Proper receptor signalling in a mutant catfish gonadotropin-releasing hormone receptor lacking the highly conserved Asp^{90} residue

Marion Blomenröhr[a], Ronald Kühne[b], Eveline Hund[a], Rob Leurs[c], Jan Bogerd[a,*], Ton ter Laak[c]

[a] *Department of Experimental Zoology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[b] *Institute for Molecular Pharmacology, Robert-Roessle-Str. 10, 13125 Berlin, Germany*
[c] *Department of Pharmacochemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

Received 22 May 2001; accepted 13 June 2001

First published online 4 July 2001

Edited by Gianni Cesareni

Abstract The negatively charged side chain of an Asp residue in transmembrane domain 2 is likely to play an important role in receptor signalling since it is highly conserved in the whole family of G protein-coupled receptors, except in mammalian gonadotropin-releasing hormone (GnRH) receptors. In this paper we show that the conserved Asp^{90} of the catfish GnRH receptor can be substituted by a neutral Asn^{90} without abolishing receptor signalling if another negatively charged Glu^{93} is introduced in a proximal region of the receptor interior, thereby mimicking the Glu^{90}–Lys^{121} salt bridge of mammalian GnRH receptors.

Key words: G protein-coupled receptor; Receptor activation; Site-directed mutagenesis; Molecular model

1. Introduction

Gonadotropin-releasing hormones (GnRHs) play a central role in the biology of reproduction [1]. To exert their action, GnRHs interact with receptors, which belong to the large rhodopsin-like family of G protein-coupled receptors (GPCRs) and which transduce the GnRH signal by coupling to the phospholipase C pathway [2]. Synthetic agonists and antagonists of GnRH receptors have proven to be valuable tools in the treatment of a wide range of reproductive disorders [3]. Detailed information about the ligand–receptor complex and about the mechanism of GnRH receptor activation, in turn, will help the rational design of more efficient GnRH analogs. So far, sequence comparisons and functional effects of site-directed receptor mutants formed the basis for computational three-dimensional molecular models of GPCR–ligand complexes [4]. Direct structural information of GPCRs was restricted to the projection map of the electron density of bovine and frog rhodopsin [5]. Recently, however, the 2.8-Å resolution X-ray structure of bovine rhodopsin has been reported [6].

We have proposed three-dimensional models of the human GnRH receptor binding the superagonist D-Trp^6-GnRH and the antagonist Cetrorelix [4]. In these models, Glu^{90} in transmembrane domain 2 (TM 2) forms a salt bridge with Lys^{121} in TM 3 in the absence of a GnRH ligand. After docking the D-Trp^6-GnRH agonist into the receptor, Lys^{121} makes an additional interaction with pGlu^1 of the agonist. In contrast, in the model of Cetrorelix binding to the receptor, Lys^{121} interacts only with Glu^{90} and is not involved in binding the antagonist. Both models are in accordance with previous studies, demonstrating that the amino-terminal domain of GnRH agonists is predominantly responsible for GnRH receptor activation [7]. We hypothesized that binding of a GnRH agonist to the Lys^{121} residue of the human receptor destabilizes the interaction of Glu^{90} in TM 2 and Lys^{121} in TM 3, thereby contributing to the process of GnRH receptor activation.

Although mammalian and non-mammalian GnRH receptors can be activated by GnRHs, they have several structural differences. Table 1 shows that fish GnRH receptors have an uncharged Met residue at the position homologous to Glu^{90} in TM 2 of mammalian GnRH receptors, whereas one helical turn beneath in TM 2 the fish GnRH receptors contain a highly conserved charged Asp residue at the position corresponding to Asn^{87} in mammalian GnRH receptors. In this respect, fish GnRH receptors are more akin to all other GPCRs than to mammalian GnRH receptors. A Lys residue in TM 3, on the other hand, is conserved in all GnRH receptors at the position corresponding to the conserved Asp residue of cationic amine receptors (Table 1).

In the present study, we investigated whether we could transpose the human model of GnRH receptor activation to the catfish GnRH receptor in order to test the role of negatively charged residues in TM 2 on the process of GnRH receptor activation. For this purpose, we used site-directed mutagenesis of the Asp^{90}, Met^{93} and Lys^{124} residues generating single, double as well as triple mutant catfish GnRH receptors. All receptor constructs were tested for their ability to be expressed in the plasma membrane, to bind GnRH and to couple to the phospholipase C pathway. In addition to the existing human GnRH receptor model with D-Trp^6-GnRH docked into the binding pocket of this receptor [4], we developed a three-dimensional molecular model of the catfish GnRH receptor and docked chicken GnRH-II (cGnRH-II)

*Corresponding author. Fax: (31)-30-2532837.
E-mail address: j.bogerd@bio.uu.nl (J. Bogerd).

