Docking of Linear Peptide Antagonists into the Human V1a Vasopressin Receptor

IDENTIFICATION OF BINDING DOMAINS BY PHOTOAFFINITY LABELING*

(Received for publication, December 16, 1998, and in revised form, April 26, 1999)

Sylvie Phalipou‡, René Seyer‡, Nathalie Cotte‡, Christophe Breton‡, Claude Barberis‡, Marcel Hibert‡, and Bernard Mouillac‡

From ‡U469 INSERM and §UPR 9023 CNRS, CCIPÆ, 141 rue de la Cardonille, 34094 Montpellier cedex 5, France and ¶ERS 655 CNRS, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch cedex, France

A novel photoactivatable linear peptide antagonist selective for the V1a vasopressin receptor, \( ^{125}\text{I} \text{[Lys}(3\text{N}_3\text{Phpa})\text{HO-LVA} \), was synthesized, characterized, and used to photolabel the human receptor expressed in Chinese hamster ovary cells. Two specific glycosylated protein species at 85–90 and 46 kDa were covalently labeled, a result identical to that obtained with a previous photoaffinity ligand, \( ^{125}\text{I} \text{[3N}_3\text{Phpa-LVA} \) (Phalipou, S., Cotte, N., Carnazzi, E., Seyer, R., Mahe, E., Jard, S., Barberis, C., and Mouillac, B. (1997) J. Biol. Chem. 272, 26536–26544). To identify contact sites between the new photoreactive analogue and the V1a receptor, the labeled receptors were digested with Lys-C or Asp-N endopeptidases and chemically cleaved with CNBr. Fragmentation with CNBr, Lyc-C, and Asp-N used alone or in combination, led to the identification of a restricted receptor region spanning the first extracellular loop. The results established that sequence Asp\(^{112}\)–Pro\(^{120}\) could be considered as the smallest covalently labeled fragment with \( ^{125}\text{I} \text{[Lys}(3\text{N}_3\text{Phpa})\text{HO-LVA} \) (covalent attachment to transmembrane domain VII), three-dimensional models of the antagonist-bound receptors were constructed and then verified by site-directed mutagenesis studies. Strikingly, these two linear peptide antagonists, when bound to the V1a receptor, could adopt a pseudocyclic conformation similar to that of the cyclic agonists. Despite divergent functional properties, these peptide antagonists could interact with a transmembrane-binding site significantly overlapping that of the natural hormone vasopressin.

Over the past few years, interest in locating ligand-binding sites in G protein-coupled receptors has increased exponentially. Indeed, identification of these binding sites is of prime importance both for a better understanding of the structure and the function of the G protein-coupled receptor superfamily and for facilitating rational design of potential therapeutic agents. Extensive mutational analysis and receptor three-dimensional molecular modeling have led to valuable information concerning “small ligand” and peptide/protein ligand-receptor-binding sites (for review, see Refs. 1–6).

In 1995, we published the mapping of arginine-vasopressin (AVP)\(^1\)-binding site in the V1a receptor subtype and described a major localization within transmembrane regions (TMR) in a position equivalent to that defined for the cationic neurotransmitters (7). Because all receptor residues potentially interacting with AVP are conserved in the different members of the AVP/oxytocin (OT) receptor family, we proposed that the binding pocket identified in the V1a might be common to V2, V1b, and OT receptor subtypes. Extracellular residues responsible for receptor-selective and species-selective binding have also been identified (8–10). Unfortunately, these first analyses of AVP receptor structure/function relationships did not provide much information on AVP receptor antagonist-binding domains (Refs. 11 and 12, and for review see Ref. 13). The photoaffinity labeling technique is an essential complement to modeling and mutagenesis approaches and allows direct unambiguous identification of the contact regions between a receptor and its specific photoactivatable ligands (for review see Ref. 14). At the present time, very few photoaffinity labeling studies have led to the direct determination of labeled amino acid residues in peptide G protein-coupled receptor; remarkable results with bovine V2 receptor (15), human NKA tachykinin receptor (16), and rat type A cholecystokinin receptor (17) allowed identification of covalently labeled residues with photoactivatable agonist analogues of AVP, substance P, and cholecystokinin, respectively.

Very recently, a first radiiodinated photoreactive linear peptide antagonist has been used in our laboratory to photolabel the human and rat V1a receptors (12, 18, 19). Our results have clearly indicated that covalent attachment of the \( ^{125}\text{I} \text{[3N}_3\text{Phpa-LVA} \) occurs in a restricted domain of the human receptor including TMR VII. Based both on this photolabeling result and on the hypothetical three-dimensional model of the human V1a receptor, residues potentially involved in binding and affinity of the antagonist ligand have been targeted. An interaction between the hydrophobic N terminus of the \( ^{125}\text{I} \text{[3N}_3\text{Phpa-LVA} \) ligand and an aromatic cluster of residues in the TMR VI has thus been experimentally verified. However, because of the lack of structural and conformational data for...
this family of V_{1a} selective ligands and because of their peptidic nature and highly variable linear structure, the determination of a single contact point between the peptide antagonist and the receptor does not provide enough information to propose a docking mode of the ligand into the receptor.

To allow a more complete location of the binding sites for this family of V_{1a} linear peptide antagonists and to generate meaningful information on receptor-antagonist interactions, we thus decided to label the human V_{1a} receptor with a second radiolabeled photoreactive antagonist. In the present study, properties of [^{[125]}I]Lys[3N_{3}Phpa]^{[2]}HO-LVA, an antagonist containing an azido group at a position (side chain of lysine residue 8) likely to covalently bind another domain of the receptor are described. Combining photo-labeling with this new ligand of the human V_{1a} receptor, cyanogen bromide cleavage and endoproteinase digestions of the receptor, a restricted photolabeled domain has been identified which spans the first extracellular proteinase digestions of the receptor, a restricted photolabeled...the precedent compound (2 10 ^{4} M, 6.5 tetrachloro-3... 25 °C). Pellets were washed in Buffer A (50 mM Tris-HCl, pH 7.4, 5 mM MgCl_{2}, 0.3 mM EDTA) and centrifuged at 44,000 × g for 20 min at 4 °C. Membranes were suspended in a small volume of Buffer A, and protein contents were determined.

