Identification of the Binding Sites of the SR49059 Nonpeptide Antagonist into the V1a Vasopressin Receptor Using Sulfydryl-reactive Ligands and Cysteine Mutants as Chemical Sensors*

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To identify the binding site of the human V1a vasopressin receptor for the selective nonpeptide antagonist SR49059, we have developed a site-directed irreversible labeling strategy that combines mutagenesis of the receptor and use of sulfydryl-reactive ligands. Based on a three-dimensional model of the antagonist docked into the receptor, hypothetical ligand-receptor interactions were investigated by replacing the residues potentially involved in the binding of the antagonist into cysteines and designing analogues of SR49059 derivatized with isothiocyanate or o-chloroacetamide moieties. The F225C, F308C, and K128C mutants of the V1a receptor were expressed in COS-7 or Chinese hamster ovary cells, and their pharmacological properties toward SR49059 and its sulfydryl-reactive analogues were analyzed. We demonstrated that treatment of the F225C mutant with the isothiocyanate-derivative compound led to dose-dependent inhibition of the residual binding of the radio-labeled antagonist [125I]HO-LVA. This inhibition is probably the consequence of a covalent irreversible chemical modification, which is only possible when close contacts and optimal orientations exist between reactive groups created both on the ligand and the receptor. This result validated the three-dimensional model hypothesis. Thus, we propose that residue Phe225, located in transmembrane domain V, directly participates in the binding of the V1α selective nonpeptide antagonist SR49059. This conclusion is in complete agreement with all our previous data on the definition of the agonist/antagonist binding to members of the oxytocin/vasopressin receptor family.

The neurohypophysial antiuretic hormone arginine vasopressin (AVP) is involved in the regulation of body fluid osmolality, blood volume, and blood pressure via the stimulation of specific receptors currently classified into V1a vascular (V1aR) and V2 renal (V2R) receptors. In addition, AVP modulates the adrenocorticotropic hormone secretion through V1b pituitary (V1bR) receptors. These different receptor subtypes along with the oxytocin receptor (OTR), which is classified in the same subfamily, possess distinct pharmacological profiles and intracellular second messengers (1, 2). Moreover, AVP belongs to the family of vasoactive and mitogenic peptides involved in physiological and pathological cell growth and differentiation (3). AVP has been shown to be one of the most powerful in vitro vasoconstrictor substances, and its vasoconstrictor and mitogenic actions may contribute to the pathogenesis of arterial hypertension, heart failure, and atherosclerosis (4, 5). AVP plays a role in the maintenance of blood pressure in several conditions, including upright posture, dehydration, hemorrhage, adrenal insufficiency, cardiac failure, and during surgery (6, 7). An abnormal vascular reactivity specific for AVP has been noted in models of genetic and experimental hypertension, and AVP is instrumental in the genesis and maintenance of several models of experimental hypertension (4, 5, 7). AVP implication in the development or maintenance of hypertension, or both, is based on measurements of plasma and urinary AVP levels and responses to specific AVP antisera or peptide or nonpeptide antagonists (8–10). The first potent and selective V1aR antagonist to be synthesized is a cyclic peptide, d(CH2)5[Tyr(Me)2]AVP (11). In addition to cyclic peptide antagonists, linear peptide antagonists have been developed, such as HO-phenylacetyl-o-Tyr(Me)2-Phe-Gln-Arg-Pro-Arg-NH2 (HO-LVA) (12). However, the lack of oral bioavailability and short half-life of these peptide compounds have limited their use in clinical medicine. During the last decade, nonpeptide AVP antagonists were discovered through random screening of chemical libraries (13, 14). The availability of these orally active compounds now facilitates the assessment to the potential therapeutic applications of AVP receptor blockade in human diseases. One of these compounds, SR49059 (C52S1-(2R, 3S)-5-chloro-3-(2-chloro-phenyl)-1-(3,4-dimethoxy-benzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbolinylpyrrolidine-2-carboxamide), is presently the most potent and selective orally active V1aR antagonist described so far (14). It has a marked affinity, selectivity, and efficacy toward both animal and human V1aR and is devoid of partial agonist activity. In healthy human volunteers, SR49059 inhibits exogenous AVP-induced platelet aggregation and vasoconstriction (15, 16).

