Synthetic Rat V1a Vasopressin Receptor Fragments Interfere with Vasopressin Binding via Specific Interaction with the Receptor*

(Received for publication, April 16, 1997, and in revised form, June 4, 1997)

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To study the vasopressin receptor domains involved in the hormonal binding, we synthesized natural and modified fragments of V1a vasopressin receptor and tested their abilities to affect hormone-receptor interactions. Natural fragments mimicking the external loops one, two, and three were able to inhibit specific vasopressin binding to V1a receptor. In contrast, the natural N-terminal part of the V1a vasopressin receptor was found inactive. One fragment, derived from the external second loop and containing an additional C-terminal cysteine amide, was able to fully inhibit the specific binding of both labeled vasopressin agonist and antagonist to rat liver V1a vasopressin receptor and the vasopressin-sensitive phospholipase C of WRK1 cells. The peptide-mediated inhibition involved specific interactions between the V1a receptor and synthetic V1a vasopressin receptor fragment since 1) it was dependent upon the vasopressin receptor subtype tested (Ki(app) for the peptide: 3.7, 14.6, and 64.5 µM for displacing [3H]vasopressin from rat V1a, V1b, and V2 receptors, respectively; 2) it was specific and did not affect sarcosin 1-angiotensin II binding to rat liver membranes; 3) it was not mimicked by vasopressin receptor unrelated peptides exhibiting putative detergent properties; and 4) no direct interaction between [3H]vasopressin and synthetic peptide linked to an affinity chromatography column could be observed. Such an inhibition affected both the maximal binding capacity of the V1a vasopressin receptor and its affinity for the labeled hormone, depending upon the dose of synthetic peptide used and was partially irreversible. Structure-activity studies using a series of synthetic fragments revealed the importance of their size and cysteinylation composition. These data indicate that some peptides mimicking extracellular loops of the V1a vasopressin receptor may interact with the vasopressin receptor itself and modify its coupling with phospholipase C.

Vasopressin (AVP),† a small polypeptidic neurohypophysial hormone, exerts different biological effects in mammals. At the periphery, its major physiological role is played in regulating water and solute excretion by the kidney. This hormone is also involved in blood pressure control, platelet aggregation, corticotropin and aldosterone secretion (by the adrenocortical system), hepatic glycogenolysis, and uterine motility. In the central nervous system, AVP is also involved in interneuronal communication.

These distinct biological functions are mediated, in mammals, by at least three distinct receptor subtypes: V1a, V1b, and V2. V2 receptors, involved in the antidiuretic response, are positively coupled to adenyl cyclase (4). V1a and V1b receptors, involved in multiple peripheral responses and in corticotropin release, stimulate phospholipase C and activate protein kinase C (5, 6). Cloning the different subtypes of receptors (7–9) confirmed that these peptidic receptors belong to the family of the G-protein-coupled receptors.

Small amounts of vasopressin receptors in natural tissues and difficulties in solubilizing and purifying vasopressin receptors (10) led to restricted information on the topology of the hormonal binding domain of AVP receptors. However, molecular biology and biochemical approaches have recently headed the way to characterize the vasopressin receptor binding domain. Using a tritium-labeled photoreactive vasopressin agonist, Kojro et al. (11) demonstrated that residues Arg106 and Thr102 present in the second loop of the bovine kidney V2 receptor are involved in AVP binding. Chini et al. (12) showed that replacement of Tyr115 by an alanine, in the first loop of the human V2 vasopressin receptor, greatly affects its ligand selectivity toward a series of vasopressin analogues. More recently, three-dimensional computer modeling of rat V1a vasopressin receptor structure, combined with directed site-mutagenesis experiments, has indicated that the transmembrane domains of the receptor are also involved in the vasopressin binding site (13). However, the precise location of vasopressin binding to the three receptor isoforms remains incompletely characterized.

Another approach to the study of hormone-receptor interactions involves the use of small synthetic peptides mimicking the sequence of the supposed active region of the receptor. This approach allows the use of pure and well-characterized fragments, available in large amounts, to determine their possible interaction with the specific ligand or with the receptor itself. This approach has been successfully used in the case of the thyrotropin-stimulating hormone receptor and luteotropin human choriogonadotropin receptor, where synthetic peptides mimicking the N-terminal extracellular sequence of the receptor were able to bind the hormone (14, 15). Similarly, peptide fragments corresponding to the N-terminal segment of the peptide-mediated inhibition involved specific interactions between the V1a receptor and synthetic V1a vasopressin receptor fragment since 1) it was dependent upon the vasopressin receptor subtype tested (Ki(app) for the peptide: 3.7, 14.6, and 64.5 µM for displacing [3H]vasopressin from rat V1a, V1b, and V2 receptors, respectively; 2) it was specific and did not affect sarcosin 1-angiotensin II binding to rat liver membranes; 3) it was not mimicked by vasopressin receptor unrelated peptides exhibiting putative detergent properties; and 4) no direct interaction between [3H]vasopressin and synthetic peptide linked to an affinity chromatography column could be observed. Such an inhibition affected both the maximal binding capacity of the V1a vasopressin receptor and its affinity for the labeled hormone, depending upon the dose of synthetic peptide used and was partially irreversible. Structure-activity studies using a series of synthetic fragments revealed the importance of their size and cysteinylation composition. These data indicate that some peptides mimicking extracellular loops of the V1a vasopressin receptor may interact with the vasopressin receptor itself and modify its coupling with phospholipase C.

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The abbreviations used are: AVP, vasopressin; GLP, glucagon-like peptide-(17–37) amide; OH-LVA, hydroxyl linear vasopressin antagonist; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline; NT, N-terminal peptide; Sar-AngII, sarcosin 1-angiotensin II; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry.