**Ahniquens of membranes were used immediately in binding assays and photo-labelling experiments or stored at −80 °C.**

**Radioligand Binding Assays**—As described in previous papers (7, 12), binding assays were performed at 30 °C using [^{[125]}I]HO-LVA, [^{[125]}I]Lys[3N_{3}Phpa]^{[2]}HO-LVA, [^{[125]}I]3N_{3}Phpa-LVA, or [^{[3]H}]AVP as the radioligands and 1–3 μg (for assays with [^{[125]}I]-labeled antagonists) or 10–15 μg (for assays with [^{[3]H}]AVP) of membrane protein. Membrane were incubated in Buffer A with 1 mg/ml bovine serum albumin and with radiolabeled and displacing peptides for 30 min (with [^{[3]H}]AVP) or 1 h (with [^{[125]}I]HO-LVA, [^{[125]}I]3N_{3}Phpa-LVA, and [^{[125]}I]Lys[3N_{3}Phpa]^{[2]}HO-LVA) and washed with Buffer A (100 mM NaCl, 5 mM MgCl_{2}, 10 mM EDTA, pH 7.4) to remove free radioligands. Membranes were resuspended in 100 mM LiCl in the presence or absence of increasing concentrations of [Lys(3N_{3}Phpa)]^{[2]}HO-LVA or 3N_{3}Phpa-LVA (from 10 ^{12} to 10 ^{6} M) and incubated for 10 min in PBS supplemented with 10 mg/ml LiCl in the absence of AVP (a concentration close to the K_{act} value determined in CHO cells). After stopping the reaction with perchloric acid, total IPs were extracted and purified by anion exchange chromatography on a Bio-Rad HPX-87H column, formate form, 200–400 mesh (Bio-Rad). For each sample, a fraction containing total IPs was collected and counted. K_{act} constants were calculated as K_{act} = IC_{50}/(1 + [AVP]/K_{act}), in which IC_{50} is the concentration of antagonist leading to 50% inhibition, [AVP] = 1 mM and K_{act} is the concentration of AVP inducing half-maximal accumulation of IP (K_{act} = 0.32 mM in CHO cells expressing the wild-type human V_{1a} receptor (12)).

**Photoaffinity Labeling Experiments**—These experiments were conducted as described before for the previous photoactivatable antagonist [^{[125]}I]3N_{3}Phpa-LVA (12). Briefly, membranes (500 μg) were resuspended in 4 ml of binding Buffer A containing bovine serum albumin (0.5 mg/ml) and were incubated for 1 or 3 h in the dark in the presence of [^{[125]}I]Lys[3N_{3}Phpa]^{[2]}HO-LVA (1–2 μg) with or without vasopressin (10 μM) to define specific labeling. Membranes were separated from unbound ligand by two subsequent centrifugations (20 min, 44,000 × g, 4 °C) and washed with Buffer A. The final pellet was resuspended in 1 ml of Buffer A and irradiated with UV light (254 nm) for 1 min on ice. After photoysis, membranes were washed twice (2 × 1 ml of Buffer A) and finally resuspended in Laemmli buffer (29). Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 1-mm-thick 12% cross-linked polyacrylamide gels (Bio-Rad) and fixed in methanol:Me_{2}SO:water (16:40:2:42), dried, and exposed to Kodak XAR-5 film at ~80 °C. In order to evaluate the yield of covalent binding, dried gels were also cut into slices, and radioactive contents in the slices were determined. Covalent binding was calculated as percentage of the total number of receptor pmol expressed in the membrane preparation.
Antagonist-binding Domains of the \( V_{1a} \) Vasopressin Receptor

**Fig. 1. Structure of the photoreactive antagonist \([^{125}\text{I}][\text{Lys(3N3Phpa)}_8]\)-HO-LVA: comparison with \([^{125}\text{I}][3\text{N3Phpa}]\)-HO-LVA.** The photoreactive azido group of \([^{125}\text{I}][\text{Lys(3N3Phpa)}_8]\)-HO-LVA is in the meta position on the aromatic ring of the Phpa moiety at the side chain of residue Lys. As for its parent compound HO-LVA (23), the octapeptide was radiiodinated on the phenolic substituent of the phenylpropionyl moiety considered as position 1. Residues 2–7 are common to both photoactivatable linear peptide antagonists.

**Electroelution of the Photolabeled \( V_{1a} \) Receptors—**Photolabeled membranes were subjected to SDS-PAGE using 12% cross-linked gels. The labeled bands were excised from the preparative gel and the human \( V_{1a} \) vasopressin receptor was electroeluted with electrophoretic model 422 (Bio-Rad) in Tris/glycine running buffer (25 mM Tris, 182 mM glycine, pH 8.3, 0.1% SDS). Samples containing “partially purified” \( V_{1a} \) receptors were washed and concentrated using Microcon-30 (Amicon). Deglycosylation with \( N \)-glycosidase F and fragmentation with cyanogen bromide and endoproteases were performed on these concentrated samples.

**Deglycosylation of the Photolabeled \( V_{1a} \) Receptors—**The photolabeled CHO membranes or the partially purified receptors were resuspended in deglycosylation buffer (100 mM Na\(_2\)HPO\(_4\), pH 8.0, 10 mM EDTA, 1% digitonin, 1% 2-mercaptoethanol, 5 \( \mu \)g/ml leupeptin, 0.1% SDS) and digested with 2 units of \( N \)-glycosidase F (Roche Molecular Biochemicals) for 2 days at 37°C. Deglycosylated receptors were analyzed by SDS-PAGE using 12% cross-linked gels.

**Cyanogen Bromide and Endoproteinase Digestions—**The electroeluted receptors were subjected to digestion with CNBr, Lys-C protease, or Asp-N protease. Double digestions were also performed (first digestion with CNBr followed by a second one with Lys-C or Asp-N protease). CNBr (a few crystals) cleavage of the electroeluted \( V_{1a} \) receptors was carried out on samples in a 100-\( \mu \)l volume of 70% (v/v) formic acid. The mixture was incubated in the dark for 24 h at room temperature under argon, and the reaction was then stopped by adding 500 \( \mu \)l of water. Sample volume was reduced under vacuum, and solvent exchange (removing formic acid) with water was accomplished. Endoproteinase Lys-C (sequencing grade from *Lysobacter enzymogenes*, Roche Molecular Biochemicals) was used at 0.2 \( \mu \)g/assay in a final 50-\( \mu \)l volume. The digestion was performed in 25 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.1% SDS at 37°C for 16–24 h and stopped by addition of Laemmli buffer. Endoproteinase Asp-N (sequencing grade from *Pseudomonas fragi*, Roche Molecular Biochemicals) was used at 0.1 \( \mu \)g/assay for 48 h at 25°C in a final 50-\( \mu \)l volume of 25 mM Na\(_2\)HPO\(_4\), pH 7.8. To perform double fragmentations, the CNBr digests were washed several times with the same volume sample was reduced before dilution in Lys-C or Asp-N buffer. Results of CNBr and protease digestions were analyzed by a Tricine discontinuous SDS-PAGE system (10–16.5% cross-linked gels) applied to the separation of small molecular mass species (30). Gels were then fixed in glacial acetic acid:methanol:Me\(_2\)SO:water (10:50:2:38), dried, and exposed to Kodak XAR-5 film at 80°C.

