A disulfide bond between conserved cysteines in the extracellular loops of the human VIP receptor is required for binding and activation

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Abstract The importance of two highly conserved cysteines in the human vasoactive intestinal peptide receptor 1 (hVIPR 1) was examined. By site-directed mutagenesis each Cys residue was converted into Ala or Ser. The mutant and wild-type genes were transfected into HEK293 cells and tested for the ability to bind VIP and to activate cAMP production. Cys^{215}-Ala/Ser and Cys^{285}-Ala/Ser showed at least a 10-fold decrease in binding affinity and receptor potency when compared to the wild type. In contradiction to the wild-type receptor, both mutations were insensitive to dithiothreitol (DTT). The results indicate the existence of a disulfide bond between Cys^{215} and Cys^{285}, which is important for stabilising the receptor in the correct conformation for ligand binding and activation.

Key words: G protein-coupled receptor; Site-directed mutagenesis

1. Introduction

The human vasoactive intestinal peptide receptor 1 (hVIPR1) is a member of the secretin receptor family, which belongs to the group of G protein-coupled receptors having seven transmembrane helices (TM) [1–3]. hVIPR1 is activated by interaction with the widely distributed neuropeptide vasoactive intestinal polypeptide (VIP) [4]. The active conformation results in stimulation of adenylate cyclase via G protein coupling and intracellular production of cAMP. Several structural features are conserved in the entire family of G protein-coupled receptors (GPCR), forming the basis of a fundamental relationship in their functioning. Among the conserved structural features, the covalent linking of the first and second extracellular loop by a disulfide bond is considered to be important for the generation and stabilisation of receptor structure in the rhodopsin family, the major and best studied family of GPCRs [5]. Convincing evidence for the existence of a disulfide bridge between extracellular loop 1 and 2 in the secretin receptor family has not yet been provided.

The purpose of the present study was to examine the importance of cysteines and disulfide bonding for the binding and activation of hVIPR1. In addition, the effect of a disulfide reducing agent, DTT, on VIP binding was examined. Our findings suggest that a disulfide bond is present in the human VIP receptor 1 between Cys^{215} and Cys^{285}, and that this linkage is critical for maintaining a functional conformation.

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Abbreviations: hVIPR 1, human vasoactive intestinal peptide receptor 1; HEK293, human epithelial kidney 293 cell; G protein, GTP-binding protein; DTT, dithiothreitol

2. Materials and methods

2.1. Receptor constructs and site-directed mutagenesis

The cDNA encoding the human VIP receptor 1 was kindly donated by Drs. A. Couvineau and M. Laburthe, INSERM U410, Paris, France. The coding region was subcloned into the expression vector pcDNA3 from Invitrogen. Mutagenesis was carried out by the overlap extension method using Pwo-polymerase (Boehringer Mannheim). Identification of all the mutants was confirmed by sequencing (Perkin Elmer).

2.2. Cell culture and transient transfection

HEK293 cells were grown in MEM (Earl's salt), supplemented with 10% fetal bovine serum and 1‰ gentamicin. HEK293 cells were plated in 200-mm dishes (4×10^6 cells/dish) and transiently transfected with wild-type cDNA and mutant cDNA, using the $CaPO_4$ precipitation method. Cells were harvested 72 h after transfection for membrane preparation and VIP-stimulated cAMP production.

2.3. Preparation of plasma membranes

Cells from confluent 200 mm culture plates were rinsed with PBS buffer and scraped off in the same buffer. After centrifugation, the supernatant was resuspended in 15 ml binding buffer containing 20 mM HEPES, 2 mM $CaCl_2$, 150 mM NaCl, 5 mM EDTA, 1 mg/ml Bacitracin and 200 μg/ml bovine serum albumin. The cell suspension was homogenised with a polytron for 30 s, centrifuged at $20\,000\times g$ for 20 min and resuspended in binding buffer. The procedure was repeated once and the membranes were resuspended in 10 ml binding buffer and stored until use at −80°C. Protein concentration was determined by the Bradford analysis (Bio-Rad).

2.4. Binding of [^{125}I]VIP to plasma membrane

The functional properties of wild-type and mutant VIP receptors were analysed by [^{125}I]VIP binding to plasma membranes. VIP was labelled and purified as described by Martin et al. [6]. Binding reactions were carried out on 10 μg of membranes in a total volume of 150 μl with a constant amount of radioiodinated VIP (10^{-10} M) and increasing concentrations of unlabeled VIP (0–10^{-6} M) for 60 min at room temperature. Bound and free radioligands were separated by centrifugation. To examine the effect of the reducing agent DTT on [^{125}I]VIP binding, wild-type and mutant receptors were incubated with 10 mM DTT. Data were analysed by non-linear least-square regression [7].

