Constitutive Activation of the Human Vasoactive Intestinal Peptide 1 Receptor, a Member of the New Class II Family of G Protein-coupled Receptors*

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The human vasoactive intestinal peptide (VIP) 1 receptor belongs to the new class II subfamily of G protein-coupled receptors. Specific change by mutagenesis of a strictly conserved histidine into arginine at position 178 of the human VIP1 receptor resulted in its constitutive activation with respect to cAMP production. Transfection of the H178R mutant into COS cells resulted in a 3.5-fold increase in the cAMP level as compared with cells transfected with the wild type receptor or the vector alone. This increase was proportional to the amount of transfected cDNA. The H178R mutant exhibited an otherwise normal cAMP response to VIP as well as a dissociation constant similar to that of the wild type receptor. Other mutants at position 178 such as H178K, H178A, and H178D were not constitutively activated. They were otherwise expressed at the cell surface of transfected nonpermeabilized cells. Double mutants were then constructed in which the H178R mutation was associated with a point mutation in the the N-terminal extracellular domain that totally abolished VIP binding or VIP-stimulated cAMP production, i.e. E36A or D68A. The corresponding double mutants H178R/E36A and H178R/D68A were no longer constitutively activated. A control double mutant (H178R/D132A) with an unaltered dissociation constant for VIP and cAMP response to VIP was still constitutively activated. Our findings demonstrate that constitutive activation of the VIP1 receptor by mutation of His178 into R requires the functional integrity of the N-terminal extracellular VIP binding domain. They might provide interesting generalities about the activation process of G protein-coupled receptors.

G protein-coupled receptors with seven transmembrane domains, which constitute a large multigene family of eucaryotic proteins, interact with alkaloids, biogenic amines, peptides, glycoprotein hormones, light, and odorants (1). During the past few years, a subfamily of the superfamily of G protein-coupled receptors has emerged that shares the seven-membrane-spanning domain topography but has a low overall amino acid sequence homology (<20%) with other members of the superfamily (2, 3). This subfamily, now referred to as the class II G protein-coupled receptor family, comprises receptors for a family of structurally related peptides that includes vasoactive intestinal peptide (VIP),1 pituitary adenylate cyclase-activating polypeptide, glucagon, secretin, glucagon-like peptide 1, gastric inhibitory polypeptide, and growth hormone-releasing peptide and more unexpectedly also comprises receptors for parathyroid hormone (PTH) and calcitonin (2–4). Recent studies have extended this subfamily (3) with the discovery of subtypes of the above mentioned receptors as well as two new members having an extraordinary long N-terminal domain: the putative EGF module-containing, mucin-like hormone receptor EMR1 (5) and the leukocyte activation antigen CD97 (6).

Class II G protein-coupled receptors for peptides have homologies ranging between 30 and 50% and among several common structural properties have a large N-terminal extracellular domain (>120 amino acid residues) that contains highly conserved amino acids, including numerous cysteine residues and several potential N-linked glycosylation sites (2, 3). Taking the human VIP1 receptor (7), which activates adenyl cyclase via stimulatory Gs proteins (8), as a prototype of class II G protein-coupled receptors, we recently provided evidence for an important role of the N-terminal domain for ligand binding with several crucial residues (9) probably positioned in a tertiary structure maintained by multiple disulfide bonds (10). We also demonstrated the mandatory role of two glycosylation sites in this domain for correct delivery of the receptor to the plasma membrane (11). Other functional domains for ligand recognition do exist, since we showed, by constructing receptor chimeras, that a structural determinant for peptide selectivity was made of three nonadjacent amino acid residues in the first extracellular loop and third transmembrane domain (12). The role of extracellular domains in natural ligand binding has now been documented for several members of class II G protein-coupled receptors such as rat VIP1 and secretin (13, 14), pituitary adenylate cyclase-activating polypeptide (15), calcitonin (16), PTH (17, 18), glucagon (19, 20), glucagon-like peptide 1 (21, 22), or growth hormone-releasing peptide (23) receptors.

