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The Human Vasoactive Intestinal Peptide/Pituitary Adenylate Cyclase Activating Peptide Receptor 1 (VPAC1): Constitutive Activation by Mutations at Threonine 343

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The human vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide receptor1 (VPAC1) belongs to the class II subfamily of G protein-coupled receptors. Specific changes by mutagenesis of a strictly conserved threonine (H) into lysine (K), proline (P) or alanine (A) at position 343 of the human VPAC1 receptor resulted in its constitutive activation with respect to cAMP production. Transfection of these mutants into Cos cells evoked a 3.5 fold-increase in the cAMP level as compared to cells transfected with the wild-type receptor. In contrast other mutants such as T343C, T343E or T343F were not constitutively activated. They were otherwise expressed at the cell surface of transfected nonpermeabilized cells. Double mutants were then constructed in which the T343K 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 T343K-E36A and T343K-D68A were no longer constitutively activated. A control double mutant (T343K-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 VPAC1 receptor can be evoked by specific mutations of T343 at the junction of the second intracellular loop and fourth transmembrane segment. This constitutive activation appears to require the functional integrity of the N-terminal extracellular VIP binding domain.

The neuropeptide vasoactive intestinal peptide (VIP) has two different receptors now referred to as VPAC1 and VPAC2 because they display very similar affinity for VIP and another regulatory peptide, the pituitary adenylate cyclase activating polypeptide (PACAP) [see Refs 1-3 for reviews]. VPAC receptors belong to the class II subfamily of G protein-coupled receptors together with receptors for VIP-related peptides such glucagon and secretin, receptors for calcitonin and parathyroid hormone (PTH) [1, 2], the so-called EGF 7TM receptors (1, 2, 4) and a α-latrotoxin receptor (5). Class II G protein-coupled receptors for peptides have homologies ranging between 30-50% and have several common structural properties including a large N-terminal extracellular domain that contains six highly conserved cysteine residues (1, 2). Taking the human VPAC1 receptor as a model class II receptor we showed that the N-terminal domain is crucial for VIP binding (1, 2, 6–8) and correct delivery of the receptor to plasma membrane (9). We also identified other functional domains for VIP binding (7, 10) or peptide selectivity (11) in extracellular loops and third transmembrane segment.

While many constitutively active mutants of G protein-coupled receptors have been characterized experimentally or have been described as disease-causing in humans (12), no such mutants were described until recently for class II receptors. In 1995, Shipani et al (13) described a constitutively active mutant of the PTH-PTH-related peptide receptor in Jansen-type metaphyseal chondrodysplasia affecting a strictly conserved histidine residue in the first intracellular loop of the receptor. We recently reported that mutation of this conserved histine residue into arginine in the human VPAC1 receptor also resulted in its constitutive activation with respect to stimulation of cAMP production (14). Another constitutively active mutant of the PTH-PTH-related peptide receptor was further described in Jansen's metaphyseal chondrodys-

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plasia affecting a threonine residue (Thr à Pro) located at the junction of the second intracellular loop and fourth transmembrane segment (15, 16). Since this threonine residue is also conserved in class II G protein-receptors for peptides, including the VPAC1 receptor (1, 2), we have determined whether mutants at this locus in the human VPAC1 receptor could be constitutively activated. In this paper, we provide evidence that mutations of threonine 343 in the VPAC1 receptor into proline, lysine or alanine do evoke constitutive activation.

MATERIALS AND METHODS

Materials. Enzymes for cloning, sequencing, and oligonucleotide-directed mutagenesis were obtained from Promega (Madison, WI, USA) or GIBCO-BRL (Cergy-Pontoise, France) and synthetic oligonucleotides from Eurogentec (Seraing, Belgium). [α-^{35}S]dATP (1000 Ci/mmol) and other radioactive reagents were obtained from Amersham (Buckinghamshire, England). Synthetic porcine VIP was purchased from Neosystem (Strasbourg, France) and culture medium and horse fetal serum from GIBCO-BRL (Cergy-Pontoise, France). [^{125}I]-VIP was prepared and purified as described (17). The monoclonal anti-Flag antibodies were obtained from Kodak (New Haven, CT, USA) and [^{125}I]antimouse IgG whole antibody from goat was purchased from NEN (Boston, MA, USA). 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 VPAC1 receptor (18) was subcloned into the EcoRI site of the pAlter-1 vector and single stranded DNA (+ strand) was produced in Escherichia coli JM109. Full-length VPAC1 receptor mutants were generated by oligonucleotide-directed mutagenesis as described (9–11). Identification of the desired mutations was obtained by direct double strand sequencing of the regions encompassing mutations. Inserts encoding mutant sequences were subcloned in the 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 A30 and A31 as described (14).

