# Rat RAMP domains involved in adrenomedullin binding specificity

Kenji Kuwasako*, Kazuo Kitamura, Hisamitsu Onitsuka, Tomohiko Uemura, Yasuko Nagoshi, Johji Kato, Tanenao Eto

*First Department of Internal Medicine, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*

Received 28 March 2002; accepted 11 April 2002

First published online 24 April 2002

Edited by Jacques Hanoune

**Abstract When coexpressed with receptor activity-modifying protein (RAMP)2 or -3, calcitonin receptor-like receptor (CRLR) functions as an adrenomedullin (AM) receptor (CRLR/RAMP2 or -3). Coexpression of rat (r)CRLR with rRAMP deletion mutants in HEK293T cells revealed that deletion of residues 93–99 from rRAMP2 or residues 58–64 from rRAMP3 significantly inhibits high-affinity [$^{125}$I]AM binding and AM-evoked cAMP production, despite full cell surface expression of the receptor heterodimer. Apparently, these two seven-residue segments are key determinants of high-affinity agonist binding to rAM receptors and of receptor functionality. Consequently, their deletion yields peptides that are able to serve as negative regulators of AM receptor function.** 

*Key words:* Adrenomedullin; Receptor activity-modifying protein; Calcitonin receptor-like receptor

## 1. Introduction

In 1998, McLatchie et al. [1] first described three accessory proteins, termed receptor activity-modifying proteins (RAMP1, -2 and -3), which mediate translocation of calcitonin receptor-like receptor (CRLR) to the cell surface and determine its ligand specificity. Coexpression of RAMP2 or -3 with CRLR leads to both proteins being presented at the plasma membrane as an adrenomedullin (AM) receptor, while coexpression with RAMP1 allows CRLR to function as a calcitonin gene-related peptide (CGRP) receptor [1–4]. Although RAMP2 and -3 share only 30% sequence identity and differ in their tissue distributions, they generate essentially equivalent AM receptors when coexpressed with CRLR in mammalian cells [3–5].

Hilairet et al. [5] demonstrated that the specific pharmacology conferred by each RAMP is independent of the CRLR glycosylation state. Instead, they suggest RAMPs determine ligand specificity by directly contributing to the structure of the ligand-binding pocket or by allosteric modulation of the conformation of the receptor. Consistent with that idea, we recently showed that a seven-residue segment in human (h)RAMP2 (amino acids 86–92) and hRAMP3 (amino acids 59–65) is essential for high-affinity agonist binding to hAM receptors, but not for the interaction of RAMP with CRLR [6]. It was also of interest to us that each of these seven-residue segments is located between three conserved residues (Trp, Cys and Tyr) common to humans, rats and mice [7]. In this study, therefore, we used appropriate deletion mutants to examine whether the seven-residue segments situated between the three conserved residues in rat (r)RAMP2 and rRAMP3 (amino acids 93–99 and 58–64, respectively) are also necessary for high-affinity agonist binding to rAM receptors.

## 2. Materials and methods

### 2.1. Reagents and antibody

[$^{125}$I]hAM (specific activity, 2 μCi/pmol) was produced in our laboratory [8]. Human αCGRP and AM were purchased from Peptide Institute (Osaka, Japan). Mouse anti-*myc*-fluorescein isothiocyanate (FITC) antibody was from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers.

### 2.2. Plasmids

Rat CRLR [9] and RAMP2 and -3 [10] were cloned from the lungs of Wistar rats by polymerase chain reaction (PCR) using the appropriate primers and then modified to provide a consensus Kozak sequence as previously described [11]. Expression vector pCAGGS-rCRLR was constructed by cloning rCRLR cDNA into the mammalian expression vector pCAGGS/Neo [4] using the 5′ *Xho*I and 3′ *Not*I sites. In addition, a myc epitope tag (EQKLISEEDL) was ligated, in-frame, to the 5′ end of the two RAMP cDNAs and the native signal sequences were replaced with MKTILALSTYIFCLVFA [12], after which the Myc-hRAMPs were cloned into pCAGGS/Neo. Deletion mutants were created using a Quick Change kit (Stratagene) according to the manufacturer's instructions. The PCR products were all sequences using an Applied Biosystems 310 Genetic Analyzer.

