythmic and antianginal drug. It also profoundly affects peripheral thyroid hormone metabolism. The drug causes a dose-dependent decrease of serum triiodothyronine (T_3) concentrations due to diminished hepatic thyronxine (T_4) 5′-deiodination secondary to inhibition of T_4 transport across the plasma membrane [1]. It also decreases gene expression of thyroid hormone-dependent genes such as α-myosin heavy chain and the low density lipoprotein receptor [2,3]. The hypothesis that amiodarone is a thyroid hormone antagonist has been further supported by the finding that desethylamiodarone (DEA), the major metabolite of amiodarone, inhibits the binding of T_3 to its nuclear receptor. DEA acts as a competitive antagonist with respect to binding of T_3 to the thyroid hormone receptor isoform α_1 ($TR\alpha_1$) but interestingly as a non-competitive antagonist with respect to the thyroid hormone receptor isoform β_1 ($TR\beta_1$) [4,5]. Further insight into this antagonistic effect of DEA on T_3 receptor binding can be obtained by evaluating the changes in the molecular constitution of either the drug or the receptor. We have previously reported the results of competition studies with amiodarone analogues: the bulky iodine atoms, the hydrophobicity, the electric charge and the overall size of the analogues markedly influenced the nature and potency of their inhibition on T_3 receptor binding [6]. These studies, however, did not provide further information on the localization of the DEA binding site on the receptor. Therefore we studied the effect of naturally occurring and artificial mutations in $TR\beta_1$ which are known to decrease T_3 affinity (R429Q, P453A and P453T), hormone-dependent transactivation (E457A), homodimerization (R429Q) or heterodimerization (L421R, 9th heptad mutant).

2. Materials and methods

2.1. Chemicals

Non-radioactive 3,5,3′-T_3 was obtained from Henning GmbH, Berlin, Germany. [^{125}I]T_3 (spec. act. 2200 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA. DEA was a kind gift of Sanofi Recherche (Montpellier, France). All reagents were of the highest grade possible.

2.2. Receptor expression

Receptors were expressed in *Escherichia coli* as a GST fusion protein using pGEX-2TK vector (Pharmacia Biotech, Sweden) containing either wild type human TRβ1, naturally occurring mutant human $TR\beta_1$ Y321C, R429Q, P453A, P453T, or artificial mutant human $TR\beta_1$ L421R and E457A (residues 174–461)[7,8]. The cells were grown overnight in 2×YT-G medium (yeast extract 10 g/l, tryptone 16 g/l, NaCl 5 g/l and 10% (w/v) glucose) and then diluted 1:10 in prewarmed (37°C) medium until an optical density of 1–1.2 was reached. After IPTG (isopropyl-β-D-thiogalactoside) 1 mM F.D. was added the cells were grown for another 1.5 h. Cells were then lysed by sonification in PBS (2×20 s, 50 W on ice). Each receptor protein preparation was purified using glutathione-Sepharose 4B affinity resin (Pharmacia Biotech, Sweden) according to the manufacturer's instructions with the following modifications [9–11]: Triton X-100 was used in a final concentration of 0.5%, PBS used as a wash solution contained 2 mM DTT and 0.2 mM PMSF. The proteins were stored at a high concentration in incubation buffer (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, 5 mM DTT pH 7.6) in liquid nitrogen, thawed on ice and diluted to the desired concentration just before use.

2.3. T_3 receptor binding assay

The receptor proteins were incubated with [^{125}I]T_3 (10^{-11} M) for 30 min at 22°C in a shaking water bath in incubation buffer containing 0.025% Triton X-100, 0.05% BSA and 1% ethanol (v/v). These additions to the incubation buffer were necessary to solubilize DEA in a hydrophilic environment as demonstrated previously [5]. Total incubation volume was 0.5 ml. Reactions were stopped by chilling on

*Corresponding author. Fax: (31) (20) 691 7682.
E-mail: h.c.vanbeeren@amc.uva.nl

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Table 1
Inhibition of the binding of [^{125}I]T_3 to wild type and mutant human TRβ_1 by DEA

