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A mutation in the second intracellular loop of the pituitary adenylate cyclase activating polypeptide type I receptor confers constitutive receptor activation

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Abstract The pituitary adenylate cyclase activating polypeptide (PACAP) type I receptor belongs to the glucagon/secretin/vasoactive intestinal polypeptide (VIP) receptor family. We mutated and deleted an amino acid residue (E261) which is located within the second intracellular loop of the rat PACAP type I receptor and which is highly conserved among the receptor family. The wild-type receptor and the mutant receptors were efficiently expressed at the surface of COS-7 cells at nearly the same level and revealed the same high affinity for the agonist PACAP-27. The cAMP contents of COS cells transfected with the E261A, E261Q, and the deletion mutant receptor were 4.6-, 5.7-, and 6.7-fold higher as compared with COS cells transfected with the wild-type receptor. Thus, all the mutant PACAP receptors were constitutively active. The data suggest that the glutamic acid in the second intracellular loop of the PACAP receptor may be a key residue to constrain the receptor in the inactive conformation with respect to its coupling to G_s proteins.

Key words: Pituitary adenylate cyclase activating polypeptide; Peptide hormone receptor; Site-directed mutagenesis; Constitutive activity; cAMP; Signal transduction

1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide originally isolated from ovine hypothalamic tissue [1]. PACAP is one of most potent stimulators of the adenylate cyclase and exists in two forms: PACAP-38 with 38 amino acids and PACAP-27 corresponding to the N-terminal 27 residues of PACAP-38. Both forms were found to be equipotent in receptor binding and adenylate cyclase assays [2]. PACAP is widely distributed in mammalians with the highest concentration in the central nervous system [3]. It is a hypophysiotropic hormone and also functions as neurotransmitter, neuromodulator and neurotrophic factor in the central nervous system [4,5].

Three PACAP receptor types have been pharmacologically characterized. The type I receptor is distributed mainly in the central nervous system and has a high affinity for PACAP, but a 1000-fold lower affinity for VIP [6]. In contrast, the type II and III receptors which are widely distributed in peripheral tissues recognize PACAP and VIP with similar affinities and are therefore identical with the VIP receptors. It has been recommended to use the terms PAC1 for type I receptor, VPAC1 for type II receptor and VPAC2 for type III receptor [7].

PACAP receptors belong to a new protein subfamily within the seven-transmembrane domain G protein coupled receptor (GPCR) superfamily [8–12]. This family, now referred to as class II GPCRs, includes, among others, receptors for secretin, glucagon, glucagon-like peptide 1, growth hormone releasing hormone, gastric inhibitory peptide, parathyroid hormone and calcitonin [13]. The receptors of this family all couple primarily to G_s to stimulate adenylate cyclase. However, the putative intracellular receptor domains which are involved in G protein interaction have not been defined. Sequence alignment analysis shows a glutamic acid in the putative center of the second intracellular loop which is highly conserved among the various receptors of the class II GPCRs (Fig. 1). This prompted us to test whether this residue plays a crucial role for G protein coupling and signal transduction. We have constructed several Glu-261 mutants of the rat PAC1 receptor and studied its functional role in G protein activation. The results show that all mutations at this position lead to constitutive active PACAP receptors. Hence, this glutamic acid may be involved in stabilizing the PAC1 receptor in an inactivated state which does not allow the receptor to bind to G_s proteins.

2. Materials and methods

2.1. Materials

^{125}I-labeled goat anti-mouse immunoglobulin G and ^{125}I-PACAP-27 were from NEN (Köln, Germany). PACAP-27 was from Novabiochem (Bad Soden, Germany). Soybean trypsin inhibitor and BSA were from Serva (Heidelberg, Germany). All other chemicals and culture media were purchased from Sigma (Deisenhofen, Germany).