Transfection of cells. Wild-type and mutant VIP receptors were transfected into Cos-7 cells by electroporation as described (9–11). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 UI/ml penicillin, 100 mg/ml streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. Then, 4.10^6 cells were preincubated in ice for 5 min with 15 μg of salmon sperm DNA used as carrier and 15 μg of wild type or mutant receptor cDNA constructs in phosphate-buffered saline (PBS). After electroporation, cells were put on ice for 5 min and then transfered into culture medium before seeding in Petri dishes for binding assay, 12-well trays for the cAMP assay or 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 used 48 h after transfection.

Ligand binding assay. The functional properties of wild-type and mutant VIP receptors were analyzed by [^{125}I]-VIP binding to transfected cell membranes as described (9–11). 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 nM [^{125}I]-VIP in the presence of increasing concentrations of unlabeled VIP. Specific binding was calculated as the difference between the amount of [^{125}I]-VIP bound in the absence and the presence of 1 μM unlabeled VIP. Binding data were analyzed using the LIGAND computer program (19). Protein content in membrane preparations was evaluated by the procedure of Bradford (20) with bovine serum albumin as standard.

cAMP experiments. Transfected Cos-7 cells were grown in 12-well trays as described above. The culture medium was discarded and attached cells were gently rinsed with PBS (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 3-isobutyl-1-methylxanthine as described (18). At the end of the incubation (30 min at room temperature) the medium was removed and cells lysed by 1M perchloric acid. The cAMP present in the lysate was measured by radioimmunoassy as described (17, 18). Cell number was determined in parallel wells.

Confocal laser scanning microscopy. Transfected cells were grown on 12-mm glass coverslips for 48 h as described above. After washing with PBS, nonpermeabilized cells were incubated for 60 min at room temperature with the mouse monoclonal anti-Flag antibodies, then washed three times with PBS and exposed for 60 min to the secondary antibody [FITC-goat anti-mouse IgG (Fab specific). Cells were then fixed for 5 min in PBS containing 2% (w/v) paraformaldehyde. The coverslips were mounted in 50% (v/v) glycerol in PBS and selected fields were scanned using a True Confocal Scanner Leica TCS 4D as described (14).

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 (14). 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 KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum and 0.5% heat-inactivated fetal bovine serum (binding buffer), incubated with anti-Flag antibodies, then washed three times with binding buffer and exposed to the radiolabeled (400,000 cpm/well) second antibodies ([^{125}I-labeled goat anti-mouse IgG). Cells were rinsed again four times with binding buffer, then lysed with NaOH and the radioactivity of the lysate was counted. Nonspecific binding was determined with cells that were incubated only with the [^{125}I-labeled second antibody. Binding of anti-Flag antibodies to epitope-tagged mutant receptors was given as a percentage of specific anti-Flag antibodies binding to epitope-tagged wild-type receptor (14).

RESULTS

We mutated threonine 343 which is located at the junction of the second intracellular loop and fourth transmembrane segment of the VPAC1 receptor (see Fig. 1) into several other residues. The corresponding mutants were transfected into Cos-7 cells, and intracellular cAMP was measured (Fig. 2). It appeared that for three mutants including T343K, T343P and T343A mutants, the basal cAMP levels were significantly higher than the basal cAMP level measured after transfection of the wild-type receptor. A 3.5-fold increase was observed for the three mutants which were constitutively activated thereby. In contrast, other mutants including T343C, T343E and T343F did not provoke alteration of basal cAMP level following transfection into Cos-7 cells (Fig. 2). In order to verify that all mutants were expressed by transfected cells, we carried out Scatchard analysis of VIP binding to cell membranes. All mutants bound VIP with dissociation constants which were similar to that of the wild-type receptor (Table 1). Although we observed variations in the binding capacities, the absence of con-