### 2.3. Cell culture and DNA transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B at 37°C under a humidified atmosphere of 95% air/5% $CO_2$. For experimentation, cells were seeded into 24-well culture plates and, upon reaching 70% confluence, were transiently transfected with rCRLR and myc-rRAMPs or their mutant expression constructs using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were incubated for 3 h in 250 μl Optimem 1 medium containing 200 ng/well plasmid DNA and 2 μl/well Lipofectamine. As a control, some cells were transfected with empty vector (pCAGGS/Neo). All experiments were performed 48 h after transfection.

### 2.4. Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry was performed to assess levels of cell surface expression of myc-rRAMPs and their deletion mutants coexpressed with

*Corresponding author. Fax: (81)-985-85 6596.
*E-mail address:* kuwasako@fc.miyazaki-med.ac.jp (K. Kuwasako).

*Abbreviations:* AM, adrenomedullin; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RAMP, receptor activity-modifying protein

0014-5793/02/$22.00 
PII: S0014-5793(02)02721-7

rCRLR in HEK293T cells. Following transient transfection, the cells were harvested, washed twice with phosphate-buffered saline (PBS), resuspended in ice-cold FACS buffer [4] and incubated for 60 min at 4°C in the dark with monoclonal anti-*myc*-FITC antibody (1:500 dilution). Following two successive washes with FACS buffer, the cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter) and analyzed using EXPO 2 software (Beckman Coulter).

*2.5. Radioligand binding assays*

To assess whole-cell radioligand binding, transfected HEK293T cells in 24-well plates were washed twice with warmed PBS and incubated for 20 min at 37°C with 0.1% bovine serum albumin (BSA)/PBS to reduce endogenous AM binding, after which the remaining adherent cells were washed with ice-cold PBS. The cells were then incubated for 3 h at 4°C with 20 pM [$^{125}$I]hAM in the presence (for non-specific binding) or absence (for total binding) of the indicated concentration of unlabeled hAM in modified Krebs–Ringers–HEPES medium [4], washed twice with ice-cold PBS, and harvested with 0.5 M NaOH. In addition, competitive inhibition of [$^{125}$I]hAM binding was evaluated by determining total binding in the presence of the indicated concentrations of unlabeled hAM. The associated cellular radioactivity was measured in a γ-counter. Specific binding was defined as the difference between total binding and non-specific binding.

*2.6. cAMP measurements*

Cells were exposed to hαCGRP or hAM in Hanks' buffer containing 20 mM HEPES, 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) for 15 min at 37°C, after which the reactions were terminated by addition of lysis buffer (Amersham). The lysates were then centrifuged at 2000 rpm for 10 min at 4°C, and the cAMP contents in samples of the supernatants were assayed using a commercial enzyme immunoassay kit according to the manufacturer's (Amersham) instructions for a non-acetylation protocol.

*2.7. Statistical analysis*

Results are expressed as means ± S.E.M. of at least three independent experiments. Differences between two groups were evaluated with Student's *t*-tests; differences among multiple groups were evaluated with a one-way analysis of variance followed by Scheffe's test. Values of $P < 0.05$ were considered significant.

## 3. Results and discussion

*3.1. Cell surface expression of rRAMP deletion mutants*

We recently demonstrated that hRAMP2 and hRAMP3 each contain a seven-residue segment (amino acids 86–92 and 59–65, respectively) essential for agonist binding to hAM receptors, and that these segments are situated between three residues (Trp, Cys and Tyr) conserved among humans, rats and mice (Fig. 1) [7]. Therefore, to examine whether the corresponding seven-residue segments of rRAMP2 and rRAMP3 confer ligand-binding specificity to the rAM receptor, HEK293T cells were transiently cotransfected with rCRLR and one of two deletion mutants (rRAMP2 D93–99 or rRAMP3 D58–64), after which detailed pharmacological characterization of the rAM receptor was carried out.

