Identification of Residues Responsible for the Selective Binding of Peptide Antagonists and Agonists in the V2 Vasopressin Receptor*

Nathalie Cotte‡, Marie-Noëlle Ballestre‡, Sylvie Phalipou‡, Marcel Hibert‡, Maurice Manning¶, Claude Barberis‡, and Bernard Mouillac‡

From the ‡Unité INSERM 469, Centre CNRS-INSERM de Pharmacologie-Endocrinologie, 141 rue de la Cardonille, 34094 Montpellier Cedex 5, France, the ¶Laboratoire de Pharmacochimie de la Communication Cellulaire, Faculté de Pharmacie de Strasbourg, 74 route du Rhin, B. P. 24, 67401 Illkirch Cedex, France, and the ©Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43614-5804

To improve our understanding of the functional architecture of G protein-coupled receptors, we have taken advantage of differences among mammalian species in ligand binding to search for the rat versus human selectivity determinants of the V2 vasopressin receptor and of its peptide ligands. Our data indicate that residue 2 of cyclic and linear peptides (2) as well as nonpeptides (3, 4). By a combination of receptor three-dimensional molecular modeling and site-directed mutagenesis approaches, our preceding study has led to the mapping of the AVP agonist-binding domain in the V1a receptor subtype (5). The hormone-binding site is localized in a narrow cleft delimited by most of the transmembrane regions −15 Å away from the extracellular surface. Because of the high conservation of residues involved in the interaction with the hormone, this binding site has been proposed to be common to the V1b, V2, and oxytocin receptors. An extracellular residue responsible for receptor subtype agonist selectivity has also been identified (6–8).

These first analyses did not provide much information on the definition of AVP/oxytocin receptor antagonist-binding domains. To date, only the upper part of transmembrane region VII and a hydrophobic cluster of aromatic residues situated in transmembrane region VI have been shown to be part of the antagonist-binding sites of the oxytocin and V1a receptor subtypes, respectively (9, 10). The purpose of this work was to increase our knowledge about the antagonist-binding sites of the V2 AVP receptor for a better understanding of the mechanisms of action of this important G protein-coupled receptor (11, 12). To conduct the study, we have taken advantage of the important differences in peptide antagonist-binding selectivity among mammalian species. Indeed, although many peptide V2 antagonists have been shown to be highly potent in the rat, the two lead candidates were found to be ineffective in humans (13). These findings provided the first clear evidence of rat/human species variability of the V2 receptors. Based on previous intensive structure-function activity and analog design of AVP/oxytocin peptide hormones (14), we have used a series of cyclic peptide antagonists displaying species selectivity or not for the rat and human V2 receptors (15). These antagonists, such as d(CH2)4-[d-Ile2,Ile4,Tyr-NH2]3AVA, highly selective for the rat V2 receptor, all share a closely related structure with AVP and its V2-selective agonist analogs, such as [1-deamino,d-Arg8]arginine vasopressin (dDAVP).

Human and rat V2 receptors (16, 17) share a very high degree of homology, and sequence identity reaches 88%. Divergent residues may represent potential major determinants responsible for the binding of species-selective AVP antagonists. Taking into account our previous data describing the three-dimensional model of AVP receptors (5), only residues localized within extracellular loops and at the top of the transmembrane regions have been considered. Those of the human V2 receptor have been subjected to site-directed mutagenesis and substituted for their homologs present in the rat receptor. The pharmacological and functional properties of the mutant V2 receptors were examined. Interestingly, we have demonstrated that binding determinants responsible for species selectivity of peptide antagonists also play an equivalent role in the selective

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† To whom correspondence should be addressed. Tel: 33-4-67-14-29-22; Fax: 33-4-67-54-24-32; E-mail: mouillac@u469.montp.inserm.fr.

1 The abbreviations used are: AVP, arginine vasopressin; dDAVP, [1-deamino,d-Arg8]arginine vasopressin; dDAVP, [d-Arg8]arginine vasopressin.
binding of peptide agonists. These results are of broad interest and should contribute to the deciphering of the molecular mechanisms of transduction/inactivation of the V2 receptor and more generally to other G protein-coupled receptors.