Constitutively active mutants of several G protein-coupled receptors have been characterized experimentally by site-directed mutagenesis (24) and have been also described as disease-causing in humans (25, 26). A constitutively active mutant of a class II G protein receptor has been reported for the PTH-PTH-related peptide receptor in Jansen-type metaphy-
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Seal chondrodysplasia affecting a strictly conserved histidine residue in the first intracellular loop of this class of receptor (27). In view of the fact that class II G protein-coupled receptors display an original structure-function relationship with respect to ligand recognition and very low sequence homology with other G protein-coupled receptors (2, 3), we further investigated their constitutive activation in the human VIP1 receptor whose structure-function relationship has been previously documented (3). In this paper, we demonstrate that specific mutation of histidine 178 in the VIP receptor causes its constitutive activation. Moreover, we take advantage of the fact that the VIP binding site is located, at least in part, in the N-terminal extracellular domain to construct double mutants in which the histidine-to-arginine mutation critical for constitutive activation has been associated with point mutations in the N-terminal domain that prevent VIP binding. This new approach allowed us to show that the agonist-independent constitutive activation of the histidine-to-arginine mutant appears to require the integrity of the natural ligand binding site. These data provide new insight into the constitutive activation of G protein-coupled receptors.

EXPERIMENTAL PROCEDURES

Materials—Enzymes for cloning, sequencing, and oligonucleotide-directed mutagenesis were obtained from Promega (Madison, WI) or Life Technologies, Inc. (Cergy-Pontoise, France), and synthetic oligonucleotides were from Eurogentec (Seraing, Belgium). [α-32P]dATP (1000 Ci/mmol) and other radioactive reagents were obtained from Amersham (Buckinghamshire, United Kingdom). Synthetic porcine VIP was purchased from NeoScience (Strasbourg, France), and culture medium and horse fetal serum from Life Technologies. [125I]VIP was prepared and purified as described (28). The monoclonal anti-Flag antibodies were obtained from Eastman Kodak Co., and [125I]-labeled antimouse IgG whole antibody from goat was purchased from NEN Life Science Products. All other chemicals of the highest quality commercially available were purchased from Sigma (Saint-Quentin Fallavier, France).

Site-directed Mutagenesis—The 1.4-kilobase EcoRI fragment containing the entire coding sequence of the human VIP1 receptor (7) was subcloned into the EcoRI site of the pALTER-1 vector, and single-stranded DNA was produced in E. coli. Oligonucleotide-directed mutagenesis was obtained by using double-stranded DNA (29). VIP receptor mutants were generated by oligonucleotide-directed mutagenesis as described (11). Identification of the desired mutations was obtained by direct double-stranded sequencing of the regions encompassing mutations. Inserts encoding mutant sequences were subcloned in the eucaryote expression vector pCDNA3. The wild type and mutant receptors were all tagged in the N-terminal extracellular domain by inserting the marker octapeptide DYKDDDDK (Flag) between Ala30 and Ala31. The Flag sequence was inserted by oligonucleotide-directed mutagenesis as described above using 5′-GGGCGCGGCCCCCGGCA-GGCCGACTACAGAGGACGATGCACGAGCTCAG-G-3′ oligonucleotide. This site of insertion was selected because it is localized between the end of the putative signal peptide (3, 7) and Glu36 which has been shown to be the first crucial amino acid residue for human VIP1 receptor functional properties (29). It was verified that insertion of the Flag octapeptide sequence between Ala30 and Ala31 did not modify the dissociation constant for VIP or the dose response of VIP to stimulate cAMP production, as compared with the native human VIP1 receptor.

Transfection of Cells—Wild type and mutant VIP receptors were transfected into COS-7 cells or CHO cells. Cells were grown in medium (Dulbecco's modified Eagle's medium for COS-7 or Ham's F-12 for CHO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. Cells were transfected by the electroporation method using an Electroporator II apparatus (Inventron). Briefly, 4 × 106 cells were preincubated in ice for 5 min with 15 μg of wild type or mutant receptor cDNA constructs in phosphate-buffered saline. After electroporation (330 V, 500 microfarads for COS-7 cells or 1,000 microfarads for CHO cells, infinite resistance), cells were put on ice for 5 min and then transferred into culture medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin before seeding in Petri dishes for binding assay, 12-well trays for the cAMP assay or 24-well trays for antibody binding experiments, or on glass slides in 24-well trays for immunofluorescence studies. The culture medium was changed 16–18 h after transfection, and cells were used 48 h after transfection.