We initially analyzed the cell surface expression of epitope-tagged mutants using FACS (Fig. 2). Surface immunoreactivity was detected in only 0.99 ± 0.06% of cells expressing the empty vector, which is well within the 2% limit of resolution characteristic of FACS analysis. When expressed alone, myc-rCRLR, -rRAMP2 and -rRAMP3 appeared at the surface of 8.6 ± 0.95%, 3.93 ± 0.05% and 8.47 ± 0.65% of cells, respectively. With respect to myc-rCRLR and myc-rRAMP2, this almost certainly reflects association with corresponding endogenous hRAMP2 and hCRLR, yielding AM receptors (data not shown). In similar fashion, expressed myc-rRAMP3 is known to associate with endogenous calcitonin receptor to produce amylin and CGRP receptors [13]. When myc-rRAMP2 or rRAMP3 was coexpressed with rCRLR, the frequency of cell surface immunoreactivity increased to 33.6 ± 0.93% and 27.5 ± 0.09%, respectively. In addition, coexpression of deletion mutant D93–99 or D58–64 with rCRLR led to their full expression at the cell surface in 19.1 ± 0.1% and 23.9 ± 1.78% of cells, respectively.

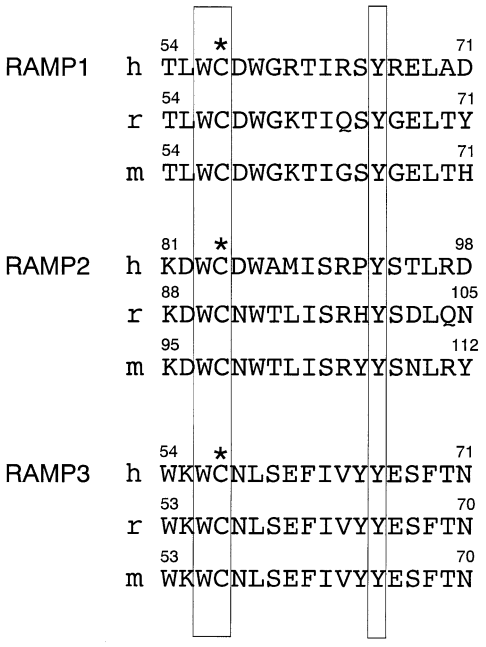


Fig. 1. Alignment of the amino acid sequences of the N-terminal domains of human (h), rat (r) and mouse (m) RAMP. Conserved amino acids are boxed; the asterisk indicates conserved cysteine residues.

*3.2. Radioligand binding to mutant receptors*

We next examined the binding of [$^{125}$I]AM to receptors comprised of rCRLR complexed with the indicated mutant (Fig. 3A). No remarkable differences were detected among cells expressing empty vector, rCRLR, rRAMP2 or rRAMP3. In cells coexpressing myc-rRAMP2 or -3 and rCRLR, the specific AM binding was about 15-fold higher than in control cells (Mock), an effect that was completely blocked by expression of the D93–99 or D58–64 mutant.

To determine whether these deletion mutants can act as dominant negative forms, we assessed the competitive inhibition of [$^{125}$I]AM binding to HEK293T cells coexpressing rCRLR with empty vector, D93–99 or D58–64 (Fig. 3B). The total AM binding in cells coexpressing D58–64 with rCRLR was significantly lower (by 16–20%) than in cells expressing rCRLR alone and approximated the level of binding seen under the Mock condition. Thus, D58–64 completely blocked the activity of endogenous RAMP2 in HEK293T cells. Coexpression of D93–99 and rCRLR elicited a similar effect, though total AM binding was only partially inhibited.

*3.3. cAMP production mediated via mutant receptors*

The functionality of AM receptors made up of rCRLR complexed with one of the deletion mutants was assessed by examining AM-evoked cAMP production in HEK293T cells coexpressing rCRLR and D93–99 or D58–64 (Fig. 4). Both AM and αCGRP elicited concentration-dependent increases

