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Functional roles of conserved transmembrane prolines in the human $VPAC_1$ receptor

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Abstract The importance of three conserved transmembrane prolines of the human vasoactive intestinal polypeptide $(VPAC)_1$ receptor was examined by single alanine substitution. P266A, P300A and P348A reduced the expression level, but maintained the binding to VIP. P266A showed decreased ability to stimulate cAMP, while P300A and P348A displayed an increased potency in cAMP production combined with a high sensitivity towards GTP compared to the wild type receptor. In addition, substitutions of two conserved leucines located in position −2 and +1 from P348 were investigated. L346A and L349A reduced the receptor expression, influenced the G protein coupling and decreased the receptor activity. These observations, which are the first on conserved transmembrane prolines within this family of receptors, indicate that these residues are important for receptor expression, G protein coupling and receptor activity.

Key words: G protein-coupled receptor; Site-directed mutagenesis; Proline; Transmembrane region; Human embryonic kidney 293 cell

1. Introduction

The human vasoactive intestinal polypeptide (VIP)/pituitary adenylate cyclase activating polypeptide (PACAP) receptor 1 ($hVPAC_1R$) is a member of the Class B family (the secretin receptor family) of seven transmembrane, GTP binding protein (G protein)-coupled receptors [1]. Receptors within this family are characterized by large N-terminal extracellular domains, which major functions are binding to peptide ligands. $hVPAC_1R$ is activated by interaction with the widely distributed neuropeptides VIP and PACAP [2,3]. The $VPAC_1$ receptor binds VIP and PACAP with similar affinities and stimulates the production of cAMP via coupling to a G_s protein and stimulation of adenylate cyclase. The secretin receptor family has certain structural features in common with the large rhodopsin family (Class A), such as seven hydrophobic transmembrane helices arranged in the same anticlockwise manner (seen from the extracellular space) [4], a disulfide bridge between extracellular loop 1 and loop 2 [4,5] and few conserved positions in the transmembrane regions [4]. Therefore, it seems reasonable to postulate a kind of structural and functional similarity between these families. While there is considerable data supporting important roles of conserved prolines in TM5, TM6 and TM7 in the rhodopsin family, less is known about these residues, which are localized in TM4, TM5 and TM6, within the secretin receptor family. Although both families have conserved prolines in TM5 and TM6, the location is different. Compared to the rhodopsin family, the conserved proline of TM5 in the secretin receptor family is placed eight residues towards the extracellular space and the proline of TM6 is placed eight residues towards the intracellular space [4]. From our suggested arrangement of the seven transmembrane helices within the secretin receptor family [4], P266 is supposed to face the interhelical region between TM3, TM4 and TM5, while P300 (TM5) presumably is in close contact with TM4 near the extracellular surface and finally, P348 in TM6 may be facing the membrane bilayer in the middle of the helix.

Proline is unique among the amino acids, as its cyclic pyrrolidine ring structure prevents it from forming the normal backbone hydrogen bond of a α-helix. This property is believed to introduce a kink in the α-helical structure, which due to helix–helix interactions may contribute to a dynamic protein structure [6]. Several studies of receptors within the rhodopsin family have shown that the proline residues are involved in conformational changes occurring during receptor activation [7]. Especially, the conserved proline in TM6 participates when receptor activation changes the kink angle, from a large kink in the inactive receptor form to a significantly smaller kink in the active form [8,9]. This property of flexibility emerges as a key factor in the G protein coupling and signal transduction.

Until now, there is no information of this unique amino acid in the human $VPAC_1$ receptor or within the secretin receptor family as general. In this study we examined the role of these conserved transmembrane prolines in $hVPAC_1R$ for receptor expression, ligand binding, G protein coupling and receptor activation by site-directed mutagenesis. We substituted single prolines with alanine to introduce a neutral and small size amino acid with a helix forming proportion in contrast to the helix destabilizing property of proline. In addition and due to earlier observation concerning the importance of residues in TM6 for receptor activation, we also changed two conserved leucines in the proximity of P348 (TM6).