**EXPERIMENTAL PROCEDURES**

**Drugs—**AVP and dDAVP came from Bachem. DAVP was from Ferring Research. The V2 receptor antagonists d(CH)2-[d-Tyr(2)-Ile(4),Tyr-NH2](AVP, d(CH)2-[d-Ile(4)-Tyr-NH2](AVP, d(CH)2-[d-Ile(4)-Tyr(2)-Tyr-NH2](AVP, d(CH)2-[d-Tyr(2)-Ile(4)-Tyr-NH2](AVP, and d(CH)2-[d-Ile(4)-Tyr-NH2](AVP were synthesized in the laboratory of Dr. M. Manning. SR 12146IA and OPC-31260 came from Sanofi Recherche. [3H]AVP (60–80 Ci/mmol) was from NEN Life Science Products.

**Cloning of the Human V2 Vasopressin Receptor cDNA—**Extraction of total RNA from Chinese hamster ovary cells expressing the human V2 receptor was performed by homogenization with RNaseol reagent (Biogrobe Systems), followed by precipitation with isopropl alcohol. Total RNA (1.5 µg) was reverse-transcribed into single-stranded cDNA (First Strand cDNA Kit for reverse transcription-polymerase chain reaction, Boehringer Mannheim). Polymerase chain reaction amplifications of cDNA were carried out with the following sequence-specific primer pair. The upstream primer was 5′-GGAATTCGATCCCGCA-CATCACCTCCAG-3′, consisting of an EcoRI restriction site and nucleotides complementary to the 5′-untranslated region from base pairs −50 to 30 of the human V2 receptor gene (16); the downstream primer, 5′-CAGGAAGGAGCTGAGTCGATTCTAGACAGAG-3′, was complementary to the 3′-untranslated region from base pairs 30 to 56 after the stop codon. Polymerase chain reaction conditions were 30 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1.5 min, followed by a final 7-min extension step at 72 °C. Agarose gel analysis (1% Sea Kem, FMC Biotech). The upstream primer was 5′-TATCCTGTGCAGGAGTCCCCGATCACCTCCAG-3′, consisting of an EcoRI restriction site and nucleotides complementary to the 5′-untranslated region from base pairs −50 to 30 of the human V2 receptor gene (16); the downstream primer, 5′-CAGGAAGGAGCTGAGTCGATTCTAGACAGAG-3′, was complementary to the 3′-untranslated region from base pairs 30 to 56 after the stop codon. Polymerase chain reaction conditions were 30 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1.5 min, followed by a final 7-min extension step at 72 °C. Agarose gel analysis (1% Sea Kem, FMC Corp. Bioproducts) of the polymerase chain reaction products revealed the presence of a single 1.2-kilobase pair expected DNA fragment. The cDNA was then subcloned into the EcoRI restriction site of the expression vector pCMV with the QuickChange™ site-directed mutagenesis kit (Amersham Pharmacia Biotech).

**Site-directed Mutagenesis—**Point mutations introduced into the human V2 vasopressin receptor cDNA sequence were directly done on the eukaryotic expression vector pCMV with the QuickChange™ site-directed mutagenesis kit (Stratagene). All mutations were verified again by direct dideoxynucleotide sequencing.

**Expression of Receptors and Cell Culture—**The rat V2 receptor cDNA (17) was kindly provided by Dr. J.-M. Elalouf. Wild-type and mutant V2 vasopressin receptors were transiently expressed in COS-7 cells by electroporation. Cells (107/0.3 ml) suspended in electroporation buffer were usually incubated with plasmid DNA (20 µg of carrier DNA (expression vector without any insert) and 200 ng of expression vector containing a cDNA insert) for 10 min at room temperature before being pulsed (280 V, 950 microfarads; GeneZapp, Kodak Scientific Imaging Systems). After electroporation, cells were split into six-well clusters or plated on 150-mm Petri dishes depending upon the experiment to be conducted. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, inc.) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in an atmosphere of 95% air and 5% CO2 at 37 °C. Cells were harvested 72 h after transfection either for cAMP assays or membrane preparations.

