Effect of inactivating mutations on phosphorylation and internalization of the human VPAC₂ receptor

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Abstract

The VPAC₂ receptor, as all members of the G-protein-coupled receptor (GPCR)-B family, has two highly conserved motifs in the third intracellular (IC₃) loop: a lysine and a leucine located at the amino-terminus and two basic residues separated by a leucine and an alanine at the carboxyl-terminus. This study evaluates the involvement of those conserved amino acid sequences in VPAC₂ signal transduction and regulation. The residues were mutated into alanine and mutants were expressed in Chinese hamster ovary (CHO) cells stably transfected with Gα₁₆ and aequorin. Mutation of L310 reduced efficacy of vasoactive intestinal polypeptide (VIP) to stimulate adenylate cyclase activity through Gas coupling by 75%, without affecting VIP capability to stimulate an increase in [Ca²⁺]_i through Gα₁₆ coupling. Mutation of R325 and, to a lesser extent, K328 reduced VIP efficacy to stimulate [Ca²⁺]_i increase and VIP potency to stimulate adenylate cyclase. The combination of mutations of both amino- and carboxyl-terminus located conserved motifs of the IC₃ loop generates an inactive receptor with respect to [Ca²⁺]_i increase and adenylate cyclase activation, but also with respect to receptor phosphorylation and internalization that were indeed directly correlated with the potency of inactivation of the receptors.

The amino-terminus of the VPAC₂ receptor IC₃ loop is thus involved in adenylate cyclase activation and the carboxyl-terminus of the IC₃ loop participates in both Gas and Gα₁₆ coupling. The mutations studied also reduced both receptor phosphorylation and internalization in a manner that appeared directly linked to the alteration of Gas and Gα₁₆ coupling.

Journal of Molecular Endocrinology (2005) **34**, 405–414

Introduction

Vasoactive intestinal polypeptide (VIP) is a neuropeptide that contributes to regulation of intestinal secretion and motility, of exocrine and endocrine secretions (Fahrenkrug 1993), and to homeostasis of the immune system (Gomariz *et al.* 2001). The effects of VIP are mediated through interaction with two receptor subclasses named VPAC₁ and VPAC₂ receptors (Harmar *et al.* 1998). These receptors are members of the G-protein-coupled receptor (GPCR)-B family, that also includes the receptors for PACAP, secretin, glucagon, GLP-1, calcitonin, parathyroid hormone and GRF, and are preferentially coupled to Gas protein that stimulates adenylate cyclase activity and induces cyclic AMP (cAMP) increase (Harmar *et al.* 1998). We previously demonstrated that both VPAC₁ and VPAC₂ receptors are also able to couple to the IP₃/[Ca²⁺]_i pathway (Langer *et al.* 2001). In CHO cells expressing a comparable density of recombinant receptors, VIP induced [Ca²⁺]_i increase is more efficient in VPAC₁ than in VPAC₂ receptor-expressing cells (Langer *et al.* 2001). While VPAC₁ receptor-mediated response can be attributed to both Gα_q and Gα_i coupling (Langer *et al.*

2002). Efficient [Ca²⁺]_i increase following VPAC₂ receptor activation can be achieved in the presence of Gα₁₆, a member of the Gα_q family that allows the coupling of a wide variety of receptors to phospholipase C (Langer *et al.* 2001) and whose expression is restricted to hematopoietic and immune cells that express functional VPAC₂ receptors (Langer *et al.* 2001).

The study of a wide variety of chimeric or mutated seven transmembrane (TM) receptors demonstrated that the second (IC₂) and third intracellular (IC₃) loops, and to a lesser extent the proximal part of the carboxyl-terminal tail, can be directly involved in G-protein/receptor interactions (Wess 1997). However, the diversity of sequences and loop sizes, even among related receptors, has made the identification of a specific set of amino acid residues to define the coupling profile difficult. In the present study we investigated the contribution of two highly conserved amino acid motifs among all members of the GPCR-B family located at the junction of TM₅ and IC₃ loop (K³⁰⁹-I³¹⁰-T³¹¹) and of IC₃ loop and TM₆ (R³²⁵ and K³²⁸), in human recombinant VPAC₂ receptors expressed in Chinese hamster ovary (CHO) cells. Like most GPCRs, VPAC₂ receptor was reported to be rapidly phosphorylated,

desensitized and internalized after agonist exposure (McDonald *et al.* 1998). Since receptor activation is also an important determinant of the agonist-induced receptor phosphorylation and internalization and as the IC₃ loop is also an important domain for the binding of arrestin after receptor phosphorylation (Ferguson 2001, Perry & Lefkowitz 2002), we evaluated the effect of mutations that impair VPAC₂ receptor coupling to signalling pathway on receptor regulation. This study revealed that: 1) mutations in IC₃ that affect the proximal residues significantly reduce VIP-induced adenylate cyclase activation (from 70 to 85%) without affecting VPAC₂ receptor ability to couple to the IP₃/[Ca²⁺]_i pathway through Gα16 interaction; 2) mutations that affect the distal residues affect both VIP-mediated cAMP and [Ca²⁺]_i increase; 3) combination of mutations in both domains inactivates VPAC₂ receptor and 4) G-protein coupling-inactivating mutations also reduced both receptor phosphorylation and internalization in a manner that appeared directly linked to the alteration of Gαs and Gα16 coupling.