Human TRβ_1	Number of experiments	IC_{50} μM (mean ± S.D.)
Wild type TRβ_1	8	56 ± 15
Y321C mTRβ_1	B_o too low to determine IC_{50}	
L421R mTRβ_1	B_o too low to determine IC_{50}	
P453A mTRβ_1	7	50 ± 11
P453T mTRβ_1	9	55 ± 16
R429Q mTRβ_1	9	32 ± 7*
E457A mTRβ_1	4	17 ± 3*

B_o, initial binding.
*$P < 0.001$ vs. wild type, determined by *t*-test.

ice-water. Bound and unbound [^{125}I]T_3 were separated at 4°C using a small Sephadex G25 medium column (bed volume 2 ml, swollen in incubation buffer with 0.05% BSA) in a Pasteur pipette. Four 0.8 ml fractions, containing the bound hormone fraction, were collected using incubation buffer as eluent. Specific binding was determined by calculating the difference between the counts bound in the absence and presence of an excess (10^{-7} M) of non-radioactive T_3. All incubations were done in duplicate. The proteins were diluted to provide a maximal binding capacity (MBC) of the T_3 receptor preparations between 3 and 10×10^{-10} M.

The potency of DEA to inhibit the binding of T_3 to the wild type and the mutated β_1-T_3 receptors was tested over a concentration range of 10^{-7} to 10^{-4} M. DEA solubilized as a stock solution of 10^{-2} M in ethanol was incubated in various concentrations with receptor proteins and [^{125}I]T_3 as described above. In all tubes the final ethanol concentration was 1% (v/v). From these experiments the IC_{50} values of DEA for the wild type and the mutated β_1-T_3 receptors were calculated.

Scatchard analyses were performed with DEA concentrations around the IC_{50} values. Receptor proteins and [^{125}I]T_3 were incubated with increasing amounts of non-radioactive T_3 (1×10^{-10} to 33×10^{-10} M) in the absence or presence of DEA. Four to six separate experiments were done for wild type and mutant receptor proteins; in each experiment the inhibitory effect of DEA was tested at two or three concentrations. MBC and K_a values were calculated using the non-linear curve-fitting computer program Ligand (Biosoft, Cambridge, UK). Changes in MBC and K_a as a function of DEA concentration were analyzed by two-way ANOVA. Langmuir plots were prepared from the data of the Scatchard analyses.

3. Results

3.1. Inhibitory potency of DEA for wild type and mutant TRβ_1

The relative affinities for T_3 of all TRβ_1-GST fusions (wild type and mutants) are similar to results obtained before [7,8]. The IC_{50} value of DEA for the wild type human TRβ_1 is 56 ± 15 μM (Table 1), in good accordance with the figure reported for rat TRβ_1 [5]. No reliable IC_{50} values for the mutant β_1 receptors Y321C and L421R could be determined because the initial binding of [^{125}I]T_3 was too low. The IC_{50} values of DEA for the mutant receptors P453A and P453T are similar to those of the wild type, implying that there is no effect of P435 mutants, which however do influence T_3 binding, on DEA binding. The IC_{50} values for the mutant receptors R429Q and E457A are significantly lower indicating that their affinity for DEA is increased compared to wild type (Table 1, Fig. 1). The inhibitory potency (IC_{50}) of DEA on T_3 binding bears no relation to the affinity (K_a) of the different mutants for T_3 binding (Table 2).