**Binding Assays—**Binding experiments were performed on COS-7 cell membrane preparations as described previously (5). The cyclic peptide antagonists were radiiodinated at position 9 on the tyrosylamide as described previously (19, 20) and were used for Scatchard analysis. Membrane preparations (3–12 µg of protein) were incubated for 60 min at 30 °C with increasing concentrations of these radioligands (from 100 to 3000 pM). D-[H]AVP was also used as a radioligand; membrane proteins (20–30 µg) were incubated for 30 min at 30 °C with [H]AVP at a concentration range of 0.5–30 nM for Scatchard analysis or with 1–2 nM [H]AVP (a concentration equaling the Kd value determined in the saturation experiments) and unlabeled ligands at a concentration range of 10–5 to 10–3 M for competition experiments. In each set of experiments, nonspecific binding was determined in the presence of a 2500- to 10000-fold excess of unlabeled analogs. Bound and free radioactivities were separated by filtration over Whatman GF/C filters presoaked with 10 mg/ml bovine serum albumin. The ligand binding data were analyzed by nonlinear least-squares regression using the computer program LIGAND (21). All assays were performed in triplicate on three separate batches of electroporated cells.

**cAMP Assays—**Transfected COS-7 cells were grown on six-well clusters. For the last day of culture (48 h after cell electroporation), [3H]adenine (Amersham International, Buckinghamshire, United Kingdom) was added to the culture medium at a final concentration of 2 µCi/ml. Cells were washed twice in phosphate-buffered saline. To evaluate the adenylate cyclase activity stimulated by AVP or dDAVP, phosphate-buffered saline supplemented with 5.5 mM glucose and 1 mM isobutylmethylxanthine was added to the well with or without each agonist at a concentration range of 10–12 to 10–6 M. To test the antagonist properties of the d(CH)2-[d-Ile(4),Tyr-NH2](AVP compound, the supplemented phosphate-buffered saline with or without the peptide (at a concentration ranging from 10–11 to 10–5 M) was added to the wells 15 min before adding 10–9 M AVP. After a further 10-min incubation period, the reaction was stopped by replacing the incubation medium with 1 ml of 5% trichloroacetic acid. ATP and cAMP were added to the acid extracts at a concentration of 0.5 mM. Relative intracellular cAMP levels were determined by measuring the formation of [3H]cAMP from the prelabeled adenine nucleotide pool. Labeled cAMP was separated by sequential chromatography on Dowex and alumina columns by the method of Salomon et al. (22). Radioactivity present in the cAMP fractions was expressed as percent of the sum of radioactivity recovered in the cAMP fraction and radioactivity that was not retained by the Dowex column. The latter mainly corresponds to labeled ATP. The data were analyzed by means of nonlinear regression using a sigmoidal dose-response equation (KaleidaGraph™ software).

**RESULTS**

**Determination of Antagonist Residues Responsible for Species-selective and High Affinity Binding—**Based on all the preceding vasopressin analog structure-activity studies (see Refs. 14 and 15 for review), the contribution of residues at positions 2 and 4 in the vasopressin cyclic peptide antagonists to high affinity binding and
TABLE I  
Pharmacological properties of rat and human V2 vasopressin receptors: binding to AVP and cyclic peptide antagonists

| Rat V2 | Human V2 |
|--------|----------|
| A. [3H]AVP | | |
| 125I-d(CH2)5-[D-Tyr(Et)2,Val4,Tyr-NH2]AVP | 1.75 ± 0.70 (3) | 1.48 ± 0.08 (3) |
| 125I-d(CH2)5-[D-Tyr(Et)2,Ile4,Tyr-NH2]AVP | 0.37 ± 0.02 (3) | 0.75 ± 0.08 (3) |
| 125I-d(CH2)5-[D-Ile2,Val4,Ile4,Tyr-NH2]AVP | 0.51 ± 0.13 (3) | 0.89 ± 0.21 (3) |
| 125I-d(CH2)5-[D-Ile2,Val4,Tyr-NH2]AVP | 0.32 ± 0.05 (3) | — |
| 125I-d(CH2)5-[D-Ile2,Val4,Tyr-NH2]AVP | 0.66 ± 0.13 (3) | — |

| B. d(CH2)5-[L-Ile2,Val4]-Tyrosylamide | 3.89 ± 0.63 (3) | 128 ± 6 (3) |
| d(CH2)5-[D-Ile2,Val4,Tyr-NH2]AVP | 2.66 ± 0.13 (3) | 55 ± 6 (3) |

*— nonmeasureable.

