photoaffinity labeling demonstrates physical contact between vasoactive intestinal peptide and the N-terminal ectodomain of the human VPAC1 receptor*

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Vasoactive intestinal peptide (VIP) is a prominent neuropeptide whose actions are mediated by VPAC receptors belonging to class II G protein-coupled receptors. To identify contact sites between VIP and its VPAC1 receptor, an analog of VIP substituted with a photoreactive para-benzoyl-L-Phe (Bpa) at position 22 has been synthesized and evaluated in Chinese hamster ovary cells stably expressing the recombinant human receptor. Bpa22-VIP and native VIP are equipotent in stimulating adenylyl cyclase activity in cell membranes. Cyanogen bromide cleavage of the covalent 125I-[Bpa22-VIP]:hVPAC1 complex yielded a single labeled fragment of 30 kDa that shifted to 11 after deglycosylation, most consistent with the 67–137 fragment of the receptor N-terminal ectodomain. Further cleavage of this fragment with V8 endoproteinase and creation of receptor mutants with new CNBr cleavage sites (Xa ` Met) demonstrated that 125I-[Bpa22-VIP] was covalently attached to the short receptor 109–120 fragment (GWTHLEPG-

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§ The abbreviations used are: VIP, vasoactive intestinal peptide; Bpa, para-benzoyl-L-Phe (Bpa); CHO, Chinese hamster ovary cells; PNGase F, peptide N-glycosidase F; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyglycine; TM, transmembrane; h, human; VPAC, VIP pituitary adenylyl cyclase-activating peptide receptor; LNB, long N-terminal type B.

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contact with the N-terminal ectodomain of the receptor. Moreover, we show that amino acid in position 22 of VIP is in the environment of the short sequence 109–120 within this ectodomain when VIP is bound to the receptor.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and culture medium were obtained from Invitrogen. Eucaryotic expression vector was from Clontech (Palo Alto, CA). DNA sequencing kit and radioactive reagents were from Amersham Biosciences. Site-directed mutagenesis kit was from Promega. Synthetic oligonucleotides were from Invitrogen. The human VPAC1 receptor cDNA was cloned in our laboratory (16). A receptor construct containing an inserted FLAG sequence between Ala20 and Ala31 (17) and fused in the C-terminal position with the green fluorescent protein (18) was used in all of the studies. It has the same phenotype as the wild-type receptor with respect to VIP binding and activation of adenyl cyclase (17, 18). The CHO-F7 clone expressing the human VPAC1 construct (Bmax, 5 pmol/mg protein; Kd, 0.6 nM VIP) was used in most experiments. VIP and [Ala17, para-benzoyl-L-Phe22]VIP-28 referred to as Bpa22-VIP were obtained by custom synthesis from NeoSystem (Strasbourg, France). The substitution of alanine for Met17 does not alter binding affinity for hVPAC1 receptor (15) and prevents undesirable cleavage of Bpa22-VIP upon CNBr treatments. Neither is Bpa22-VIP cleavable upon V8 endoproteinase treatments since there is no ChTextures residue in its sequence. [3H]VIP (radioiodinated on Tyr75) and [3H]cAMP were prepared and purified in our laboratory as described previously (15). [3H]-Bpa22-VIP was prepared and purified following the method used for native VIP (15). All of the highly purified chemicals used were from Sigma.

Site-directed Mutagenesis and Cell Transfection—Oligonucleotide-directed mutagenesis was performed as previously described (10). Identification of the desired mutations was obtained by direct sequencing of the mutated region (10). Mutants were stably transfected in CHO cells as described previously (15).