2.2. Construction of mutant receptors

The construction of the expression plasmid pPACAPR was performed as previously described [14]. This plasmid encodes an amino-terminal c-myc epitope tag and the full-length rat PAC1 receptor. Oligonucleotide-directed mutagenesis was performed by in vitro mutagenesis. The *Eco*RI/*Xba*I fragment encoding the c-myc tagged full-

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Abbreviations: DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, 50% effective concentration; G protein, guanine nucleotide binding protein; GPCR, G protein coupled receptor; HBSS, Hanks' balanced salt solution; IC_{50}, median inhibitory effect; PACAP, pituitary adenylate cyclase activating polypeptide; PAC1 receptor, PACAP type I receptor; PMSF, phenylmethylsulfonyl fluoride; PTH, parathyroid hormone; PTHrP, PTH-related protein; VIP, vasoactive intestinal peptide; VPAC receptor, VIP-PACAP receptor

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length type I receptor cDNA was subcloned into the multiple cloning site of the mutagenesis vector pAlter1 (Promega). Single strand DNA was prepared. To create the mutants, the nucleotide sequence encoding the amino acid Glu, was deleted or exchanged by Ala or Gln. For this purpose, oligonucleotides which each introduce a unique restriction site were used: original coding nucleotide sequence, 5′-GAG ACC TTC TTC CCT *GAG* AGG AGA TAT TTC TAC T-3′; oligonucleotide for deletion mutation with *Arv*II sequence underlined, 5′-GAG ACC TTC TTC CCT AGG AGA TAT TTC TAC T-3′; oligonucleotide for E261A exchange with *Bss*HII sequence underlined, 5′-GAG ACC TTC TTC CCT GCG CGC AGA TAT TTC TAC T-3′; oligonucleotide for E261Q exchange with *Bsm*BI sequence underlined, 5′-GAG ACC TTC TTC CCT CAG AGA CGA TAT TTC TAC T-3′. The vectors containing the PAC1 receptor mutant constructs were transformed into DH5α using ampicillin and tetracyclin selection. The mutant cDNAs were subcloned into the *Bam*HI and *Hpa*I sites of the pPACAP plasmid and the mutations were confirmed by dideoxy DNA sequencing.

2.3. Cell culture and transient transfection

Hybridoma cells producing the anti-c-myc monoclonal antibody 9E10 were cultivated as described [14]. The hybridoma supernatant was used undiluted as antibody source for the subsequent immunodetection procedures. COS-7 cells were grown in 100 mm petri dishes or 24-well plates using DMEM containing 10% fetal calf serum at 37°C. For transient transfection of COS-7 cells, we used a modified DEAE-dextran/chloroquine method as previously described [15].

2.4. Membrane preparation

COS-7 cells were harvested about 60 h after the transfection by scraping with phosphate buffered saline (PBS) containing 1 mM EDTA. The cell pellets were suspended in buffer A (5 mM HEPES, pH 7.4, 1 mM EDTA) and homogenized using a Elvjehm homogenizer. The homogenate was centrifuged at $40\,000\times g$ for 20 min. The pellet was resuspended in buffer B (20 mM HEPES, pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 0.1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor, 5 μg/ml antipain, 5 μg/ml leupeptin and 1 μg/ml pepstatin) and was then recentrifuged. The membrane pellet was resuspended at a protein concentration of 2–5 mg/ml in buffer B. The designated membrane fragments were frozen in aliquots and stored at −70°C. Protein content was quantified by the Bradford method using BSA as a standard.

2.5. Receptor binding studies

Binding experiments were performed as previously described [16]. Briefly, competitive binding assays were carried out with membranes containing PAC1 receptors or its mutants with ^{125}I-PACAP-27 and various amounts of PACAP-27 at 25°C for 40 min in binding buffer (20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% BSA, 0.5 mg/ml bacitracin, 0.04 mg/ml soybean trypsin inhibitor, 1 mM PMSF). Non-specific binding was determined by incubation in the presence of a 1000-fold excess of unlabeled PACAP-27. This value was subtracted from total binding to yield the specific PACAP binding.

2.6. Intracellular cAMP assay

About 24 h after transfection, the COS-7 cells were transferred to 24-well culture plates ($\sim 1\times 10^5$ cells/well) and were grown for further 36 h. The cells were rinsed with assay solution (Hanks' balanced salt solution (HBSS) containing 0.1% BSA) and were incubated for 30 min at 37°C in the absence or presence of increasing concentrations of PACAP-27. A concentration of 1 mM of 3-isobutylmethylxanthine was present in the assay solution. Reactions were terminated by aspirating the medium, washing the cells with HBSS and adding 0.24 ml of ice-cold 5% trichloroacetic acid. Intracellular accumulation of cAMP was determined using the Amersham kit (TRK 432), as described previously [17].