Fig. 2. Alignment of rat and human V2 vasopressin receptor sequences. Only 44 residues out of 371 are different. These residues are boxed.

selectivity for the rat and human V2 receptors was investigated. Both residues are localized in the cyclic part of the antagonists (Fig. 1). To evaluate the role of only one substitution at a time, four different antagonist compounds were tested: d(CH2)5-[D-Tyr(Et)2,Val4,Tyr-NH2]AVP, d(CH2)5-[D-Ile2,Ile4,Tyr-NH2]AVP, and their hybrid analogs, d(CH2)5-[D-Tyr(Et)2,Ile4,Tyr-NH2]AVP and d(CH2)5-[L-Ile2,Val4,Tyr-NH2]AVP.² The binding properties of these molecules were analyzed in membrane preparations from COS-7 cells transiently expressing rat and human V2 vasopressin receptors. The ligand affinity values were determined either directly in saturation experiments with the radiolabeled compounds or in competition binding assays by displacement of [3H]AVP (1–2 nM). The binding assays were conducted on membrane preparations of COS-7 cells expressing the different wild-type receptors. All values are the means ± S.E. of three different experiments performed in triplicate.

In conclusion, only the residue at position 2 determines the species-selective properties of the antagonists. d(CH2)5-[L-Ile2,Ile4,Tyr-NH2]AVP appears to be the most appropriate tool for searching V2 receptor residues involved in the binding selectivity of this cyclic peptide antagonist family.

Determination of V2 Receptor Residues Involved in the Selective Binding of Cyclic Peptide Antagonists—The peptide sequence comparison of rat and human V2 receptor subtypes shows that only 44 residues out of 371 are different (Fig. 2). To choose among the different residues those potentially involved in the selective binding of the cyclic peptide antagonists, we have taken into account our previous results describing three-dimensional models of vasopressin receptors (5). Thus, only residues localized in the extracellular loops and at the top of transmembrane regions of the human V2 subtype have been considered. Using a site-directed mutagenesis approach, these residues were substituted with their homologs present into the rat V2 subtype in order to measure gain in function (Fig. 3). In the first series of experiments, six receptors were constructed with one or several residues mutated at a time to rapidly target the regions possibly involved in the binding selectivity. For each mutant receptor transiently expressed in COS-7 cells, the affinity for [3H]AVP was determined by Scatchard analysis (Table II); Kd values for AVP were equivalent to those of the wild-type human V2 receptor (Kd = 1.48 ± 0.08 nM). Furthermore, no change in the B max values was observed with respect to the wild-type human V2 receptor (B max = 2–4 pmol/mg of protein), indicating a comparable level of expression of the different mutant receptors.

The affinity of the rat V2 receptor-selective antagonists (d(CH2)5-[L-Ile2,Ile4,Tyr-NH2]AVP and d(CH2)5-[L-Ile2,Val4,Tyr-NH2]AVP) was measured in competition binding assays by displacement of [3H]AVP (Table II). Only mutant human V2

² M. Manning and L. L. Cheng, unpublished data.
receptors R202L, T204A, and G304R, A305P displayed a gain in affinity for these ligands ($K_i = 18.8 \pm 1.8$ and $29.3 \pm 2.6$ nM for the d(CH$_2$)$_5$-[d-Ile$^2$,Ile$^4$,Tyr-NH$_2$$^9$]AVP compound, respectively), whereas the $K_i$ value for the wild-type human V2 receptor was higher ($128 \pm 6$ nM). To precisely determine the residues responsible for selective binding, a second series of single-point mutant receptors (R202L, T204A, G304R, or A305P) was studied (Table II). The restoration of high affinity binding observed for both R202L, T204A, and G304R, A305P mutant receptors was exclusively due to the substitution of residue 202 in the second extracellular loop and of residue 304 in transmembrane domain VII. Each single mutation (R202L or G304R) equally influenced the binding selectivity for the antagonists possessing a d-isoleucyl residue at position 2, whatever the residue present at position 4 (valyl or isoleucyl). Indeed, an equivalent gain in affinity was observed: 7.0–7.4-fold with the d(CH$_2$)$_5$-[d-Ile$^2$,Ile$^4$,Tyr-NH$_2$$^9$]AVP ligand and 5.3–5.9-fold with the d(CH$_2$)$_5$-[d-Ile$^2$,Val$^4$,Tyr-NH$_2$$^9$]AVP analog.