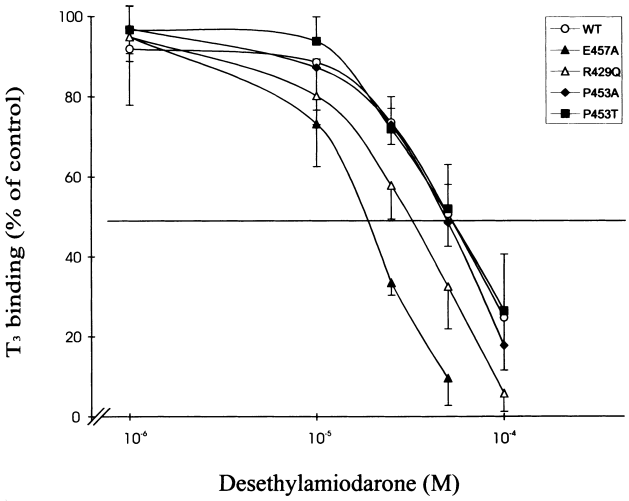

Fig. 1. Binding of T_3 to the wild type and human mutant TRβ_1. Wild type (○), R429Q (△), P453A (◆), P453T (■) and E457A (▲) were incubated with [^{125} I]T_3 in the presence of increasing concentrations DEA. Binding is expressed as a percentage of the control value without DEA. Data are presented as the mean ± S.D.

3.2. Nature of inhibition of T_3 binding by DEA on wild type and mutant TRβ_1

Representative Scatchard plots of the effect of DEA on T_3 binding to the different TRβ_1s are depicted in Fig. 2. DEA decreased both MBC and K_a in a dose-dependent manner as evident from the Scatchard plots for the binding of T_3 to the wild type and the mutant TRβ_1s (P453A, P453T and E457A) (Table 2). Langmuir plots demonstrated non-competitive inhibition by DEA for the wild type and these three mutated

Table 2
Characteristics of the inhibition of the binding of T_3 to the wild type and mutant human β_1-T_3 receptors by DEA, as evident from Scatchard plots

DEA	–	10^{-5} M	2.5×10^{-5} M	5×10^{-5} M	*P* value
MBC 10^{-10} M					
Wild type	5.86 ± 1.0		5.21 ± 0.73	4.57 ± 0.66	0.03
R429Q	5.60 ± 0.41	4.57 ± 0.59	2.96 ± 1.00		0.0003
P453A	7.47 ± 1.94		6.34 ± 1.58	5.06 ± 1.82	0.03
P453T	8.82 ± 1.24	8.78 ± 1.22	7.83 ± 1.23	6.32 ± 2.39	0.007
E457A	3.96 ± .38	2.94 ± 0.16	1.62 ± 0.30		0.002
K_a 10^9 l/M					
Wild type	1.17 ± 0.18		0.84 ± 0.11	0.56 ± 0.10	0.002
R429Q	0.99 ± 0.30	0.83 ± 0.21	0.82 ± 0.19		0.15
P453A	0.50 ± 0.09		0.40 ± 0.07	0.31 ± 0.04	0.005
P453T	0.36 ± 0.09	0.33 ± 0.05	0.27 ± 0.07	0.21 ± 0.05	0.005
E457A	1.00 ± 0.12	0.90 ± 0.15	0.72 ± 0.22		0.03

Values are given as the mean ± S.D. (wt $n = 4$; R429Q $n = 4$; P453A $n = 4$; P453T $n = 6$; E457A $n = 4$).