Ligand Binding Assays—The functional properties of wild type and mutant VIP receptors were analyzed by [125I]VIP binding to transfected cell membranes. Transfected COS-7 cells were washed twice with cold phosphate-buffered saline. Then they were harvested with a rubber policeman and centrifuged at 3,000 rpm for 5 min at 4 °C, and the cell pellets were incubated for 30 min on ice in a hypotonic 5 mM HEPES buffer, pH 7.4. Thereafter, cells were homogenized as described (28), and the homogenate was centrifuged at 11,000 rpm for 15 min at 4 °C. The pellet was washed with 20 mM HEPES buffer and stored at −80 °C until use. This pellet was referred to as the membrane preparation. Membranes (200 μg of protein/ml) were incubated for 60 min at 30 °C in 20 mM HEPES buffer, pH 7.4, containing 2% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, 0.05 mM 125I-VIP in the presence of increasing concentrations of unlabeled VIP. The reaction was stopped as described (28). Specific binding was calculated as the difference between the amount of 125I-VIP bound in the absence and the presence of 1 μM unlabeled VIP. Binding data were analyzed using the LIGAND computer program (30). Protein content in membrane preparations was evaluated by the procedure of Bradford (31) with bovine serum albumin as standard.

cAMP Experiments—Transfected COS-7 cells or CHO cells were grown in 12-well trays as described above. The culture medium was discarded, and attached cells were gently rinsed with phosphate-buffered saline (pH 7). They were then incubated with or without VIP under continuous agitation in 0.5 ml of phosphate-buffered saline containing 2% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, 0.01 mg/ml aprotinin, and 1 mM iso-butyl-1-methylxanthine as described (7). At the end of the incubation (30 min at room temperature), the medium was removed, and cells were lysed by 1% percolloric acid. The cAMP present in the lysate was measured by radiomunnoassay as described (32). Cell number was determined in parallel wells, and data are reported as pmol of cAMP/106 cells.

Confocal Laser Scanning Microscopy—Transfected cells were grown on 12-mm glass coverslips for 48 h as described above. After they were washed three times with phosphate-buffered saline (PBS), nonpermeabilized cells were incubated for 60 min at room temperature with the mouse monoclonal anti-Flag antibodies diluted 1:50 in PBS containing 1% (w/v) bovine serum albumin. The cells were then washed three times with PBS and exposed for 60 min to the secondary antibody (FITC-goat anti-mouse IgG [Fab-specific] to a 1:250 dilution). Cells were then fixed for 5 min in PBS containing 2% (w/v) paraformaldehyde. The coverslips were washed once in 90% (v/v) PBS and once in PBS, and they were scanned using a Leica TCS 4D true confocal scanner composed of a Leica Diaplan microscope equipped with an argon-crytron-argon laser (488 nm) with an output power of 2–50 milliwatts and a VME bus MC 68020/68881 computer system coupled to an optical disc for image storage (Leica Lasertechnik GmbH). The emitted light was collected through a long pass filter on the target of the photo multiplier. Each sample was treated with a calman filter to increase the ratio signal versus background. All image generating and processing operations were carried out using the Leica CLSM software package. Screen images were taken on Kodak Ektachrome film using a 35-mm camera.