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Abbreviations: $hVPAC_1R$, human vasoactive intestinal polypeptide/pituitary adenylate cyclase activating polypeptide receptor 1; HEK293 cells, human embryonic kidney 293 cells; G protein, GTP binding protein

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2. Materials and methods

2.1. Materials

The cDNA encoding the human $VPAC_1$ receptor was kindly donated by Dr. A. Couvineau, Institute National de la Santé et de la Recherche Médicale, INSERM, France. VIP was purchased as custom synthesis (Bachem; Torrance, CA, USA), Peninsula Laboratories (Belmont, CA, USA). Restriction enzymes were from Medinova, Amersham and Promega. Primers were synthesized by DNA Technologies (Aarhus, Denmark). Pwo-polymerase used for PCR was purchased from Boehringer Mannheim. Human embryonic kidney 293 cells (HEK293 cells) were obtained from ATCC (American Type Culture Collection). Cell culture reagents and buffers were purchased from Life Technologies (Roskilde, Denmark) and In Vitro (Fredensborg, Denmark). The multiscreen assay system and the filter plates were from Millipore, Denmark. The cAMP radioimmunoassay kit was from Amersham Life Science.

2.2. Receptor constructs and site-directed mutagenesis

The coding region of the human $VPAC_1$ receptor cDNA was subcloned as a *Hin*dIII-*Xba*I fragment into the expression vector pcDNA3 (Invitrogen). $hVPAC_1$ receptor mutants were generated by site-directed mutagenesis made by overlap extension method [10] using two unique restriction sites *Pfl*MI–*Sse8387*I (P300A, L346A, P348A and L349A) and *Bsu*36I–*Nhe*I (P266A) to introduce the mutated segment. In several of the mutant primers a silent mutation was introduced, which either includes or excludes a restriction enzyme site. Identification of all the mutants was made after the first transformation step by restriction enzyme digestion and later confirmed by dideoxy chain termination DNA sequencing.

2.3. Cell culture and transient transfection

HEK293 cells were grown in MEM – minimum essential medium (with Earl's salts and without glutamine), supplemented with 10% fetal calf serum, 2 mM glutamine and 1‰ gentamicin or penicillin/streptomycin. HEK293 cells were plated in 200 mm dishes (4×10^6 cells/dish) and transiently transfected with $hVPAC_1R$ cDNA encoding wild type/mutant receptors, using the calcium-phosphate precipitation method. Control plates were made by transfecting HEK293 cells with 5 μg of vector DNA (pcDNA3)/200 mm plate. Wild type and mutant producing cells were made by transfection with 2–10 μg of cDNA/200 mm plate. Cells were harvested 72 h after transfection. Transfection efficiency was estimated by co-transfection of all the mutants/wild type with cDNA encoding β-galactosidase. The amount of β-galactosidase was defined as unit β-galactosidase/mg protein. The protein concentration was estimated by the Bradford method (Bio-Rad).

2.4. Preparation of plasma membranes

Cells from a confluent 200 mm culture plate were rinsed with phosphate buffered saline (PBS) and scraped off in the same buffer for centrifugation. After centrifugation, the supernatant was resuspended in 15 ml binding buffer containing 20 mM HEPES, 2 mM $CaCl_2$, 1.5 mM NaCl, 5 mM EDTA and 1 mg/ml bacitracin. The cell suspension was homogenized with a polythron for 30 s, centrifuged at $20\,000\times g$ for 20 min and resuspended in 15 ml binding buffer. The procedure was repeated once and the membranes were resuspended in binding buffer and stored at −80°C.