**Computer Three-dimensional Molecular Modeling and Docking of Photoactivatable Antagonists—**The three-dimensional model of the human \( V_{1a} \) vasopressin receptor hosting AVP was constructed using the procedure already extensively described in a previous publication for the rat \( V_{1a} \) receptor (7). Briefly, the transmembrane part of the \( V_{1a} \) receptor was constructed by using the three-dimensional model first developed on the bacteriorhodopsin experimental structure (31, 32) and refined on the bovine rhodopsin footprint (33). The extracellular regions of the receptor were then built in an acceptable conformation. The rat residues were exchanged for the corresponding human residues. Transmembrane domain VII was rotated of about 20° to bring residues Thr710 and Ala711,\(^2\) pointing both toward the core of the binding cleft.\(^3\) The whole receptor structure was then energy minimized in order to relax the structure and to remove unfavorable steric constraints. AVP

\(^2\) Thr710 and Ala711 positions 10 and 11 in TMR VII.
\(^3\) N. Cotte, M. N. Balestre, A. Aumelas, E. Mahé, S. Phalipou, D. Morin, C. Barberis, M. Hibert, and B. Mouillac, manuscript in preparation.

**Site-directed Mutagenesis of the Human \( V_{1a} \) Receptor—**The construc-

---

\[ \text{CO}^1\text{-DTyr(Me)_2-Ph_2-Gln}^4\text{-Asn}^5\text{-Arg}^6\text{-Pro}^7\text{-Lys}^8\text{-NH}_2 \]

\[ \text{[^{125}\text{I}][\text{Lys}(3\text{N3Phpa})_8]\text{HO-LVA}} \]

---

**Fig. 2. Reverse phase HPLC monitoring during coupling of the aryiazido group to the [Lys\(^8\)]HO-LVA peptide.** An aliquot of the reaction mixture was injected onto the analytical column and eluted at 2 ml/min, in linear gradient mode, by CH\(_3\)CN in water (1% min, shown by the dotted line), both acidified with 0.1% CF\(_3\)CO\(_2\)H. The compounds were detected by 214 nm (upward) and 254 nm (downward) UV absorption and characterized by their percentage of elution (corrected for the void volume of the apparatus) and their \( \epsilon_{254}/\epsilon_{214} \) ratio. Hydroxybenzotriazole came from PyBOP. If needed, aryazido acid and PyBOP were added in order to substitute a maximum of peptide [Lys\(^8\)]HO-LVA. The peak of target compound is *shaded*.
tion of mutants W613A, F616V, and F617L (see legend of Fig. 8 and Table IV for numbering) has been reported in a previous paper (12). Point mutations Y225D, Q218A, K308A, and Q413A were introduced in the human V1a vasopressin receptor using the QuickChange site-directed mutagenesis kit (Stratagene). These substitutions were directly done on the eukaryotic expression vector pCMV (34) and verified by direct dideoxynucleotide sequencing (T7 Sequencing™ kit, Amersham Pharmacia Biotech). All the mutant receptors were transiently expressed in COS7 cells. In all cases, cell membrane preparations and radioligand binding assays were conducted as described above.

RESULTS

Chemical, Pharmacological, and Functional Properties of the Photoactivatable Linear Peptide—Considering firstly that all V1a structurally related AVP linear peptide antagonists probably bind the V1a receptor in the same way and secondly that introduction of an arylazido group at the N terminus of the ligand led to covalent binding into the TMR VII of the V1a receptor (12), we designed, synthesized, and characterized a novel photoactivatable linear peptide compound (Fig. 1) containing an azido group at a position likely to covalently bind another domain of the receptor. Among the different possibilities in the series of linear peptide antagonists (35), we chose a peptide with a phenylpropionyl substitution at position 1, on the basis that this alkyl chain length confers a high affinity for the receptor (18). Instead of an Arg8 as in the highly potent V1a
antagonist HO-LVA (23), we introduced a Lys8 derivatized with a 3-azidophenylpropionyl group in order to photolabel the V1a receptor. The peptide part of the molecule was synthesized on p-methylbenzhydroxylamine resin (18), and the arylazido group was attached in solution with PyBOP under a dual wavelength reverse phase HPLC monitoring (22), allowing the identification of the target compound \([\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) (Fig. 2). This ligand was purified by semi-preparative reverse phase HPLC and characterized by mass spectroscopy (fast atom bombardment mass spectrometry, \(M+1 = 1287\)) and UV spectroscopy (azido characteristic peak at 250 nm, \(e = 7000\) cm\(^{-1}\) M\(^{-1}\)), destroyed by 254-nm UV irradiation. As for the HO-LVA, this compound can be iodinated on its phenolic N-terminal blocking group (4HO-Phpa) using ICl and purified by HO-LVA, this compound can be iodinated on its phenolic N-terminal blocking group (4HO-Phpa) using ICl and purified by HPLC in order to obtain the nonradioactive probe as a chromatographic reference (fast atom bombardment mass spectrometry, \(M+1 = 1412\)). As shown in Fig. 3A, the corresponding radiiodinated peptide \([\text{125I}][\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) exhibited a high affinity for the human V1a receptor stably expressed in CHO cells; \(K_d\) mean value was 141.5 ± 25 pm (\(n = 4\)). This affinity was equivalent to that measured \((K_d = 137 ± 37\) pm) for \([\text{125I}][\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) (see Figs. 3D and 1 for comparison of the ligand structures) and close to that of its parent compound \([\text{125I}]\text{HO-LVA}\ (K_d = 38 ± 7\) pm). As reported in Table I, affinities \((K_i)\) of the unlabeled \([\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) for other human AVP/OT receptors expressed in CHO cells \((V_{1b}, V_2,\) and OT subtypes) have been calculated from competition binding experiments using the \([\text{125I}]\text{AVP}\) as the radioligand and compared with that deduced for the V1a subtype. As observed in Fig. 3B, displacement of \([\text{125I}]\text{AVP}\) with the photoactivatable peptide allowed the measurement of \(K_i\) values of 11, 574, and >2000 nM for human OT, V1b, and V2 receptor subtypes, respectively. These affinities were 60–10,000-fold lower than that measured for the human V1a receptor \((0.18\) nM), establishing the \([\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) as a selective ligand. This photosensitive ligand displays competitive pharmacological properties equivalent to those of the 3N3Phpa-LVA antagonist (Table I and Fig. 3E for comparison of the selectivity profiles of these two peptides). As shown in Fig. 3C, the photoactivatable peptide potently inhibited the IP accumulation induced by AVP \((1\) nM, a concentration producing a half-maximal response) in CHO cells in a concentration-dependent manner. The average \(K_{\text{inact}}\) calculated from experimental IC\(_{50}\) values was 80 ± 25 pm \((n = 3)\), a value close to that determined for 3N3Phpa-LVA \((130 ± 40\) pm \((n = 3)\); see Fig. 3F for comparison). This \(K_{\text{inact}}\) value is also in agreement with the \(K_i\) determined \((180\) pm; Table I) in binding experiments, a result equally obtained for 3N3Phpa-LVA \((130\) pm for \(K_{\text{inact}}\) versus 240 pm for \(K_i\)). Moreover, no residual agonistic activity of the \([\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) was detected (accumulation of IPs measured in the presence of \(10^{-6}\) M of the photoactivatable peptide was equivalent to that of basal level). Both peptides behave equivalently toward the human V1a vasopressin receptor. Taken together, these results indicate that they competitively inhibit AVP binding and block the AVP-induced signal generation (for review see Ref. 6). In conclusion, the novel linear photoactivatable peptide \([\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) could be considered as a potent and selective antagonist for the human V1a receptor and appeared to be a valuable tool to further investigate its covalent binding sites in the receptor.