2.5. Intracellular cAMP assay

Intracellular cAMP levels were assayed with a [^{125}I]cAMP assay system from Amersham. HEK293 cells were transfected and seeded to 2×10^5 cells/well 48 h after transfection. After a further 24 h cells were washed with MEM and incubated with 500 μl MEM containing 0.1 mM isobutyl-methylxanthine (IBMX) for 20 min at 37°C and for a further 25 min at 37°C with VIP in increasing concentrations (0 M–10^{-6} M). cAMP was extracted by incubating the cells with 50 μl trichloroacetic acid and neutralised 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 according to the manufacturer's instructions. Data were analysed by non-linear least-square regression [7].

3. Results and discussion

Several sequence alignments have been made in the rhodop-

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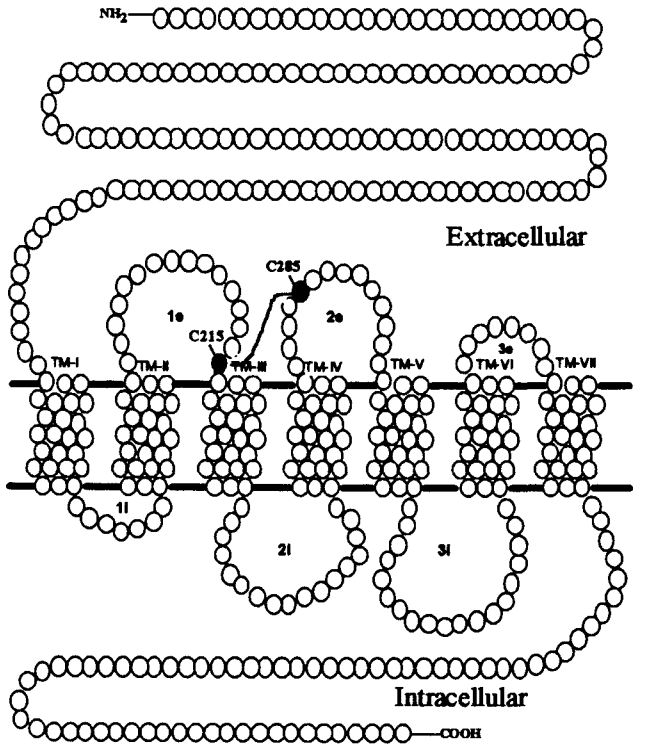

Fig. 1. Schematic representation of the human VIP receptor 1, consisting of seven transmembrane helices (TMI–TMVII), an extracellular N-terminal domain and a C-terminal domain located inside the cell. 1e–3e corresponds to the extracellular loops and 1i–3i to the intracellular loops. The investigated cysteine residues C215 and C285 are presented in black.

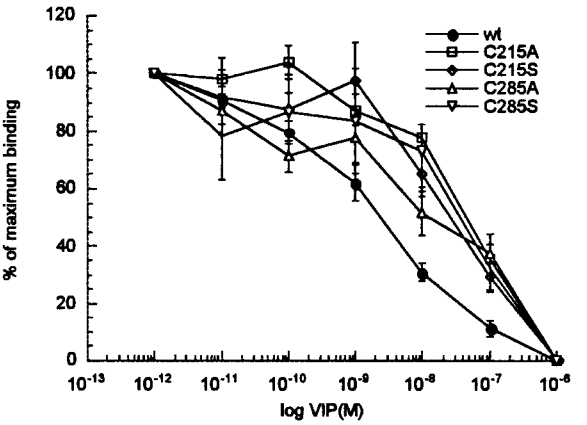

Fig. 2. Competitive inhibition of [^{125}I]VIP binding to membrane preparation from HEK293 cells, transiently expressing hVIPR1 (wild type) and mutants, by unlabelled VIP. Data points represent the mean ± S.E.M. of 8–13 separate experiments, each made in duplicate. The IC_{50} values for wild type and mutants are given in Table 1.

sin family and a common feature is the presence of two conserved cysteines in the first and second extracellular loops [8,9]. These two positions are supposed to form a covalent linking by a disulfide bridge thereby stabilising the structure of the receptor. Two residues, Cys^{215} in extracellular loop 1 and Cys^{285} in extracellular loop 2 of the human VIP receptor 1 (Fig. 1), corresponding to the highly conserved positions in the rhodopsin family, were mutated into alanine (A) or serine (S) using site-directed mutagenesis. The wild-type and mutant receptors were transfected into HEK293 cells.