Abbreviations: TM, transmembrane domain; GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; cGnRH-II, chicken GnRH-II; ELISA, enzyme-linked immunosorbent assay; IP, inositol phosphate

0014-5793/01/$20.00
PII: S0014-5793(01)02647-3

into the catfish and the human GnRH receptor, respectively. Both models, which are in accordance with the recently published 2.8-Å resolution crystal structure of bovine rhodopsin [6], were used to explain the results from our present mutagenesis studies on the catfish GnRH receptor.

This comparative study in which we reconstitute receptor signalling of the $Asp^{90}Asn$ mutant catfish GnRH receptor by introducing an additional $Met^{93}Glu$ mutation one helix turn higher in TM 2 is aimed at a better understanding of the relationship between structure and function of GnRH receptors as a model of the rhodopsin-like GPCR family in general.

2. Materials and methods

2.1. Peptide

cGnRH-II ([His^5,Trp^7,Tyr^8]-GnRH) was synthesized at the Institute of Molecular Pharmacology (Berlin, Germany) by the solid-phase method.

2.2. Mutant catfish GnRH receptor constructs

Mutations in the catfish GnRH receptor cDNA insert [8] were introduced using the pALTER-1 in vitro mutagenesis system (Promega, Madison, WI, USA) according to the manufacturer's instructions. After sequence confirmation, the cDNA inserts were subcloned into pcDNA3 (Invitrogen, San Diego, CA, USA) for expression studies.

2.3. Cell culture and transfection

HEK 293T cells were cultured as described previously [9] and transiently transfected with wild-type or mutant catfish GnRH receptor cDNA (5 μg DNA/100-mm^2 dish, or 2.5 μg DNA/60-mm^2 dish) using the SuperFect transfection method (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.4. Biochemical measurements

The detection of GnRH receptor expression at the cell surface of transfected HEK 293T cells was performed by an enzyme-linked immunosorbent assay (ELISA) as described by Blomenröhr et al. [10]. Ligand binding assays were carried out on cell membranes from HEK 293T cells expressing receptors as described [11]. Total inositol phosphate (IP) was extracted and separated as described by Millar et al. [12].

2.5. Statistical analysis

All data are presented as mean ± S.E.M. of three independent experiments. Statistical analysis was performed using one-way analysis of variance and, where $P < 0.05$, followed by the Bonferroni test. A $P < 0.05$ was considered to be significant.

2.6. Computational methods

The catfish GnRH receptor model was based on Baldwin's template of the electron density structure of bovine rhodopsin [5] and built from an existing human GnRH receptor model [4] by substituting the appropriate amino acids in the transmembrane regions using the program SYBYL 6.4. Start coordinates of the docked cGnRH-II peptide were also abstracted from the human GnRH receptor model and the catfish GnRH receptor-cGnRH-II complexes were minimized with AMBER 4.IFF before running AMBER 4.1 Molecular Dynamics simulation of 500 ps in vacuo.

3. Results and discussion

3.1. A negatively charged residue in TM 2 is pivotal for receptor functioning

We hypothesized that the Asp^{90} residue of the catfish GnRH receptor, which is homologous to Asn^{87} of mammalian GnRH receptors, might take over the role of the Glu^{90} residue in TM 2 of mammalian GnRH receptors in interacting with the conserved Lys residue in TM 3. This would be in accordance with our previous study which demonstrated that Asp^{90} of the catfish GnRH receptor is critical for agonist binding [9]. In the present study, we showed that the $Asp^{90}Asn$ mutant receptor completely lacks the capacity to bind cGnRH-II (Table 2), although it is detected at the cell surface at 49% of wild-type receptor expression levels (Table 3). Recently, the Asn^{87} residue of the mouse GnRH receptor has also been reported to be involved in receptor expression [13]. The lack of GnRH binding to the $Asp^{90}Asn$ mutant catfish receptor also resulted in the inability of this receptor to respond to GnRH with an accumulation of IP (Table 3). However, the $Asp^{90}Asn/Met^{93}Glu$ mutant catfish receptor, harboring the arrangement found in TM 2 of mammalian GnRH receptors (Table 1), regained this ability (Table 3). Thus, apparently a negatively charged residue in this region of TM 2 is sufficient to preserve functionality in the catfish GnRH receptor.