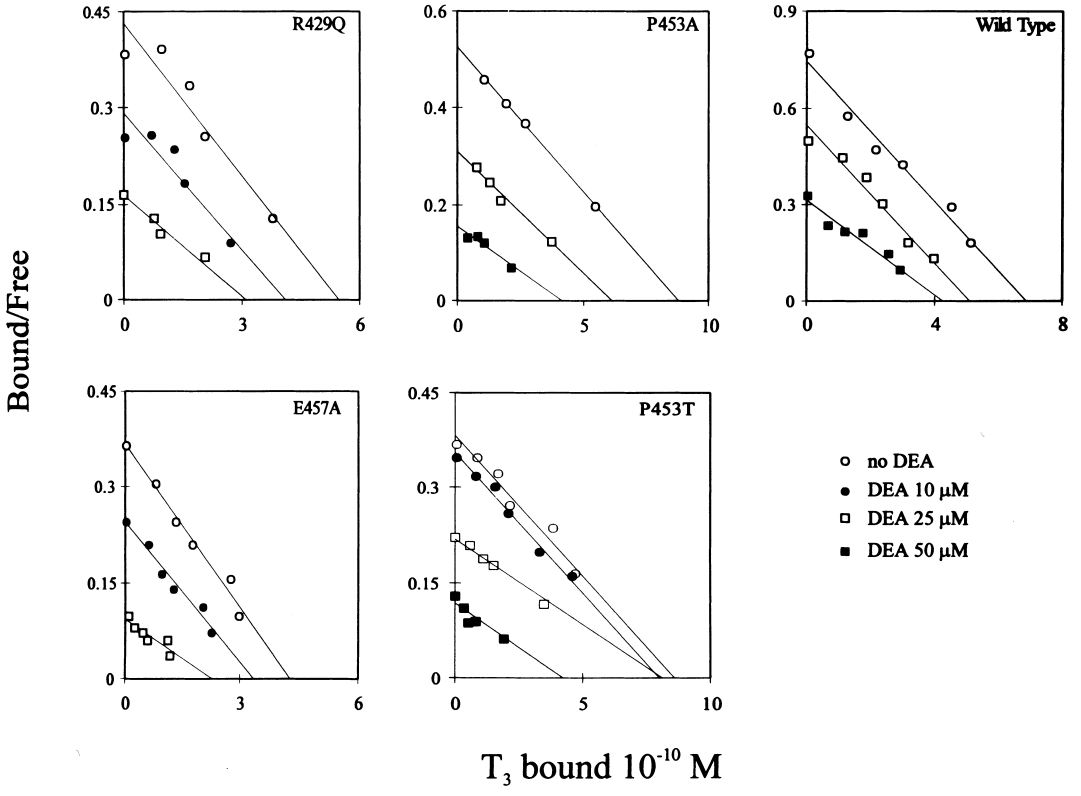

Fig. 2. Scatchard analyses of the binding of T_3 to wild type and human mutant TRβ_1 in the absence (○) or presence of DEA 10 μM (●), 25 μM (□) or 50 μM (■).

receptors. In the case of mutant R429Q, DEA decreased MBC in a dose-dependent manner, but the observed decrease in K_a did not reach statistical significance. After plotting the data in a double reciprocal plot (Langmuir plot), it was however clear that DEA is a non-competitive inhibitor for T_3 binding to this mutant as well (Fig. 3).

4. Discussion

Whereas amiodarone itself has almost no inhibitory effect on the binding of T_3 to its nuclear receptors, the major metabolite DEA does inhibit the binding of T_3 [4,5]. The results of the present study indicate that some mutations in the ligand binding domain of human TRβ_1 change the potency of DEA to inhibit T_3 binding to the thyroid hormone receptor. Whereas no change in inhibitory potency was observed for the naturally occurring mutants P453A and P453T relative to wild type TRβ_1, a stronger inhibitory potency of DEA was observed for the mutant TRβ_1s R429Q and E457A. This differential behavior of mutant TRβ_1 with respect to the antagonistic (inhibitory) effect of DEA allows us to postulate a putative location of the DEA binding site in the ligand binding domain of TRβ_1.

The amino acid sequences of the hormone binding domain of the rat TRα_1 and the human TRβ_1 show 88% identity and are identical from the start of helix 11 (H362 in rTRα_1 and H416 in hTRβ_1), except for the last three amino acids of rTRα_1 which are lacking in hTRβ_1 (Fig. 4). It is assumed that the three-dimensional structure of the hormone binding domain of rTRα_1 and hTRβ_1 is comparable [12]. Therefore the position of amino acids R429, P453 and E457 in hTRβ_1 reflects the position of R375, P399 and E403 in rTRα_1 (Fig. 4).

Inhibition of T_3 binding by DEA was non-competitive in nature for the wild type and all four mutant TRβ_1s tested, indicating that the DEA binding site on the receptor is not equivalent to the T_3 binding site of TRβ_1. The hormone appears to fit tightly in the binding pocket, and is completely buried inside the receptor molecule [12]. Its fit is such that there is no apparent extra room inside the binding pocket. This, combined with the fact that DEA is larger and that its ring structures are not perpendicular, supports the notion that the DEA binding site may be different from that of T_3.