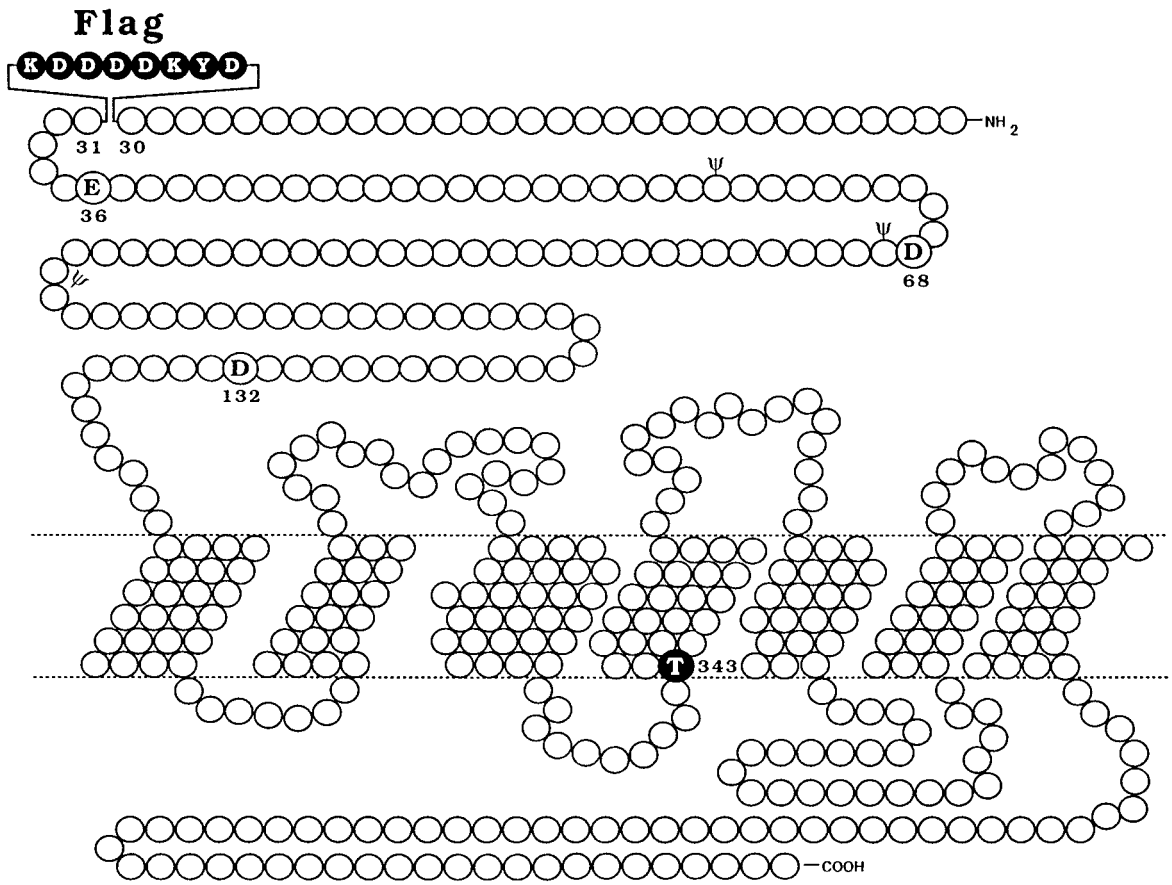

FIG. 1. Putative seven-transmembrane-segment topography of the human VPAC1 receptor showing residues that have been mutated in this study. This includes threonine 343, glutamate 36, aspartate 68 and aspartate 132. The position and sequence of a Flag in the N-terminal domain of the receptor are also shown.

stitutive activity for the T343C, T343E and T343F mutants could not be ascribed to a low level of expression since their Bmax were similar to that of the wild-type receptor (Table 1). We also tested the ability of VIP to stimulate cAMP production in Cos-7 cells transfected with mutants (Fig. 3). It appeared that VIP (1 μM) stimulated cAMP production whatever the mutant tested. The T343K, T343A and T343F mutants responded like the wild-type receptor whereas a reduced efficacy of VIP was observed for other mutants including T343C, T343P and T434E. Finally, immunofluorescence studies were carried out owing to the insertion of a Flag epitope in the extracellular N-terminal domain of the mutant receptors (see Fig. 1 and ref. 14). They revealed similar expression of all mutants and wild-type receptor to the cell surface of transfected Cos-7 cells (not shown) in accordance with binding studies (see Table 1).