Materials and methods

Construction and expression of VPAC₂ mutant receptors

The cell line expressing VPAC₂ receptor has been detailed in previous a publication (Langer *et al.* 2001). Generation of mutated receptors was achieved using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions as described (Langer *et al.* 2002). Confirmation of the expected mutation was achieved by DNA sequencing on an ABI automated sequencing apparatus, using the BigDye Terminator Sequencing Prism Kit from ABI (Perkin-Elmer, CA, USA). Following DNA amplification using a midiprep endotoxin-free kit (Stratagene), the complete nucleotide sequence of the receptor coding region was verified by DNA sequencing. 20 µg of receptor coding region was transfected by electroporation into the CHO cell line expressing aequorin and Gα16 (kindly provided by Vincent Dupriez, Euroscreen SA, Brussels, Belgium) as described previously (Langer *et al.* 2001). Selection was carried out in culture medium [50% HamF12; 50% DMEM; 10% fetal calf serum; 1% Penicillin (10 mU/ml); 1% Streptomycin (10 µg/ml); 1% L-Glutamine (200 mM), Life Technologies Ltd, Paisley, UK.], supplemented with 600 µg Geneticin (G418)/ml culture medium. After 10 to 15 days of selection, isolated colonies were transferred to 24-well plates and grown until confluence, trypsinized and further expanded in 6-well plates, from which cells were scraped and membranes prepared for identification of receptor expressing clones by an adenylate cyclase activity assay in presence of 1 µM VIP. Selection of KLT-AAA R325A-K328A

and ΔKLT R325A-K328A mutants was carried out by binding assay with [¹²⁵I]-Ro 25-1553 (see below).

Membrane preparation

Membranes were prepared from scraped cells lysed in 1 mM NaHCO₃ by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4 °C for 10 min at 400 g and the supernatant was further centrifuged at 20 000 g for 10 min. The resulting pellet, resuspended in 1 mM NaHCO₃, was used immediately as a crude membrane fraction.

Adenylate cyclase activation assay

Adenylate cyclase activity was determined by the procedure of Salomon *et al.* (1974), as described previously. Membrane proteins (3–15 µg) were incubated in a total volume of 60 µl containing 0.5 mM [α³²P]-ATP, 10 µM GTP, 5 mM MgCl₂, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho(enol)pyruvate, 30 µg/ml pyruvate kinase and 30 mM Tris-HCl at a final pH of 7.8. The reaction was initiated by the addition of the membranes and was terminated after 15 min incubation at 37 °C by adding 0.5 ml of a 0.5% sodium dodecyl-sulfate solution containing 0.5 mM ATP, 0.5 mM cAMP and 20 000 c.p.m. [³H]-cAMP. cAMP was separated from ATP by two successive chromatographies on Dowex 50W x 8 and neutral alumina.

Binding studies

Binding studies were performed by using the selective VPAC₂ receptor ligand [¹²⁵I]-Ro 25-1553. The non specific binding was defined as residual binding in the presence of 1 µM unlabeled Ro 25-1553. [¹²⁵I]-Ro 25-1553 was preferred for quantification of receptors as the specific binding and the ratio between total and non specific binding was higher than with [¹²⁵I]-VIP. Binding was performed for 30 min at 23 °C in a total volume of 120 µl containing 20 mM Tris-maleate, 2 mM MgCl₂, 0.1 mg/ml bacitracin, 1% BSA (pH 7.4) buffer. 3–30 µg of protein was used per assay. Bound and free radioactivity were separated by filtration through glass-fiber GF/C filters presoaked for 24 h in 0.01% polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) sodium phosphate buffer containing 0.8% BSA. The binding sites density was estimated by analysis of homologous competition curves assuming that the labeled and unlabeled ligands had the same affinity for the receptors.

[Ca²⁺]_i increase assay

[Ca²⁺]_i increase was measured by a functional assay based on the luminescence of mitochondrial aequorin/

coelenterazine following $[Ca^{2+}]_i$ increase as described previously (Brini *et al.* 1995, Stables *et al.* 1997). Briefly, cells were collected from plates with PBS containing 5 mM EDTA, pelleted and resuspended at $5 \cdot 10^6$ cells/ml in DMEM-F12 medium supplemented with 0.5% BSA, incubated with 5 μ M coelenterazine H (Molecular Probes, Eugene, OR, USA) for 3 h at room temperature under light agitation. Cells were then diluted at a concentration of $5 \cdot 10^5$ cells/ml and incubated for one more hour. 50 μ l of cell suspension was added to agonists diluted in a volume of 50 μ l DMEM-F12. $[Ca^{2+}]_i$ increase was evaluated by measuring for 20 s the luminescent signal (integration of area under the curve) resulting from the activation of the aequorin–coelenterazine complex using a Victor luminometer (Perkin Elmer, Wellesley, MA, USA). The data were normalized for basal (0%, background removal) and maximal luminescence (100%) corresponding to the signal measured following exposure to 50 μ M of digitonin.