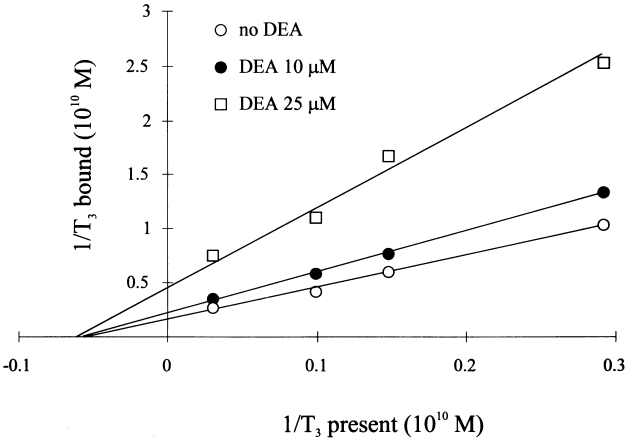

Fig. 3. Langmuir plot of the binding of T_3 to mutant TRβ_1 R429Q in the absence (○) or presence (● and □) of DEA, constructed from the data obtained in the Scatchard analysis.

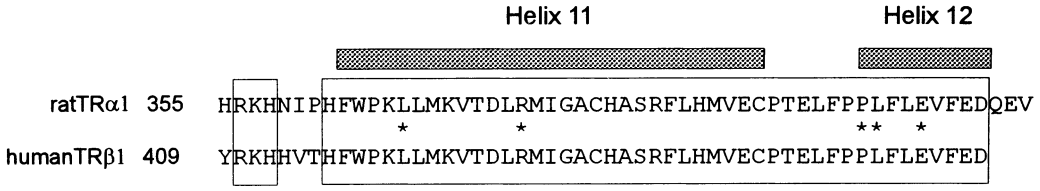

Fig. 4. Amino acid sequences of the rTRα_1 (H355–V410) and the hTRβ_1 protein (Y409–D461) from the start of helix 11 until the C-terminal end.

The results with mutant hTRβ_1 presented in this paper permit a more precise delineation of the DEA binding site. Amino acid P453 is situated at the beginning of the amphipathic helix 12 in a hydrophobic region at the outer surface of the receptor protein. The change from proline to threonine (P453T) or from proline to alanine (P453A), although decreasing T_3 binding (Table 2), does not grossly affect the hydrophobicity on the outside of the TRβ_1. The structure of the protein could differ, however, because proline is thought to be important in angle formation. There is no influence of these mutations on the affinity for DEA, suggesting that it may not be the precise receptor structure that is important for DEA binding but its surface hydrophobicity.

Amino acid E457 is a negatively charged spot within a hydrophobic surface facing outward into the solvent [12]. Mutant E457A, which may be deficient in coactivator interaction [13], gives a change from negatively charged glutamate to hydrophobic alanine, resulting in an increased inhibitory potency of DEA. This again is compatible with a greater affinity of DEA for a more hydrophobic outer surface of the TRβ_1. To further prove that hydrophobicity is important in DEA binding, 9th heptad [14] mutant L421R, also facing outward into the solvent, was tested. A decrease in inhibitory potency of DEA for this mutant was expected on the basis of the change from the hydrophobic amino acid leucine to the positively charged arginine. Unfortunately the initial binding of T_3 was too low to determine the IC_{50} values of DEA for this mutant hTRβ_1 and perform the other experiments.

In our study the ligand binding affinity of mutant R429Q was not affected but the inhibitory potency of DEA was almost two times higher compared to wild type (IC_{50} 32 μM vs. 56 μM). Despite the normal ligand binding affinity and coactivator recruitment [15] the RTH mutant R429Q [16,17] is impaired in corepressor release giving rise to increased negative transcription regulation [15]. The arginine residue 429 (TRβ_1) participates in a hydrophilic interaction with the charged pair arginine 383 (TRβ_1) and glutamic acid 311 (TRβ_1) forming a polar invagination [15]. We recently argued that (part of) the mechanism of action of DEA could be an increase in corepressor binding by the TR [18]. An interesting parallel is therefore present between the supposed mechanisms of action behind R429Q and DEA.