2.7. Evaluation of receptor cell surface expression

Wild-type or mutant PAC1 receptors contain the myc epitope at the N-terminus and thus, if correctly expressed, expose the epitope at the extracellular surface of transfected COS-7 cells. The cells were rinsed three times with PBS (pH 7.4) containing 5% FBS in a 24-well plate. They were then incubated with the hybridoma supernatant of the anti-myc monoclonal antibody 9E10 at 15°C for 2 h. The cells were washed four times before adding ^{125}I-labeled goat anti-mouse immunoglobulin G (1:500). After 2 h at room temperature, the incubation was terminated by washing four times with the same buffer and the cells were lysed with 5 N NaOH. The radioactivity was counted in the gamma spectrometer.

2.8. Analysis of data

Results are expressed as means ± S.E.M. All experiments were done at least twice and each determination was done in triplicate. The LIGAND computer program (Biosoft) was used for the mathematical analysis of the binding data. Figures and curve fits were drawn using the SigmaPlot software package (Jandel Scientific).

3. Results

3.1. Construction and expression of the Glu-261 mutants of PAC1 receptor

As indicated in Fig. 1, a highly conserved glutamic acid (E261 in rat PAC1 receptor) is found within the second intracellular loop of the glucagon/secretin/VIP receptor family. To evaluate a potential interaction of this glutamic acid with G proteins, we performed site-directed mutagenesis at this position. Three mutant PAC1 receptors were created: E261 was deleted or exchanged by Gln or Ala, referred to as 'DEL', 'EQ', and 'EA' mutants. The constructs which encode PAC1 wild-type and mutant receptors were expressed in COS-7 cells. Each of the constructs encodes a myc epitope (EQLISEEDL) at the N-terminus of the receptor which was exploited to assess receptor surface expression in intact COS cells. Fig. 2 shows that the wild-type receptor as well as the three mutant

RECEPTORS	SECOND INTRACELLULAR LOOP
r(h,b,m)PACR1	...V E T F F P **E** R R Y F Y W Y T...
r(h,p)VPACR1	...A V S F F S **E** R K Y F W G Y I...
rSECR	...A I S F F S **E** R K Y L Q A F V...
hSECR	...A I S F F S **E** R K Y L Q G F V...
rabSECR	...V V S F F S **E** R K C L Q G F I...
rGLUR	...S I T T F S **E** K S F F S L Y L...
hGLUR	...G L A T C P **E** K S F F S L Y L...
mGLUR	...S L A T F S **E** K S F F S L Y L...
rGLPR	...A F S V L S **E** Q R I F K L Y L...
hGLPR	...A F S V L S **E** Q W I F R L Y V...
mGLPR	...A F S V F S **E** Q R I F K L Y L...
r(h,m,p)PTH/PTHrPR	...F M A F F S **E** K K Y L W G F T...
r(m)CTR	...V M A V F T **E** D Q R L R W Y Y...
hCTR	...V V A V F T **E** K Q R L R W Y Y...
pCTR	...V V S V F A **E** G Q R L W W Y H...
r(h)CGRPR	...V V A V F A **E** K Q H L M W Y Y...
rGIPR	...V V V R R S **E** K G H F R C Y L...
hGIPR	...V L V G G S **E** E G H F R Y Y L...
hamGIPR	...V I V G G S **E** K G H F R C Y L...
r(m)CRHR2	...V M T Y S T **E** H L R K W L F L...
hCRHR2	...V M T Y S T **E** R L R K C L F L...
achdoDHR	...V A T F T G **E** K V K L Q I Y I...
manseDHR	...V E T F T A **E** N I K L K V Y T...
r(m)CRHR	...V L T Y S T D R L R K W M F V...
r(h)PTHR2	...F V S F F S D T K Y L W G F I...
rGHRHR	...A S T S P R S K P A F W W L V...
r(m)VPACR2	...V A I L P P S R C F L A Y L L...
hVPACR2	...V A M L P P R R C F L A Y L L...