Finally, for a mutant receptor combining both substitutions (R202L and G304R), we observed that the pharmacological properties toward the cyclic peptide antagonists were totally switched to those of the rat V2 receptor. Indeed, as for the wild-type rat V2 receptor, the affinity of the four antagonists could be directly determined by Scatchard analysis using their radioiodinated analogs (Table II). The restoration of high affinity binding observed on the rat V2 receptor ($K_i = 2.16 \pm 0.33$ nM compared with $8.16 \pm 2.12$ nM measured on the double-mutant receptor for dDAVP) was not achieved, indicating that another region of the receptor is probably involved in the peptide agonist selectivity. All of the mutants described in Table II were subsequently tested for their ability to contribute to the selective binding of dDAVP and DAVP. For both compounds, only mutant K100D displayed a gain in affinity equivalent to that observed on mutant R202L or G304R (Table III; data not shown for other mutants). A mutant receptor combining these three substitutions was then analyzed; its affinity for dDAVP was equivalent to that measured for the rat V2 vasopressin receptor ($K_i = 3.5 \pm 0.4$ nM compared with $2.2 \pm 0.3$ nM) (Table III). Surprisingly, the combination of these three mutations did not allow the total restoration of high affinity for DAVP. As observed for cyclic peptide antagonists, these three substitutions act in an additive manner to control species selectivity of dDAVP. In conclusion, two residues determine the bind-

**Fig. 3.** Targeting the residues potentially involved in the receptor selectivity for the cyclic peptide antagonists. The primary sequence and the possible transmembrane topology of the human V2 receptor are shown. Nonconserved residues between rat and human V2 receptors are highlighted in black. Residues selected for the site-directed mutagenesis are labeled according to their peptide sequence number. Human V2 receptor residues were substituted with the residues present at the equivalent positions in the rat V2 receptor.

**Determination of V2 Receptor Residues Involved in the Selective Binding of Cyclic Peptide Agonsists**—We then looked at the possible implication of residues 202 and 304 in the selective binding of cyclic peptide agonists. DAVP and dDAVP, two slightly modified analogs of AVP (Fig. 1), display species selectivity for the rat versus human V2 receptors (Table III). These findings indicate that replacement of l-Arg at position 8 with d-Arg is sufficient to provide selectivity to these molecules. The affinity of these two compounds has been measured on the R202L, G304R mutant V2 receptor (Table III). Our results show that substitution of both residues led to a gain in affinity for each agonist: 7.4- and 4-fold for dDAVP and DAVP, respectively. To evaluate the influence of each substitution on the binding selectivity, both agonists were tested on single-point mutant receptors (R202L and G304R). The calculated affinity values indicated that each single mutation induced an equivalent gain in affinity: 4.6–5.3-fold with dDAVP and 1.4–1.9-fold with DAVP. However, for both peptide agonists, a total restoration of the high affinity observed on the rat V2 receptor ($K_i = 2.16 \pm 0.33$ nM compared with $8.16 \pm 2.12$ nM measured on the double-mutant receptor for dDAVP) was not achieved, indicating that another region of the receptor is probably involved in the peptide agonist selectivity. All of the mutants described in Table II were subsequently tested for their ability to contribute to the selective binding of dDAVP and DAVP. For both compounds, only mutant K100D displayed a gain in affinity equivalent to that observed on mutant R202L or G304R (Table III; data not shown for other mutants). A mutant receptor combining these three substitutions was then analyzed; its affinity for dDAVP was equivalent to that measured for the rat V2 vasopressin receptor ($K_i = 3.5 \pm 0.4$ nM compared with $2.2 \pm 0.3$ nM) (Table III). Surprisingly, the combination of these three mutations did not allow the total restoration of high affinity for DAVP. As observed for cyclic peptide antagonists, these three substitutions act in an additive manner to control species selectivity of dDAVP. In conclusion, two residues determine the bind-
The label of the mutant receptors corresponds to the peptide sequence number of the substituted residues. The affinities of AVP, d(CH$_2$)$_5$-[Lys$_2$,Ile$_4$,Tyr-NH$_2$]AVP, and d(CH$_2$)$_5$-[D-Ile$_2$,Ile$_4$,Tyr-NH$_2$]AVP were obtained in competition binding experiments by displacement of [H]AVP, except for the R202L,G304R mutant, for which the affinities of these two compounds were also measured directly in saturation experiments with the radiiodinated molecules (see values indicated by an asterisk). All values are the means ± S.E. The number of independent determinations is given in parentheses.