Genetic immunization and generation of monoclonal antibody (mAb) for VPAC₂ receptor identification

Six week old Balb/c female mice were anaesthetized by injections of 6–10 mg/kg ketamin HCl and 0.1 ml/kg Rompum. The anterior tibialis muscle of each leg was injected at day 0 with 100 μ l of 10 mM cardiotoxin (Latoxan, Rosans, France) and 5 days later with 50 μ g of the plasmid construct in a final volume of 100 μ l 0.09% NaCl. Injections were repeated 3 and 6 weeks thereafter. Blood samples were collected from retro-ocular puncture 7 weeks after the initial immunization and serum tested by FACS and Western Blotting for the presence of antibodies against VPAC₂ receptor. The mouse selected for mAb production, was boosted by i.v. injection of 10^6 CHO cells expressing the human VPAC₂ receptor resuspended in 100 μ l saline solution. Three days later, splenocytes were fused with SP2O, a non-secreting myeloma cell line, at a 3:1 ratio in presence of polyethylene glycol. Fused cells were then delivered into 96-well plates and selected with 100 μ M hypoxanthine, 400 μ M aminopterin and 16 μ M thymidine. Irradiated macrophages from mouse peritoneum were also added to supply cytokines and growth factors. After 10 days, culture supernatants were screened by FACS (see below) and the cells producing antibodies cloned by dilution. The monoclonal antibody selected (mAb VPAC₂) was purified using ImmunoPure IgG Purification kit (Pierce, Rockford, IL, USA). Using the mouse mAb isotyping kit (Isotrip, Roche) we established that it was of the IgG 2a subtype.

Immunoprecipitation and determination of receptor phosphorylation levels

Cells were cultured in phosphate-free DMEM medium for 16 h and then incubated for 2 h at 37 °C in the

same medium containing 200 μ Ci acid-free [³²P]-orthophosphate. At the end of this labelling period agonist was added. When kinase inhibitors were tested, they were added 2 h prior to agonist addition. After agonist exposure, cells were washed three times with ice-cold buffer consisting of 10 mM HEPES, 4.2 mM NaHCO₃, 11.7 mM glucose, 1.2 mM MgSO₄, 4.7 mM KCl, 118 mM NaCl and 1.3 mM CaCl₂, pH 7.4, and subsequently lysed in 1 ml of a buffer consisting of 20 mM Tris, 100 mM (NH₄)₂SO₄ and 10% glycerol, pH 7.5. The cell lysate was centrifuged at 600 *g* at 4 °C for 10 min and the supernatant centrifuged at 19 000 *g* for 30 min. The resulting pellet was resuspended in the same buffer containing 1% dodecylmaltoside and solubilized for 45 min at 4 °C. The remaining insoluble material was eliminated by a further centrifugation. The supernatant was added to 50 μ l of a 10% protein A sepharose suspension previously coated during 2 h with 2 μ g of purified monoclonal antibody. After 150 min incubation under light agitation at 4 °C, the sepharose beads were separated by centrifugation and washed successively with the concentrated lysis buffer, then with a two fold diluted buffer and finally with water. The final beads pellet was resuspended in a buffer consisting of 125 mM Tris, 10% β -mercaptoethanol, 4% SDS, 20% glycerol and 0.02% bromophenol blue pH, 6.8. After heating at 60 °C for 10 min, the samples were resolved by SDS page using 10% gel. The gel was fixed, dried and the phosphorylated bands detected and quantified by phosphoimaging.

Receptor internalization

Receptor internalization was defined as the percentage of cell surface receptors that were no longer accessible to the mAb, after agonist exposure. Cells expressing VPAC₂ or mutated receptors were incubated with 1 μ M VIP for 20 min at 37 °C. After washing 3 times with ice-cold PBS, cells were detached from the plates using a 5 mM EDTA/EGTA PBS solution, harvested by centrifugation (500 *g*, 4 °C, 4 min), washed once with PBS solution and resuspended at density of $3 \cdot 10^5$ cells/assay in 100 μ l PBS-BSA 0.1%, containing 0.1 μ g purified anti-VPAC₂ receptor mAb. After 30 min incubation at 4 °C, the cells were washed with PBS-BSA 0.1% and then incubated with FITC-conjugated- γ -chain-specific goat anti-mouse IgG (Sigma Chemicals) for 30 min on ice in the dark. The labelled cells were washed and resuspended in 250 μ l PBS-BSA 0.1%. The fluorescence level was analyzed using a FACScalibur (Becton Dickinson, Franklin Lanes, NJ, USA) and the data processed using the cell quest software (Becton Dickinson, Franklin Lanes, NJ, USA). Basal fluorescence was determined from a sample of untransfected CHO cells. Propidium iodide (10 μ g/ml) was used to exclude debris and dead cells from our analysis.