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follitropin receptor were shown able to bind this hormone (16). More recently, Howl and collaborators (17) also described the inhibitory properties of some synthetic peptides mimicking neurohypophyseal hormone receptor. A 20-amino acid synthetic peptide derived from the tumor necrosis factor receptor inhibited the binding and the cytolytic activity of the corresponding recombinant human hormone (18). This approach has also been successfully used in characterizing hormonal receptor-G-protein second intracellular loop, such as synthetic peptides mimicking the third extracellular loop of the 5HT_{1a} receptor which prevent hormonal adenyl cyclase inhibition (19), and also the interactions between other classes of proteins like actin-tropomyosin and calponin (20).

To further delineate the vasopressin binding site of the V_{1a} vasopressin receptor, we elected to follow an approach similar to that described above. Thus, we synthesized peptides corresponding to extracellular regions of the rat V_{1a} vasopressin receptor (N-terminal sequence and hydrophilic extracellular loops) and examined their ability to alter specific vasopressin binding to V_{1a} vasopressin receptor and to modify vasopressin-stimulated phospholipase C activity. Our data indicate that some synthetic peptides and particularly one fragment of the second extracellular loop with an additional cysteine residue exhibited antagonistic properties. We studied the mechanisms involved in such inhibition.

**MATERIALS AND METHODS**

- **Chemicals—**Triitated [Arg^{8}]vasopressin (60 Ci/mmol) ([^3]H)AVP and myo[^3]H]inositol (10–20 Ci/mmol) were obtained from NEN Life Science Products; OH-LVA, a specific V_{1a} vasopressin antagonist (2000 Ci/mol), and Sar-AngII, an angiotensin II antagonist (2000 Ci/mmol), were radiiodinated using the IODO-GEN technique as described previously (21, 22). [Arg^{8}]vasopressin was obtained from Bachem. The peptid Nt-(50–79) (deriving from the N-terminal part of the bovine endothelin A receptor) and GLP (a glucagon-like-peptide) were synthesized and characterized in the laboratory by solid phase peptide synthesis as described previously (23, 24). Dowex AG-X8 (100–200 mesh), chloride form, was obtained from Fluka. Affinity chromatography was performed with Affi-Gel[^6] 10 gel from Bio-Rad. All other chemicals were of A-grade purity.

- **Solid-Phase Purification of Receptor Fragments—**All peptides were obtained by solid phase peptide synthesis, using two different procedures. The fluoromethoxy carbonyl strategy consisted in using a continuous flow procedure in an automated solid-phase synthesizer (PepSynthesizer 9050, from Perseptive Biosystems, Millipore) (25). The resin was Fmoc-Amino-Acid-PAL-PEG-PSP[^8] from Perseptive Biosystems, Millipore (1 g of 0.2 mmol NH_{2}g). We used free cO{H}II amino acids (0.6 mmol) with temporary protection on the cNH_{2} and the side-chain protections as follows: pentamethylchromanesulfonyl for Arg; trityl for Cys, Asn, and Gln; tertioxy carbonyl for Lys; tertiobutyl ether for Ser, Thr, and Tyr; tertioxybutyl ester for Asp and Glu. The coupling agent used was 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (0.6 mmol) and diisopropylamine as base (1.2 mmol). After the completion of the synthesis, the peptides were deprotected and cleaved from the support using K reagent (CF_{3}COOH/phe

- **Plasma Membrane Preparations—**Rat mammary tumor cell line, were cultured as described previously (32). Briefly, cells were plated at a density of 10^5 cells/dish in a modified minimum essential medium, containing 5% calf serum, 1% rat serum, 290 mg/ml glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. They were cultured at 37 °C in a humidified atmosphere containing 5% CO_{2}, 95% air. Two days after plating, the culture medium was removed and replaced by a fresh one containing 1 μCi/ml myo[^3]H]inositol and cells were used 2 days thereafter.

- **Binding Experiments—**[^3]H]AVP binding to plasma membrane preparations was performed as described previously (4, 6, 33). Briefly, kidney, adenylyl cyclase or liver membrane preparations (30–60 μg of protein) were incubated 1 h at 37 °C in 200 μl of a buffer containing 50 mm Tris-HCl, pH 7.4, 3 mm MgCl_{2}, 1 mm EDTA, 0.1 mm phenethylsulfonyl fluoride, using a glass/glass Potter-Elvehjem. The extracts were then centrifuged at 4 °C for 15 min at 3500 rpm. The supernatants were discarded and pellets resuspended in a large volume of hypotonic buffer, the composition of which was similar to the one described above, but without sucrose. After a 20-min incubation at 0 °C, extracts were centrifuged under the same conditions. Resulting pellets were resuspended in the hypotonic medium and centrifuged again. These pellets were collected, resuspended in hypotonic medium (1 mg of protein/ml), and used immediately for binding experiments.

- **Cell Culture—**WRK1 cells, a rat mammary tumor cell line, were cultured as described previously (32). Briefly, cells were plated at a density of 10^5 cells/dish in a modified minimum essential medium, containing 5% calf serum, 2% rat serum, 290 mg/ml glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. They were cultured at 37 °C in a humidified atmosphere containing 5% CO_{2}, 95% air. Two days after plating, the culture medium was removed and replaced by a fresh one containing 1 μCi/ml myo[^3]H]inositol and cells were used 2 days thereafter.