The expression level, binding affinity on purified membranes and production of cAMP on cell lysates from C215A/S, C285A/S and wild type were examined. Using the equation of Akera and Cheng [10] to calculate the expression level (B_{max}), we obtained similar data for C215A/S, C285A/S and the wild type (Table 1). However, a marked decrease in binding affinity of C215A/S and C285A/S compared to the wild-type receptor was observed (Fig. 2). The effect of the disulfide reducing agent DTT was examined on wild-type and mutant receptors. Incubation of the wild type with 10 mM DTT caused, in agreement with the findings described by Robberecht et al. [11], a loss in VIP binding and receptor number, whereas the binding characteristics of the mutant receptors were unaffected (data not shown). In accordance with our binding data, the dose–response curves for VIP-induced cAMP production in the mutants were shifted 30- to 300-fold to the right (Fig. 3).

In a previous study, Gaudin et al. [12] performed a number of point mutations in the hVIPR1 including C215G and C285G. They examined the effect on VIP binding and found similar binding affinity of wild type and C215G, whereas binding to C285G was not detectable. C215G showed a 30-fold decrease in expression level compared to the wild type, which is in contrast to our findings of a similar expression level for wild type and mutants. Their conclusion was, however, that a disulfide bridge is not present. A number of explanations can be offered to explain the discrepancies between theirs and our results: (1) different methods of labelling and purifying the radioligand; (2) different buffer systems; (3) different preparations of plasma membranes; and (4) different cell lines. Their binding data were not, as in the present study, supported by cAMP production to demonstrate receptor activity. Furthermore, our findings are in accordance with the reported importance of the cysteine bond between extracellular loops 1 and 2 in the rhodopsin family [13–17].

In conclusion, our data suggest the existence of a disulfide bond between the two conserved extracellular cysteines (C215

Table 1
Binding affinity (IC_{50}), activation (EC_{50}) and receptor number (B_{max}) for human VIP receptor 1 wild type and Cys residue mutations, transiently expressed in HEK293 cells

Receptor mutation	IC_{50} (nM) ± S.E.M.	EC_{50} (nM) ± S.E.M.	B_{max} (pmol/mg protein) ± S.E.M.
Wild type	3.1 ± 0.6	0.6 ± 0.4	2.5 ± 0.5
C215A	42 ± 6	18 ± 3	1.5 ± 0.8
C215S	61 ± 28	40 ± 21	3.6 ± 0.8
C285A	60 ± 25	75 ± 35	2.4 ± 0.9
C285S	62 ± 31	232 ± 30	4.0 ± 0.8

Data represent the mean ± S.E.M. of at least five experiments, each made in duplicate.

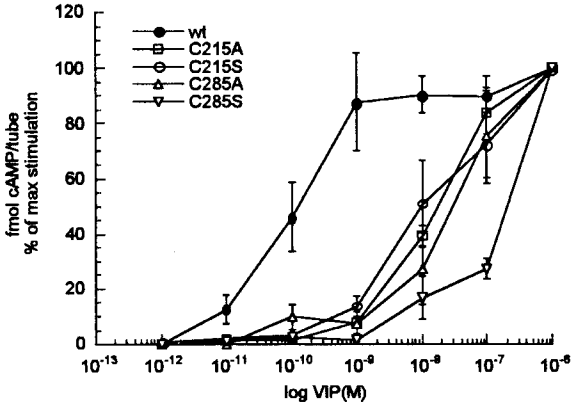

Fig. 3. VIP-induced cAMP production in HEK293 cells expressing wild-type and mutant receptors for hVIPR1. Data points represent the mean ± S.E.M. of 4–6 separate experiments, each made in duplicate. The EC_{50} values for wild type and mutants are given in Table 1.

and C285) in the human VIP receptor 1. By point mutation of the cysteines, the expression level of the receptor was unaffected, but binding and activation were severely impaired. It is likely that the cysteines and the disulfide bridge are important for the maintenance of the correct high-affinity structure of the receptor.

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