VPAC ₂	-KLTSPDVGGNDQSQYKRLAK-
K309A	- A LTS PDVGGNDQSQYKRLAK-
L310A	-K A TSPDVGGNDQSQYKRLAK-
T311A	-KL A SPDVGGNDQSQYKRLAK-
KLT-AAA	- AAA SPDVGGNDQSQYKRLAK-
ΔKLT	- --- SPDVGGNDQSQYKRLAK-
R325A	-KLTSPDVGGNDQSQYK A LAK-
K328A	-KLTSPDVGGNDQSQYKRL AA -
R325A-K328A	-KLTSPDVGGNDQSQYK AA L AA -
KLT-AAA R325A-K328A	- AAA SPDVGGNDQSQYK AA L AA -
ΔKLT R325A-K328A	- --- SPDVGGNDQSQYK AA L AA -

Figure 1 Amino acid sequences of IC₃ loop of VPAC₂ wild type and mutated receptors.

Data analysis

All competition curves, dose–response curves, IC₅₀ and EC₅₀ values were calculated using non linear regression (GraphPad Prism software, San Diego, CA, USA). Statistical analyses were performed with the same software.

Results

Binding studies

Receptor density was evaluated by [¹²⁵I]-Ro 25–1553/Ro 25–1553 binding studies. This molecule, a VIP analogue with a lactam bridge in the carboxyl-terminus was developed first as a potent long-acting bronchodilator (O'Donnell *et al.* 2004). It was later found to be a selective agonist that preferentially recognizes VPAC₂ to VPAC₁ receptor and a had higher affinity for VPAC₂ receptor than VIP (Gourlet *et al.* 1997). When iodinated it also appeared to be a better ligand than iodinated VIP to label the VPAC₂ receptors, as the ratio total binding/non specific binding was higher for iodinated Ro 25–1553. All the mutants (Fig. 1) were expressed at the membrane. Screening of several cell clones was performed to select a material expressing a comparable density of sites and therefore an easier comparison of the data (Table 1). However four clones studied (KLT-AAA, ΔKLT, KLT-AAA R325A-K328A and ΔKLT R325A-K328A) expressed a 6- to 12-fold higher receptor density than those expressing the wild type receptor. VIP IC₅₀ values were not significantly different from the wild type receptor value (3 nM) for the K309A and T311A mutants. For all other mutants, VIP IC₅₀ values were significantly higher (between 10 and 50 nM, see Table 1

and Fig. 2). The effect of GTP was also evaluated. In competition binding assays GTP inhibited about 30% of tracer binding except for KLT-AAA, ΔKLT, R325A-K328A, KLT-AAA R325A-K328A and ΔKLT R325A-K328A mutants that display a lower tracer binding inhibition (Table 1).

Adenylate cyclase activation

VIP dose-effect curves of adenylate cyclase stimulation were performed on each clone. VIP EC₅₀ values for K309A and T311A mutants were identical to the wild type receptor value (2–3 nM). L310A mutant displayed a moderate decrease of VIP potency (10 nM) and efficacy (70%) to stimulate cAMP production. When deleting or mutating the K³⁰⁹-L³¹⁰-T³¹¹ sequence into alanine, VIP efficacy (but not potency) was slightly further reduced. However KLT-AAA and ΔKLT expressing cell lines had a significant higher receptor density (6- to 9-fold), and it is therefore possible that the effect of those combined modifications and the potential contribution of K³⁰⁹ and T³¹¹ were underestimated. In the same line, the discrepancy between VIP EC₅₀ and VIP IC₅₀ values of the KLT-AAA mutant could be explained by spare receptors. Mutation of R³²⁵ or K³²⁸ into alanine reduced VIP potency (about 15-fold) without affecting the maximal stimulatory effect. Combining those mutations (R325A-K328A) dramatically decreased both VIP efficacy (90%) and potency (25-fold). cAMP production could not be detected for the KLT-AAA R325A-K328A and ΔKLT R325A-K328A mutants despite the expression of a very high receptor density of 37.7 and 26.1 pmole/mg protein respectively (Table 1 and Fig. 2).

Table 1 Summary of binding and functional studies performed on wild type and mutated VPAC₂ receptors

	Receptor density (pmole/mg protein)	Binding		cAMP		[Ca ²⁺] _i	
		IC ₅₀ (nM)	GTP effect [§]	EC ₅₀ (nM)	E _{max} [#]	EC ₅₀ (nM)	E _{max} [°]
VPAC ₂	3.0±0.6	2.6±0.3	27±2	2.2±0.5	228±16	120±16	82±2
Mutation in N-terminus of IC₃ loop							
K309A	4.0±0.4	4.0±0.6	30±3	3.3±0.3	202±9	90±14	70±4
L310A	5.8±0.3*	11.2±0.8*	29±3	10.0±0.9*	60±7*	150±20	74±4
T311A	3.4±0.3	3.7±0.6	33±2	3.8±0.6	189±10	113±15	89±3
KLT-AAA	26.0±2.3*	10±1*	20±2*	2.0±0.2	68±4*	157±21	96±4*
ΔKLT	18.0±1.2*	20±1*	7±2*	61±5*	34±7*	185±30	72±3
Mutation in C-terminus of IC₃ loop							
R325A	4.2±0.4	17±1*	27±2	27±3*	247±32	216±34	24±1*
K328A	2.7±0.2	33±4*	26±2	30±2*	201±34	284±39	54±3*
R325A-K328A	8.7±0.3*	49±3*	9±1*	550±35*	22±5*	—	—
Combined mutations							
KLT-AAA R325A-K328A	37.7±1.5*	43±3*	5±1*	—	6±2*	314±57	25±2*
ΔKLT R325A-K328A	26.1±1.5*	47±3*	—	—	7±2*	—	—