### Table II

**Pharmacological properties of wild-type and mutant V2 receptors: binding to peptide agonists and nonpeptide antagonists**

| Mutant receptor | $K_d$ for [H]AVP | $K_i$ for d(CH$_2$)$_5$-[Lys$_2$,Ile$_4$,Tyr-NH$_2$]AVP | $K_i$ for d(CH$_2$)$_5$-[D-Ile$_2$,Ile$_4$,Tyr-NH$_2$]AVP |
|-----------------|------------------|---------------------------------|---------------------------------|
|                 | Val$_4$          | Ile$_4$                          | Val$_4$                          |
| K100D           | 2.74 ± 0.45 (3)  | 0.69 ± 0.09 (3)                  | ND                              |
| R106H           | 3.22 ± 0.48 (3)  | 0.77 ± 0.03 (3)                  | ND                              |
| N182D,E184G,G185N | 2.13 ± 0.15 (3) | 0.88 ± 0.03 (3)                  | ND                              |
| T190F,C185R     | 2.36 ± 0.05 (3)  | 0.85 ± 0.19 (3)                  | ND                              |
| R202L,T204A     | 2.64 ± 0.13 (3)  | 0.51 ± 0.04 (3)                  | ND                              |
| G304R,A305P     | 2.19 ± 0.25 (3)  | 0.74 ± 0.09 (3)                  | ND                              |
| R202L           | 1.67 ± 0.14 (3)  | 0.47 ± 0.02 (3)                  | 0.83 ± 0.03 (3)                |
| T204A           | 2.50 ± 0.40 (3)  | 0.97 ± 0.05 (3)                  | ND                              |
| G304R           | 2.24 ± 0.57 (3)  | 0.60 ± 0.08 (3)                  | 0.93 ± 0.07 (3)                |
| A305P           | 3.90 ± 0.67 (3)  | 0.76 ± 0.05 (3)                  | ND                              |
| R202L,G304R     | 0.85 ± 0.20 (3)  | 0.34 ± 0.03 (3)                  | 0.61 ± 0.10 (3)                |

* ND, not determined.

### Table III

**Pharmacological properties of the human V2 mutant receptors: binding to peptide agonists and nonpeptide antagonists**

| Mutant receptor | [H]AVP | dDAVP | DAVP | OPC-31260 | SR 121463A |
|-----------------|--------|-------|------|-----------|------------|
|                 | $K_i$  |       |      |           |            |
|                 | Val$_4$ | Ile$_4$ | Val$_4$ |            |            |
| Wild-type rat V2 | 1.75 ± 0.70 (3*) | 2.2 ± 0.3 (3) | 5.1 ± 0.8 (3) | 37.9 ± 3.8 (3) | 0.45 ± 0.08 (3) |
| Wild-type human V2 | 1.48 ± 0.08 (3*) | 60.5 ± 1.3 (3) | 51.0 ± 5.0 (3) | 36.3 ± 0.4 (3) | 1.20 ± 0.11 (3) |
| Human R202L,G304R V2 | 0.85 ± 0.20 (3*) | 8.2 ± 2.1 (3) | 17.4 ± 2.3 (3) | 50.0 ± 3.4 (3) | 0.52 ± 0.02 (3) |
| Human R202L V2 | 1.67 ± 0.14 (3*) | 15.3 ± 1.5 (3) | 36.8 ± 0.7 (3) | ND*        | ND          |
| Human G304R V2 | 2.24 ± 0.57 (3*) | 11.4 ± 0.3 (3) | 27.3 ± 0.8 (3) | ND*        | ND          |
| Human K100D V2 | 2.74 ± 0.45 (3*) | 22.8 ± 1.0 (3) | 31.7 ± 1.7 (3) | ND          | ND          |
| Human K100D,R202L,G304R V2 | 0.83 ± 0.16 (3*) | 3.5 ± 0.4 (3) | 15.4 ± 2.2 (3) | ND          | ND          |

* ND, not determined.
that residues 202 and 304 are most likely involved in the ligand binding process and do not affect the coupling efficacy of the V2 vasopressin receptor.