Cell Culture and Membrane Preparation—The CHO-F7 cells expressing the wild-type VPAC1 receptor construct and the CHO cells expressing stably transfected receptor mutants were cultured in Ham’s F-12 medium supplemented with 10% decomplemented fetal calf serum and 100 units/ml penicillin G and 100 µg/ml streptomycin in a humidified atmosphere containing 95% air and 5% CO2 at 37 °C. Cells were grown to confluence. After removing the culture medium, attached cells were washed twice with phosphate-buffered saline and then harvested with a rubber policeman and centrifuged for 10 min at 3,000 × g. The cell pellet was exposed for 30 min at 4 °C to hypoosmotic 5 mM HEPES buffer, pH 7.4, and membranes were obtained as described previously (15) and stored at −80 °C until use. Protein content was measured by the procedure of Bradford (19) with bovine serum albumin as standard.

Ligand Binding Assay and Measurement of Adenylyl Cyclase Activity—The binding assay was as described previously (15). Membranes (100 μg of proteins/ml) derived from transfected CHO cells were incubated for 1 h at 30 °C with 0.05 nM [3H]VIP in the presence of increasing concentrations of VIP or Bpa22-VIP in 20 mM HEPES buffer, pH 7.4, containing 2% (w/v) bovine serum albumin. The reaction was stopped by transferring the incubation medium to an excess of ice-cold buffer. Bound and free peptides were separated by centrifugation (14,000 × g for 10 min), and membrane pellets were washed twice with 10% (w/v) sucrose in ice-cold 20 mM HEPES. The radioactivity was then assayed in a γ-counter. Specific binding was calculated as the difference between the amount of [3H]VIP bound in the absence (total binding) and the presence (nonspecific binding) of 1 µM unlabeled VIP. Binding data were analyzed using the LIGAND computer program. The computer analysis of peptides that elicited half-maximal inhibition of specific [3H]VIP binding (Ki) was determined by computer analysis. Adenyl cyclase activity in membranes from clone F7 cells was assayed in the presence of increasing concentrations of VIP or Bpa22-VIP as described previously (15). Dose-response curves were fitted, and concentrations of peptides giving half-maximal responses (EC50) were calculated using the Prism software suite (GraphPad Software, San Diego, CA).

Data Analysis—The Prism software suite (GraphPad Software, San Diego, CA). Dose-response curves were fitted, and concentrations of VIP or Bpa22-VIP in 20 mM HEPES buffer, pH 7.4, containing 2% (w/v) bovine serum albumin. The reaction was stopped by transferring the incubation medium to an excess of ice-cold buffer. Bound and free peptides were separated by centrifugation (14,000 × g for 10 min), and membrane pellets were washed twice with 10% (w/v) sucrose in ice-cold 20 mM HEPES. The radioactivity was then assayed in a γ-counter. Specific binding was calculated as the difference between the amount of [3H]VIP bound in the absence (total binding) and the presence (nonspecific binding) of 1 µM unlabeled VIP. Binding data were analyzed using the LIGAND computer program. The computer analysis of peptides that elicited half-maximal inhibition of specific [3H]VIP binding (Ki) was determined by computer analysis. Adenyl cyclase activity in membranes from clone F7 cells was assayed in the presence of increasing concentrations of VIP or Bpa22-VIP as described previously (15). Dose-response curves were fitted, and concentrations of peptides giving half-maximal responses (EC50) were calculated using the Prism software suite (GraphPad Software, San Diego, CA).

Photoaffinity Labeling—Transfected cells were incubated in darkness with 10 nM [3H]-Bpa22-VIP in 10 ml of 20 mM HEPES buffer, pH 7.4, containing 0.2% (w/v) ovalbumin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM TLCK. After a 1-h incubation at room temperature, cells were centrifuged and the pellets resuspended in 4 ml of 20 mM HEPES with 1 mM phenylmethylsulfonyl fluoride and 0.1 mM TLCK. 1 ml of labeled cells was then added to each well of a six-well tissue culture plate, and the photoreaction (λ = 365 nm) was carried out on ice at a distance of ~2 cm. After 40-min UV exposure, cells were collected into individual 1.5-ml Eppendorf tubes and washed twice with 10 mM HEPES containing 25 mM glycine, 75 mM NaCl buffer, pH 2.5, and one time with 20 mM HEPES. The photolabeled receptors were then analyzed by electrophoresis or after chemical or enzymatic cleavage.