**P*<0.05 evaluated by Mann–Whitney test (*n*=3) as compared to wild type receptor value. [§]Expressed in % of tracer binding inhibition. [#]E_{max} in response to 1 μM VIP expressed in over basal production of cAMP/min/mg protein. [°]E_{max} in response to 10 μM VIP expressed in % of digitonin response. Results expressed as means±S.E.M. of three independent experiments in duplicate.

[Ca²⁺]_i increase

VIP induced [Ca²⁺]_i increase was evaluated by a functional assay based on the luminescence of aequorin/coelenterazine following [Ca²⁺]_i increase. As mentioned before, VPAC₂ receptors display efficient [Ca²⁺]_i increase in presence of Ga16 only. All mutants tested were thus stably transfected in CHO cells expressing constitutively the Ga16 protein. No significant differences in VIP potency (EC₅₀ values) were observed between wild type VPAC₂ receptor and all the mutants that elicited a [Ca²⁺]_i increase allowing EC₅₀ determination (Table 1 and Fig. 2). In all cases, VIP concentrations required for half-maximal [Ca²⁺]_i increase were higher than that required for adenylate cyclase activation. This has been regularly observed in that system as [Ca²⁺]_i measurement is performed within 20 s after agonist addition and thus essentially reflects the association constant rate of the ligand rather than the equilibrium constant (Blanpain *et al.* 1999, Dethoux *et al.* 2000). The K309A, L310A, T311A and ΔKLT mutants were also undistinguishable from the wild type receptor with respect to the maximal stimulatory effect of VIP. Maximal VIP response was higher in the KLT-AAA mutant (increase of 17% as compared with wild type receptor) and significantly lowers in R325A, K328A and KLT-AAA R325A-K328A mutants (decrease of 70%, 35% and 70% respectively). No [Ca²⁺]_i increase was observed following VIP stimulation of the R325A-K328A and ΔKLT R325A-K328A mutants (Table 1 and Fig. 2).

Receptor phosphorylation

Like most GPCR, VPAC₂ receptors undergo rapid phosphorylation, desensitization and internalization following agonist exposure. Receptor phosphorylation was evaluated after preincubation of the cells with inorganic [³²P] followed by VIP stimulation and receptor immunoprecipitation with anti-VPAC₂ receptor mAb. In a first set of experiments we characterized the wild type VPAC₂ receptor phosphorylation by performing time course and dose-effect studies (Fig. 3). VIP induced a rapid, dose-dependent stimulation of [³²P] incorporation into a protein of an apparent molecular size of 65 kDa that immunoprecipitated with the VPAC₂ monoclonal antibody. This band was not observed in control cells that do not express the human VPAC₂ receptor (data not shown). The time course of VPAC₂ receptor phosphorylation was bell-shaped reaching a maximum after 5 min treatment with 1 μM VIP. VPAC₂ receptor phosphorylation was undetectable in absence of VIP. A dose related study performed after 5 min incubation with VIP indicated a progressive increase in receptor phosphorylation up to 1 μM VIP. We next evaluated the effect of PKA and PKC inhibitors on VPAC₂ receptor phosphorylation by pre-treating cells for 2 h with 10 μM Rp-8-Br-cAMP or 300 nM calphostin. As shown in Fig. 3 no significant differences were observed in VPAC₂ receptor phosphorylation following these pre-treatments (evaluated densitometry in arbitrary units: 44615 ± 3054 (VIP); 36879 ± 3578, *P*=0.57 (VIP+Rp-8-Br-cAMP); 37824 ± 4534 *P*=0.31

(VIP+calphostin)) indicating that it was essentially an agonist-dependant phenomenon.

Following 5 min treatment with 1 μ M VIP, K309A, L310A, T311A mutants exhibited receptor phosphorylation levels similar to that of wild type receptor (Fig. 4). Stimulation of cells expressing KLT-AAA and Δ KLT mutants results in an increase of receptor phosphorylation levels (6- and 5-fold as measured by densitometry in arbitrary units on gels loaded with the same amount of protein: 254290 ± 27855 , $P=0.028$ (KLT-AAA);

Figure 3 VIP induced phosphorylation of VPAC₂ receptor. Receptor phosphorylation levels evaluated after preincubation of the cells with inorganic [³²P] followed by VIP stimulation and receptor immunoprecipitation with anti-VPAC₂ receptor mAb. (A) Time course study following 1 μ M VIP stimulation. (B) Dose-effect study following 5 min VIP stimulation. (C) Effect of 2 h preincubation with Rp-8-Br-cAMP 10 μ M and calphostin 300 nM on receptor phosphorylation induced by 5 min stimulation with 1 μ M VIP. Representative of three independent experiments.

