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Asn^{229} in the third helix of VPAC$_1$ receptor is essential for receptor activation but not for receptor phosphorylation and internalization: Comparison with Asn^{216} in VPAC$_2$ receptor

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Abstract

After stimulation with agonist, G protein coupled receptors (GPCR) undergo conformational changes that allow activation of G proteins to transduce the signal, followed by phosphorylation by kinases and arrestin binding to promote receptor internalization. Actual paradigm, based on a study of GPCR-A/rhodopsin family, suggests that a network of interactions between conserved residues located in transmembrane (TM) domains (mainly TM3, TM6 and TM7) is involved in the molecular switch leading to GPCR activation.

We evaluated in CHO cells expressing the VPAC$_1$ receptor the role of the third transmembrane helix in agonist signalling by point mutation into Ala of the residues highly conserved in the secretin-family of receptors: Y^{224}, N^{229}, F^{230}, W^{232}, E^{236}, G^{237}, Y^{239}, L^{240}. N^{229}A VPAC$_1$ mutant was characterized by a decrease in both potency and efficacy of VIP stimulated adenylate cyclase activity, by the absence of agonist stimulated $[Ca^{2+}]_i$ increase, by a preserved receptor recognition of agonists and antagonist and by a preserved sensitivity to GTP suggesting the importance of that residue for efficient G protein activation. N^{229}D mutant was not expressed at the membrane, and the N^{229}Q with a conserved mutation was less affected than the A mutant. Agonist stimulated phosphorylation and internalization of N^{229}A and N^{229}Q VPAC$_1$ were unaffected. However, the re-expression of internalized mutant receptors, but not that of the wild type receptor, was rapidly reversed after VIP washing. Receptor phosphorylation, internalization and re-expression may be thus dissociated from G protein activation and linked to another active conformation that may influence its trafficking.

Mutation of that conserved amino acid in VPAC$_2$ could be investigated only by a conservative mutation (N^{216}Q) and led to a receptor with a low VIP stimulation of adenylate cyclase, receptor phosphorylation and internalization. This indicated the importance of the conserved N residue in the TM3 of that family of receptors.

Keywords: VPAC$_1$ receptor; VPAC$_2$ receptor; TM helix; G protein activation; Receptor phosphorylation; Receptor internalization

1. Introduction

The human VPAC$_1$ receptor is expressed in liver, breast, kidney, prostate, bladder, pancreatic ducts, thyroid gland, lymphoid tissues and gastrointestinal mucosa and in most of the tumours derived from these tissues [1]. The physiological ligands are the Vasoactive Intestinal Polypeptide (VIP) and the Pituitary Adenylate Cyclase Activating Peptide (PACAP) [2]. Adenylate cyclase activation is the main signalling pathway of that receptor through coupling to Gαs although a coupling to the phospholipase C and the calcium/IP3 pathway through either Gαq and Gαi is also effective [3]. The VPAC$_1$ receptor is a member of the class B seven transmembrane G protein coupled receptors (7 TM GPCR) that encompass also the VPAC$_2$-, the secretin-, the PACAP-, the glucagon-, the glucagon like-peptide 1 and 2-, the calcitonin- and the parathormone receptors [4,5]. Data on the 3D structure, on the positioning of the ligand and on the molecular mechanisms leading to cell signalling are much

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Table 1
Summary of binding and functional studies performed on wild type and mutated $VPAC_1$ receptors

Receptors	VIP		[R^{16}]-VIP		$VPAC_1$ agonist		$VPAC_1$ antagonist
	pIC_{50}	pEC_{50}	pIC_{50}	pEC_{50}	pIC_{50}	pEC_{50}	pIC_{50}
$VPAC_1$	8.70±.0.09	8.30±0.08	9.30±0.20	9.52±0.13	8.50±0.17	8.52±0.07	8.70±0.14
$Y^{224}L$	8.56±0.30	8.54±0.09	9.50±0.30	9.18±0.09	9.28±0.08	8.93±0.14	8.68±0.12
$Y^{224}F$	8.81±0.11	8.66±0.26	9.40±0.23	9.50±0.31	8.97±0.08	8.85±0.18	8.38±0.08
$N^{229}A$	**8.79±0.40**	**7.26±0.19***	**9.47±0.23**	**8.25±0.25***	**9.18±0.09**	**8.26±0.12**	**8.71±0.10**
$F^{230}A$	8.43±0.09	8.01±0.23	9.65±0.19	8.74±0.49	8.87±0.07	7.83±0.21	8.63±0.07
$W^{232}A$	9.08±0.08	8.26±0.08	9.75±0.32	9.33±0.08	9.12±0.08	9.46±0.17	8.54±0.08
$E^{236}A$	8.71±0.38	8.90±0.17	9.31±0.35	9.51±0.78	8.91±0.27	9.04±0.34	8.58±0.31
$G^{237}A$	8.26±0.09	8.62±0.09	9.30±0.08	9.95±0.16	8.76±0.09	9.15±0.52	8.48±0.09
$Y^{239}A$	8.49±0.09	8.61±0.34	9.09±0.09	9.53±0.32	8.48±0.17	8.79±0.34	9.28±0.47
$L^{240}A$	8.66±0.16	8.50±0.12	9.57±0.11	9.83±0.09	8.98±0.09	8.70±0.57	8.70±0.10

pIC_{50} of binding and pEC_{50} of adenylate cyclase activation for VIP and analogs on membranes from CHO cells expressing the wild type $hVPAC_1$ or mutated receptor. The values derived from experiments performed on cell membranes from the clones used during the present study. Values are given with the standard error of the mean (three determinations). *$p<0.05$ evaluated by Mann–Whitney test. Mutant different from the WT receptor is shown in bold.