Chemical Cleavage of Photoaffinity-labeled VPAC1 Receptor Constructs—The photolabeled receptors in cells were incubated overnight in darkness with 10 mg/ml CNBr in 80% formic acid at room temperature. Cyanogen bromide present in the reaction mixture was then removed by Waters C18 Sep-Pak purification. After extensive washing of the Sep-Pak cartridge with 40% acetonitrile in 0.1% trifluoroacetic acid, CNBr-generated peptide fragments were eluted with 2 ml of 100% acetonitrile. Fractions were counted for radioactivity and evaporated under vacuum (Speed-Vac). The resulting material was then analyzed directly by electrophoresis or after enzymatic treatments (see below).

Enzymatic Treatments—For protein deglycosylation, the material obtained after CNBr treatment was incubated for 2 h at 37 °C with PNGase F (3 units/ml) as described previously (20). V8 endopeptidase digestion of proteins in this material was obtained by incubation for 6 h at 25 °C in 25 mM ammonium bicarbonate, 0.01% SDS, 5% acetonitrile, pH 7.8, as described previously (21).

Electrophoresis and Autoradiography—Samples were analyzed by Tricine/SDS-PAGE performed according to the method of Schagger and Von Jagow (22) under reducing conditions in the presence of 0.2 M diithiothreitol. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of the broad range protein markers from BioLabs and the Rainbow™ colored protein molecular weight markers from Amersham Biosciences versus the log values of their masses. After electrophoresis, gels were dried and exposed to x-ray film for 3–10 days with intensifying screens at −80 °C as described previously (20).