In conclusion, these findings lead us to postulate that amino acids R429 and E457 are at or close to the binding site for DEA. This putative DEA binding site on hTRβ_1 thus differs from the T_3 binding site, in accordance with the non-competitive nature of the inhibition of T_3 binding to TRβ_1 by DEA. The involvement of amino acids R429 and E457 in cofactor binding [13,15] combined with their apparent role in DEA binding (this study) may help to clarify the mechanism of action of the drug amiodarone.

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References

[1] Wiersinga, W.M. (1997) in: Pharmacotherapeutics of the Thyroid Gland (Weetman, A.P., and Grossman, A., Eds.), pp. 225–287, Springer-Verlag, Berlin.
[2] Paradis, P., Lambert, C. and Rouleau, J. (1991) Can. J. Physiol. Pharmacol. 69, 865–870.
[3] Hudig, F., Bakker, O. and Wiersinga, W.M. (1997) J. Endocrinol. 152, 413–421.
[4] van Beeren, H.C., Bakker, O. and Wiersinga, W.M. (1995) Mol. Cell. Endocrinol. 112, 15–19.
[5] Bakker, O., van Beeren, H.C. and Wiersinga, W.M. (1994) Endocrinology 134, 1665–1670.
[6] van Beeren, H.C., Bakker, O. and Wiersinga, W.M. (1996) Endocrinology 137, 2807–2814.
[7] Tone, Y., Collingwood, T.N., Adams, M. and Chatterjee, V.K.K. (1994) J. Biol. Chem. 269, 31157–31161.
[8] Collingwood, T.N., Adams, M., Tone, Y. and Chatterjee, V.K.K. (1994) Mol. Endocrinol. 8, 1262–1277.
[9] Guan, K.L. and Dixon, J.E. (1991) Anal. Biochem. 192, 262–267.
[10] Ball, E.H., Shephard, L.B. and Gill, G.N. (1995) Protein. Express. Purif. 6, 33–38.
[11] Yen, P.M., Sugawara, A., Liu, Y., Whang, J. and Chin, W.W. (1995) Thyroid 5, 309–313.
[12] Wagner, R.L., Apriletti, J.W., McGrath, M.E., West, B.L., Baxter, J.D. and Fletterick, R.J. (1995) Nature 378, 690–697.
[13] Collingwood, T.N., Rajanayagam, O., Adams, M., Wagner, R., Cavailles, V., Kalkhoven, E., Matthews, C., Nystrom, E., Stenlof, K., Lindstedt, G., Tisell, L., Fletterick, R.J., Parker, M.G. and Chatterjee, V.K.K. (1997) Proc. Natl. Acad. Sci. USA 94, 248–253.
[14] Forman, B.M., Yang, C., Au, M., Casanova, J., Ghysdael, J. and Samuels, H.H. (1989) Mol. Endocrinol. 3, 1626.
[15] Clifton-Bligh, R.J., de Zeger, F., Wagner, R.L., Collingwood, T.N., Francois, I., Van Helvoirt, M., Fletterick, R.J. and Chatterjee, V.K.K. (1998) Mol. Endocrinol. 12, 609–621.
[16] Flynn, T.R., Hollenberg, A.N., Cohen, O., Menke, J.B., Usala, S.J., Tollin, S., Hegarty, M.K. and Wondisford, F.E. (1994) J. Biol. Chem. 269, 32713–32716.
[17] Hayashi, Y., Weiss, R.E., Sarne, D.H., Yen, P.M., Sunthornthepvarakul, T., Marcocci, C., Chin, W.W. and Refetoff, S. (1995) J. Clin. Endocrinol. Metab. 80, 3246–3256.
[18] Bakker, O., Hudig, F., Meijssen, S. and Wiersinga, W.M. (1998) Biochem. Biophys. Res. Commun. 249, 517–521.