**Summary**

- Introduction of a Lys8 derivatized with a 3-azidophenylpropionyl group to photolabel the V1a receptor.
- Synthesis and purification of the nonradioactive probe (4HO-Phpa) using ICl.
- Purification of the radiiodinated peptide (4HO-Phpa) using HPLC.
- Measurement of affinities for the human V1a receptor.
- Comparison of affinities for other human AVP/OT receptors.
- Measurement of \(K_{\text{inact}}\) for the photoactivatable peptide.

**Table I**

| Receptors | \(K_d\) [nM] | \([\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\) | 3N3Phpa-LVA |
|-----------|-------------|---------------------|-------------|
| V1a       | 0.7 ± 0.17  | 0.18 ± 0.05         | 0.24 ± 0.07 |
| OT        | 1.36 ± 1.00 | 11 ± 4              | 29.8 ± 11.3 |
| V1b       | 0.37 ± 0.05 | 574 ± 88            | 54.7 ± 19.5 |
| V2        | 1.36 ± 0.45 | >2000               | >2000       |

**Fig. 4.** Photoaffinity labeling of the human V1a receptor with \([\text{125I}]\text{Lys}(3\text{N}3\text{Phpa})8]\text{HO-LVA}\). The autoradiograms of dried gels are representative of at least three distinct experiments.
Fig. 5. Schematic representation of the human $V_{1a}$ receptor. The primary sequence of the receptor, deduced from the cDNA cloning (24), is shown as well as the possible arrangement of the protein through the cell membrane. The three potential N-glycosylation sites are shown at Asn$^{14}$, Asn$^{27}$, and Asn$^{196}$. White squares indicate a consensus sequence (residues 103–108) spanning the cleavage site for a metalloproteinase (15, 36). Potential cleavage sites for Lys-C (gray triangles), Asp-N (gray squares), and CNBr (gray diamonds) are also indicated. As described previously (12), fragment photolabeled with the previous $[^{125}I]$3N3Phpa-LVA antagonist including the TMR VII is shown (gray circles). The smallest photolabeled fragment with $[^{125}I]3N3Phpa$HO-LVA resulting from Asp-N digestion is represented by black circles and spans the first extracellular loop of the receptor.

Fig. 6. Fragmentation of the human $V_{1a}$ receptor with CNBr and endoproteinases. CHO cell membranes (500 µg) expressing the $V_{1a}$ receptor were incubated with the $[^{125}I]3N3Phpa$HO-LVA for 3 h at 30 °C without ZnCl$_2$ and protease inhibitors, a condition that favors the preferential accumulation of the 46-kDa receptor species. Membrane proteins were then separated on a preparative 12% gel, and the photolabeled 46-kDa species was electroeluted, washed, and concentrated as described under “Experimental Procedures.” Equivalent amounts of electroeluted photolabeled receptors were used in each digestion or chemical cleavage assays (30,000 cpm). The samples were then loaded on discontinuous 10–16.5% Tricine gels. A, CNBr chemical cleavage. The partially purified receptor was treated (lane 2) or not (lane 1) with CNBr for 24 h in the dark at room temperature. B, Lys-C protease digestion. The 46-kDa species was treated (lane 2) or not (lane 1) with the enzyme for 24 h at 37 °C. C, Asp-N protease digestion. The partially purified receptor was treated (lane 2) or not (lane 1) with the enzyme for 48 h at 25 °C. The figure shows autoradiograms of dried gels exposed to Kodak XAR-5 film at −80 °C for 48 h. Molecular mass markers are indicated on the left in each panel. Each assay is representative of at least three distinct experiments.

1 Fig. 4A) could be significantly reduced (~20–25%) in incubation conditions (1 h at 4 °C in the presence of ZnCl$_2$ and protease inhibitors leupeptin, benzamidine, and soybean trypsin inhibitor) reducing the action of endogenous proteases present in cell membrane preparations (data not shown). This observation confirmed that the $V_{1a}$ receptor was proteolyzed during incubation with the $[^{125}I]3N3Phpa$HO-LVA probe. Deglycosylation of the Photolabeled Human $V_{1a}$ Receptor—As seen in Fig. 4B, the treatment of photolabeled membranes with N-glycosidase F before SDS-PAGE reduced apparent molecular masses of the 85–90- and 46-kDa protein bands (lane 1) to approximately 50 and 34 kDa, respectively (lane 2), indicating that they were both glycosylated and contained at least one N-glycosylated site. As shown in Fig. 5, potential N-glycosylation sites (Asn-Xaa-Ser/Thr) are located at Asn$^{14}$, Asn$^{27}$, and Asn$^{196}$. 50 kDa is a molecular mass very close to the theoretical mass of the receptor core deduced from the cDNA sequence (48.2 kDa, including the 1.4-kDa antagonist mass), whereas 34 kDa is significantly smaller. This observation confirmed once again that the photolabeled glycosylated protein band at 46 kDa effectively corresponds to a proteolytic truncated form of the receptor and that the photolabeled species at 85–90 kDa likely represents the native glycosylated state of the receptor expressed in the CHO cell system. Looking at the primary sequence of the receptor (Fig. 5) and at the localization of the different N-glycosylation sites (Asn$^{14}$, Asn$^{27}$, and Asn$^{196}$), a
deglycosylated 34-kDa protein (or the glycosylated 46-kDa species counterpart) could only account for a large truncated receptor fragment including Asn\textsuperscript{196} and spanning the protein to the C terminus. Taking into account both the present results of deglycosylation and sensitivity of the receptor to endogenous membrane protease degradation, we concluded that proteolytic cleavage must occur at a site located between Asn\textsuperscript{27} and Asn\textsuperscript{196}. As mentioned before (12), sequence Phe\textsuperscript{103}–Gln\textsuperscript{108} in the V1\textsubscript{a} receptor (Fig. 5) corresponds to a potential metalloprotease cleavage site. This enzyme has been clearly shown (36) to digest the bovine renal V\textsubscript{2} receptor in membrane preparations between Gln\textsuperscript{92} and Val\textsuperscript{93} (Gln\textsuperscript{104} and Val\textsuperscript{105} in the V1\textsubscript{a} sequence). The 34-kDa molecular mass of the deglycosylated photolabeled band is consistent with that of a proteolytic fragment that could be generated from such a cleavage in this receptor region. We also concluded that photolabeling of the V1\textsubscript{a} receptor with [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA occurred at residue(s) distal to this proteolytic cleavage site.