Finally, we compared the coupling efficiency of the triple-mutant receptor with that of the wild-type human V2 receptor when the adenylyl cyclase activity was stimulated with dDAVP (Fig. 4C). The half-maximum response (EC$_{50}$) was reached at a dDAVP concentration of 17.0 ± 9.7 μM for the mutant receptor, whereas a 21 times higher concentration was necessary to elicit an equivalent response with the wild-type receptor (EC$_{50}$ = 365 ± 152 μM). The gain in efficacy (21-fold) for the triple-mutant receptor was on the same order of magnitude as the gain in affinity observed by displacement of [3H]AVP with dDAVP in binding studies (17-fold). In conclusion, the substitution of residues 100, 202, and 304 does not modify the coupling efficacy of the human V2 vasopressin receptor for the $G_s$ protein, indicating that they are most likely involved in the agonist binding process.

DISCUSSION

This study demonstrates that two residues localized in the upper part of the V2 vasopressin receptors (residue 202 in the second extracellular loop and residue 304 at the top of the transmembrane region VII) are responsible for species-selective binding of cyclic peptide antagonist ligands and contribute, in association with a third residue (residue 100 at the top of transmembrane region II), to the species-selective binding of peptide agonists (Fig. 3).

The binding pocket of AVP/oxytocin receptors for their natural endogenous ligands and related agonists has already been identified (5). The combination of three-dimensional molecular modeling and site-directed mutagenesis approaches allowed us to propose and validate a transmembrane localization involving several highly conserved residues in AVP/oxytocin receptors. In the proposed model, only the side chain of AVP residue 8 projects outside the transmembrane core of the receptor and interacts with a residue located in the first extracellular loop (6–8). This receptor residue in the V2 subtype (Asp$^{103}$) is responsible for the subtype-selective binding of agonists dDAVP and DAVP and does not influence the binding of non-selective molecules. Due to the presence of a d-Arg$^8$ residue instead of a natural l-Arg$^8$ residue in the structure of dDAVP or DAVP and to a deaminated residue 1 for dDAVP, the conformation and docking of these ligands in the V2 receptors are likely to be slightly different from those of AVP. However, it seems reasonable to propose that most of the interactions between AVP or dDAVP/DAVP with the V2 receptor agonist-binding site are common. From the results described in this study, we cannot conclude whether residues 100, 202, and 304 interact directly or indirectly with the agonist before its entry in the transmembrane pocket or once it is docked in the receptor. However, it is clear that the presence of Asp$^{100}$, Leu$^{202}$, and Arg$^{304}$ in the rat V2 receptor facilitates the binding of dDAVP and DAVP and that each residue plays an equivalent role. In contrast, the presence of Lys$^{100}$, Arg$^{202}$, and Gly$^{304}$ is unfavorable for their binding in the human V2 receptor. These residues may represent the molecular basis of an exclusion mechanism. Such a process has already been proposed for agonist binding in other protein and peptide receptor families. Indeed, the existence of distinct negative determinants that restrict the ligand-receptor interactions was first reported in receptors for the luteinizing hormone and follicle-stimulating hormone (24). Agonist species selectivity in opioid receptors may be imparted through a mechanism of exclusion rather than specific pharmacophore recognition within the extracellular loops and the N-terminal domain (25). Such a hypothesis has been proposed to explain neurokinin receptor selectivity, which could be mostly influenced by the presence or absence of inhibitory binding domains (26). In conclusion, several upper regions of the human V2 vasopressin receptor play a role in the selective binding of agonist ligands: 1) Asp$^{103}$ in the first extracellular loop (7); and 2) Lys$^{100}$ at the top of transmembrane domain II, Arg$^{202}$ in the second extracellular loop, and Gly$^{304}$ at the top of the transmembrane domain VII. Each of these residues could influence the binding of agonists by interacting directly or indirectly with ligand residues at positions 1 and 8.