[^1]: Peptides were purified by reverse phase HPLC. Analytical HPLC was performed using a C_{18} cartridge and linear CH_{3}OH/C_{6}H_{4}O_{2}/0.1% CF_{3}COOH (v/v) gradients as described previously (28). Preparative runs were performed using linear gradients of CH_{3}OH in H_{2}O, acidified (pH 2.5) with 0.1% CF_{3}COOH (v/v). The purified fractions were checked via amino acid analysis, electrospray ionization mass spectrometry (ESI-MS, VG FISONs, TRIO 2000) as described previously (29) or by fast atom bombardment (FAB-MS, JEOL, SX 102). For the ESI-MS method, determination of the peptide true molecular weight from the raw m/z data was performed using VG TRIO 2000 mass spectrometer software (29). For the FAB-MS, determination of the true molecular weight was derived from the (MH)+ peak of the fragmentation profile. Peptide quantification was performed by weighing, assuming CF_{3}COOH as counter-ion in the lyophilized compounds, but without considering any hydration of the peptides.

[^2]: Rapic Thiol Blocking Procedure—The e2-(194–218)K peptide was treated with iodoacetamide, as described previously (30), to block its cysteine residues. This technique was preferred to N-ethylmaleimide blockade, since it introduced a reduced steric modification with no additional charge on the native peptide.

[^3]: Vasopressin Receptor Fragments Interact with Receptor
peptides and the plasma membranes, prior addition of [125I]OH-LVA. As shown in Fig. 1A, a 30-min preincubation at 37 °C did not modify the ability of e2-(194–218)C to inhibit [125I]OH-LVA specific binding. These results validate the experimental protocol chosen for measuring the interaction between the synthetic peptides and the hormonal receptor. To further characterize such interactions, we also calculated the Hill coefficient for the e2-(194–218)C peptide, as described previously (33).

To further characterize such interactions, we also calculated the Hill coefficient for the e2-(194–218)C peptide, as described previously (33). The Hill coefficient (n) is indicated in parentheses. Determination of the apparent dissociation constant (Kd(app)) for the vasopressin receptor fragments was calculated as described previously (33), assuming a direct competition between the vasopressin receptor fragment and labeled AVP or OH-LVA molecules for the vasopressin receptor binding sites. Briefly, the concentration of vasopressin fragment leading to half-maximal specific binding inhibition of labeled hormone (ED50) was determined by concentration-displacement experiments and its Kd(app) was calculated according to the Cheng-Prusoff equation: Kd(app) = ED50 × Kd/Ka + [H+]), where Kd is the dissociation constant of the labeled ligand used for the vasopressin receptor considered and [H+] the concentration of the labeled hormone used in the assay.

**RESULTS**

**Sequences and Purity of V1α Vasopressin Receptor Fragments**—We prepared synthetic fragments of the rat V1α vasopressin receptor from the primary sequence published (8). These peptides (from 13 to 51 residues) mainly corresponded to hydrophilic extracellular sequences of the rat V1α receptor. The C termini of the synthetic peptides, for which length did not exceed 26 residues, were amidated to suppress the carboxyl negative charge not present in the corresponding domain of the native protein. The putative importance of cysteine residues in the receptor binding domain (35), led us to synthesize “modified peptides.” They were either cysteinylated (addition of a C-terminal extra-cysteinyl residue) or alkylated by iodoacetamide (blockade of cysteiny1 residues).

The sequences and the abbreviations of the peptides are summarized in Table I. Peptide purity was determined by analytical HPLC and evaluated to be higher than 98%, as can be shown for e2-(194–218)C peptide (Fig. 2, inset). Moreover, amino acid analysis of each peptide is in good agreement with its sequence (data not shown). Finally, the mass of each peptide was confirmed using mass spectrometry. As shown in Table II, the experimental mass values found for each peptide corresponded to the calculated mass. Fig. 2 illustrates a typical result of mass spectrometry for e2-(194–218)C synthetic fragment.

HPLC control experiments performed on the synthetic fragment e2-(194–218)C, containing cysteiny1 residues, showed that it was not able to dimerize or to be cyclized (data not shown).

**Influence of V1α Vasopressin Receptor Fragments on the Iodinated OH-LVA Specific Binding to Rat V1α Vasopressin Receptor**—Fig. 3 summarizes the effects of some receptor fragments on the specific binding of [125I]OH-LVA on rat liver plasma membranes, a preparation that contained only the V1α vasopressin receptor subtype (33). Short fragments of the first loop (e1-(111–124)), of the second loops (e2-(205–218), e2-(206–218), e2-(194–218)), and of the third loop (e3-(319–335)) inhibited, in a concentration-dependent fashion, the specific binding for [125I]OH-LVA. The concentration of labeled hormone used in this assay was around 0.2 nM, a value similar to the Kd of this analogue for rat liver AngII receptor (22).

**Vasopressin Receptor Fragments Interact with Receptor**

In this series of experiments, we have chosen to examine the interaction between the vasopressin receptor and a synthetic fragment, e2-(194–218), that had been previously synthesized and characterized (33). The fragment e2-(194–218), containing cysteinyl residues, showed an affinity for the receptor that was approximately 5 times lower than those of peptides that corresponded to the second and third loops, respectively (33). Nevertheless, this fragment was able to dimerize in the presence of a reducing agent and did not show any cyclization in mass spectrometry experiments. Moreover, the ability of this fragment to interact with the vasopressin receptor subtype (33), led us to synthesize “modified peptides.” They were either cysteinylated (addition of a C-terminal extra-cysteinyl residue) or alkylated by iodoacetamide (blockade of cysteiny1 residues).