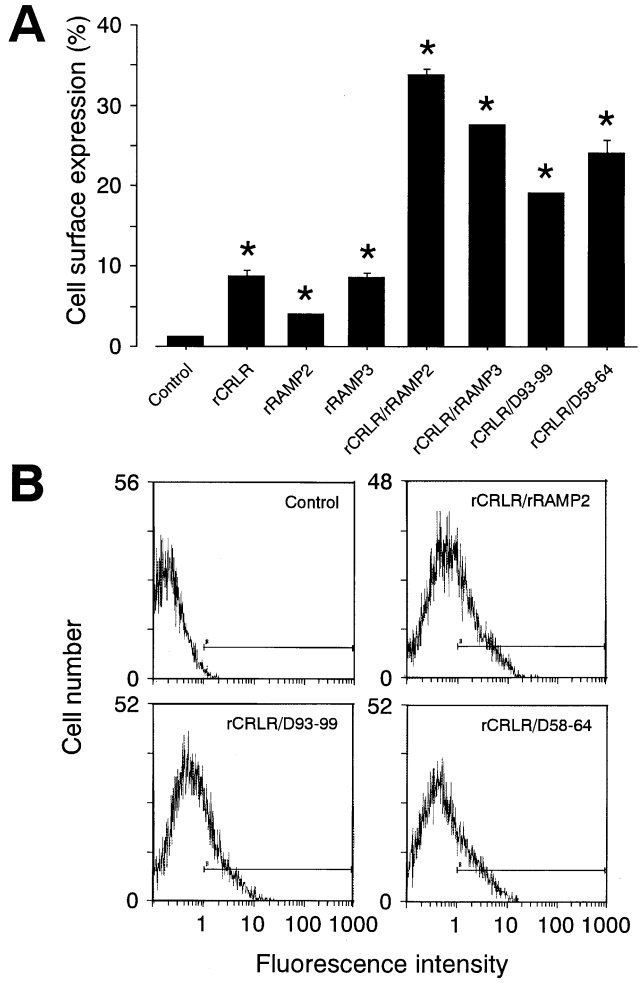


Fig. 2. FACS analysis of HEK293T cells expressing myc-rCRLR, -rRAMPs and rRAMP deletion mutants. A: Cell surface expression of the indicated myc-tagged proteins. Forty-eight hours after transfection, cells were incubated for 1 h at 4°C with monoclonal anti-*myc*-FITC antibody; mock incubation with the antibody served as the control. Cell surface expression of each construct was estimated by flow cytometry. Bars represent means ± S.E.M. of three independent experiments; $*P < 0.002$ vs. control. B: Representative results obtained from cells coexpressing rCRLR with myc-rRAMP2, -rRAMP3 or rRAMP deletion mutants D93–99 or D58–64. Cell numbers were estimated by flow cytometry. Fluorescence intensities (arbitrary units) from a total of 10 000 cells are shown in each panel; the *y*-axis indicates the absolute number of immunoreactive cells at each intensity.

in cAMP in cells expressing rCRLR and myc-rRAMP2 or -3, though they responded more selectively to AM ($EC_{50} = 1.70$ or 0.23 nM, respectively) (Fig. 4A). AM had a small effect on cells expressing empty vector, with maximal cAMP levels reaching ca. three-fold over baseline (Fig. 4B), which is indicative of the endogenous expression of hRAMP2. In cells transfected with rCRLR alone, AM elicited significant, concentration-dependent increases in cAMP (Fig. 4B). And consistent with the above-mentioned binding data (Fig. 3B), AM-evoked cAMP production was completely blocked by cotransfection of D58–64 and partially inhibited by D93–99 (Fig. 4B).

Our earlier studies showed that seven-residue segments situated between three residues conserved in both hRAMP2 and hRAMP3 (amino acids 86–92 and 59–65, respectively) are crucially involved in the agonist binding to the hAM receptors [6]. It is notable that these segments show little sequence identity, and that substituting an alanine for each of the amino acids in the RAMP2 segment caused no significant change on AM-evoked cAMP production. It thus seems unlikely that any single amino acid residue is responsible for determining the AM selectivity of the receptor, or that AM binds directly to these sequences. In the present study, expression of an rRAMP deletion mutant (D93–99 or D58–64) significantly diminished both [$^{125}$I]AM binding and AM-evoked cAMP production, despite full cell surface expression of the receptor heterodimer. Because the seven-residue segment of RAMP2, unlike that of RAMP3, is not highly conserved between human and rat (Fig. 1), it seems likely that these sequences confer selectivity by contributing to the structure of the ligand binding pocket, or perhaps through allosteric modulation of the conformation of CRLR, rather than directly binding AM.

In the same study [6], we also observed that two hRAMP2 deletion mutants (D83–85 and D93–96) failed to appear at the