**Fragmentation of the Photolabeled Human V\textsubscript{1a} Receptor**—To identify antagonist-binding domains covalently bound to the photoactivatable linear peptide [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA, photolabeled receptors were partially purified from a preparative SDS-PAGE by electroelution and then subjected to fragmentation with either CNBr, endoproteinase Lys-C, or endoproteinase Asp-N. Because CNBr, Lys-C, and Asp-N cleave proteins specifically at the C terminus of methionine and lysine residues and at the N terminus of aspartic acid residues, respectively (Met, Lys, and Asp in the V1\textsubscript{a} receptor are shaded in Fig. 5), the photoactivatable peptide ligand itself is thus expected to be protected against these different fragmentations (Lys\textsuperscript{a} is modified and constitutes the amidated C terminus end of the antagonist). The 46-kDa photolabeled band derives from the 85–90-kDa photolabeled species, only the truncated receptor at 46 kDa was excised from gels, electroeluted, and subjected to the chemical cleavage and enzymatic digestions.

As seen in Fig. 6A, CNBr cleavage of the photoaffinity-labeled V\textsubscript{1a} receptor yielded a major labeled fragment migrating at an apparent mass ~5.5 kDa and a higher minor labeled band at ~7.5 kDa. A similar cleavage pattern was observed when electroeluted 46-kDa fragment was first deglycosylated with N-glycosidase F before CNBr treatment (data not shown), indicating that radiolabeled fragments at ~5.5 and 7.5 kDa do not contain N-glycosylation sites. This result eliminates fragments Arg\textsuperscript{2}–Met\textsuperscript{109} (a fragment that could also be eliminated on the basis that this sequence is not included in the truncated 46-kDa photolabeled receptor) and Ile\textsuperscript{192}–Met\textsuperscript{220} as the sites of covalent attachment of the [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA ligand (see primary structure of the V1\textsubscript{a} receptor on Fig. 5). There are only three CNBr fragments of the V1\textsubscript{a} receptor that could account for a ~5.5-kDa molecular mass (including the 1.4-kDa mass of the antagonist itself): His\textsuperscript{87}–Met\textsuperscript{109} (probably not entirely included in the 46-kDa truncated form of the receptor), Cys\textsuperscript{110}–Met\textsuperscript{135}, and Thr\textsuperscript{146}–Met\textsuperscript{170} (equivalent to the second intracellular loop).

As shown in Fig. 6B, digestion of the photolabeled 46-kDa receptor with Lys-C endoproteinase yielded a major fragment at ~5 kDa and two minor higher labeled bands at ~8 and 14.5 kDa. The smallest labeled fragment with an apparent 10.5-kDa molecular mass (Met\textsuperscript{292}–Lys\textsuperscript{370}) resulting from the Lys\textsubscript{C} cleavage of the 46-kDa species photolabeled with a previously described photoactivatable peptide [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA (12), was not produced when the receptor was photolabeled with the [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA peptide antagonist. This indicated that fragment Met\textsuperscript{292}–Lys\textsuperscript{370} cannot account for covalent binding of [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA. Only three fragments, Leu\textsuperscript{53}–Lys\textsuperscript{82} (obviously not present in the truncated 46 kDa species), Val\textsuperscript{105}–Lys\textsuperscript{128} (assuming that the N terminus of this fragment would result from proteolytic digestion of the receptor by a membrane protease during incubation with the photoactivatable ligand), and His\textsuperscript{129}–Lys\textsuperscript{158} could be in agreement with a ~5-kDa labeled band produced by Lys-C digestion of the receptor.

As seen in Fig. 6C, digestion of the radioiodinated antagonist-bound 46-kDa photolabeled species with Asp-N endoproteinase yielded an intense major ~2–2.5-kDa labeled fragment and very minor higher labeled bands at ~6, 12.5, and 18 kDa. Only one possible Asp-N fragment of the V1\textsubscript{a} receptor could account for a labeled band with an electrophoretic migration at 2–2.5 kDa; this fragment, Asp\textsuperscript{112}–Pro\textsuperscript{120}, spans the first extracellular loop of the V1\textsubscript{a} receptor. The localization of this short receptor domain, as the site of covalent binding site for [\textsuperscript{125}I]\textsubscript{[Lys(3N\textsubscript{3}Phpa)\textsubscript{8}]}HO-LVA, was consistent with the results of CNBr and Lys-C cleavages described above. In order to confirm this localization, CNBr cleavage was also used in combination with either Lys-C or Asp-N protease digestion. Successive treatment of the 46-kDa photolabeled receptor with CNBr and Lys-C (Fig. 7A, lane 2) generated a new fragment slightly smaller than the 5 kDa obtained with Lys-C alone (lane 1) or the 5.5 kDa produced with CNBr alone (lane 3). This labeled receptor fragment with a molecular mass (~4.5 kDa) could correspond to Cys\textsuperscript{110}–Lys\textsuperscript{128} sequence. Successive treatment of the 46-kDa labeled species with CNBr and Asp-N endoproteinase (Fig. 7B, lane 2) demonstrated that fragments obtained with CNBr alone (lane 1) were converted into smaller ones with Asp-N protease to yield the smallest fragment with an apparent mass (2–2.5 kDa) equivalent to that generated with Asp-N protease alone (lane 3). The results of double fragmentations were consistent with those of CNBr cleavage and...
Lys-C or Asp-N proteinase digestions and confirmed the identity of the Asp112–Pro120 fragment as the photolabeled receptor domain with [125I][Lys(3N3Phpa)8]HO-LVA.

Docking of the Photoactivatable Linear Peptide Antagonists into the Human V1a Receptor—The putative models of [125I][Lys(3N3Phpa)8]HO-LVA and [125I][3N3Phpa]LVA docked into the human V1a receptor are displayed in Fig. 8. The residues putatively involved in their binding are listed in Tables II and III. The hypotheses presented here are based on the above photoaffinity labeling study and the previous photolabeling data obtained with [125I][3N3Phpa]LVA (12). The two linear ligands differ from AVP in a number of positions: no disulfide bridge; a substituted phenylpropionyl N-terminal; an O-methylated DTyr in position 2 instead of a Tyr; an Arg in position 6; and a modified C-terminal. However, they retain the same residues in positions 3, 4, 5, and 7. In the models, side chains of the four conserved residues and of the DTyr(Me)2 could bind in a similar way compared with homologous features in AVP. Very interestingly, the Arg6 basic moiety of [125I][3N3Phpa]LVA is proposed to be located at the same position as the basic glycineamide terminus of AVP, whereas the alkyl part of the side chain occupies the same domain as Cys6 in AVP. Interestingly, the guanidinium group of Arg6 could also establish intramolecular interactions with the C-terminal amide group and the HO-Phpa group of [125I][Lys(3N3Phpa)8]HO-LVA (Fig. 8C) or with the [125I]Tyr9 and the Phpa group of [125I][3N3Phpa]LVA (Fig. 8A), thus stabilizing the linear peptides in a conformation similar to that of the cyclic AVP.