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**Inositol Phosphate Accumulation Measurements**—As described previously (34), myo-[3H]inositol-pretreated WRK1 cells were incubated for 45 min at 37 °C in a culture medium deprived of myo-[3H]inositol and sera, washed twice with 1.5 ml of phosphate-buffered saline (PBS) and then preincubated 15 min at 37 °C with 0.7 ml of PBS supplemented with: 1 g/liter glucose, 10 mM LiCl, 1 mg/ml BSA, and the vasopressin receptor fragments tested. AVP (1 nM) was then added to the incubation medium for an additional 6-min period. Perchloric acid (5% final concentration) together with 0.1 ml of BSA (20 mg/ml) were added to the incubation medium to stop the reaction. Cells were scraped and cellular extracts centrifuged. Labeled mono-, bis-, and triphosphate inositol present in the supernatant were separated from inositol and glycero-phosphoinositides using Dowex AG1-X8 columns and measured by liquid scintillation spectrometry as described previously (34).

**Data Analysis**—Data presented are the mean of triplicate determinations. The standard error (S.E.) associated to each experimental value never exceeded 10% both for binding and inositol phosphate accumulation measurements. The number of distinct experiments performed (n) is indicated in parentheses. Determination of the apparent dissociation constant (Kd(app)) for the vasopressin receptor fragments was calculated as described previously (33), assuming a direct competition between the vasopressin receptor fragment and labeled AVP or OH-LVA molecules for the vasopressin receptor binding sites. Briefly, the concentration of vasopressin fragment leading to half-maximal specific binding inhibition of labeled hormone (ED50) was determined by concentration-displacement experiments and its Kd(app) was calculated according to the Cheng-Prusoff equation: Kd(app) = ED50 × [Kd/Ka + [H+]), where Kd is the dissociation constant of the labeled ligand used for the vasopressin receptor considered and [H+] the concentration of the labeled hormone used in the assay.
of 125I-OH-LVA with an apparent inhibition constant ranging between 7 and 61 μM (see Table III). Finally, the N-terminal part of the V1a vasopressin receptor, Nt-(1–51) peptide, had no effect even up to 300 μM. From these results, it appears that among the V1a vasopressin receptor fragments tested, the peptides corresponding to the second extracellular loops were the most active. More interestingly, their size and cysteine composition seemed to represent crucial parameters for their activities. Thus, increasing the size of peptide e2-(205–218) resulted in peptide e2-(194–218) with a reduced activity (see Table III). The importance of the cysteine 205 residue in e2-(194–218) is further evidenced in Fig. 4. Its replacement by a serine in e2-(194–218)S reduced by at least 10-fold the peptide activity. Moreover, peptide e2-(205–218), which contained only one N-terminal cysteine more than e2-(206–218), was found more potent (see Table III).

Influence of Active V1a Vasopressin Receptor Fragments on the [3H]AVP Specific Binding to Rat V1a Vasopressin Receptor—As the agonist and antagonist binding sites are probably distinct, we compared the activity of some of the most active vasopressin receptor fragments on the specific binding of 125I-OH-LVA (a vasopressin linear antagonist) and [3H]AVP (the natural agonist) to the V1a vasopressin receptor. e2-(194–218), e2-(194–218)C, and e2-(205–218) peptides were able to fully inhibit the specific [3H]AVP binding to rat liver membranes in a concentration-dependent manner (Fig. 5). The rank order of potency to inhibit [3H]AVP binding was found to be similar to that observed for the inhibition of 125I-OH-LVA. However, the e2-(194–218)C peptide was found to be more active in displacing the iodinated OH-LVA than the tritiated AVP (Ki(app) = 0.55 and 3.7 μM, respectively) (Table III). In contrast, e2-(205–218) and e2-(194–218)C peptides exhibited a similar efficiency in inhibition of both antagonist and agonist specific binding (Table III).

Selectivity of the e2-(194–218)C Fragment on the [3H]AVP Specific Binding to Various Vasopressin Receptor Subtypes—To further test the specificity of the e2-(194–218)C peptide, we tested its ability to inhibit specific hormone binding on the two other vasopressin receptor subtypes, earlier characterized: V1b present in rat adenohypophysis (6) and V2 present in rat kidney inner medulla (4). We also compared these values to those
obtained with the rat liver V₁ₐ vasopressin receptor. For these studies, we used [³H]AVP as radioligand, since this hormone exhibited the same affinity for all receptor subtypes studied (2, 6). As illustrated in Fig. 6, the e₂-(194–218)C peptide was found to be more active to inhibit [³H]AVP binding to the V₁ₐ vasopressin receptor than to the other subtypes. However, it entirely suppressed the specific binding to the V₁b receptor, but with a lower potency (Kᵢ(app) = 3.7 ± 1.4, n = 3 and 14.6 ± 4.5 μM, n = 4 for V₁a and V₁b receptors, respectively). It also weakly inhibited the [³H]AVP binding to the V₂ vasopressin receptor (Kᵢ(app) = 64.5 ± 3.5 μM, n = 4).