2.5. Binding assay

The binding affinities of wild type and mutant $hVPAC_1$ receptors were analyzed by [^{125}I]VIP binding to plasma membranes from transfected cells. VIP was labeled and purified as described by Martin et al. [11]. Competitive binding analysis, with a constant amount of radioiodinated peptide (10^{-10} M) and increasing amount of non-radiolabeled peptide (0–10^{-6} M) was set up in order to determine the total number of binding sites (B_{max}) and the binding affinity, represented by the inhibitory constant, IC_{50}. The binding assay was performed using a multiscreen assay system with 0.45 μm surfactant-free mixed cellulose, ester membranes. The membrane plates were preincubated with 200 μl 0.5% polyethyleneimine (PEI) for a minimum of 3 h at 4°C. After preincubation, the coating solution was removed by filtration and the plates were washed three times with ice cold binding buffer. Binding was performed using 100 μl membrane preparation (10–100 μg membrane protein), VIP tracer and increasing concentrations of unlabeled VIP in a total volume of 150 μl, and were incubated for 60 min at room temperature under gentle mixing. The binding assay was terminated by applying vacuum to the filter plates and washed with ice cold binding buffer. The filters were dried, punched out and the bound radioactivity was quantified in a γ-counter. To determine the effect of GTP on VIP binding, membranes were incubated with GppNHp or GTPγS in a total concentration of 10 μM and with [^{125}I]VIP and non-radiolabeled VIP as mentioned above.

2.6. Biological activity

The functional properties of wild type and mutant receptors were investigated by intracellular cAMP measurements using a [^{125}I]cAMP assay kit. HEK293 cells were transfected with the human $VPAC_1$ receptor and seeded to 2×10^5 cells/well in 24 well culture dishes coated with poly-D-lysine 48 h after transfection. Cells were washed after further 24 h with Dulbecco's PBS medium (DPBS) and incubated with 500 μl (DPBS)-medium containing 0.1 mM isobutyl-methylxanthine (IBMX) for 15 min at 37°C and for a further 20 min at 37°C with VIP in increasing concentrations (0–10^{-6} M). cAMP was extracted by incubating the cells with 50 μl trichloroacetic acid (10%) and neutralized by 50 μl 0.8 M Tris-base. The generation of standard curves and the measurement of cAMP levels in supernatant of cell lysates were performed following the manual from Amersham Life Science. All the mutants had a basal cAMP level comparable with the wild type receptor, excluding detectable changes in constitutive activity. HEK293 cells transfected with pcDNA3 without receptor insert did not show any VIP-stimulated cAMP production, indicating absence of endogenous VIP-sensitive receptors.

2.7. Data analysis

This study is a comparison of wild type and alanine substitutions, investigated under the same assay conditions. The apparent binding affinity was measured as IC_{50}, while the effect of a particular mutation is presented relative to wild type, i.e. IC_{50}(mutant)/IC_{50}(wild type). To obtain the actual IC_{50}, the binding curves were fitted to the following four parameter, one step equation: $Y=A+(B-A)/((1+X/IC_{50})^D)$, where A is non-specific binding, B is the maximum binding, D is the slope of the curve and X is log VIP(M). The VIP-induced cAMP production was measured as EC_{50}, while the functional effect of a particular mutation is presented relative to wild type, i.e. EC_{50}(mutant)/EC_{50}(wild type). The cAMP curves were fitted to the same equation as the binding curves to obtain the actual EC_{50} value, but with A as the basal level of produced cAMP and B as the maximum level of produced cAMP. All observations were repeated at least four times in independent experiments.