We further investigated constitutive activation by mutation at threonine 343 by constructing double mutants in which a mutation resulting in constitutive activation of the receptor (T343K) was associated with point mutations in the N-terminal extracellular domain of the receptor, which abolished VIP binding as previously shown (6, 8). First we chose a mutation affecting an amino acid residue highly conserved in the class II G protein-coupled receptors, i.e. D68A (6). As expected the T343K-D68A mutant no longer bound VIP like the single mutant D68A (Table 1). Nor did it mediate the stimulation of cAMP production by VIP (Fig. 3). This double mutant is no longer constitutively activated when expressed in Cos cells (Fig. 2). It was verified that the epitope-tagged T343K-D68A mutant like the D68A mutant exhibited a cell surface expression similar to that of the wild type receptor as assessed by confocal microscopy (not shown) and antibody binding experiments (Table 2). Next we chose an amino acid residue which was not conserved in the class II G protein-coupled receptors but the mutation of which abolished VIP binding i.e. E36A (8). Like the single mutant E36A, the T343K-E36A mutant no longer bound VIP (Table 1) and did not mediate the stimulation of cAMP production by VIP (Fig. 3). The T343K-E36A double mutant was not constitutively activated when expressed in Cos cells (Fig. 2). Again, it was verified that the epitope-tagged T343K-E36A mutant like the E36A mutant exhibited a cell surface

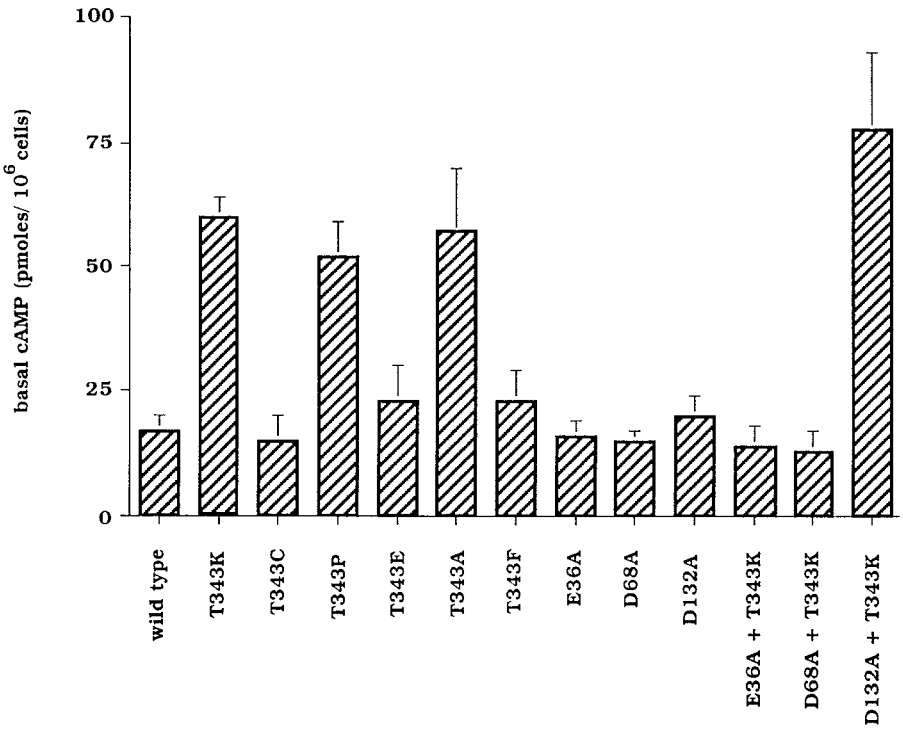

FIG. 2. Basal cAMP levels in Cos cells expressing the wild-type or single or double mutants of the human VPAC1 receptor. Cells were incubated for 30 min at room temperature and intracellular cAMP was measured as descibed in Materials and Methods. Data are means ± SE of three experiments.