Assessment of Cell Surface Expression of Mutated Receptors—Cell surface expression of mutated receptors was assessed using the mouse monoclonal anti-Flag antibodies as described (33) with modifications. Transfected cells grown in 24-well trays (see above) were rinsed twice with 50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 5 mM KC1, 2 mM CaCl2, 5% heat-inactivated horse serum, and 0.5% heat-inactivated fetal bovine serum (binding buffer), incubated for 4 h at room temperature with anti-Flag antibodies diluted 1:50 in binding buffer. Cells were then washed three times with binding buffer and exposed for 2 h at room temperature to the radiolabeled (400,000 cpm/well) secondary antibodies [125I]-labeled goat anti-mouse IgG. Cells were rinsed again four times with binding buffer and then lysed with 250 μl of 0.5 M NaOH, and the radioactivity of the lysate was counted. Nonspecific binding was determined with cells that were incubated only with the [125I]-labeled secondary antibody. Binding of anti-Flag antibodies to epitope-tagged mutant receptors was given as a percentage of anti-Flag antibodies binding to epitope-tagged wild type receptor.

RESULTS

Fig. 1 shows a schematic representation of the wild type human VIP1 receptor, pointing out the amino acid residues.
that have been changed by site-directed mutagenesis in the present study. We first mutated histidine 178, which is highly conserved in class II G protein-coupled receptors. The histidine-to-arginine mutant (H178R) of the human VIP1 receptor, which mimics the situation found in constitutively activated PTH receptors (27), was transfected into monkey kidney COS-7 cells, and intracellular cAMP was measured. The basal cAMP level was 3.5-fold higher than the basal cAMP level measured after transfection of the wild type human VIP1 receptor (Fig. 2) or the vector alone (not shown). This latter observation suggests that the wild type receptor was not constitutively activated. Similar constitutive activity of the H178R mutant was observed upon transfection of the CHO cell line derived from Chinese hamster ovary (not shown). Indeed, basal cAMP level in CHO cells transfected with the H178R mutant was 5 times higher than that observed for the wild type receptor, i.e. 19.5 ± 5.6 and 3.7 ± 1.4 pmol/10^6 cells (three experiments), respectively. Therefore, constitutive activation does not appear to be dependent on the nature of the cell line expressing the mutated receptor cDNA. Fig. 3 shows the cAMP response in COS-7 cells transfected by H178R mutant and wild type receptors upon stimulation by VIP. Maximal cAMP responses were identical. Half-maximal stimulations above basal level were obtained for similar concentrations of VIP in cells transfected with the H178R receptor or the wild type receptor, i.e. 1.5 ± 0.5 × 10^{-10} M and 0.4 ± 0.1 × 10^{-10} M, respectively. Scatchard analysis of VIP binding to COS-7 cell membranes indicated that the H178R receptor mutant bound VIP with a similar dissociation constant as compared with the wild type receptor (Table I). In these experiments, the concentration of VIP binding sites was higher in cells transfected with the wild type receptor than in cells transfected with the H178R receptor mutant (Table I). This observation did not favor the hypothesis that the higher basal cAMP level in cells transfected with H178R could be merely related to a higher expression of the receptor as compared with cells transfected with the wild type receptor inasmuch as the wild type receptor is not constitutively activated by itself (see above). However, to document this issue, we transfected COS cells with increasing concentrations of cDNA encoding the H178R mutant or wild type receptor. For the receptor mutant, it was observed that basal cAMP levels increased when increasing amounts of cDNA were transfected in COS cells (Fig. 4). In sharp contrast, the basal cAMP level in COS cells transfected with the wild type receptor was constant regardless of the amount of cDNA transfected (Fig. 4). This latter observation further argued against constitutive activity of the wild type human VIP1 receptor itself. For both the mutated and wild type receptors, we verified that VIP (10^{-6} M)-stimulated cAMP levels increased with the amount of transfected cDNA (Fig. 4). Likewise, the ligand binding assay showed that the amount of 125I-VIP specifically bound to trans-
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 Constitutive activation of the human VIP1 receptor by mutation of His178 into Arginine did not happen with mutations into alanine, aspartate, and even lysine, and that such mutations also abolished VIP binding suggests that such constitutive activation might be dependent on the integrity of the VIP binding site. To investigate this issue, we constructed double mutants in which the mutation resulting in the constitutive activation of the receptor (H178R) was associated with point mutations in the N-terminal extracellular domain of the receptor, which abolished VIP binding as shown previously (9, 29). First, we chose a mutation affecting an amino acid residue highly conserved in the class II G protein-coupled receptors, i.e. D68A (9). As expected, the H178R/D68A mutant no longer bound VIP like the single mutant D68A (Table I); nor did it mediate the stimulation of cAMP production by VIP (Fig. 6).