The molecular interactions between antagonists such as d(CH$_2$)$_5$-[D-Ile$^2$,Ile$^4$,Tyr-NH$_2$]$^9$AVP and the V2 vasopressin receptor are necessarily different from those of AVP or dDAVP due to their antagonist properties and differences in structure.

Fig. 4. Functional characterization of wild-type, R202L,G304R, and K100D,R202L,G704R human V2 receptors expressed in COS-7 cells: coupling to adenylyl cyclase. A, vasopressin-induced cAMP accumulation mediated by the wild-type (W.T.; ○), double-mutant (●), and triple-mutant (▲) V2 receptors. cAMP formation is expressed as percentage of maximal response to 1 μM AVP. B, effect of the d(CH$_2$)$_5$-[D-Ile$^2$,Ile$^4$,Tyr-NH$_2$]$^9$AVP antagonist on cAMP accumulation. The adenylyl cyclase was stimulated with 1 nM vasopressin. Increasing doses of d(CH$_2$)$_5$-[D-Ile$^2$,Ile$^4$,Tyr-NH$_2$]$^9$AVP (10$^{-11}$ to 10$^{-5}$ M) were added to observe its inhibitory effects on the wild-type and double-mutant receptors. C, dDAVP-induced cAMP accumulation mediated by the wild-type and triple-mutant V2 receptors. cAMP formation is expressed as percentage of maximal response to 1 μM dDAVP. Each curve on A–C is taken from one experiment and is representative of at least three distinct experiments. EC$_{50}$ (concentration of agonist leading to half-maximum stimulation) values for AVP and dDAVP were calculated from three distinct dose-response curves. $K_i$ for the antagonist was deduced from three different determinations of IC$_{50}$ (concentration of antagonist conducting to the half-maximum inhibition).
However, the present results clearly demonstrate that in the V2 receptor, species-selective binding of cyclic peptide ligands containing a D-isoleucyl residue at position 2 is also totally controlled by residues 202 and 304. To date, we do not know how these antagonists bind to the receptor and lead to its inactivation, but several binding hypotheses could be proposed. 1) Like their agonist counterparts, these peptide antagonists could enter the transmembrane-binding region and establish their own network of molecular interactions. This possibility would be consistent with the marked hydrophobic nature of these ligands and of the bottom of the receptor-binding cleft (5). In this case, residues localized in the upper part of the receptor and responsible for receptor species selectivity would act in a primary fashion and play a discriminatory role in ligand binding toward certain antagonists via a possible direct interaction with the ligand or not. 2) The vasopressin peptide antagonists could not be able to enter the hydrophobic transmembrane core of the receptor. In this case, one can consider two possibilities. The high affinity binding of d(CH₂)₅-[D-Ile²,Ile⁴,Tyr-NH₂]³AVP could be due to a direct interaction between ligand residue 2 (D-Ile) with receptor residues 202 and 304. The most favorable situation is achieved when the residue couple Leu²₀² and Arg³₀⁴ is present, as in the rat V₂ receptor. Alternatively, the cyclic peptide antagonists could interact with the upper part of the receptor without any direct interaction with residues 202 and 304. This suggests that these residues may then be involved in stabilizing the receptor in conformations that could prevent or favor the binding of peptide antagonists displaying species differences.

To date, understanding the structural basis of species-selective binding has only been successfully undertaken for nonpeptide antagonists. For instance, construction of chimeric human/canine cholecystokinin-B/gastrin receptors (27) or chimeric rat/human neurokinin-1 receptors (28) has led to the identification of transmembrane residues that are critical for their selective binding process. However, as these compounds are smaller than peptides and usually of highly hydrophobic nature, their binding process could be different from that of peptide antagonist ligands. The present study is the first report in which residues of the extracellular loops and at the top of the transmembrane domains of a peptide hormone receptor are shown to provide the molecular basis for both peptide antagonist- and agonist-binding species selectivity. Moreover, as all the agonists and antagonists used in this study have a closely related structure to vasopressin (see Fig. 1), the present findings constitute a new step in the comprehension of subtle molecular peptide-receptor interactions defining agonism (for transduction of the signal) or antagonism (for inactivation of G protein-coupled receptors).

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