expression similar to that of the wild type receptor as assessed by confocal microscopy (not shown) and antibody binding experiments (Table 2). As a control, we developed another double mutant in which T343K was associated with a point mutation (D132A) in the N-terminal extracellular domain which was previously shown not to alter VIP binding (8). As expected the T343K-D132A mutant, like the single mutant D132A, bound VIP with a dissociation constant similar to that of the wild type receptor (Table 1). This mutant also mediated VIP-stimulated cAMP production (Fig. 3) and exhibited cell surface expression similar to that of wild type receptor as assessed by confocal microscopy (not shown) and antibody binding experiments (Table 2). Most interestingly the double mutant H178R-D132A did exhibit constitutive activation upon transfection in Cos cells (Fig. 2).

TABLE 1

Parameters of VIP Binding for Wild-Type and Mutated Human VPAC1 Receptors after Transfection of cDNAs into Cos Cells

Constructs	Kd (nM)	Bmax (pmoles/mg of protein)
Wild-type	1.5 ± 0.2	8.3 ± 2.7
T343K	1.5 ± 0.5	2.8 ± 0.9
T343C	3.3 ± 1.4	7.9 ± 3.1
T343P	0.7 ± 0.2	1.7 ± 0.5
T343E	2.0 ± 0.8	5.5 ± 1.4
T343A	0.3 ± 0.1	5.7 ± 2.6
T343F	3.0 ± 0.1	11.7 ± 1.7
E36A	ND	ND
D68A	ND	ND
D132A	0.7 ± 0.1	1.6 ± 0.3
E36A/T343K	ND	ND
D68A/T343K	ND	ND
D132A/T343K	1.7 ± 0.4	4.3 ± 1.2

Note. Dissociation constants (Kd) and binding capacities (Bmax) were determined by Scatchard analysis of binding data as described in Materials and Methods. ND, Binding Not Detectable. Results are means ± SE of three experiments.

DISCUSSION

This work demonstrates that mutations at threonine 343 in the human VPAC1 receptor result in ligand-independent, constitutive activation with respect to cAMP production which constitutes its signalling pathway (1, 2). It also provides evidence supporting a role for the N-terminal extracellular VIP binding domain in conveying the signal from the mutated threonine to the activation of cAMP production.

Our data indicate that mutations of threonine 343 into lysine, proline or alanine evoke constitutive activation of the VPAC1 receptor whereas mutations into cysteine, glutamate or phenylalanine do not. From the