209892 $\pm$ 2444, $P=0.028$ (Δ KLT)), but as mentioned before the two clones studied express high receptor density, and phosphorylation appeared comparable to that of the wild type receptor when normalized to the amount of receptors. Cells expressing the R325A and K328A mutants displayed a comparable 50% reduced phosphorylation level (evaluated densitometry in arbitrary units: 21193 $\pm$ 787, $P=0.028$ (R325A); 22493 $\pm$ 1641, $P=0.028$ (K328A)). Receptor phosphorylation was undetectable following stimulation of the R325A-K328A double mutant (evaluated densitometry in arbitrary units: 640 $\pm$ 165, $P=0.028$). Surprisingly, the levels of unstimulated (basal) phosphorylation of KLT-AAA R325A-K328A and Δ KLT R325A-K328A mutants were significantly higher than those of the wild type receptor or of the mutant expressing large amount of receptors, and were not increased in the presence of VIP (evaluated densitometry basal levels in arbitrary units: 1060 $\pm$ 100 (VPAC₂); 6043 $\pm$ 500 (KLT-AAA);

Figure 2 Pharmacological characterization of VPAC₂ wild type receptor and L310A, Δ KLT, R325A-K328A or Δ KLT R325A-K328A mutants. VIP competition curves of specific [¹²⁵I]-Ro 25-1553 binding (top panel), dose-effect curves of VIP induced cAMP (middle panel) and [Ca²⁺]_i increases (bottom panel) in CHO cells expressing wild type (close circles), L310A (open circles), Δ KLT (open squares), R325A-K328A (open triangles) or Δ KLT R325A-K328A (open diamonds) mutants. Results represent the mean of three independent experiments in duplicate.

Figure 4 VIP induced phosphorylation of VPAC₂ wild type and mutated receptors. Receptor phosphorylation levels evaluated after preincubation of the cells with inorganic [³²P] followed by 5 min stimulation with 1 μM VIP and receptor immunoprecipitation with anti-VPAC₂ receptor mAb. Representative of three independent experiments.

1280 ± 150 (ΔKLT); 114659 ± 5297, $P=0.028$ (KLT-AAA R325A-K328A); 64678 ± 7391, $P=0.028$ (ΔKLT R325A-K328A)). Basal and VIP stimulated receptor phosphorylation of wild type and ΔKLT R325A-K328A mutant were evaluated in the presence of serine/threonine kinase inhibitor A3 and tyrosine kinase inhibitor genistein (Fig. 5). Two hour preincubation with 200 μM A3 did not modify basal or VIP induced receptor phosphorylation levels of wild type receptor or ΔKLT R325A-K328A mutant. Two hour preincubation with 100 μM genistein reduced VIP stimulated wild type

Figure 5 Effect of kinase inhibitors and VPAC₂ antagonist on phosphorylation of VPAC₂ and ΔKLT R325A-K328A receptors. Receptor phosphorylation levels evaluated after preincubation of the cells with inorganic [³²P] followed by 5 min stimulation with VIP and receptor immunoprecipitation with anti-VPAC₂ receptor mAb. Top panel, effect of 2 h preincubation with A3 200 μM and genistein 200 μM on basal or 1 μM VIP induced VPAC₂ or ΔKLT R325A-K328A receptor phosphorylation levels. Bottom panel, effect of 20 min preincubation with VPAC₂ antagonist 1 μM (PG 99-465) on basal or 0.1 μM VIP induced VPAC₂ or ΔKLT R325A-K328A receptor phosphorylation levels. Representative of three independent experiments.

Receptor internalization

Figure 6 VIP induced internalization of VPAC₂ wild type and mutated receptors. Receptor internalization upon 20 min exposure to 1 μM VIP assessed by flow cytometry analysis on viable non-permeabilized cells using anti-VPAC₂ receptor antibody. The results are expressed as percent of receptors remaining accessible to the antibody (mean ± S.E.M., $n=3$). * $P<0.05$ as compared to wild type receptor response evaluated by Mann-Whitney test.

receptor phosphorylation levels (evaluated densitometry in arbitrary units: 26789 ± 2871, $P=0.028$) but not the basal or VIP stimulated ΔKLT R325A-K328A receptor phosphorylation levels. The effect of the VPAC₂ receptor antagonist PG 99-465 (Moreno *et al.* 2000) was also evaluated; it did not affect basal phosphorylation, but inhibited the VIP stimulation of the wild type receptor (evaluated densitometry in arbitrary units: 978 ± 100, $P=0.028$), and had a slight inhibitory effect on the basal phosphorylation levels of ΔKLT R325A-K328A mutant (evaluated densitometry in arbitrary units: 51140 ± 3218, $P=0.028$) (Fig. 5).