CGRP: calcitonin gene related protein, CRH: corticotrophin releasing hormone, CT: calcitonin, DH: diuretic hormone, GHRH: growth hormone releasing hormone, GIP: gastric inhibitory peptide, GLP: glucagon-like peptide 1, GLU: glucagon, PACR: pituitary adenylate cyclase activating polypeptide receptor, PTH: parathyroid hormone, PTHrP: PTH-related protein, R: receptor, SEC: secretin, VPACR: VIP-PACAP receptor, achdo: *Acheta domesticus* (house cricket), b: bovine, ham: hamst, h: human, m: mouse, manse: *Manduca sexta* (tobacco hornworm), r: rat, rab: rabbit.

Fig. 1. Sequence alignment of the second intracellular loop of class II GPCRs. The glutamic acid (E261, outlined in black) of the rat PAC1 receptor was mutated in this study.

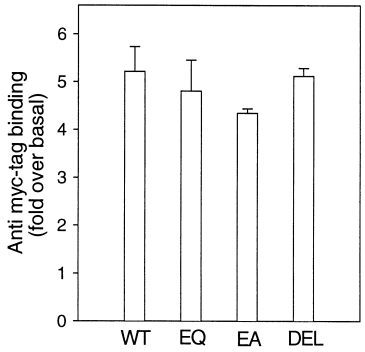

Fig. 2. Surface expression of the wild-type and mutant PAC1 receptors in transfected COS-7 cells. Anti-myc monoclonal antibody (9E10) binding to myc epitope tagged receptors was used to quantitate the levels of receptors expressed at the cell surface. Mock-transfected COS-7 cells were used as control, i.e. basal binding of the antibody. Data shown are the means ± S.D. of three measurements performed in triplicate. WT, wild-type receptor; EQ and EA, E261 substituted by Q and A; DEL, E261 deletion mutation.

receptors were efficiently expressed at nearly the same level on the surface of the cells.

3.2. Ligand binding affinity of the Glu-261 mutants of PAC1 receptor

To characterize the expressed PAC1 receptors in membranes of transfected COS cells, competitive binding experiments were performed using ^{125}I-PACAP-27 as radioligand and increasing amounts of PACAP-27 as homologous displacer. As shown in Fig. 3, the doses of unlabeled PACAP-27 required to produce half-maximal inhibition (IC_{50}) of ^{125}I-PACAP-27 binding activity were 0.79 nM for the wild-type PAC1 receptor, 0.69 nM for the EQ mutant, 0.94 nM for the EA mutant, and 0.84 nM for the DEL mutant. The statistical evaluation of the fitted data revealed that the calculated

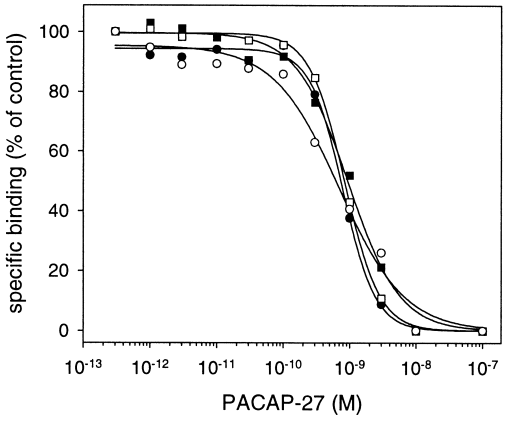

Fig. 3. Competition binding isotherms of ^{125}I-PACAP-27 by PACAP-27 in membranes of COS-7 cells transfected with the PAC1 wild-type receptor (closed circle) and the receptor mutants E261Q (open circle), E261A (closed square) and E261 deletion mutant (open square). Membranes (40 μg/ml) were incubated with 0.1 nM ^{125}I-PACAP-27 and increasing concentrations of PACAP-27 in a final volume of 0.1 ml. Binding is expressed as the percentage of specific binding obtained in the absence of the competitor PACAP-27. The IC_{50} values and slope factors of the fitted curves were as follows: 0.79 nM and 1.76 (closed circle), 0.69 nM and 0.88 (open circle), 0.94 nM and 1.12 (closed square), 0.84 nM and 1.65 (open square).