B_{max} was calculated according to the equation presented by Akera and Cheng [12]. Due to transient transfection, the transfection efficiency varied from experiment to experiment. Therefore the average

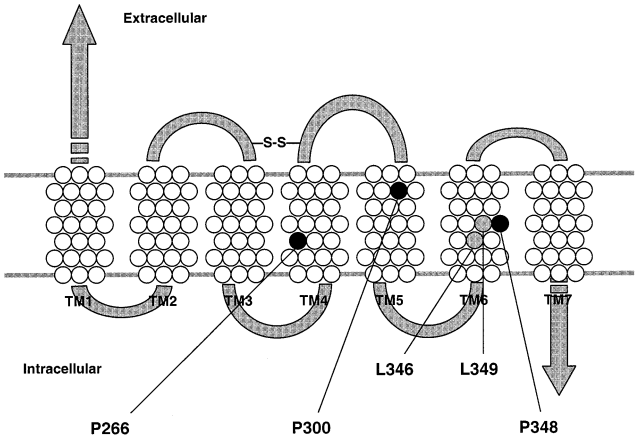

Fig. 1. Schematic secondary topology model of the human $VPAC_1$ receptor. The investigated residues of transmembrane helices TM4, TM5 and TM6 are indicated as black circles (P266, P300 and P348) and gray circles (L346, L349) with projection to letters in bold below the model. All helices are defined to have a length of 24 residues and residue numbers were set by determining the center of each helix in the lipid membrane as previously described [4].

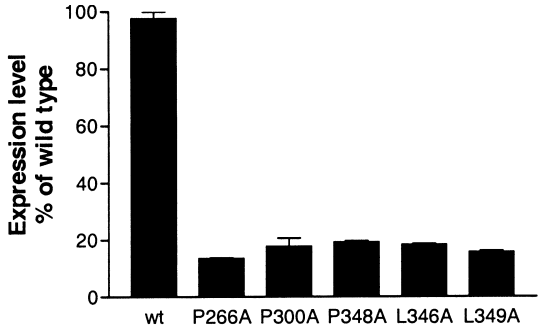

Fig. 2. Receptor expression, B_{max}, of wild type and mutant receptors. The expression level of the wild type receptor was normalized to 100%, while all the mutant data were presented relative to this value. Data points represent the mean ± S.E.M. of four or five experiments.

B_{max} value for the wild type receptor was normalized to 1 for each transfection, and the values for all the mutants are presented as fractions of wild type.

3. Results

To elucidate the role of conserved transmembrane prolines of the $hVPAC_1$ receptor (for model see Fig. 1), three prolines (P266, P300 and P348) located in TM4, TM5 and TM6, respectively, were substituted individually with alanine.

The expression levels of all the selected positions were reduced to 15–20% of the wild type receptor expression (Fig. 2). None of the investigated mutants did affect the VIP binding, but several of them disturbed the G protein coupling and receptor activity. By adjusting the amount of DNA in the transfection step, it was possible to obtain a similar expression level (2–3 pmol/mg protein) for all the investigated mutants and the wild type receptor. Thereby it became possible to