**Nature of the Interaction between the e₂-(194–218)C and the V₁ₐ Vasopressin Receptor**—To further study the mechanisms by which e₂-(194–218)C peptide inhibited the hormone specific binding, we analyzed the effects of this peptide on the binding parameters of ¹²⁵I-OH-LVA to rat liver membranes. As shown in Fig. 7, and as described previously (21), ¹²⁵I-OH-LVA interacts with a single class of receptor intermediates, present in rat liver membranes. The addition of e₂-(194–218)C in the incubation medium drastically reduced the specific binding whatever the concentration of iodinated antagonist used. Scatchard representations of dose-dependent binding experiments indicated that e₂-(194–218)C affected both the affinity of ¹²⁵I-OH-LVA for the V₁ₐ vasopressin receptor and its maximal specific binding capacity. As illustrated in Table IV, when membranes were incubated with 3.16 μM e₂-(194–218)C peptide, the apparent affinity was reduced by 2.3-fold. The maximal binding capacity (Bₘₐₓ) was slightly reduced, but this effect was not statistically significant. Increasing the amounts of synthetic peptide in the incubation medium up to 10 μM greatly increased the Kᵢ by 7-fold and reduced the Bₘₐₓ significantly (by 2-fold). Such results indicate that e₂-(194–218)C peptide inhibits ¹²⁵I-OH-LVA in an uncompetitive fashion, which suggests interaction between the receptor and the peptide. We also tested the reversible nature of the interaction between e₂-(194–218)C fragment and the V₁ₐ vasopressin receptor. For this purpose, rat liver membranes were first preincubated with or without a concentration of synthetic peptide allowing an almost maximal inhibitory effect and washed to eliminate the synthetic peptide. The kinetic binding parameters of the V₁ₐ vasopressin receptor were then measured in control and preincubated membranes. As illustrated in Fig. 8A, the inhibitory effect of e₂-(194–218)C peptide still persisted even after washing the membranes previously incubated with the synthetic receptor fragment. However, at variance with membrane directly incubated with the synthetic receptor fragment, only the effect on the maximal binding capacity was observed. Fig. 8B also showed that membranes preincubated with e₂-(194–218)C peptide and further washed were still sensitive to the synthetic receptor fragment. A new addition of e₂-(194–218)C peptide always strongly reduced the remaining specific binding of ¹²⁵I-OH-LVA. Altogether, these results indicate that the interactions between e₂-(194–218)C peptide and the V₁ₐ vasopressin receptor are partly irreversible.

**Influence of V₁ₐ Vasopressin Receptor Fragments on Inositol Phosphate Accumulation**—As the
The ability of synthetic fragments of V₁a vasopressin receptor to inhibit [³H]AVP or [¹²⁵I]-OH-LVA binding to rat liver membranes was determined as described in legend of Figs. 3 and 5. The apparent inhibition constant of each peptide was calculated as indicated under "Materials and Methods." The antagonistic properties of each synthetic peptide was determined as described in legend of Fig. 9. Values in the table corresponded to the inhibition of vasopressin-stimulated inositol phosphate accumulation induced by synthetic peptides. Results were expressed as: 100 – (100 × inositol phosphate accumulated in the presence of 1 nM AVP plus 30 µM peptide over basal/inositol phosphate accumulated in the presence of 1 nM AVP over basal). Results were the mean ± S.E. of the number of distinct experiments indicated in parentheses (undetectable = no significant inhibition observed for 10⁻⁶ M peptide). ND, not determined.

**TABLE III**

| Vasopressin Receptor Fragments Interact with Receptor |
|-----------------------------------------------------|
| Biological properties of natural and modified synthetic fragments of rat V₁a vasopressin receptor |

| Abbreviations | Apparent inhibition constant of [³H]AVP binding (K<sub>app</sub>) | Apparent inhibition constant of [¹²⁵I]-OH-LVA binding (K<sub>app</sub>) | Inhibition of AVP-stimulated inositol phosphate accumulation induced by 30 µM peptide |
|---------------|-------------------------------------------------|-------------------------------------------------|---------------------------------------------------------------------|
| Nt-(1–51)     | Undetectable (n = 3)                            | ND                                             | Undetectable (n = 2)                                                 |
| e1-(111–124)  | 27 ± 6 (n = 4)                                  | ND                                             | 25 (n = 1)                                                          |
| e3-(319–335)  | 61 ± 22 (n = 4)                                 | ND                                             | ND                                                                  |
| e2-(206–218)  | 22 ± 3 (n = 3)                                  | ND                                             | ND                                                                  |
| e2-(205–218)  | 7 ± 2 (n = 3)                                   | 17.0 ± 3 (n = 2)                               | 37 ± 12 (n = 3)                                                     |
| e2-(194–218)  | 33 ± 6 (n = 5)                                  | 56 ± 7 (n = 3)                                 | 9 ± 2 (n = 3)                                                       |
| e2-(194–218)S | >300 (n = 2)                                    | ND                                             | ND                                                                  |
| e2-(194–218)C | 0.55 ± 0.15 (n = 5)                             | 3.7 ± 1.4 (n = 5)                              | 90 ± 7 (n = 3) for 10 µM peptide                                   |
| e2-(203–218)C | >100 (n = 3)                                    | ND                                             | ND                                                                  |
| e2-(201–218)C | 65 ± 51 (n = 3)                                 | ND                                             | ND                                                                  |
| e2-(194–218)C(cam)<sub>1</sub> | >80 (n = 1) | ND | ND |

**FIG. 4.** Influence of modified extracellular fragments of rat V₁a vasopressin receptor on [¹²⁵I]-OH-LVA specific binding. Rat liver membranes were incubated 60 min at 37 °C with 1 nM [³H]AVP (total binding), or the same amount of labeled AVP plus 1 µM unlabeled AVP (nonspecific binding) in the absence (control) or presence of increasing amounts of V₁a vasopressin receptor fragments (e2-(194–218) (■), e2-(194–218)C (▲), e2-(203–218)C (●), e2-(194–218)S (○)). Specific binding were calculated and expressed as % of control as described in Fig. 1. Results were the mean of triplicate determinations from a single experiment representative of three.