3.2. Lys^{124} in TM 3 is important for GnRH binding and GnRH-induced IP accumulation

We demonstrated that the Lys^{124} residue of the catfish GnRH receptor is important for high-affinity binding of GnRH agonists, since the $Lys^{124}Met$ mutant receptor showed a 148-fold increase in its IC_{50} value for cGnRH-II compared to the wild-type receptor (Table 2). We choose to replace Lys^{124} of the catfish receptor by the flexible hydrophobic Met ($Lys^{124}Met$), thereby keeping the sterical properties of the side chain identical, but removing the strong hydrogen bond donor function of Lys^{124}. When the homologous positively charged Lys^{121} of the mouse GnRH receptor was replaced by a more bulky branched side chain ($Lys^{121}Leu$) or by a negatively charged side chain ($Lys^{121}Asp$), mouse GnRH receptor activation was totally abolished [14]. Replacement of Lys^{121} by the weak hydrogen bond donor Gln^{121} resulted in a more than three orders of magnitude higher EC_{50} value of this $Lys^{121}Gln$ mutant mouse receptor for the accumulation of IP. This increase in EC_{50} was most likely due to a decrease in receptor affinity for agonists, since variation in receptor expression could account maximally for one order of magnitude higher EC_{50} values [14]. Thus, the two homologous residues Lys^{124} of the catfish GnRH receptor and Lys^{121} of the

Table 1
Alignment of amino acid sequences in TM 2, 3 and 7 of selected members of the GPCR family

Receptor	TM2	TM3	TM7
Catfish GnRH-R	ASA **D** LV **M** TFV	CFL **K** LFA	FVF **G** NLNTCC **D** PVI
Goldfish GnRH-R A	VSA **D** LM **M** TFI	CFL **K** LFA	FVF **G** NLNTCC **D** PVI
Goldfish GnRH-R B	ASA **D** LV **M** TFV	CFL **K** LFA	FVF **G** NLNTCC **D** PVI
Rat GnRH-R	TLA **N** LL **E** TLI	SYL **K** LFS	FLF **A** FLNPCF **D** PLI
Human GnRH-R	TLA **N** LL **E** TLI	SYL **K** LFS	FLF **A** FLNPCF **D** PLI
Mouse GnRH-R	TLA **N** LL **E** TLI	SYL **K** LFS	FLF **A** FLNPCF **D** PLI
Human β_2-adrenergic R	ACA **D** LV **M** GLA	TSI **D** VLC	NWI **G** YVNSGF **N** PLI
Bovine rhodopsin	AVA **D** LF **M** VFG	GFF **A** TLG	AFF **A** KTSAVY **N** PVI

D90, M93, K124, G314 and D321 of the catfish GnRH receptor and the homologous residues in the other receptors are in bold.

Table 2
Summary of displacement binding studies

Construct	IC_{50} (log *M*)	$IC_{50\ (mutant/wt)}$
Wild-type	-8.97 ± 0.09	
$Asp^{90}Asn$	ND[a]	
$Asp^{90}Asn/Met^{93}Glu$	-7.76 ± 0.14[b]	16
$Lys^{124}Met$	-6.80 ± 0.21[b]	148
$Asp^{90}Asn/Met^{93}Glu/Lys^{124}Met$	-6.08 ± 0.31[b]	776

Results shown are mean ± S.E.M. of three independent experiments. $IC_{50\ (mutant/wt)}$ is the ratio of the IC_{50} value for a particular mutant receptor to the IC_{50} value for the wild-type receptor and indicates the fold decrease in apparent receptor affinity for cGnRH-II.
[a]ND, not detectable.
[b]Indicates a significant difference ($P < 0.05$) compared to the wild-type receptor.

mouse GnRH receptor are both important for agonist binding.

ELISA results indicate that the diminished IP response of the $Lys^{124}Met$ mutant receptor most likely results from a decrease in membrane expression (Table 3). The latter phenomenon might be due to either less efficient biosynthesis and trafficking of the receptor to the plasma membrane or to instability and degradation of expressed receptors.