The 3-azido-phenylpropionic moiety of [125I][3N3Phpa]LVA is found buried in a hydrophobic pocket of the binding cleft, in
Antagonist-binding Domains of the V$_{1a}$ Vasopressin Receptor

In the human V$_{1a}$ receptor column, the numbering of the residues is taken from the G protein-coupled receptor alignment and does not correspond to the numbering of the amino acids in the primary sequence. The first digit corresponds to the helical TMR, and the next two digits indicate the rank of the residue in the considered helix. Tyr225 is numbered according to the same rule but is no longer in the second TMR. It is located in the first extracellular loop between TMR II and TMR III (residue 115 in the primary sequence). The abbreviations used are: B, backbone; SC, side chain; Ar, aromatic; H bond, hydrogen bond; CT, change transfer.

| Table II | Putative ([^{125}I]3N$_3$Phpa LVA/human V$_{1a}$ receptor interactions as observed in the model) |
|-----------------|---------------------------------------------------|
| Residue [^{125}I]3N$_3$Phpa-LVA | Residue Human V$_{1a}$ receptor | Bond, chemical type |
| [^{125}I]Tyr$^a$ | SC; Ar | Phe213 | SC; Ar | Ar-Ar |
| | SC; Ar-Oh | Gln214 | SC; CONH$_2$ | H bond |
| Arg$^b$ | SC; guanidinium | Tyr225 | SC; Ar-Oh | Ct, H bond, ionic? |
| Pro$^c$ | Cycle | Trp434 | SC; Ar | hydrophobic |
| Arg$^d$ | B; CO | Lys308 | SC; NH$_3$ | ionic H bond |
| | B; CO | Lys308 | SC; NH$_3$ | ionic H bond |
| Asn$^e$ | SC; guanidinium | Thr710 | SC; OH | ionic H bond |
| | SC; guanidinium | Gln214 | SC; CONH$_2$ | H bond |
| Gln$^f$ | B; CO | Lys308 | SC; NH$_3$ | ionic H bond |
| | B; NH | Gln311 | SC; CO | H bond |
| | SC; CONH$_2$ | Gln413 | SC; CONH$_2$ | H bond |
| | SC; CONH$_2$ | Tyr414 | SC; Ar-Oh | H bond |
| Phe$^g$ | SC; Ar | Phe509 | SC; Ar | Ar-Ar |
| | | Trp613 | Sc; Ar | Ar-Ar |
| D Tyr(Me)$^h$ | SC; Ar | Trp613 | SC; Ar | Ar-Ar |
| 3N$_3$Phpa$^i$ | SC; N$_3$ | Phe616 | SC; Ar | Ar-Ar |
| | | Thr710 | SC; OH | contact |
| | | Ala711 | SC | contact |
| | | Gly714 | B | contact |
| | | Ser715 | SC | contact |
| | | Asn717 | SC | contact |
| | | Ser718 | SC | contact |

Contact with TMR VII that has been found to be photolabeled (12). According to the model, the residues that might be covalently bound are Thr710, Ala711, Glys14, Ser715, and possibly Asn717 and Ser718 (Fig. 8F). Phe616 is also found in the direct neighborhood in agreement with site-directed mutagenesis data (12). As in AVP (7), only Arg$^b$ side chain protrudes toward the extracellular loop 1 of the human V$_{1a}$ receptor in order to potentially interact with Tyr225.

In the model, the 4-hydroxy-3-[^{125}I]phenylpropionyl group of [^{125}I]Lys3N$_3$Phpa$^a$HO-LVA is found in contact with residues of TMR VII, in a position equivalent to that proposed for the 3-azidophenylpropionic moiety of [^{125}I]3N$_3$Phpa-LVA. In contrast, the ninth residue is not present in [^{125}I]Lys3N$_3$Phpa$^a$HO-LVA, and Arg$^b$ has been replaced by the 3-azidophenylpropionylated lysyl. The backbone constraints are such that the modified lysyl side chain has to point toward the extracellular part of the receptor. Consequently, the azidophenyl moiety is found directly in contact with residues of the first extracellular loop between TMR II and TMR III (Fig. 8D), in the neighborhood of residues Tyr225, Arg226, Phe227, and Arg228, in full agreement with the labeling data reported here.

Site-directed Mutagenesis of the Human V$_{1a}$ Receptor—In order to verify the proposed docking of both linear peptide antagonists into the V$_{1a}$ receptor, the role of some receptor residues (Tables II and III) potentially interacting with the ligands, was investigated. These residues were mutated, and binding properties of the mutant receptors transiently expressed in COS7 cells were studied (Table IV). Because we have already demonstrated important aromatic/ aromatic interactions between [^{125}I]3N$_3$Phpa-LVA and the aromatic residue cluster (particularly Phe616) of TMR VI (12), affinities of [Lys3N$_3$Phpa$^a$]HO-LVA for mutant receptors W613A, F616V, and F617L were measured. Mutation of Phe616 into a valine led to a dramatic loss in binding affinity (1266-fold reduction, 228 nM compared with 0.18 nM for the wild-type receptor). This result confirmed the crucial role played by Phe616 in defining high affinity of the V$_{1a}$-selective linear peptide antagonists. Consequences of Trp613 and Phe617 mutations in binding affinity were not significant. Conserved hydrophilic residues Gln218, Lys308, and Gln413 that have been demonstrated to control the binding of AVP in the rat V$_{1a}$ receptor (7) were substituted with alanine residues. Binding properties of the mutants were measured using [^{125}I]HO-LVA as the radioligand (Table IV); a significant decrease in the affinity of [Lys3N$_3$Phpa$^a$]HO-LVA and 3N$_3$Phpa-LVA for the three mutants was observed (5–28-fold reduction in $K_i$ values). Finally, we decided to investigate a potential role for Tyr225, located in the first extracellular loop (corresponding to Tyr115 in the primary sequence), in the affinity and selectivity of the photoactivatable antagonist ligands because (i) this residue has been shown (9) to control receptor subtype selectivity and participate in agonist high affinity binding by interacting with hormone residue 8; (ii) photolabeling of the receptor with [^{125}I]Lys3N$_3$Phpa$^a$HO-LVA and 3N$_3$Phpa-LVA at the side chain of residue 8 occurred in the first extracellular loop. Tyr225 in the human V$_{1a}$ vasopressin receptor was mutated into an Asp, the residue naturally occurring at the same position in the V$_2$ receptor and the properties of the Y225D mutant studied. When compared with the wild-type V$_{1a}$ receptor, displacement of [^{3}H]AVP by [Lys3N$_3$Phpa$^a$]HO-LVA led to a $K_i$ increased from 0.18 to 0.9 nM (Table IV). This small reduction in binding affinity was emphasized for 3N$_3$Phpa-LVA, the previous photoactivatable antagonist ligand: in this case, $K_i$ shifted from 0.24 to 4.9 nM (20-fold reduction in affinity). In conclusion, Tyr225 could participate in the binding of both [Lys3N$_3$Phpa$^a$]HO-LVA and 3N$_3$Phpa-LVA peptides, but its role in the receptor-selective binding of these two ligands is only minor. Taken together, these mutagenesis data suggest that residues Gln218, Tyr225, Lys308, Gln413, and Phe616 contribute to the binding of 3N$_3$Phpa-LVA and [Lys3N$_3$Phpa$^a$]HO-LVA and validate the
proposed docking of the photoactivatable antagonists into the human V1a receptor.