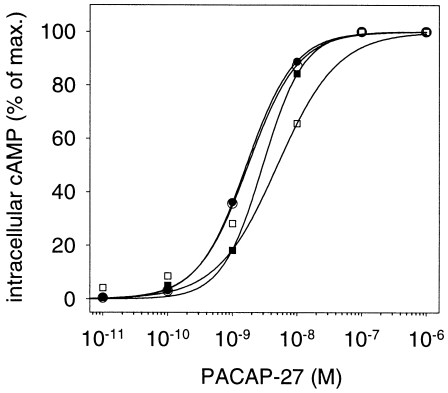

Fig. 4. Ability of PACAP-27 to increase intracellular cAMP in COS-7 cells expressing rat PAC1 wild-type (closed circle) and mutant receptors E261Q (open circle), E261A (closed square) and E261 deletion mutant (open square). The cells were incubated with increasing concentrations of PACAP-27 at 37°C for 30 min (for details see Section 2.6). The produced cAMP accumulations are presented as percentage of the maximal stimulation level. The data represent averages of three determinations. The corresponding EC_{50} values were 1.6 nM (closed circle), 1.7 nM (open circle), 2.9 nM (closed square), and 5.0 nM (open square).

IC_{50} values for the three mutant receptors were not significantly different from the IC_{50} value calculated for the wild-type PAC1 receptor. In case of the fitted data for the mutants EA and EQ, slightly shallower displacement curves (see slope factors in the legend of Fig. 3) were obtained. This prompted us to test for the potential presence of two or more affinity states in case of these two mutants. However, even in experiments with more data points, a one-site fit was always significantly better than a two- or more-site fit (not shown). Overall, these results demonstrate that the ligand binding affinities of the mutant PAC1 receptors were not significantly affected as compared to the wild-type PAC1 receptor.

3.3. Intracellular cAMP production of the Glu-261 mutants in COS cells

Next, we determined whether the E261 mutations in the second intracellular loop of the PAC1 receptor influence the stimulation of the adenylate cyclase. For that purpose, the intracellular cAMP production was measured in COS cells transfected with the wild-type or the mutant receptors following stimulation with PACAP-27 in the presence of isobutylmethylxanthine. Using 0.1 μM PACAP-27, the same maximal intracellular cAMP content was produced in COS cells expressing either wild-type or mutant PAC1 receptors. In case of the wild-type receptor, the PACAP-27-induced cAMP level was 38-fold higher than the basal, i.e. uninduced cAMP level of the cells.

Fig. 4 shows the dose–response curves for the wild-type and mutant receptors with respect to the intracellular cAMP production in dependence of various PACAP-27 concentrations. For COS cells transfected with the wild-type receptor, half-maximal stimulation (EC_{50}) of cAMP production was obtained with 1.64 nM PACAP-27. The corresponding EC_{50} values for the mutant receptors are given in the legend to Fig. 4. In COS cells transfected with the EA and DEL mutant receptors, slightly higher (1.7- and 3-fold) concentrations of PACAP-27 were necessary to achieve half-maximal stimulation of the intracellular cAMP level. Overall, the EC_{50} values (Fig. 4) for the PACAP-27 activation of the wild-type and

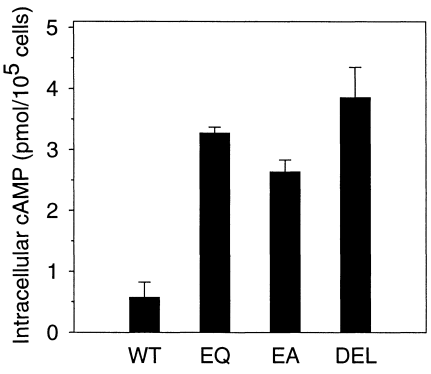

Fig. 5. Basal, i.e. ligand-independent cAMP production in COS-7 cells expressing rat PAC1 wild-type (WT) and mutant receptors E261Q (EQ), E261A (EA) and E261 deletion mutant (DEL). The cells were incubated in the absence of agonist at 37°C for 30 min (for details see Section 2.6). Each of the PAC1 receptors increased intracellular cAMP to nearly the same level when stimulated with 100 nM PACAP-27. Data are means ± S.D. of three measurements performed in triplicate.

mutant PAC1 receptors are in good agreement with the corresponding IC_{50} values (Fig. 3) that were obtained in the competition binding assays.