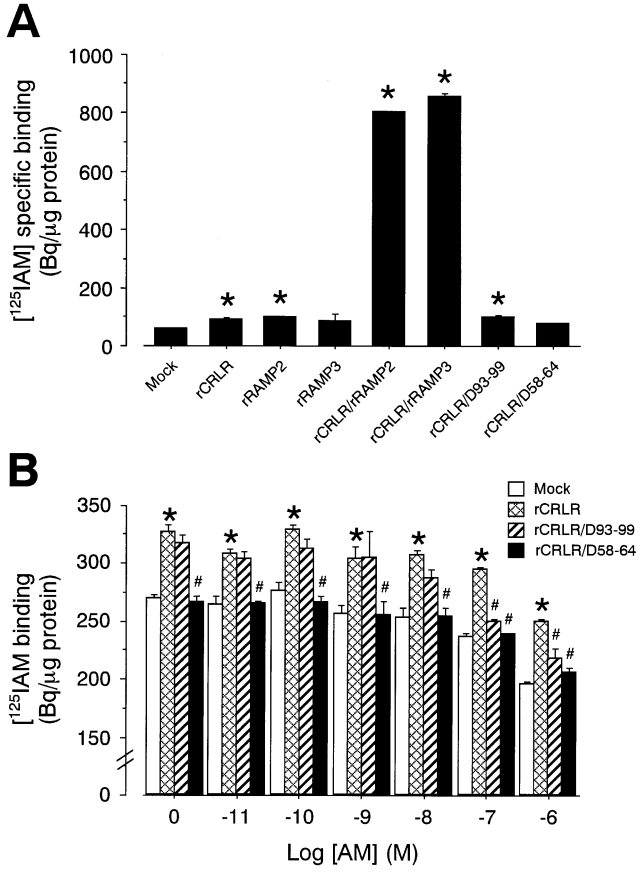


Fig. 3. Effects of rRAMP deletion mutants on [$^{125}$I]AM binding and agonist-induced cAMP production in HEK293T cells expressing rCRLR. A: Specific [$^{125}$I]AM binding. Cells were transiently transfected with the indicated myc-tagged genes and then incubated for 4 h at 4°C with 20 pM [$^{125}$I]hAM in the presence or absence of 1 μM unlabeled hAM. Non-specific binding was ~298 Bq/μg protein. The non-specific/total binding ratio in cells coexpressing rCRLR and myc-rRAMP2 or -rRAMP3 was ~30%. Bars represent means ± S.E.M. of three experiments; $*P < 0.04$ vs. Mock. B: Competitive inhibition of [$^{125}$I]hAM binding to HEK293T cells coexpressing rCRLR with empty vector (Mock), D93–99 or D58–64. All experiments were carried out using 20 pM [$^{125}$I]hAM. Bars represent means ± S.E.M. of three experiments; $*P < 0.03$ vs. Mock; $^{\#}P < 0.05$ vs. rCRLR-expressing cells.