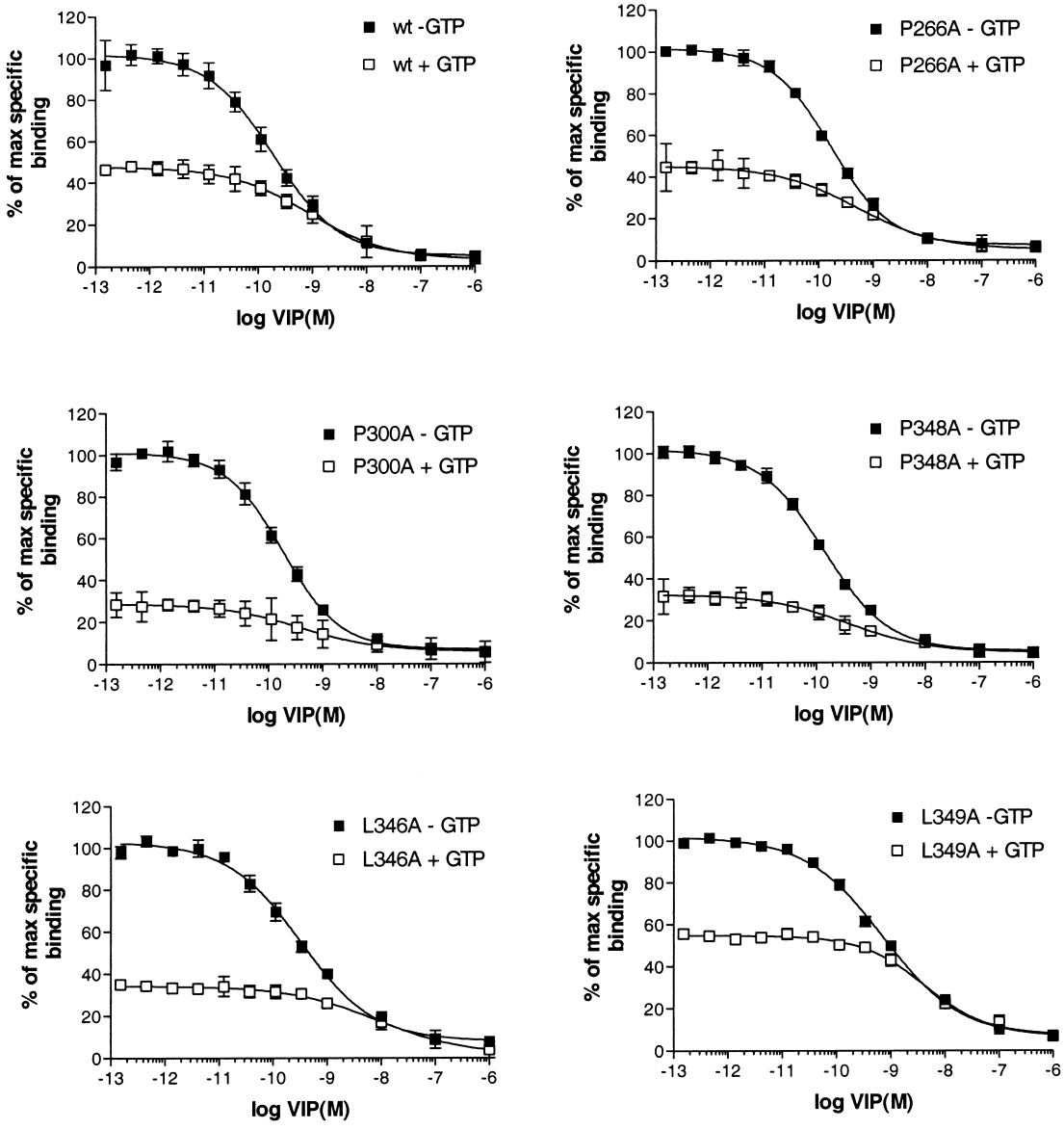

Fig. 3. Effects of GTPγS on [^{125}I]VIP binding to wild type and mutant $VPAC_1$ receptors. Binding to membranes prepared from transient transfected HEK293 cells expressing wild type and mutant receptors in the absence or presence of GTPγS as described under Section 2.

Table 1
Binding (IC_{50}) and functional (EC_{50}) characteristics of wild type and P266A, P300A, P348A, L346A and L349A mutant receptors transiently expressed in HEK293 cells

Amino acid substitution	Localization	IC_{50} (relative to wild type)	EC_{50} (relative to wild type)
Wild type	–	1.0	1.0
P266A	TM4	2.8 ± 1.0	24 ± 5
P300A	TM5	1.8 ± 0.8	0.3 ± 0.2
P348A	TM6	3.1 ± 0.2	0.1 ± 0.2
L346A	TM6	1.7 ± 0.1	20 ± 2
L349A	TM6	3.3 ± 0.2	104 ± 11

Data are means ± S.E.M. of four or five independent experiments.