3.3. Asp^{90} and Lys^{124} independently contribute to GnRH binding and GnRH-induced IP accumulation

Since our results indicate that both, Asp^{90} in TM 2 and Lys^{124} in TM 3, have a role in agonist binding and/or catfish GnRH receptor activation, a $Asp^{90}Asn/Met^{93}Glu/Lys^{124}Met$ mutant receptor construct was generated in order to study if these residues have a correlated contribution to these events. This triple mutant receptor had a 776-fold decreased affinity for cGnRH-II compared to the wild-type receptor (Table 2). This difference in affinity seems to represent an additive effect of the $Asp^{90}Asn/Met^{93}Glu$ and the $Lys^{124}Met$ mutant receptors (Table 2), indicating that these residues have not a correlated contribution to agonist binding. Moreover, the $Asp^{90}Asn/Met^{93}Glu/Lys^{124}Met$ mutant receptor was well expressed at the cell surface and yet nearly uncoupled from phospholipase C activation (Table 3). This is in contrast to the $Lys^{124}Met$ mutant receptor, which exhibited a low maximal IP response resulting most likely from the decreased level of mutant receptor expression (Table 3). We have, therefore, an additional argument that the contributions of Asp^{90} in TM 2 and Lys^{124} in TM 3 to catfish GnRH receptor activation are independent. Moreover, if Asp^{90} and Lys^{124} stabilize the inactive state of the catfish receptor via a salt bridge, the substitution of either or both residues by an uncharged residue should destabilize the salt bridge, as GnRH binding to Lys^{124} does. If this destabilization indeed contributes to the process of receptor activation, we might expect the $Asp^{90}Asn$ and the $Lys^{124}Met$ mutants to be constitutively active receptor constructs. However, basal IP levels of all mutant receptors were similar to those of the wild-type receptor (680 ± 71 dpm).

3.4. Computational models

In order to integrate the experimental observations on the mutant catfish GnRH receptors, we generated a three-dimensional molecular model of the catfish GnRH receptor. Previously we reported that the molecular model of the human GnRH receptor was in accordance with published and new data from mutagenesis studies on mammalian GnRH receptors [4]. In the human GnRH receptor model we found that the hydrogen bond donor function of Lys^{121} is in contact with the amino-terminal $pGlu^1$ of cGnRH-II (Fig. 1A). At the same time, the primary amino function of Lys^{121} makes an ionic hydrogen bond with Glu^{90} (N···O distance 2.59 Å; Fig. 1A). Since Glu^{90} is replaced by a methionine in fish GnRH receptors (Table 1; Met^{93}), we investigated whether Asp^{90} one helical winding deeper in TM 2 of the fish receptor could replace the ionic hydrogen bond acceptor function of Glu^{90} as observed in the human GnRH receptor. Molecular dynam-