**DISCUSSION**

In the present study, we have characterized \[^{125}I\][Lys(3N3Phpa)]HO-LVA as a useful V1a-selective photoaffinity ligand. Compared with \[^{125}I\]3N3Phpa-LVA, the previous photoactivatable antagonist used for mapping peptide-binding domains of the rat and human V1a vasopressin receptors (18, 19, 12), this novel antagonist analogue also combines high affinity, selectivity, possibility of radiiodination and high covalent binding yield. The novel ligand allowed us to photolabel the receptor which migrated on SDS-PAGE as two protein bands with apparent molecular masses of 85–90 and 46 kDa, respectively. This result is comparable with that previously observed with \[^{125}I\]3N3Phpa-LVA (12), thus confirming the identity of the receptor and the specificity of the signal. Once again, the human V1a vasopressin receptor was degraded during incubation with the ligand. As demonstrated in our previous study, the 46-kDa species deriving from the larger protein at 85–90 kDa is cleaved by endogenous proteases present in the CHO membrane preparations. Deglycosylation of the photolabeled receptors with N-glycosidase F confirmed this observation. Fragmentation of the photolabeled V1a vasopressin receptor with a combination of chemical CNBr cleavage and Lys-C and Asp-N protease digestions led to the identification of a restricted receptor region that likely spans the first extracellular loop. The position of the photosensitive azido group in the \[^{125}I\][Lys(3N3Phpa)]HO-LVA, at the side chain of residue lysine 8, was chosen in order to covalently bind a receptor domain different from the TMR VII labeled with \[^{125}I\]3N3Phpa-LVA (12) and then to propose docking of these antagonists. The present results validate our strategy. The Asp112–Pro120 sequence, identified as the smallest photolabeled fragment with \[^{125}I\][Lys(3N3Phpa)]HO-LVA, spans the first extracellular loop of the V1a receptor. This region has already been shown to constitute the site of covalent attachment in the bovine renal \(V_2\) receptor with a photoactivatable agonist (15). In this case, the AVP analogue \(^{3}\text{H}1\text{-deamino[Lys}^8\text{]vasopressin contained the photoreactive arylazido group at the side chain of Lys}^8\).

The position of the photosensitive azido group in the \[^{125}I\][Lys(3N3Phpa)]HO-LVA, at the side chain of residue lysine 8, was chosen in order to covalently bind a receptor domain different from the TMR VII labeled with \[^{125}I\]3N3Phpa-LVA (12) and then to propose docking of these antagonists. The present results validate our strategy. The Asp112–Pro120 sequence, identified as the smallest photolabeled fragment with \[^{125}I\][Lys(3N3Phpa)]HO-LVA, spans the first extracellular loop of the V1a receptor. This region has already been shown to constitute the site of covalent attachment in the bovine renal \(V_2\) receptor with a photoactivatable agonist (15). In this case, the AVP analogue \(^{3}\text{H}1\text{-deamino[Lys}^8\text{]vasopressin contained the photoreactive arylazido group at the side chain of Lys}^8\).

Determination of the first extracellular loop of the \(V_2\) receptor as the site of interaction with AVP analogue residue 8 led to the identification of Asp103 as the residue responsible for agonist binding specificity (8). Independently and at the same time, equivalent result was demonstrated in our laboratory for the V1a receptor: Tyr115 (homologue to Asp103 in the V2) was shown to behave as a crucial residue for agonist high affinity binding

**Table III**

Putative \[^{125}I\][Lys(3N3Phpa)]HO-LVA/human V1a receptor interactions as observed in the model

| Residue | Chemical function | Human V1a receptor |
|---------|------------------|--------------------|
| Lys(3N3Phpa) | SC, Ar-N3 | Tyr225 B; SC contact |
| Arg226 | B; SC | contact |
| Phe227 | B; SC | contact |
| Arg228 | B; SC | contact |
| Pro7 | Cycle | Trp434 GC, Ar hydrophobic |
| Arg6 | B, CO | Lys308 SC, NH3 ionic bond |
| Asn5 | SC; Lys308 | Gly311 SC, ConH2 H bond |
| Gln4 | SC; Lys308 | Gly314 SC, ConH2 H bond |
| Phe3 | SC, Ar | Lys308 SC, NH3 ionic bond |
| Tyr(Me)7 | SC, Ar | Trp613 SC, Ar Ar-Ar |
| 3-[^125]I4-OH-Phpa | SC, Ar | Trp613 SC, Ar Ar-Ar |

See Table II for details.
and also for receptor selectivity (9). The position of the photo-reactive moiety (azido phenyl) in the [125I][Lys(3N3Phpa)8]HO-LVA antagonist used in the present study is again at the side chain of peptide residue 8. Interestingly, both peptide agonist and antagonist photoaffinity ligands allow covalent attachment of the first extracellular loop in the V2 and V1a vasopressin receptors respectively when containing the photoactivatable group at the side chain of Lys6. However, residues in this extracellular region of vasopressin receptors responsible for agonist receptor subtype selectivity only play a minor role for antagonists. Other binding selectivity determinants for these compounds have to be discovered elsewhere in the receptor.

Photolabeling of the human V1a receptor with two different selective linear photoactivatable and iodinatable peptide antagonists of AVP led to the identification of two receptor regions in close proximity to the bound photoligands (Ref. 12 and the present study). Taking into account these photoaffinity labeling results, three-dimensional models for the antagonist peptide-binding sites of the V1a vasopressin receptor were then proposed and verified experimentally. The two photoactivatable ligands are linear molecules. They are both very flexible and can adopt a quasi-infinite number of conformations within an acceptable energy window. Therefore, looking for the minimum energy conformers would not provide any clue regarding the receptor-bound conformations. An alternative way consists in examining the physicochemical features of both the ligands and the binding cleft and looking for complementarity. However, it is interesting to observe that [125I][Lys(3N3Phpa)8]HO-LVA and [125I][3N3Phpa]LVA presented a striking homology with AVP whose binding mode had already been probed (7). Most of the receptor residues putatively involved in the binding of these two linear photoactivatable peptide antagonists are those already demonstrated to interact with AVP. These two peptides differ from AVP in positions 1, 2, 6, and 8 and at the C terminus but retain the same residues in positions 3, 4, 5, and 7. In the models, the side chains of the 4 conserved residues bind in a similar way compared with homologous features in AVP. Interestingly, the docking procedures lead these antagonists to adopt a pseudocyclic conformation similar to that of the cyclic AVP. Based on these models, the linear peptide antagonists could enter the transmembrane-binding pocket like their agonist counterparts and establish their own network of molecular interactions. This is consistent with the marked hydrophobic nature of these ligands and that of the bottom of the receptor-binding cleft. Interestingly, as confirmed from the mutagenesis results, aromatic/aromatic contacts represent the most important interactions for antagonists, whereas hydrogen bonds with conserved hydrophilic receptor residues seem to represent the most crucial interactions for agonists like AVP (7).