3.4. The Glu-261 mutants are constitutively active PAC1 receptors

While the agonist-induced activation of the mutant PAC1 receptors lead to the same level of cAMP signal transduction, an unexpected discrepant behavior of the mutants versus wild-type receptor was observed when the ligand-independent cAMP production was analyzed. The basal levels of cAMP accumulation were substantially higher in COS cells transfected with the mutant PAC1 receptors as compared with COS cells transfected with either the wild-type receptor or with mock-transfected cells. In comparison with COS cells expressing the wild-type receptor, the basal cAMP levels were found to be 5.7-, 4.6- and 6.7-fold higher in cells expressing the EQ, EA and DEL mutant receptor, respectively (Fig. 5). These observations show that each of the E261 mutations changes the PAC1 receptor into a ligand-independent constitutively active receptor.

4. Discussion

It is known that the intracellular domains of the GPCRs are involved in G protein recognition and activation [18]. For the class II GPCR family, only few studies have been performed to identify G protein interacting domains. Deletion mutants in the third intracellular loop of the glucagon-like peptide 1 receptor resulted in a dramatic reduction of agonist-stimulated cAMP production, suggesting that this region may contain key residues involved in G protein contact [19]. Differential signal transduction has been reported for the PAC1 receptor. There are two alternatively spliced exons in the PAC1 receptor gene, rat hip and hop [8] or human SV-1 and SV-2 [20]. This results in the existence of splice variants of the PAC1 receptor which differ in the length of the third intracellular loop. A hip cassette inserting in the third intracellular loop of the rat PAC1 receptor impairs adenylate cyclase stimulation and abolishes phospholipase C stimulation [8]. This suggests that the third intracellular loop is an important determinant, at least in coupling of the rat PAC1 receptor to phospholipase C activating G proteins. On the other hand, for each of the human PAC1 receptor splice variants, PACAP-27 had similar affinity and potency to stimulate either adenylate cyclase or phospholipase C [20]. Thus, further domains seem to be required for coupling of the PAC1 receptor to adenylate cyclase stimulating G_s proteins. Our data show that the mutagenesis of a glutamic acid (E261) within the second intracellular loop of the rat PAC1 receptor results in constitutively active receptors. Thus, the mutant PAC1 receptors adopted one or more conformations that enable them to bind to G_s proteins which in turn stimulate the adenylate cyclase. This suggests but does not prove that the second intracellular loop is involved in cAMP signaling. The importance of the second intracellular loop for G_s coupling has also been demonstrated for another member of the class II GPCRs, the glucagon receptor [21]. Deletion of eight residues within the putative second intracellular loop of the human glucagon receptor resulted in a protein that was well expressed showing a high affinity for glucagon but a drastically attenuated glucagon-mediated signal activation [21].

Constitutively active class II receptors resulted from histidine to arginine exchanges at a conserved position localized in the first intracellular loop of the human VIP1 (= VPAC1) [22] receptor, the glucagon receptor [23] and the PTH-PTHrP receptor [24]. A rare form of short-limbed dwarfism, the Jansen-type metaphyseal chondrodysplasia, was found to be associated with the latter constitutively active PTH-PTHrP receptor [24]. For both the VIP1 and glucagon receptor, the replacement of this histidine by residues other than arginine did not lead to constitutive receptor activity but instead to a loss of ligand binding activity and cAMP production for the VIP1 receptor [22] and to an increased binding affinity for the glucagon receptor [23]. Hence, the constitutively active receptor phenotype created by the histidine to arginine substitution is clearly different from the mutant PAC1 receptor phenotype described in this study. There is now compelling evidence that the mechanisms by which constitutive receptor activities are generated, can differ among GPCRs. In case of the α1b-adrenergic receptor, all possible mutations at a single position within the third intracellular loop confer constitutive receptor activity in conjunction with a higher affinity for agonists [25], a behavior which was first thought to be typical for constitutively active receptors. Since then, several other constitutively active receptor mutants were described which did not show a higher agonist affinity, such as the receptors for thyrotropin [26], luteinizing hormone [27], angiotensin 1A [28], or oxytocin [29].

Conclusively, in this report, we have identified the first constitutively active PAC1 receptor. The mutated glutamic acid residue is highly conserved within the second intracellular loop of class II GPCRs and may thus be of importance for many members of this receptor class. Since the glutamic acid residue is also present in the human PAC1 receptor and mutagenesis of this residue leads to a strong basal adenylate cyclase activity, humans with naturally occurring mutations at the equivalent position may have severe pathophysiological symptoms.

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