Further experiments were carried out to determine whether mutation of His178 into residues other than arginine also resulted in constitutive activation of the human VIP1 receptor. We mutated H178 into a neutral residue (H178A), an acidic one (H178D), or a basic one (H178K). Among these mutants, none was constitutively activated after transfection in COS-7 cells (Fig. 2). Moreover, these mutants were unable to mediate VIP-stimulated cAMP production (Fig. 2), probably because they no longer bound VIP (Table I). To determine the pattern of expression of these inactive mutants in transfected COS-7 cells, immunofluorescence studies and antibody binding experiments were performed. No antibodies to native human VIP1 receptors are currently available. However, the Flag sequence DYKD-DDDK could be inserted between Ala30 and Ala31 in the N-terminal extracellular domain of the human VIP1 receptor without altering its phenotype with regard to VIP binding and VIP-stimulated cAMP production (see “Experimental Procedures”), indicating that insertion of the Flag epitope had no impact on the receptor’s functional properties. Insertion of this extracellular epitope enabled us to perform immunofluorescence studies with the wild type and mutated receptors in nonpermeabilized transfected COS cells and also to assess cell surface expression of receptors by anti-Flag antibody binding to nonpermeabilized transfected COS cells. Confocal laser microscopy of nonpermeabilized COS cells expressing the epitope-tagged wild type receptor revealed intense fluorescence when incubated with the anti-Flag antibodies and subsequently with an FITC-labeled antimouse antibody (Fig. 5). This observation supported delivery of the receptor protein at the cell surface as expected for the wild type receptor. Untransfected COS cells that were incubated with both antibodies or COS cells expressing the epitope-tagged wild type receptor that were incubated only with the FITC-labeled antimouse antibodies showed no fluorescence (Fig. 5).

The dose response of VIP in stimulating cAMP accumulation in COS cells expressing the wild type human VIP1 receptor (C) or the H178R receptor mutant (○). Cells were incubated for 30 min at room temperature in the presence of various concentrations of VIP as indicated. The cAMP was measured as described under “Experimental Procedures.” Data are means ± S.E. of three experiments.

Table I

| Constructs              | Dissociation constant (nM) | Binding capacity (pmol/mg protein) |
|------------------------|----------------------------|-----------------------------------|
| Wild type              | 0.62 ± 0.05                | 6.1 ± 1.5                         |
| H178R                  | 0.29 ± 0.08                | 2.0 ± 0.9                         |
| H178A                  | ND*                        | ND                                |
| H178D                  | ND                         | ND                                |
| H178K                  | ND                         | ND                                |
| E36A                   | ND                         | ND                                |
| H178R/E36A             | ND                         | ND                                |
| D68A                   | ND                         | ND                                |
| H178R/D68A             | ND                         | ND                                |
| D132A                  | 0.69 ± 0.08                | 1.6 ± 0.3                         |
| H178R/D132A            | 1.12 ± 0.66                | 3.0 ± 0.6                         |

*ND, binding not detectable.

Fig. 3. Dose response of VIP in stimulating cAMP accumulation in COS cells expressing the wild type human VIP1 receptor (C) or the H178R receptor mutant (○). Cells were incubated for 30 min at room temperature without (C) or with (○) 10⁻⁶ M VIP. Data are the mean ± S.E. of three experiments.

Fig. 4. Basal (A) and VIP-stimulated (B) cAMP accumulation in COS cells transfected with increasing concentrations of the plasmid DNA encoding the wild type human VIP1 receptor (C) or the H178R receptor mutant (○). Cells were incubated for 30 min at room temperature without (A) or with (B) 10⁻⁶ M VIP. Data are the mean ± S.E. of three experiments.
verified that the epitope-tagged H178R/D68A mutant like the D68A mutant (not shown) exhibited a cell surface expression similar to that of the wild type receptor as assessed by confocal microscopy (Fig. 5) and antibody binding experiments (Table II). Next, we chose an amino acid residue that was not conserved in the class II G protein-coupled receptors but the mutation of which abolished VIP binding i.e. E36A (29). Like the single mutant E36A, the H178R/E36A mutant no longer bound VIP (Table I) and did not mediate the stimulation of cAMP production by VIP (Fig. 6).