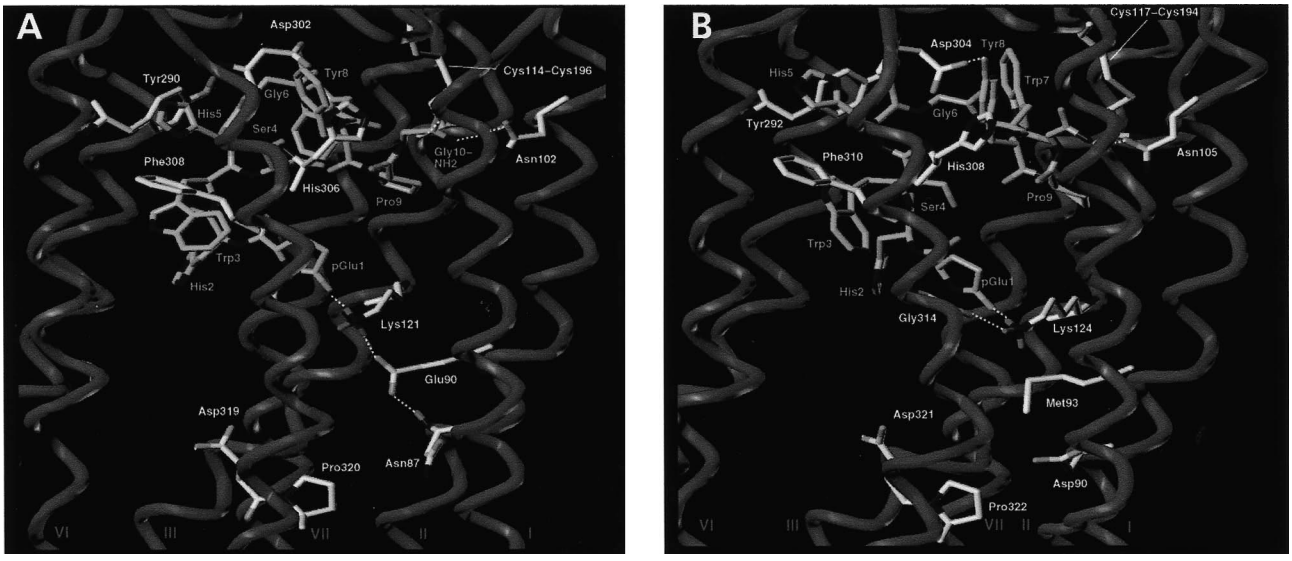

Fig. 1. Molecular models of the human (A) and the catfish (B) GnRH receptors based on Baldwin's template [5] with the cGnRH-II ligand docked into the binding pocket. Capped sticks with gray carbon atoms: cGnRH-II; tube: human and catfish GnRH receptor backbone; capped sticks with white carbon atoms: receptor side chains. For clarity, some conserved side chains (Asn^{102}/Asn^{105} in TM 2, Lys^{121}/Lys^{124} in TM 3, Asp^{319}/Asp^{321} and Pro^{320}/Pro^{322} in TM 7 in the human and catfish receptor, respectively) are shown. Tyr^8 makes a hydrogen bond with Asp^{302}/Asp^{304}, Gly^6 lays near the entrance of the receptor pocket allowing a β-turn-like conformation in cGnRH-II, the amino-terminal $pGlu^1$ interacts with Lys^{121}/Lys^{124} and the carboxy-terminal Gly^{10}-NH_2 with Asn^{102}/Asn^{105} in the first extracellular loop in agreement with experimental data (see text for details).

Table 3
Cell-surface receptor expression and cGnRH-II-induced [^{3}H]IP production

Construct	ELISA	cGnRH-II-induced [^{3}H]IP production		
	expression (% wild-type)	E_{max} (% wild-type)	EC_{50} (log M)	$EC_{50\ (mutant/wt)}$
Wild-type	100	100	-8.90 ± 0.17	
$Asp^{90}Asn$	49 ± 6[a]	ND[b]	ND[b]	
$Asp^{90}Asn/Met^{93}Glu$	159 ± 8[a]	120 ± 23	-7.69 ± 0.18[a]	16
$Lys^{124}Met$	31 ± 3[a]	41 ± 5[a]	-6.05 ± 0.04[a]	708
$Asp^{90}Asn/Met^{93}Glu/Lys^{124}Met$	100 ± 12	23 ± 5[a]	-6.05 ± 0.17[a]	708

Results shown are mean ± S.E.M. of three independent experiments. E_{max} and EC_{50} values are calculated from data received from cGnRH-II-induced [^{3}H]IP production in HEK 293T cells expressing wild-type and mutant catfish GnRH receptors. Basal [^{3}H]IP levels for wild-type and mutant receptors were similar (680 ± 71 dpm). $EC_{50\ (mutant/wt)}$ is the ratio of the EC_{50} value for a particular mutant receptor to the EC_{50} value for the wild-type receptor and indicates the fold decrease in cGnRH-II potency associated with the mutation.
[a]Indicates a significant difference ($P < 0.05$) compared to the wild-type receptor.
[b]ND, not detectable.