**FIG. 5.** Influence of extracellular fragments of rat V₁a receptor on [³H]AVP specific binding. Rat liver membranes (30–40 µg of protein/assay) were incubated 60 min at 37 °C with 1 nM [³H]AVP (total binding), or the same amount of labeled AVP plus 1 µM unlabeled AVP (nonspecific binding) in the absence (control) or presence of increasing amounts of V₁a vasopressin receptor fragments (e2-(194–218) (■), e2-(203–218) (●), and e2-(194–218)S (○)). Specific binding were calculated and expressed as percent of control values (100% = 7500 ± 600 dpm). Results were the mean of triplicate determinations from a single experiment representative of three.

V₁a vasopressin receptor subtype is positively coupled to phospholipase C activation, we tested the activity of some of the most active V₁a vasopressin receptor fragments characterized above. Such studies were performed on WRK1 cells, which expressed, as rat liver membranes only, a V₁a vasopressin receptor tightly coupled to phospholipase C (32, 34). As illustrated in Fig. 9, e2-(194–218), e2-(194–218)C, and e2-(205–218) peptides up to 300 µM did not stimulate basal inositol phosphate accumulation. However, they were able to fully inhibit in a concentration-dependent manner the vasopressin-stimulated inositol phosphate accumulation. As expected, e2-(194–218)C peptide was the most potent in inhibiting the vasopressin effect (K<sub>app</sub> = 0.44 ± 0.07 µM, 3 distinct experiments). The two other peptides, e2-(205–218) and e2-(194–218), were less active (K<sub>app</sub> = 23 and 84 µM, respectively). This rank order of potency corresponded to those found for binding studies (Table III).

**Specificity of V₁a Vasopressin Receptor Fragment Activity—**To further demonstrate that peptide e2-(194–218)C specifically inhibited the binding of [¹²⁵I]-OH-LVA to V₁a vasopressin receptor, we performed the following control experiments. First, we tested the ability of a 30-amino acid peptide derived from the N-terminal part of the endothelin A receptor (peptide Nt-(50–79)) to inhibit specific vasopressin binding. This peptide, containing a cysteiny1 residue and a size similar to e2-(194–218)C fragment with no sequence homology did not alter the binding of [¹²⁵I]-OH-LVA on rat liver membranes, even tested at 30 µM. Under the same experimental conditions, e2-(194–218)C peptide almost completely inhibited the [¹²⁵I]-OH-LVA binding (Fig. 10A). Second, we also tested the influence of GLP, known to exhibit amphiphilic properties due to its helicoidal structure (36), to exclude any nonspecific putative deterrent properties of the hormonal receptor fragments. As illustrated on Fig. 10A, GLP did not inhibit [¹²⁵I]-OH-LVA binding at a maximal dose of 30 µM. Third, we checked that e2-(194–218)C peptide was not able to alter the binding of [¹²⁵I]-Sar-AngII to the angiotensin II receptor also present in rat liver membranes. As seen on Fig. 10B, under experimental conditions where [¹²⁵I]-OH-LVA specific binding was completely suppressed (30 µM e2-(194–218)C), no significant modification
Vasopressin Receptor Fragments Interact with Receptor

**FIG. 6. Influence of e2-(194–218)C fragment on [3H]AVP specific binding to distinct rat vasopressin receptor subtypes.** Rat liver membranes (●), rat kidney membranes (▲), or rat adenohypophysis membranes (○) (30–50 μg of protein/assay) were incubated 60 min at 37 °C with 1 nM [3H]AVP in the absence (control) or in the presence of increasing amounts of e2-(194–218)C fragments of rat V1a vasopressin receptor. Specific binding was calculated as described in Fig. 5. Results, expressed as percent of corresponding control values, are the mean of triplicate determinations from a single experiment representative of three (100% = 7500 ± 600, 5200 ± 400, and 490 ± 70 dpm for rat liver, rat kidney, and rat adenohypophysis membrane preparations, respectively).

of 125I-Sar-AngII was observed in the same plasma membrane sample. Fourth, to verify that there is no direct interaction between the e2-(194–218)C peptide and the labeled hormone, we compared the elution profiles of [3H]AVP either on an Affi-Gel column where e2-(194–218)C peptide was covalently fixed by its N-terminal part or on a control Affi-Gel column. Whatever the column used, the two elution profiles were identical. All the radioactivity was recovered in a single symmetrical peak coeluting with blue dextran, a marker of the column void volume (data not shown). Control experiments that validate this negative experiment have been previously published by Pradelles and collaborators. They demonstrated that the well known protein/protein interactions between AVP and neurophysin still persist even if neurophysin was covalently linked to the Sepharose column (37). This property was routinely used in the laboratory to purify tritiated vasopressin.

**DISCUSSION**

Vasopressin receptors belong to the G-protein-coupled receptor family exhibiting an N-terminal part, seven transmembrane helices of the V1a vasopressin receptor are responsible for vasopressin anchoring (39, 40). However, additional studies have also demonstrated that the external loops of V1a vasopressin receptors may also play a role in these mechanisms. Mutation of one amino acid of the first extracellular loop of the V1a vasopressin receptor deeply affects its selectivity for two series of vasopressin analogues (12), photoaffinity labeling of V1a vasopressin receptor pointed to the second extracellular loop as a potential binding site for the hormone (11), and a peptide strategy suggested that the first extracellular loop of the V1a vasopressin receptor may represent a putative binding site for radioligands (17).

To further characterize the interaction between the vasopressin and the extracellular loops of the V1a vasopressin receptor, we synthesized a series of peptides mimicking these hydrophilic loops and tested their abilities to interfere with hormonal binding. Results presented in this study indicate that the peptide mimicking the N-terminal part of the V1a receptor is unable to alter specific agonist or antagonist binding to this receptor. This suggests either that this receptor domain is not concerned in binding or the structural determinants such as glycosylation or three-dimensional organization are lacking for this longer peptide. In contrast, fragments of V1a receptor exhibiting sequences similar to those of the external loops one, two, and three are active. They inhibit in a concentration-dependent manner the specific [3H]AVP and 125I-OH-LVA binding to the rat V1a vasopressin receptor with K_i(app) ranging between 10 and 60 μM (Table III).