It is now commonly accepted that peptide ligands, agonists or antagonists, partly bind to transmembrane domains of their receptors (for review see Ref. 6). It has been demonstrated with different approaches, such as site-directed mutagenesis, photoaffinity labeling, or spectrofluorimetric methods. However, very few studies have led to the identification of binding sites for peptide antagonists. In the NK-2 neurokinin receptor systems, the N-termini of agonists and of antagonists of similar molecular size have distinct binding sites (39). In the angiotensin AT1 and the endothelin ETA receptors, minimal overlapping binding sites between peptide agonists and antagonists have been demonstrated, respectively (40, 41). Very recently, it has been observed that structurally related peptide agonist, partial agonist, and antagonist occupy a similar binding pocket within the rat cholecystokinin CCK-A receptor (42). Similarly, we describe in the present study major transmembrane overlapping binding sites in the V1a vasopressin receptor for both peptide agonist and two linear peptide antagonists.

Acknowledgments—We are grateful to Dr. T. Durroux for critical reading of the manuscript. Many thanks to M. Passama and L. Charvet for help in the illustrations.

REFERENCES

1. Strader, C. D., Fong, T. M., Tota, M. R., and Underwood, D. (1994) Annu. Rev. Biochem. 63, 101–132
2. Schwartz, T. W. (1994) Curr. Opin. Biotechnol. 5, 434–444
3. Baldwin, J. M. (1994) Curr. Opin. Cell Biol. 6, 189–190
4. Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995) FASEB J. 9, 745–754
5. van Rhee, A. M., and Jacobson, K. A. (1996) Drug Develop. Res. 37, 1–38
6. J. H. I. Grossmann, M., and Ji, H. (1996) J. Biol. Chem. 271, 17299–17302
7. Mouillac, B., Chini, B., Ballestre, M. N., Elands, J., Trumpf-Kallmeyer, S., Hofflack, J., Hibert, M., Jard, S., and Barberis, C. (1995) J. Biol. Chem. 270, 25771–25777
8. Ufer, E., Petina, R., Gorbulov, V., and Fahrenholz, F. (1995) FEBS Lett. 362, 19–23
9. Chini, B., Mouillac, B., Ala, Y., Ballestre, M. N., Trumpf-Kallmeyer, S., Hofflack, J., Elands, J., Hibert, M., Manning, M., Jard, S., and Barberis, C. (1995) EMBO J. 14, 2176–2182
10. Cotte, N., Ballestre, M. N., Phalipou, S., Hibert, M., Manning, M., Barberis, C., and Mouillac, B. (1998) J. Biol. Chem. 273, 29462–29468
11. Petina, R., Kojro, E., and Fahrenholz, F. (1996) J. Biol. Chem. 271, 31593–31601
12. Phalipou, S., Cotte, N., Carnazzi, E., Seyer, R., Maze, E., Jard, S., Barberis, C., and Mouillac, B. (1997) FEBS Lett. 405, 305–309
13. Barberis, C., Mouillac, B., and Durroux, T. (1999) J. Endocrinol. 156, 223–229
14. Kotzyba-Hibert, F., Kipfer, I., and Goeldner, M. (1999) Angew. Chem. Int. Ed. Engl. 34, 1296–1312
15. Kojro, E., Rich, P., Gimpl, G., and Fahrenholz, F. (1993) Biochemistry 32, 13537–13544
16. Girault, S., Sagan, S., Bolbach, G., Laviolle, S., and Chassaign, G. (1996) Eur. J. Biochem. 240, 215–222
17. Ji, Z., Hadac, E. M., Henne, R. M., Patel, S. A., Lybrand, T. P., and Miller, L. J. (1997) J. Biol. Chem. 272, 24393–24401
18. Carnazzi, E., Atoussia, A., Barberis, C., Guilhon, G., and Seyer, R. (1994) J. Biol. Chem. 269, 1841–1849
19. Carnazzi, E., Atoussia, A., Phalipou, S., Mouillac, B., Guilhon, G., Barberis, C., and Seyer, R. (1994) J. Biol. Chem. 269, 13841–13849
20. Coste, J., Le Nguyen, D., and Castro, B. (1990) FEBS Lett. 272, 29462–29468
21. Seyer, R., and Atoussia, A. (1990) J. Chem. Soc. Perkin Trans. 1, 3269–3299
22. Barberis, C., Ballestre, M. N., Jard, S., Tribollet, E., Arsenojevic, Y., Freiduss, J. J., Bankovski, K., Manning, M., Chan, W. Y., Schlosser, S. S., Holsboer, F., and Elands, J. (1996) Neuroendocrinology 62, 135–146
24. Thibonnier, M., Auzan, C., Madhun, Z., Wilkins, P., Berti-Mattera, L., and Clauser, E. (1994) *J. Biol. Chem.* **269**, 3304–3310
25. Kasas, S., Henneberry, R. C., and Fishman, P. H. (1984) *J. Biol. Chem.* **259**, 4910–4916
26. Park, C., Chamberlin, M. E., Pan, C. J., and Chou, J. Y. (1996) *Biochemistry* **35**, 9807–9814
27. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
28. Berridge, M. J., Downes, C. P., and Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
29. Laemmli, U. K. (1970) *Nature* **227**, 680–685
30. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
31. Hibert, M. F., Trumpp-Kallmeyer, S., Bruinvels, A., and Hoflack, J. (1991) *Mol. Pharmacol.* **40**, 8–15
32. Trumpp-Kallmeyer, S., Hoflack, J., Bruinvels, A., and Hibert, M. (1992) *J. Med. Chem.* **35**, 3448–3462
33. Schertler, G. F., Villa, C., and Henderson, R. (1993) *Nature* **362**, 770–772
34. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raah, H., Kuhr, W. J., and Goeddel, D. V. (1990) *Cell* **61**, 361–370
35. Manning, M., Bankowski, K., Barberis, C., Jard, S., Elands, J., and Chan, W. Y. (1992) *Int. J. Pept. Protein Res.* **40**, 261–267
36. Kojro, E., and Fahrenholz, F. (1995) *J. Biol. Chem.* **270**, 6476–6481
37. Gopalakrishnan, V., McNeill, R. J., Sulakke, P. V., and Triggle, C. H. (1988) *Endocrinology* **123**, 922–931
38. Pávo, I., and Fahrenholz, F. (1990) *FEBS Lett.* **272**, 205–208
39. Turcatti, G., Vogel, H., and Chollet, A. (1995) *Biochemistry* **34**, 3972–3980
40. Ji, H., Leung, M., Zhang, Y., Catt, K. J., and Sandberg, K. (1994) *J. Biol. Chem.* **269**, 16533–16536
41. Lee, J. A., Elliott, J. D., Sutiphong, J. A., Friesen, W. J., Ohlstein, E. H., Stadel, J. M., Gleason, J. G., and Peisheff, C. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7164–7168
42. Dong, M., Ding, X. Q., Pinon, D., Hadac, E. M., Odas, R. P., Landers J. P., and Miller L. J. (1999) *J. Biol. Chem.* **274**, 4778–4785