RESULTS

The synthetic Bpa22-VIP photoaffinity probe developed in this study was first evaluated for its ability to interact with the human VPAC1 receptor stably expressed in CHO-F7 cells. As shown in Fig. 1, Bpa22-VIP has the same potency (EC50 = 0.06 nM) and efficacy as native VIP in stimulating adenyl cyclase activity in membranes prepared from CHO-F7 cells. Bpa22-VIP and native VIP have also similar affinities for the hVPAC1 receptor in the ligand binding assay (data not shown). Therefore, this probe is relevant for photoaffinity labeling of human VPAC1 receptors in CHO-F7 cells. After radiiodination, the 125I-[3H]-Bpa22-VIP probe was incubated with CHO-F7 cells, labeled cells were then exposed to UV, and the labeled proteins were analyzed by SDS-PAGE. A 95-kDa labeled band was observed, which was no more present when an excess of cold VIP was added together with the labeled probe (Fig. 2). This molecular mass is in good agreement with the expected mass.
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(92 kDa) considering the previously characterized (20, 23) mass of glycosylated VPAC1 receptor expressed in CHO cells (64 kDa) and the presence of green fluorescent protein (25 kDa) at the C terminus of the construct and of covalently bound Bpa\textsuperscript{22}-VIP (3 kDa). Treatment of the 95-kDa band with PNGase F generated a 70-kDa band (data not shown) in consonance with the previously characterized presence of 9-kDa carbohydrate moiety on each of the three consensus N-glycosylation sites in the N-terminal extracellular domain of the hVPAC1 receptor (20). The 95-kDa band corresponding to \textsuperscript{125I}-[Bpa\textsuperscript{22}-VIP]/hVPAC1R conjugate was then subjected to a series of chemical and enzymatic cleavages to identify the site of the covalent contact between VIP and the receptor. Cyanogen bromide cleavage of the 95-kDa band generated a 30-kDa labeled band (Fig. 2). This band was further shifted to 11 kDa after treatment with PNGase F (Fig. 2), indicating that it is heavily glycosylated, most probably, at two consensus N-glycosylation sites. Altogether, these data are only consistent with the covalent attachment of \textsuperscript{125I}[Bpa\textsuperscript{22}-VIP] to the Trp\textsuperscript{67}-Met\textsuperscript{137} receptor fragment (Table I) in the N-terminal extracellular domain of the receptor. This fragment contains two N-glycosylation sites on Asn\textsuperscript{69} and Asn\textsuperscript{100} (Table I) previously shown to be occupied by a 9-kDa carbohydrate (20). To further validate the Trp\textsuperscript{67}-Met\textsuperscript{137} receptor fragment as the site of attachment of the photoaffinity probe, we created new CNBr cleavage sites within this sequence by substituting methionine for Ile\textsuperscript{120} (Table I). The I120M hVPAC1 receptor mutant was then cleaved by CNBr and deglycosylated by PNGase F without or with further cleavage by V8 endoproteinase (Fig. 5). After CNBr treatment, the I120M mutant yielded a 9-kDa band as compared with a 11-kDa band for the wild-type receptor. Although the exact determination of such low molecular masses is clearly difficult by electrophoresis, it is crucial to point out that the band obtained with the mutant is systematically shifted as compared with that obtained for the wild-type receptor over four experiments. This finding strongly supported that the Gly\textsuperscript{109}-Glu\textsuperscript{133} fragment is actually the site of attachment of Bpa\textsuperscript{22}-VIP to the Gly\textsuperscript{109}-Glu\textsuperscript{133} receptor fragment (see Table I). To further validate the Gly\textsuperscript{109}-Glu\textsuperscript{133} fragment as the site of attachment of Bpa\textsuperscript{22}-VIP and to precisely determine which subdomain is concerned, we created new CNBr cleavage sites in this sequence by substituting methionine for Ile\textsuperscript{120} (Table I). The I120M hVPAC1 receptor mutant was constructed and stably expressed in CHO cells. It bound VIP with an affinity identical (K\textsubscript{i} = 0.6 nM) to that of the wild-type receptor (Fig. 3). The I120M mutant expressed in CHO cells was photolabeled with \textsuperscript{125I}-[Bpa\textsuperscript{22}-VIP], yielding a 95-kDa band upon electrophoresis (data not shown) as observed for the wild-type receptor (Fig. 2). V8 endoproteinase treatment of the 30-kDa band generated a 6-kDa band (Fig. 2). Identical data were obtained by V8 endoproteinase treatment of the 11-kDa band. Given the mass of generated products and the absence of further effect of PNGase F (data not shown) on the migration of the labeled products after V8 endoproteinase digestion, the data support the covalent attachment of the \textsuperscript{125I}-[Bpa\textsuperscript{22}-VIP] probe to the Gly\textsuperscript{109}-Glu\textsuperscript{133} receptor fragment (see Table I). Since we have previously developed a three-dimensional model for a large part of the N-terminal ectodomain of the hVPAC1 receptor (13), the location of the Gly\textsuperscript{109}-Ile\textsuperscript{120} sequence was analyzed and is highlighted in Fig. 6. It is of great interest to consider that this short sequence is within or con-
tiguous to the proposed VIP binding groove (13), which contains amino acid residues previously shown by site-directed mutagenesis (6) to be important for VIP binding (Fig. 6).

**DISCUSSION**

The present study was aimed at mapping the cross-linking site for the newly developed photolabile VIP probe $^{125}$I-[Bpa$^{22}$-VIP] in the human VPAC1 receptor. Based on various digestions of the probe-labeled receptor and the construction of receptor mutants, our data indicate that the benzophenone group present on the side chain of Bpa in position 22 of VIP is in close proximity to a small domain within the N-terminal ectodomain of the receptor. These data provide the first direct evidence that VIP physically interacts with the N-terminal ectodomain of hVPAC1 receptor. They extend previous site-directed mutagenesis and structural modelization studies supporting the crucial role of this domain for VIP binding (reviewed in Ref. 6).