To study structure-activity relationships of the most active peptide found, e2-(205–218), we increased its size and modified its cysteinyl composition by adding a C-terminal cysteine residue. These modifications were based upon the following arguments. 1) Hydropathy profile of the primary receptor sequence indicated that the second extracellular loop, determined by hydrophilic amino acid composition, is larger than the e2-(205–
plasma membranes were preincubated 30 min at 37 °C with or without of 20–30

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\text{Specific binding (Bound/Free) was calculated in each condition as the difference between total and nonspecific binding and these values plotted against the ratio (Bound/Free). Similarly, membranes preincubated with 3.16 μM e2-(194–218)C peptide were further incubated 60 min at 37 °C with increasing amounts of 125I-OH-LVA in the absence of specific binding (Bound) was calculated in each condition as the difference between total and nonspecific binding and these values plotted against the ratio (Bound/Free).}
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| Assay conditions | \( K_d \) (pmM) | \( B_{max} \) (pmol 125I-OH-LVA specifically bound·mg⁻¹ protein) |
|------------------|-----------------|---------------------------------------------------------------|
| Control membrane | 18.2 ± 6.5      | 1.54 ± 0.6                                                   |
| Membrane + 3.16 μM e2-(194–218)C peptide | 42.2 ± 5.2      | 1.32 ± 0.30                                                  |
| Control membrane | 17.2 ± 1.3      | 1.39 ± 0.10                                                  |
| Membrane + 10 μM e2-(194–218)C peptide | 123.7 ± 30.0    | 0.74 ± 0.27                                                  |

**Fig. 8. Partial irreversibility of e2-(194–218)C effect on rat liver vasopressin receptor.** Rat liver membranes (40 μg protein/ml) were preincubated 30 min at 37 °C with (●) or without (○, □ = control) 3.16 μM e2-(194–218)C peptide. Reaction was stopped by diluting 6-fold the incubation medium with an ice-cold buffer containing 50 mM Tris-HCl, 7.4, and 1 mM MgCl₂. Membranes were then quickly centrifuged for 15 min at 14,000 × g at 0 °C. The supernatants were discarded and the pellets resuspended in the ice-cold buffer to a protein concentration of 20–30 μg/ml. Panel A, control membranes (○, □) were further incubated 60 min at 37 °C with increasing amounts of free 125I-OH-LVA in the presence (□) or in the absence (○) of 3.16 μM e2-(194–218)C peptide. Specific binding (Bound) was calculated in each condition as the difference between total and nonspecific binding and these values plotted against the ratio (Bound/Free). Similarly, membranes preincubated with 3.16 μM e2-(194–218)C peptide (●) were further incubated 60 min at 37 °C with increasing amounts of 125I-OH-LVA in the absence of specific binding (Bound) was calculated in each condition as the difference between total and nonspecific binding and these values plotted against the ratio (Bound/Free). Panel B, in this set of experiments, plasma membranes were preincubated 30 min at 37 °C with or without 3.16 μM e2-(194–218)C fragment. Specific binding was calculated in each condition. Results are the mean of triplicate determinations from a single experiment representative of two.

218) sequence. 2) Cysteinyl residues of rat V₂ and, to a lesser extent, those of rat V₄, vasopressin receptors play an important role in AVP binding (35). 3) Most of our active fragments exhibited a cysteinyl residue. 4) Recent representations of the vasopressin receptor favored the existence of a disulfide bridge between the cysteinyl residues of the extracellular loops one and two (13). Results obtained indicate that increasing the size of the most active peptide, e2-(205–218), leads to e2-(194–218), 5-fold less active on 125I-OH-LVA binding assays on rat liver membranes. In contrast, increasing the size of the inactive modified e2-(203–218)C peptide leads to e2-(201–218)C and e2-(194–218)C peptides with enhanced activity. Addition of 2 or 9 amino acids to the N-terminal part of the inactive fragment, e2-(203–218)C, led to peptides active at the 50 μM and 1 μM concentration range, respectively. Moreover, the presence of cysteine residues in the synthetic fragments also greatly improve their activities; e2-(194–218)C peptide, which differs from e2-(194–218)C peptide by only one cysteinyl residue in the C-terminal part, is 60-fold more active. On the opposite, blocking its cysteinyl residues leads to a nearly inactive fragment. All together, these data suggest that the size and the cysteinyl composition of the most active peptide, e2-(194–218)C, constitute crucial parameters for its activity.

At least two reasons may explain why e2-(194–218)C peptide inhibits specific vasopressin binding. This receptor fragment may interact either with the receptor itself or with the hormone. In both cases, such interactions prevent the hormonal binding to its specific receptor. Experimental data presented in this study favor the first hypothesis for the following reasons. 1) No direct interaction between AVP and e2-(194–218)C peptide could be observed by an affinity chromatography approach. [³H]AVP was not retained on an Affi-Gel column on which e2-(194–218)C peptide was covalently linked, as neurophysin was retained, using the same experimental approach (37). 2) A direct interaction between [³H]AVP and e2-(194–218)C peptide would lead to identical inhibitory effects on the three vasopressin receptor subtypes, since they possessed the same affinity for [³H]AVP (Kₐ 3.5 ± 0.2, 1.0 ± 0.2, and 1.5 ± 0.3 nm, for V₁₉, V₁₈, and V₂ vasopressin receptors present on rat liver, rat pituitary, and rat kidney membranes, respectively). Data presented in Fig. 6 clearly indicate that it is not the case. e2-(194–218)C peptide preferentially inhibited the binding of [³H]AVP on the V₁₉ vasopressor receptor and is 4- and 17-fold less active on the V₁₈ and the V₂ vasopressor receptors, respectively. 3) The inhibitory effects of e2-(194–218)C peptide on specific 125I-OH-LVA binding were partially irreversible. They still persist even if rat liver plasma membranes preincubated with the synthetic receptor fragment were washed before the hormonal binding assay (Fig. 8).