![Confocal laser scanning microscopic detection after transfection in COS cells of the epitope-tagged wild type receptor and receptor mutants.](image)

### FIG. 5
Confocal laser scanning microscopic detection after transfection in COS cells of the epitope-tagged wild type receptor and receptor mutants. Nonpermeabilized cells were incubated with anti-Flag antibodies and then exposed to the radiolabeled second antibodies. Nonpermeabilized transfected cells were incubated with anti-Flag antibodies, washed, incubated with antimouse immunoglobulin G conjugated to FITC, and then fixed as described under “Experimental Procedures.” The following receptor constructs were shown: wild type receptor (C), H178R mutant (D), H178A mutant (E), H178D mutant (F), H178K mutant (G), H178R/E36A mutant (H), H178R/D68A mutant (I), and H178R/D132A mutant (J). Controls were carried out with untransfected COS cells (A) and COS cells expressing the epitope-tagged wild type receptor that were incubated only with the FITC-labeled antimouse antibody (B).

### TABLE II
Cell surface expression of mutated human VIP1 receptors after transfection of cDNAs into COS-7 cells

| Constructs                  | Surface expression (% of wild type) |
|-----------------------------|-------------------------------------|
| Wild type                   | 100 ± 3 (8)                         |
| H178R                       | 122 ± 15 (8)                        |
| H178A                       | 56 ± 2 (6)                          |
| H178D                       | 42 ± 7 (8)                          |
| H178K                       | 89 ± 7 (6)                          |
| H178R/E36A                  | 103 ± 11 (8)                        |
| H178R/D68A                  | 94 ± 18 (8)                         |
| H178R/D132A                 | 98 ± 8 (6)                          |

![Basal (A) and VIP-stimulated (B) cAMP levels in COS cells expressing the wild type human VIP1 receptor or single or double mutants of the receptor as indicated.](image)

### FIG. 6
Basal (A) and VIP-stimulated (B) cAMP levels in COS cells expressing the wild type human VIP1 receptor or single or double mutants of the receptor as indicated. Cells were incubated for 30 min at room temperature without (A) or with 10^{-6} M VIP (B). The intracellular cAMP was measured as described under “Experimental Procedures.” Data are means ± S.E. of three experiments.
was not constitutively activated when expressed in COS cells (Fig. 6). Again, it was verified that the epitope-tagged H178R/E36A mutant, like the E36A mutant (not shown), exhibited a cell surface expression similar to that of the wild type receptor as assessed by confocal microscopy (Fig. 5) and antibody binding experiments (Table II). As a control, we developed another double mutant in which H178R was associated with a point mutation (D132A) in the N-terminal extracellular domain, which was previously shown not to alter VIP binding (29). As expected, the H178R/D132A mutant, like the single mutant D132A, bound VIP with a dissociation constant similar to that of the wild type receptor (Table I). This mutant also mediated VIP-stimulated cAMP production (Fig. 6) and exhibited cell surface expression similar to that of wild type receptor in nonpermeabilized transfected cells as assessed by confocal microscopy (Fig. 5) and antibody binding experiments (Table II). Most interestingly, the double mutant H178R/D132A did exhibit constitutive activation upon transfection in COS cells (Fig. 6).

**DISCUSSION**

The concept of ligand-independent or constitutive activation of G protein-coupled receptors has emerged following site-directed mutagenesis of the α₁-adrenergic receptor (34). It was subsequently documented by the discovery of naturally occurring mutations in diseases (25, 26) as well as experimental mutations (24) in various G protein-coupled receptors. Following the discovery of a constitutively active mutant of the PTH-PTH-related peptide receptor in Jansen-type metaphyseal chondrodysplasia (27), this paper is the first to analyze constitutive activation of a member of the emerging class II family of G protein-coupled receptors, e.g., the human VIP1 receptor.