compare the functional data, as it is well known that the expression level influences the receptor activity [20]. The functionality was measured by VIP-mediated cAMP production in transiently transfected HEK293 cells. P300A and P348A displayed a 3 and 10 fold increased ability, respectively, while P266A, L346A and L349A decreased the potency by 24, 20 and 100 fold, respectively (Table 1). As a consequence of these results, the ability of the investigated mutant receptors to interact with G proteins was evaluated by measuring the effect of GTPγS on [^{125}I]VIP binding to cell membranes. Addition of GTPγS reduced the total agonist binding of the wild type receptor with almost 50% and thereby shifted the binding affinity from high affinity binding to low affinity binding, i.e. an uncoupling of the G protein. The GTP sensitivity of P300A and P348A was increased compared to the wild type, showing a total binding reduction of 70% (Fig. 3), consistent with the improved ability of these mutants to mediate cAMP production (Table 1). L349A was less affected by GTP treatment, showing a reduction of about 40% (Fig. 3), which agreed with the impaired cAMP activity (Table 1). Addition of GTPγS to L346A and P266A had almost similar effects as on the wild type receptor (Fig. 3).

4. Discussion

The functional role of three conserved prolines in the transmembrane helices of the human VIP/PACAP receptor 1 ($hVPAC_1R$) was investigated in terms of their surface expression, VIP binding, G protein coupling and VIP-induced activation. Mutations at the three positions had only minor influence on the binding affinity, but the signaling properties were significantly altered.

P266A, P300A and P348A reduced the receptor expression markedly, which is in agreement with studies on G protein coupled receptors within the Class A family [13–15]. The mechanism behind the impaired receptor expression of the mutants remains to be clarified, but for other receptors it has been suggested to be due to receptor misfolding in the endoplasmic reticulum and the regulation of this process by chaperones [14].

Concerning the VIP-stimulated cAMP production, P266A reduced the receptor activity by ~20 fold, suggesting a destabilizing effect on the receptor conformation. P266 is placed in a conserved motif (GWGXP), where it could be involved in a kink formation. A similar pattern has been described for the voltage-gated channel forming peptides alamethicin and melittin, where the motifs G-X-X-P and G-X-P, respectively, are found within the central portion of the helix. Alanine substitutions show that the high amplitude bending motion for alamethicin can be attributed to this motif [16].

Contrary to P266A, P300A and P348A increased the activity of cAMP production corresponding to an increase in the rate constant for shifting $R \rightarrow R^*$, i.e. P300A and P348A stabilize the high affinity receptor conformation. One interpretation could be that these prolines have a constraining role in the receptor by preventing an activation and interaction with the G protein. The experiment with addition of GTP analogues support this notion, since the GTP sensitivity of P300A and P348A were increased compared to the wild type receptor. P300 and P348 could have a structural stabilizing effect involved in the G protein coupling mechanism, which for P348 (TM6) correlate to studies of receptors within the rhodopsin family [7,9]. For further investigation, we substituted two conserved leucines, localized in position −2 and +1 from P348, with alanine. The results of L346A and L349A seem to support the importance of the area surrounding P348, when both positions affected the ligand-mediated receptor activation. Based on our suggested arrangement of the seven transmembrane helices [4], P348 is supposed to face the lipid bilayer while L346 and L349 are oriented towards TM7 and probably influenced by the bulky pyrrolidine ring of proline. Thus, L346 and L349 could have a central role in helix–helix interaction between TM6 and TM7.

Several studies within the rhodopsin family have shown transmembrane prolines to play important roles in receptor expression, ligand binding, helix flexibility, receptor activity and G protein coupling. However, it is very difficult to draw parallels to the secretin receptor family when the pattern of sequences is different, i.e. the conserved prolines in Class A receptors are in TM5, TM6 and TM7, while conserved prolines within the secretin receptor family (Class B) are localized in TM4, TM5 and TM6. [13,17–19].

In summary, this study describes the importance of conserved transmembrane prolines in receptor expression, ligand binding, G protein coupling and receptor activation within the $hVPAC_1$ receptor. The impaired activity of P266A connected to the reduced receptor expression and improved receptor activity of P300A and P348A suggest that these prolines are essential determinants in several aspects of the activating mechanism.

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