The effects we observed with the synthetic peptides mimicking the rat V₁₉, vasopressor receptor on vasopressin binding are specific and could not be explained by their putative detergent properties or cysteinyl composition. Indeed, we demonstrated that 30 μM e2-(194–218)C peptide, which completely inhibited 125I-OH-LVA binding on rat liver plasma membranes, did not affect, under the same experimental conditions, the specific
binding of $^{125}$I-Sar-AngII (Fig. 10B). Similarly, we tested the effects of unrelated V$_{1a}$ vasopressin receptor peptides exhibiting either cysteine residue (Nt-(50–79), a fragment of the endothelin A receptor) or an helicoidal structure with potential detergent properties (GLP, glucagon-like peptide) on $^{125}$I-OH-LVA binding. As observed on Fig. 10a, neither Nt-(50–79) nor GLP peptide inhibited specific $^{125}$I-OH-LVA binding to rat liver membranes even tested at 30 $\mu$M.

The mechanisms by which e2-(194–218)C peptide specifically inhibits hormonal binding to rat V$_{1a}$ vasopressin receptor are probably complex, since we observed both an irreversible loss of the maximal binding capacity and a reversible alteration of the affinity of the vasopressin receptor for its ligand. The substantial loss of specific binding site observed for high concentrations of e2-(194–218)C peptide (Table IV) is probably due to covalent interactions between the receptor itself and the synthetic peptide via cysteinyI residues since 1) both the V$_{1a}$ vasopressin receptor and the e2-(194–218)C peptide exhibited cysteinyI residues (Ref. 35 and Table I), 2) replacement of the Cys$^{205}$ residue of the natural receptor fragment e2-(194–218) by a serine led to a compound (e2-(194–218/S) more than 10-fold less active, 3) blockade of the two cysteinyl residues of e2-(194–218)C peptide led to peptide e2-(194–218/C(cam2) exhibiting a $K_{i}$ of at least 150-fold higher, and 4) the effect of e2-(194–218)C peptide on $^{125}$I-OH-LVA binding to rat liver membranes is partly irreversible (Fig. 8). The modulation of the affinity of the V$_{1a}$ vasopressin receptor for its ligand induced by e2-(194–218)C fragment also probably involved non-covalent protein-protein interactions between the synthetic peptide and the receptor, since these effects were reversible (Fig. 8). The fact that synthetic peptides devoid of any cysteinyI residue like e2-(206–218) were active reinforces this assumption (see Table III). Probably, we have synthesized a cysteinyI site-directed peptide able to interact with the V$_{1a}$ vasopressin receptor and as a consequence to block its ability to recognize its natural hormone and to transduce intracellular second messenger generation. However, as shown in Fig. 10, the cysteine is not sufficient to confer activity to a peptide, since a fragment of endothelin receptor of size similar to e2-(194–218)C contains cysteine and yet did not inhibit $^{125}$I-OH-LVA binding. This indicates that sequence-specific interactions are involved in the biological activity of the peptide studied. Such results would imply that synthetic peptides mimicking some specific regions of the extracellular domains of the V$_{1a}$ vasopressin receptor are able to interact with unidentified part of the V$_{1a}$ vasopressin receptor and block its activity. This suggests that intramolecular protein-protein interactions may represent a crucial parameter for vasopressin receptor activity. Such a hypothesis was already verified by Ridge et al. (41), who showed that co-expression of two or three complementary fragments of rhodopsin in COS cells led to the expression of a protein that reproduced the spectral properties of native rhodopsin. Alternatively, our data would be also consistent with the proposed importance of intermolecular protein-protein interactions between two receptors domains of distinct proteins. Such specific interactions may explain the formation of hormonal receptor dimers as described previously for the muscarinic, the $\beta$-adrenergic and the glucagon receptors (42, 43). Similarly, the V$_{1a}$ vasopressin receptor photolabeling, in rat liver membranes showed, by SDS-PAGE autoradiography, higher molecular weight structures suggesting the possibility of dimer formation (44). More interestingly, they may represent a new mechanism to regulate their activities (45). Thus, Bouvier and collaborators confirmed that $\beta$-adrenergic receptor may dimerize. They also demonstrate that a peptide mimicking the transmembrane domain VI of this receptor prevents dimerization and also inhibits $\beta$-adrenergic stimulation of adenylyl cyclase activity.
(46). Maggio et al. (47) also demonstrated that co-expression in
COS cells of two muscarinic/adrenergic receptor chimera, individ-
ually devoid of any binding activity, led to specific active
muscarinic and adrenergic binding sites.

In conclusion, the specific interaction evidenced in the paper
between synthetic peptide mimicking the sequence of the V1a
vasopressin receptor and the receptor itself strongly suggests
the importance of intra- or intermolecular interactions between
two receptor molecules and provides a new molecular basis to
study their regulations.

Acknowledgments—We thank M. Passama and D. Bellenoue for
drawings, M. C. Maraval and M. Chalier for English editing, Dr. J.
and D. Pouy, E. C., and Barberis, C., Jard, S., and Chan,

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