more limited for that receptor's family than for the A family that can use rhodopsin or bacteriorhodopsin as template models. Receptor domains involved in the stability of the active and inactive forms are, in almost all the GPCR class A, the E/DRY sequence located at the junction between the third transmembrane domain (TM3) and the second intracellular loop (IC2) and the NPXXY sequence located in the distal part of TM7. These sequences were not found in the GPCR-B family. It was shown however [6] in the human $VPAC_1$ receptor that a sequence YL highly conserved in the GPCR-B family and located in the position of the DRY sequence of the GPCR-A family was involved in receptor coupling to the G protein and it was also hypothesized [7] on the basis of structure prediction that a E/DRY motif could be formed by three non-adjacent residues consisting in R^{174} in the cytoplasmic end of TM2, E^{236} and Y^{239} in the distal part of TM3. It is established for the GPCR-A family that the switch between the inactive and the active receptor states operates mainly through a network of interactions between TM3, TM6 and TM7 [8–10]. As for the signature motives mentioned above, such a network was not yet proposed for the GPCR-B family. Actually, considering the $VPAC_1$ receptor as a paradigm for the class B, a large network must also be considered: indeed, mutagenesis studies suggest the participation of TM1 [11], TM2 [12,13], TM3 [6], and TM6 [14] but also of the IC3 loop [15] and of the proximal part of the C-terminal intracytoplasmic tail in signal transduction [16].

In the present work we investigated the role of conserved residues located in TM3 of $VPAC_1$ receptor by site-directed mutagenesis and identified the importance of the N^{229} residue as its mutation into alanine severely blunted agonist stimulated adenylate cyclase activity, abolished VIP stimulated $[Ca^{+}]_i$ increase, but reduced moderately agonist binding affinity that remained still inhibited by GTP. VIP stimulated receptor phosphorylation was only slightly decreased and receptor internalization was apparently not affected, but was rapidly reversed by VIP washing, a phenomenon not observed on the wild type receptor. On a whole, mutation of N^{229} impaired the capacity of $VPAC_1$ to activate the G proteins pre-coupled to the receptor and the results suggest that receptor phosphorylation and internalization may be dissociated from G protein activation. The conserved N residue in TM3 was also of importance in the $VPAC_2$ receptor but only the conservative mutation $N^{216}Q$ could be studied.

2. Materials and methods

2.1. Construction and expression of $VPAC_1$ and $VPAC_2$ mutant receptors

The cell lines expressing $VPAC_1$ and $VPAC_2$ wt receptors have been detailed in previous publications [3]. Generation of mutated receptors was achieved

Table 2
Summary of adenylate cyclase activation studies performed on wild type and mutated $VPAC_1$ receptors

Receptors	Adenylate cyclase activation				
	Basal	VIP (1 μM)	[R^{16}]-VIP (1 μM)	$VPAC_1$ agonist (1 μM)	$VPAC_1$ antagonist (1 μM)
$VPAC_1$	27±3	160±7 (100%)	165±6 (104%)	140±3 (85%)	26±5 (0%)
$Y^{224}L$	29.7±0.9	99.9±6.2 (100%)	102.6±7.4 (104%)	93.2±6.7 (90%)	20.3±3.4 (0%)
$Y^{224}F$	21.6±3.4	97.8±4.8 (100%)	98.2±6.6 (101%)	96.6±6.9 (98%)	22.6±7.4 (1%)
$N^{229}A$	**24.8±4.5**	**50.1±5.0 (100%)**	**57.3±3.9 (130%)**	**33.4±7.2 (34%)**	**26.6±4.6 (7%)**
$F^{230}A$	17.9±0.1	99.6±11.8 (100%)	106.2±10.9 (108%)	68.6±12.2 (62%)	17.6±0.5 (0%)
$W^{232}A$	30.8±0.7	119.1±11.5 (100%)	117.4±13.4 (98%)	111.7±12.2 (92%)	39.6±0.4 (10%)
$E^{236}A$	35.9±5.3	109.1±35.8 (100%)	110.0±39.3 (101%)	100.5±27.6 (88%)	46.5±9.3 (14%)
$G^{237}A$	17.8±1.9	93.5±3.8 (100%)	90.7±3.5 (96%)	85.1±9.7 (89%)	17.7±2.2 (0%)
$Y^{239}A$	38.2±3.3	144.1±18.3 (100%)	145.8±14.0 (102%)	117.9±19.1 (75%)	38.2±9.6 (0%)
$L^{240}A$	40.2±2.8	111.6±25.7 (100%)	110.1±21.9 (98%)	91.8±11.9 (72%)	33.5±3.9 (0%)

The values derived from experiments performed on cell membranes from the clones used during the present study. Values are given with the standard error of the mean (three determinations). Basal and E_{max} are expressed in production of cAMP/min mg prot. E_{max} is measured in response to 1 μM VIP and analogs. The percentage in brackets represents the activation of the different analogs compared with the VIP stimulation; considered as 100%. Mutant different from the WT receptor is shown in bold.