Receptor internalization

Receptor internalization upon 20 min exposure to 1 μM VIP was assessed by flow cytometry analysis on viable non-permeabilized cells using anti-VPAC₂ receptor antibody. The results are presented in Fig. 6 and are expressed as percent of receptors remaining accessible to the antibody at the cell surface. Following VIP stimulation, K309A, L310A, T311A mutants and the wild type receptor had a comparable 40% reduction in the number of receptors. KLT-AAA, ΔKLT, R325A and K328A mutants displayed reduced receptor internalization as VIP treatment reduced the number of accessible receptors by 20–25%. Receptor disappearance was not detected after VIP treatment of cell lines

expressing R325A-K328A, KLT-AAA R325A-K328A and Δ KLT R325A-K328A mutants.

Discussion

The current model of seven transmembrane receptor activation and paradigm of receptor regulation have essentially been developed on the basis of extensive study of the largest family of GPCRs also called the GPCR-A/rhodopsin family (Ferguson 2001, Kohout & Lefkowitz 2003, Perry & Lefkowitz 2002, Wess 1997). The mechanisms regulating the GPCR-B family signal transduction are less precisely understood due in part to the size and relative fragility of the ligands and in part to the absence of reliable receptor modelling. As all members of the GPCR-B family, VPAC₂ receptor is preferentially coupled to G_{as} protein that stimulates adenylate cyclase activity and induces cAMP increase (Harmar *et al.* 1998). We also demonstrated that, in presence of G α 16, VPAC₂ receptor may couple efficiently to IP₃/[Ca²⁺]_i pathway (Langer *et al.* 2002). A common feature of the GPCR-B family is the presence of two highly conserved amino acid motifs located in IC₃ loop: a lysine and leucine residue located at the junction of TM₅ and two basic residues located at the junction of TM₆. In the present study, we investigate the contribution of those conserved residues (K³⁰⁹, L³¹⁰, T³¹¹, R³²⁵ and K³²⁸) in VPAC₂ receptor coupling to G_{as} and G α 16 and subsequent cAMP and intracellular [Ca²⁺]_i increases. This led to the discovery of inactivating mutations and these receptors were studied in detail, particularly for receptor phosphorylation and internalization.

The present data demonstrated that the K³⁰⁹-L³¹⁰-T³¹¹ sequence (and particularly L³¹⁰) located at junction of TM₅ and IC₃ loop is involved in adenylate cyclase activation but not in G α 16 coupling. The two conserved basic residues located at junction of TM₆ and IC₃ loop (R³²⁵ and K³²⁸) were involved in both cAMP and intracellular [Ca²⁺]_i increases. Combined mutation of the two basic residues into Alanine (R325A-K328A mutant), inactivated the receptor almost completely: only a slight cAMP increase could still be detected. In order to obtain a completely inactive receptor we combined mutations altering both proximal and distal domains of IC₃ loop. This was achieved with the Δ KLT R325A-K328A mutant that did not respond to VIP. These results, in line with previous studies performed on other members of the GPCR-B family that identified the proximal domain of IC₃ loop as essential for G_{as} coupling and adenylate cyclase activation (Huang *et al.* 1996, Takhar *et al.* 1996, Chan *et al.* 2001, Couvineau *et al.* 2003), add to the original finding that this domain was not involved in G α 16 triggered IP₃/[Ca²⁺]_i pathway. Additionally we identified that the distal

domain of IC₃ loop and particularly two basic residues (R³²⁵ and K³²⁸) were involved in G α 16 coupling but also in adenylate cyclase activation. The effect of GTP on VIP affinity was evaluated by measuring the decreased binding of the iodinated agonist Ro 25-1553. GTP ability to reduce agonist binding was inversely related to the affinity of receptor/G protein complex thus reflecting G protein-coupling. Several mutations (KLT-AAA, Δ KLT, R³²⁵A-K³²⁸A and KLT-AAA R³²⁵A-K³²⁸A) significantly reduced GTP effect, suggesting an impaired interaction between the receptor and G protein. Moreover, only the completely inactive mutant (Δ KLT R³²⁵A-K³²⁸A) was insensitive to GTP, indicating that the global measure of GTP effects reflects the coupling of both G_{as} and G α 16 to the receptor. The junctions of IC₃ loop are predicted to be α -helical and it is assumed that the correct positioning of charged amino acids plays a crucial role in G protein interaction. However, our results and other data suggest that uncharged residues are also important for G protein interaction (Kosugi *et al.* 1993, Kostenis *et al.* 1997, Greasley *et al.* 2001). It is possible that IC₃ loop junctions activate G protein directly or that they may serve as regions that control the loop conformation. As mutations may change both direct interaction site and secondary structure, it is difficult to define more precisely the mechanisms involved in IC₃ loop/G protein interactions.