This work demonstrates that replacement of histidine 178 by arginine in the human VIP1 receptor results in constitutive activation of the receptor with respect to cAMP production, which constitutes its signaling pathway (35). In contrast, the wild type VIP receptor appears to be truly silent in the absence of VIP. While mutation of histidine 178 into arginine results in constitutive activation of the receptor, mutations into other residues such as alanine, aspartate, and even lysine did not confer ligand-independent activation. This suggests that the replacement by arginine evokes a subtle conformational change that cannot be mimicked by lysine and is probably not simply related to the electrical charge of arginine. This situation is quite different from that described for the α₁₁-adrenergic receptor, in which the 19 possible amino acid substitutions at a single site confer constitutive activation, suggesting that this site may function to constrain the G protein coupling of the receptor in the inactive form in the wild type receptor (36). With regard to the human VIP1 receptor, it may be suggested that the histidine to arginine exchange does not simply remove some stabilizing conformational constraints as in α₁₁-adrenergic receptors (36) or β₂-adrenergic receptors (37). In this context, it is worth pointing out that the naturally occurring mutation of the equivalent histidine in the PTH-PTHrP receptor in Jansen metaphyseal chondrodysplasia was also into arginine (27). The crucial importance of histidine 178 in the human VIP1 receptor is further suggested by the fact that substitutions other than arginine resulted in the absence of VIP binding and of VIP-stimulatable cAMP production, supporting the idea of an important structural change in these mutants. In contrast, the histidine to arginine substitution did not change the dissociation constant of the receptor, nor did it alter the potency of VIP in stimulating cAMP production. This contrasts again with the α₁₁-adrenergic receptor, since all mutated receptors that are constitutively activated demonstrate a higher affinity for the agonist, a characteristic of the active conformation of G protein-coupled receptors (36). Similar increased sensitivity to the agonist was reported for constitutively active β₂-adrenergic receptors (38) or muscarinic receptors (39). However, such an increase in the apparent affinity of constitutively activated receptors for agonists was not observed for several other receptors such as the thyrotropin receptor (40) or the luteinizing hormone receptor (41). A recent report indicates that constitutive activation of the AT₁a angiotensin II receptor by mutation in the third transmembrane domain does not modify the Kᵣ and Kₐₑₑ values for angiotensin (42). This suggests that differences regarding the mechanism of constitutive activation may exist not only between amine receptors and the class II family to which VIP receptors belong but also within the class I family of receptors for amines, peptides, or glycoprotein hormones.

Constitutive activation evoked by replacement of histidine 178 by arginine in the human VIP1 receptor is much less efficient than activation induced by the natural agonist VIP in the H178R mutant receptor or the wild type receptor. Nevertheless, the construction of double mutants supports a close relationship between the ligand-dependent activation triggered by VIP and the ligand-independent one evoked by mutation of histidine 178 into arginine. Indeed, when the H178R mutation at the junction of the first intracellular loop and second transmembrane domain was associated with mutation of aspartate 68 (D68A) in the N-terminal extracellular domain, which abolishes VIP binding and VIP-stimulated cAMP production (9), the resulting double mutant was no more constitutively activated and has the phenotype of the D68A mutant. Since aspartate 68 is highly conserved in the class II family of receptors (3), it may be argued that its mutation profoundly affects the overall structure of the receptor by disrupting the structure of the N-terminal extracellular domain. We have thus considered another residue within the N-terminal extracellular domain that is not conserved in the class II family of receptors but whose mutation abolishes VIP binding and VIP-stimulated cAMP production i.e., glutamate 36 (29). Again, the double mutant H178R/E36A was no longer constitutively activated and displayed the same phenotype as the single mutant E36A. These observations suggested that the integrity of the VIP binding site at the N-terminal extracellular domain should be maintained for constitutive activation by the H178R mutation. This was further supported by a control double mutant affecting a residue in the N-terminal extracellular domain whose mutation was previously shown not to affect the phenotype of the receptor i.e., aspartate 132 (29). The resulting double mutant H178R/D132A clearly displayed constitutive activation. From these data, it could be hypothesized that the H178R mutation mimics what happens when VIP binds to the wild type receptor and thereby triggers activation of cAMP production. Similar conclusions were previously drawn from analysis of constitutive activation of other receptors (43) such as thrombin receptors (44) or α₁-adrenergic receptors (45). However, it is worth pointing out that our original approach consisting of constructing double mutants provides new arguments indicating that the conformational change triggering constitutive activation in a receptor mutant requires the functional integrity of the ligand binding site. This double mutation approach was feasible for the VIP receptor, because the binding site in the N-terminal extracellular domain is well separated from the site of the mutation evoking constitutive activation. To the best of our knowledge, such a double mutation approach has not been described previously.