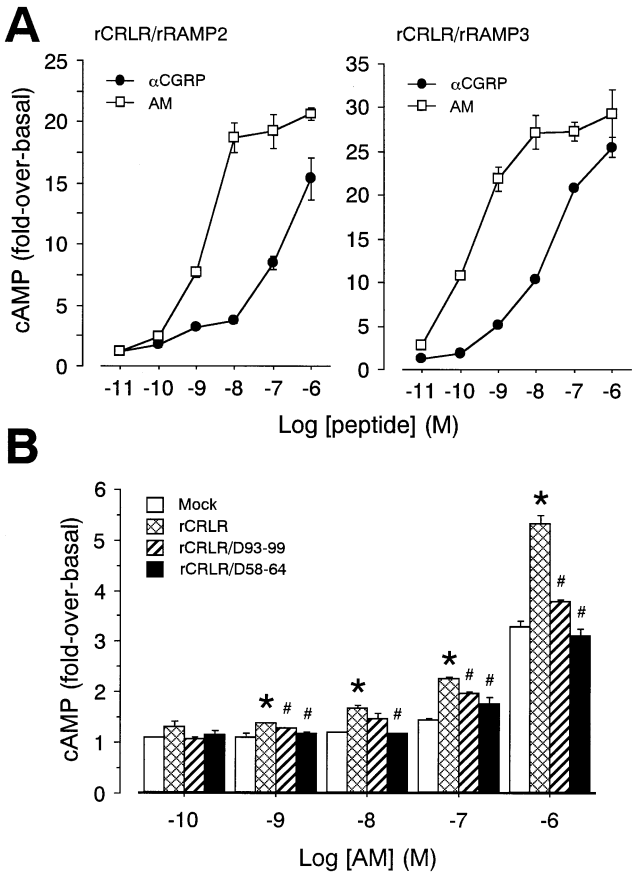


Fig. 4. Agonist-evoked cAMP production in HEK293T cells coexpressing rCRLR with empty vector, myc-rRAMP2, r-RAMP3, D93–99 or D58–64. A: Cells were transiently cotransfected with rCRLR plus myc-rRAMP2 or -rRAMP3, after which they were incubated for 15 min at 37°C with the indicated concentrations of hαCGRP or hAM and then lysed. The resultant lysates were analyzed for cAMP content. Symbols depict means ± S.E.M. of three experiments. B: Cells transiently coexpressing rCRLR with empty vector (Mock), D93–99 or D58–64 were exposed to the indicated concentrations of hAM. Bars represent means ± S.E.M. of three experiments; $^{*}P < 0.03$ vs. Mock; $^{\#}P < 0.05$ vs. rCRLR-expressing cells.

cell surface, even when coexpressed with hCRLR. That the three residues conserved in human, rat and mouse RAMPs (Trp, Cys and Tyr) were deleted in these mutants is indicative of those residues' crucial involvement in the interaction between CRLR and RAMP, or perhaps in the transport of the CRLR–RAMP complex to the cell surface.

Finally, we have shown that coexpression of rRAMP2 D93–99 or rRAMP3 D58–64 with rCRLR significantly inhibits its specific [$^{125}$I]AM binding and AM-evoked cAMP production in cells coexpressing rCRLR and endogenous hRAMP2. Like hRAMP2 D86–92 and hRAMP3 D59–65, these mutants apparently can act as negative regulators of AM receptor function. Although several studies have addressed the relative affinities of various RAMP forms for CRLR [2,14–16], additional studies will be necessary to clarify whether inhibition of receptor function by dominant negative RAMP mutants is due to competitive inhibition, formation of heterodimeric complexes or both.

## References

[1] McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G. and Foord, S.M. (1998) Nature 393, 333–339.
[2] Muff, R., Leuthauser, K., Bühlmann, N., Foord, S.M., Fischer, J.A. and Born, W. (1998) FEBS Lett. 441, 366–368.
[3] Fraser, N.J., Wise, A., Brown, J., McLatchie, L.M., Main, M.J. and Foord, S.M. (1999) Mol. Pharmacol. 55, 1054–1059.
[4] Kuwasako, K., Shimekake, Y., Masuda, M., Nakahara, K., Yoshida, T., Kitaura, M., Kitamura, K., Eto, T. and Sakata, T. (2000) J. Biol. Chem. 275, 29602–29609.
[5] Hilairet, S., Foord, S.M., Marshall, F.H. and Bouvier, M. (2001) J. Biol. Chem. 276, 29575–29581.
[6] Kuwasako, K., Kitamura, K., Ito, K., Uemura, T., Yanagita, Y., Kato, J., Sakata, T. and Eto, T. (2001) J. Biol. Chem. 276, 49459–49465.
[7] Sexton, P.M., Albiston, A., Morfis, M. and Tilakaratne, N. (2001) Cell. Signal. 13, 73–83.
[8] Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 192, 553–560.
[9] Chang, C.P., Pearse II, R.V., O'Connell, S. and Rosenfeld, M.G. (1993) Nephron 11, 1187–1195.
[10] Nagae, T., Mukoyama, M., Sugawara, A., Mori, K., Yahata, K., Kasahara, M., Suganami, T., Makino, H., Fujinaga, Y., Yoshioka, T., Tanaka, I. and Nakao, K. (2000) Biochem. Biophys. Res. Commun. 270, 89–93.
[11] Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J. and Li, Y. (1996) J. Biol. Chem. 271, 11325–11329.
[12] Guon, X.-M., Kobilka, T.S. and Kobilka, B.K. (1992) J. Biol. Chem. 267, 21995–21998.
[13] Christopoulos, G., Perry, K.J., Morfis, M., Tiakaratine, N., Gao, Y., Fraser, N.J., Main, M.J., Foord, S.M. and Sexton, P.M. (1999) Mol. Pharmacol. 56, 235–242.
[14] Foord, S.M. and Marshall, F.H. (1999) Trends Pharmacol. Sci. 20, 184–187.
[15] Husmann, K., Sexton, P.M., Fisher, J.A. and Born, W. (2000) Mol. Cell. Endocrinol. 162, 35–43.
[16] Bühlmann, N., Leuthauser, K., Muff, R., Fischer, J.A. and Born, W. (1999) Endocrinology 140, 2883–2890.