ics simulation (500 ps) of the catfish GnRH receptor with cGnRH-II docked in a binding mode similar to that of mGnRH or cGnRH-II in the human GnRH receptor showed that the amino group of Lys^{124} is not in contact with Asp^{90} in the catfish receptor (N···O average distance 8.16 Å), since their side chains are sterically hindered by the hydrophobic side chain of Met^{93} (Fig. 1B). These orientations of Asp^{90}, Met^{93} and Lys^{124} are in very good agreement with the relative positions of homologous residues (Table 1) Asp^{83}, Met^{86} and Ala^{117} in the recently published 2.8-Å resolution crystal structure of bovine rhodopsin [6]. Instead, Lys^{124} shows an additional hydrogen bond with the backbone of Gly^{314} in TM 7 (Fig. 1B). The latter residue is present in fish but not in mammalian GnRH receptors (Table 1). The helix of TM 7 is slightly distorted as glycine, with only a hydrogen atom as a side chain, can adopt a much wider range of conformations than other residues. Interestingly, this Gly is also present in all GPCRs in which endogenous ligands have their primary amino groups interacting with the conserved $Asp^{3.32}$ in TM 3 (see for example the β-adrenergic receptor; Table 1). One small subgroup of aminergic receptors, the muscarinic/acetylcholinergic receptors, lack the Gly at this position in TM 7, however, their ligands also do not contain a protonated primary amino group. Thus, we can speculate that Gly^{314} indeed helps to accommodate the primary amino group of Lys^{124} as observed in the molecular simulation of the catfish GnRH receptor (Fig. 1B), but that this Gly is absent in the mammalian GnRH receptors where Lys^{121} makes an ionic hydrogen bond with Glu^{90} (Fig. 1A). Experimental evidence from the relative positions of Ala^{117} and Ala^{298} in the latest crystal structure of bovine rhodopsin homologous to Lys^{124} and Gly^{314} in the catfish GnRH receptor model supports this hypothesis.

In summary, we demonstrated that the binding modes of GnRH into either the human or the catfish GnRH receptor are similar and that both receptors need a negatively charged residue in TM 2 for proper receptor functioning. However, it seems that the proposed model of GnRH receptor activation has no general validity. The results of this comparative study are useful for extending and refining an experimentally testable model of the structure of the GnRH receptor–ligand complex. The latter will certainly help elucidating the molecular dynamics of GnRH receptor activation. Thus, this study is an example as how comparative studies between receptors from evolutionary distant animal species may reveal similarities and differences in the molecular forms of hormone receptors and their mechanism of action. This is important to understand the functioning of hormone-controlled processes and formulate general principles.

Acknowledgements: We thank Dr. G.B. Willars for comments on the manuscript.

References

[1] Fink, G. (1988) in: The Physiology of Reproduction (Knobil, E. and Neill, J., Eds.), pp. 1349–1377, Raven, New York.
[2] Berridge, M.J. (1993) Nature 361, 315–325.
[3] Shulman, D.I. and Bercu, B.B. (1999) Mol. Cel. Pediatr. Endocrinol. 10, 179–189.
[4] Hoffmann, S.H., Ter Laak, A.M., Kühne, R., Beckers, T. and Beckers, T. (2000) Mol. Endocrinol. 14, 1099–1115.
[5] Baldwin, J., Schertler, G.F.X. and Unger, V.M. (1997) J. Mol. Biol. 272, 144–154.
[6] Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M. and Miyano, M. (2000) Science 289, 739–745.
[7] Sealfon, S.C., Weinstein, H. and Millar, R.P. (1997) Endocr. Rev. 18, 180–205.
[8] Tensen, C.P., Okuzawa, K., Blomenröhr, M., Rebers, F., Leurs, R., Bogerd, J., Schulz, R. and Goos, H. (1997) Eur. J. Biochem. 243, 134–140.
[9] Blomenröhr, M., Bogerd, J., Leurs, R., Schulz, R.W., Tensen, C.P., Zandbergen, M.A. and Goos, H.T. (1997) Biochem. Biophys. Res. Commun. 238, 517–522.
[10] Blomenröhr, M., Heding, A., Sellar, R., Leurs, R., Bogerd, J., Eidne, K.A. and Willars, G.B. (1999) Mol. Pharmacol. 56, 1229–1237.
[11] Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P.L. and Eidne, K.A. (1998) J. Biol. Chem. 273, 11472–11477.
[12] Millar, R.P., Davidson, J., Flanagan, C. and Wakefield, I. (1995) in: Receptor Molecular Biology (Sealfon S.C., Ed.), pp. 145–162, Academic Press, San Diego, CA.
[13] Flanagan, C.A., Zhou, W., Chi, L., Yuen, T., Rodic, V., Robertson, D., Johnson, M., Holland, P., Millar, R.P., Weinstein, H., Mitchell, R. and Sealfon, S.C. (1999) J. Biol. Chem. 274, 28880–28886.
[14] Zhou, W., Rodic, V., Kitanovic, S., Flanagan, C.A., Chi, L., Weinstein, H., Maayani, S., Millar, R.P. and Sealfon, S.C. (1995) J. Biol. Chem. 270, 18853–18857.