Many natural or experimental mutations resulting in constitutive activation of G protein-coupled receptors have been reported in the third intracellular loop, most probably because
this loop is involved in G protein activation by the receptor (4). Such mutations have been also described for several receptors in other domains including transmembrane segments and extracellular loops (4). Mutation in the N-terminal extracellular domain of the thrombin receptor (44) or deletion of a portion of this domain in the thyrotropin receptor (45) can also cause ligand-independent transmembrane signaling. To our knowledge, constitutively activating mutations at the junction of the first intracellular loop and second transmembrane domain have been only described for the VIP1 receptor (this paper) and the PTH-PTHrP receptor (27). Whether such atypical localization is related to a unique characteristic of the class II family of G protein-coupled receptors with respect to coupling to G proteins and their mechanism of transmembrane signaling remains unclear. What is clear is that the third intracellular loop of members of class II family of receptors such as the glucagon-like peptide 1 receptor (47) and the PTH-PTHrP receptor (48) is critical for coupling to the cAMP signal transduction pathway as has been observed for other G protein-coupled receptors (4). At present, nothing is known about the domain(s) involved in the coupling of VIP1 receptors with G proteins.

Since histidine 178 in the human VIP1 receptor is strictly conserved in all members of the class II family of receptors, it may be of general functional importance in these G protein-coupled receptors. Whether mutation of the equivalent histidine in other peptide receptor of this family results in ligand-independent activation has been only reported for the PTH receptor (27). In this context, it is worth pointing out that two members of this family are receptors having an extraordinary long N-terminal extracellular domain with unique features: the putative EGF module-containing, mucin-like hormone receptor EMR1 (5) and the leukocyte activation antigen antigen CD97 (6). Since histidine 178 in the human VIP1 receptor is strictly conserved in all members of the class II family of receptors, it may be of general functional importance in these G protein-coupled receptors. Whether mutation of the equivalent histidine in other peptide receptor of this family results in ligand-independent activation has been only reported for the PTH receptor (27). In this context, it is worth pointing out that two members of this family are receptors having an extraordinary long N-terminal extracellular domain with unique features: the putative EGF module-containing, mucin-like hormone receptor EMR1 (5) and the leukocyte activation antigen antigen CD97 (6). Since histidine 178 in the human VIP1 receptor is strictly conserved in all members of the class II family of receptors, it may be of general functional importance in these G protein-coupled receptors. Whether mutation of the equivalent histidine in other peptide receptor of this family results in ligand-independent activation has been only reported for the PTH receptor (27). In this context, it is worth pointing out that two members of this family are receptors having an extraordinary long N-terminal extracellular domain with unique features: the putative EGF module-containing, mucin-like hormone receptor EMR1 (5) and the leukocyte activation antigen antigen CD97 (6).

In conclusion, this study represents the first analysis of a constitutively active receptor within the class II G protein-coupled receptor family. Our results underscore the importance of the N-terminal extracellular agonist binding domain in the mechanism of ligand-independent constitutive activation of the VIP1 receptor. Whether the extracellular ligand binding domain is also important in the mechanism of constitutive activation of other class II G protein-coupled receptors remains to be established.

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