Like most GPCR, VPAC₂ receptors was shown to be rapidly phosphorylated, desensitized and internalized following agonist exposure (McDonald *et al.* 1998). However, the published data are scarce. Since receptor activation and intrinsic agonist activity are important determinants for agonist induced receptor phosphorylation (Ferguson 2001), we evaluated the effect of partial and complete inactivating mutations on receptor phosphorylation and internalization. We found that the VPAC₂ receptor undergoes rapid agonist-dependant phosphorylation following VIP exposure. The significant but partial inhibitory effect of genistein and the absence of evidence for implication of cAMP and phorbol esters activated kinase on receptor phosphorylation also suggest the contribution of both tyrosine kinase (the NetPhos program (Blom *et al.* 1999) predicts three potentially phosphorylatable tyrosine residues) and GRK. We found that G protein-coupling-inactivating mutations also reduced both receptor phosphorylation and internalization in a manner that appeared directly linked to the alteration of the G_{as} and G α 16 coupling. As mutants studied do not affect phosphorylatable residues, this suggests that impaired receptor phosphorylation and internalization directly reflect the reduced ability of the mutants to adopt active receptor conformation. These results gave additional findings that secondary coupling to G proteins also significantly contributes to the mechanisms involved in GPCR regulation and

trafficking. They could also explain results of some studies describing point mutations that impair the single signal transduction pathway (*Gas*, *Gai* or *Gaq*) and do not affect receptor internalization (Couvineau *et al.* 2003).

Surprisingly, we observed that the completely inactive mutant (Δ KLT R325A-K328A) had an enhanced basal receptor phosphorylation level that was unchanged in presence of VIP. This increase can not be explained by a higher receptor density of the cell clone studied as the mutant KLT-AAA had a similar receptor density but a barely detectable basal phosphorylation level. Enhanced basal receptor phosphorylation level has only been described for some constitutively active receptors that mimic the active receptor conformation and are therefore substrates for GRK-mediated phosphorylation in absence of agonist (Mhaouty-Kodja *et al.* 1999, Min & Ascoli 2000). The Δ KLT R325A-K328A mutants do not display a constitutively active phenotype: basal cAMP levels are similar to that of wild type receptor (Fig. 2), agonist binding was GTP insensitive (suggesting an impaired coupling to G proteins) and all cell clones obtained expressed very high receptor density (Table 1) while constitutively active receptors are generally known to be unstable and display low receptor density. As current paradigm of receptor regulation includes uncoupling of GPCR to G proteins following agonist induced receptor phosphorylation (Ferguson 2001, Kohout & Lefkowitz 2003), we tentatively test the hypothesis that higher basal phosphorylation was responsible for receptor inactivity. We search for a tool that reduces basal phosphorylation levels of the Δ KLT R325A-K328A mutant. We evaluated the effect of A3 (serine/threonine kinase inhibitor), genistein (tyrosine kinase inhibitor) and VPAC₂ antagonist (PG 99–465) and found that PG 99–465 only was able to slightly reduce basal phosphorylation levels of the mutant. We therefore treated cells expressing Δ KLT R325A-K328A mutant for 2 h with 1 μ M PG 99–465 and measured VIP stimulated adenylate cyclase activity. The activity was however not restored (data not shown). All together, the data suggest that the Δ KLT R325A-K328A mutant was phenotypically close to a desensitized receptor – phosphorylated, uncoupled to G protein and inactive. As kinase inhibitors do not reduce basal phosphorylation levels of Δ KLT R325A-K328A mutant and that GRK have the unique ability to recognize and phosphorylate their GPCR substrates only in their active conformation (Ferguson 2001, Kohout & Lefkowitz 2003), it is possible that enhanced basal phosphorylation levels rather reflects a decrease of affinity for phosphatases. Additionally, this inactive mutant also displays apparent impaired receptor internalization suggesting that it also fails to bind to accessory proteins responsible for receptor trafficking. Actual concepts agree that receptor domains necessary for G protein-coupling and receptor internalization are in part overlapping (Ferguson 2001,

Kohout & Lefkowitz 2003), but as this mutant does not respond anymore to VIP, it is likely that this effect is mainly due to its impairment to adopt agonist induced active conformation leading to interaction with arrestins.

In conclusion, we identify that: 1) IC₃ loop of VPAC₂ receptor is involved in G protein-coupling: the K³⁰⁹L³¹⁰T³¹¹ sequence located in the amino-domain is essential for *Gas* but not *Gai6* coupling and two basic residues (R³²⁵ and K³²⁸) located in carboxyl-domain are essential for both *Gas* and *Gai6* coupling; 2) inactivating mutations reduce both receptor phosphorylation and internalization; 3) impaired receptor phosphorylation and internalization capability is correlated with potency of inactivating mutations and impaired capability of mutants to adopt active receptor conformation.

Acknowledgments

This work was financially supported by an 'Interuniversity Poles of Attraction Program, Belgian State, Prime Minister's Office, Federal Office for Scientific, Technical and Cultural Affairs' and a grant from the Fonds Lequime-Ropsy. C Langlet is a recipient of a doctoral fellowship from the FRIA (Communauté Française de Belgique). I Langer is 'chargée de recherches' from the Fonds National de la Recherche Scientifique (FNRS), Belgium.

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Received 23 November 2004

Accepted 6 December 2004

Made available online as an

Accepted Preprint 7 December 2004