The photolabile VIP derivative with substitution of Bpa for tyrosine at position 22 of VIP is a high potency full VIP agonist as shown by its ability to stimulate adenylyl cyclase activity in CHO expressing recombinant hVPAC1 receptor. This could be
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Fig. 5. Digestions by PNGase F and V8 endoproteinase of the 125I-[Bpa22-VIP]-labeled I120M hVPAC1 receptor mutant and wild-type receptor. Receptors expressed in CHO cells were photoaffinity-labeled with 125I-[Bpa22-VIP], and cells were subjected to treatment with CNBr. They were then digested with PNGase F alone (lanes 2 and 5) or with PNGase F and V8 endoproteinase (lanes 3 and 6). Controls without enzyme digestion are shown in lanes 1 and 3. The labeled proteins were analyzed by Tricine/SDS-PAGE followed by autoradiography. See “Experimental Procedures” for details. MW, molecular mass; wt, wild type.

Fig. 6. Backbone representation of three-dimensional model of the hVPAC1-(1-139) N-terminal ectodomain highlighting the receptor fragment to which 125I-[Bpa22-VIP] is covalently attached. This model (6, 13) suggests the presence of a binding groove whose backbone is shown with ribbon representation. The receptor fragment (residues 109–120) covalently labeled by 125I-[Bpa22-VIP] is shown in red and is clearly on one edge of the groove. The location of amino acids previously shown by site-directed mutagenesis to be important for VIP binding are highlighted in green with Corey-Pauling-Koltun representation within the receptor fragment (109–120) and, for the sake of clarity only, by green color on the backbone of the remaining part of the groove. Tube representation is used for other parts of the hVPAC1 receptor ectodomain. The N-terminal extremity (residues 1–30) is mostly helical and corresponds to the signal peptide.

expected from previous alanine scanning studies, which showed that Tyr22 is not important for VIP binding to hVPAC1 receptor and subsequent adenyl cyclase activation (15). On this basis, it is reasonable to assume that the photoaffinity probe adopts a binding mode similar to that taken up by native VIP. The proposed covalent attachment of 125I-[Bpa22-VIP] to the Gly109-Ile120 segment within the N-terminal ectodomain of the hVPAC1 receptor (see Fig. 6) is unambiguously consistent with the photoaffinity labeling results. Following UV irradiation of the complex formed by the receptor construct fused in the C-terminal position with the green fluorescent protein and 125I-[Bpa22-VIP], covalent labeling of a glycoprotein of 95 kDa was observed (see Fig. 2). Upon cleavage with CNBr, the labeled material was shifted to 30 kDa. Extensive studies of hVPAC1 receptor glycosylation have shown that the receptor expressed in COS cells or CHO cells is glycosylated with three N-glycosylation sites on Asn59, Asn88, and Asn100 in the N-terminal ectodomain, each site being occupied by 9-kDa carbohydrates (20, 23). The fact that the 30-kDa band was further shifted to 11 kDa after digestion with PNGase F clearly demonstrated that the labeled receptor fragment was heavily glycosylated, most probably, on two N-glycosylation sites. Altogether, these data were consistent with the labeling of the large Trp67-Met137 receptor fragment, which contains two glycosylation sites on Asn69 and Asn100 (see Table I). Further cleavage with V8 endoproteinase and development of new CNBr cleavage sites by substituting methionine for native amino acids at four different positions in receptor mutants provided clear evidence for the covalent attachment of 125I-[Bpa22-VIP] to the Gly109-Ile120 segment of the receptor (see Figs. 4 and 5).