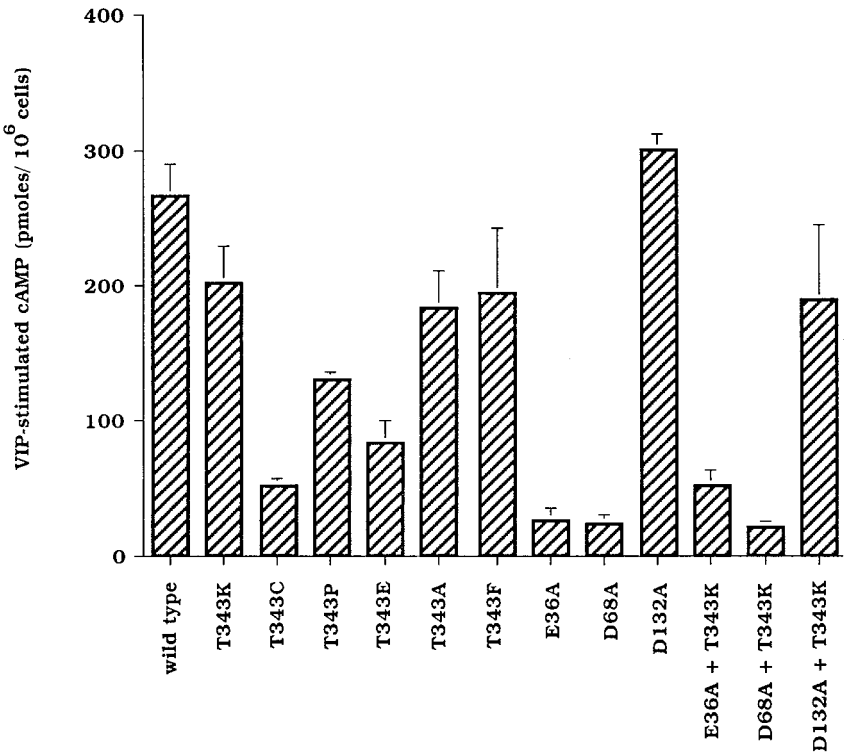

FIG. 3. VIP-stimulated cAMP levels in Cos cells expressing the wild-type or single or double mutants of the human VPAC1 receptor. Cells were incubated with 10^{-6} M VIP for 30 min at room temperature and intracellular cAMP was measured as descibed in Materials and Methods. Data are means ± SE of three experiments.

present work, it is not possible to speculate on the mechanism whereby change of threonine into lysine, proline or alanine results in constitutive activation. However, we can emphasize that: (1) mutation of threonine into proline is the natural mutation in the PTH-PTH-related peptide receptor in Jansen's metaphyseal chondrodysplasia (15, 16); (2) constitutive activation by mutation at another locus in the human VPAC1 receptor can be only achieved by the change of the histidine 178 into arginine (14); (3) This situation is quite different from that described for the α_{1B}-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 (21). With regard to the human VPAC1 receptor, it may be suggested that the exchange of threonine to lysine, proline or alanine does not simply remove some stabilizing conformational constraints like in α_{1B}-adrenergic receptors (21). The same was true for constitutive activation of the VPAC1 receptor evoked by the histidine 178 to arginine exchange (14).

TABLE 2

Cell Surface Expression of VPAC1 Receptor Mutants after Transfection into Cos-7 Cells

Constructs	Surface expression (% of wild type)
Wild-type	100 ± 4
E36A/T343K	99 ± 9
D68A/T343K	58 ± 5

Note. Nonpermeabilized transfected cells were incubated with anti-flag antibodies and then exposed to the radiolabeled second antibodies. Cells were rinsed and the radioactivity of cell lysates was counted as described in Materials and Methods. Nonspecific binding was determined with cells that were incubated only with the ^{125}I-labeled second antibody. It represented 0.1% of total radioactivity added. Binding of anti-Flag antibodies to epitope-tagged mutant receptors is given as a percentage of anti-Flag antibodies binding to epitope-tagged wild-type receptor. Results are means ± SE of three experiments.

The construction of double mutants supports a close relationship between the ligand-dependent activation triggered by VIP and the ligand-independent one evoked by mutations of threonine 178. Indeed, when the T343K mutation at the junction of the second intracellular loop and fourth transmembrane domain was associated with mutation of glutamate 36 (E36A) or aspartate 68 (D68A) in the N-terminal extracellular domain which abolishes VIP binding and VIP-stimulated cAMP production (9), the resulting double mutant were no more constitutively activated and have

the phenotype of the E36A or D68A mutant. 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 T343K mutation. Identical conclusion was previously drawn for the constitutive activation of the VPAC1 receptor evoked be the histidine 178 to arginine exchange (14). From these data, it could be hypothesized that the T343K (this paper) or H178R (14) mutations mimic 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 (22). Our approach consisting of constructing double mutants further suggests that the conformational change triggering constitutive activation in the T343K receptor mutant may transit over the ligand binding site in the N-terminal extracellular domain and requires the structural and functional integrity of this domain.

Since threonine 343 of the human VPAC1 receptor is highly conserved in class II G protein-coupled receptors for peptides (1, 2), it could be hypothesized that mutations of the equivalent threonine in other receptors also result in their constitutive activation. This has been described for the T410P mutant of the PTH-PTH-related peptide receptor (15, 16) and the T340P mutant of the glucose-dependent insulinotropic receptor (23). Only a very small constitutive activation was described for the T352A mutant of the glucagon receptor (24). In contrast, when mutations were introduced in the corresponding threonine residue of PTH2 receptor and the receptors for calcitonin, secretin, growth hormone-releasing factor, glucagon-like peptide 1 and corticotropin-releasing hormone, the resulting mutants failed to induce constitutive activity (16). It may thus be suggested that mutations of a highly conserved locus in class II G protein coupled receptors for peptides can generate different phenotypes.

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