Photoaffinity labeling identifies receptor domains in close proximity to the bound photoligand. The other privileged approach to molecular delineation of ligand-receptor interaction, site-directed mutagenesis, identifies domains necessary for function that are not necessarily proximal to the site of that function (24). As far as the VIP-hVPAC1 receptor interaction is concerned, there is not only a good agreement between photoaffinity labeling and mutagenesis experiments but also an exquisite complementarity as discussed further on. (i) The covalent attachment of 125I-[Bpa22-VIP] to the N-terminal ectodomain of the hVPAC1 receptor is consistent with previous experiments in which the receptor has been truncated or mutated. Indeed, deletion of the N-terminal ectodomain of hVPAC1 receptor abolishes its ability to bind VIP (6). Moreover, site-directed mutagenesis identified many residues within the N-terminal ectodomain as crucial or important for VIP binding including Glu36, Ser64, Met66, Trp67, Asp68, Trp73, Pro74, Pro97, Phe98, Gly109, Trp110, or Pro115 (6, 13, 25–27). These residues have been assumed to be positioned in a tertiary structure (see below) probably stabilized by the formation of three disulfide bonds formed between six cysteine residues, which are strictly conserved in class II G protein-coupled receptors (7). Indeed, mutation of all of the six cysteines clearly abolishes VIP binding to the hVPAC1 receptor (28). (ii) Site-directed mutagenesis showed that residues that are crucial for VIP binding are dispersed along the primary sequence of the N-terminal ectodomain of hVPAC1 receptor (6). A structural model of a large part of this domain has been constructed by sequence homology with a yeast lipase whose structure was available in the Protein Data Bank (13). This model confirmed the existence of a signal sequence at the N terminus (see Fig. 6) and showed most importantly that residues that are important for VIP binding are gathered around an electronegative groove with an outspanning shell of three tryptophan residues at one end (13). In this context, the fact that 125I-[Bpa22-VIP] covalently attached to the Gly109-Ile120 segment of the hVPAC1 receptor is quite meaningful. Indeed, this segment is located on one side of the groove and encompasses several crucial residues (6, 13) including Gly109, Trp110, and Pro115 (see Fig. 6). In that respect, the present study provides the first experimental basis for the current view of VIP binding to the hVPAC1 receptor. Indeed, it has been suggested that the central part of VIP with its crucial basic residues (15) may interact with the acidic residues in the electronegative groove in the N-terminal ectodomain of the receptor (6, 13). This finding fits well with the fact
that Bpa<sup>22</sup> in the photoaffinity probe is adjacent to the crucial basic residue Lys<sup>21</sup> of VIP (15). The C-terminal part of VIP may also interact with the N-terminal ectodomain of the receptor, whereas it has been suggested that the N-terminal domain of VIP interacts with a still poorly characterized binding domain on the core of the receptor that may consist of, at least, the first and second extracellular loops and the upper part of the second transmembrane helix as viewed from the outside of the cell (6). In particular, based on dual mutations in VIP and hVPAC<sub>1</sub> receptor, it was suggested that Asp<sup>3</sup> side chain of VIP penetrates in the transmembrane domain in proximity to basic amino acids from transmembrane helix 2 (29). The hypothesis that peptide-binding cleft could reside between the hVPAC<sub>1</sub> receptor N-terminal ectodomain and the receptor body is reminiscent of the situation described for two other class II receptor, the parathyroid hormone receptor (30, 31) and the secretin receptor (32). Development of new photoaffinity probes with Bpa substitutions at the N and C termini of VIP are clearly needed to further delineate the sites of interaction of the different part of this 28-amino acid neuropeptide with its receptors.

In conclusion, the results of the photoaffinity labeling study of the hVPAC<sub>1</sub> receptor provides a contact region between the receptor and the side chain at position 22 of VIP. This represents the first direct evidence for physical proximity between VIP and the hVPAC<sub>1</sub> receptor complex. The compatibility between the structural model of the N-terminal ectodomain of hVPAC<sub>1</sub> receptor (6, 13) and the present photoaffinity cross-linking study further validates this model and paves the way of future docking of VIP in its receptor. Additional studies with new photoaffinity probes of VIP are clearly needed to identify anchor points in hVPAC<sub>1</sub> receptor for interaction with other parts of the long 28-amino acid VIP. Such studies are currently in progress in our laboratory.

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