Up to now, identification of the binding site of the SR49059 into the V1aR has not been investigated at a molecular level. Several studies based on a combination of receptor three-dimensional modeling and site-directed mutagenesis experi-

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The abbreviations used are: AVP, arginine vasopressin; V1aR, vascular V1α vasopressin receptor; V2R, renal V2 vasopressin receptor; V1bR, pituitary V1b vasopressin receptor; OT, oxytocin; OTR, OT receptor; HO-LVA, HO-phenylacetyl-o-Tyr(Me)2-Phe-Gln-Arg-Pro-Arg-NH2; TM, transmembrane segment; E, extracellular loop; GA, genetic algorithm; GPCR, G protein-coupled receptor.
ment have suggested that an AVP-binding pocket is buried into a 15–20 Å-deep central cavity of the V₁ₐ receptor, defined by the transmembrane helices and surrounded by the extracellular loops (17, 18). Extracellular residues also play a role in the binding of the hormone (19, 20). Binding domains for synthetic peptide antagonists overlap those for peptide agonists into the APV/OT receptors; however, discrimination of agonist versus antagonist ligands is achieved by conserved aromatic residues (Trp₃₀⁴, Phe₃₀⁷, and Phe₃₀⁸) located at the bottom of the binding pocket in the transmembrane helix VI (21–24). Although the binding mode of SR49059 into the V₁ₐR is not yet defined, nonconserved residues Thr₁₃₃ and Ala₁₃₄ located in transmembrane region VII have been shown to control the V₁ₐR/V₁ₐR receptor binding selectivity for SR49059 and for cyclic peptide antagonists as well (21). To precisely investigate the SR49059-V₁ₐR binding interactions at a molecular level, we first constructed a three-dimensional model of the antagonist docked into the receptor based on the x-ray crystal structure of bovine rhodopsin (25). To validate or invalidate the hypotheses of potential ligand-receptor interactions, we developed a site-directed irreversible labeling strategy that combines mutagenesis of the receptor and binding of chemically reactive probes derived from SR49059. This approach generates a chemical bond between the nucleophilic moiety of a cysteine residue incorporated into the receptor and the electrophilic moiety of a sulfydryl-reactive group (isothiocyanate or an α-chloroacetamide) in the SR49059 analogue. Because such a chemical bond formation is only possible when a close contact exists between reactive elements created both on the ligand and on the protein, the covalent link can be taken as a proof of direct interaction versus a long range effect (26). This strategy combines the advantages of three-dimensional modeling and site-directed mutagenesis techniques with those of a direct unambiguous identification of contact regions between a receptor and a specific ligand, like in photoaffinity labeling studies. This is an elegant complement to receptor photolabeling; however, it is much easier to develop than photolabeling, because it does not require the use of a radiolabeled ligand and the overproduction of the target receptor. However, this strategy necessitates introduction of a mutation in the receptor, which could be by itself detrimental to the overall structure of the protein. In this study, we demonstrated that the use of cysteinyl mutants of V₁ₐR allows the chemical characterization of sulfydryl-reactive ligands and an unambiguous localization of the SR49059 nonpeptide-binding site and offers a reliable molecular model of the V₁ₐR receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—SR49059 and its affinity probe derivatives were synthesized according to a SANOFI Recherche Montpellier patent (27). The synthesis will be published in a paper to come.² [³H]AVP (80–90 Ci/mmol⁻¹) was purchased from PerkinElmer Life Sciences. HO-LVA (H9251) was kindly provided by Dr. M. Manning (Toledo, OH) and was radioiodinated in our laboratory as previously described (12).

**Alignment of Amino Acid Sequences**—The amino acid sequences of the three human AVP receptor subtypes and that of the OT receptor were retrieved from the Swiss-Prot data base (accession numbers were as follows: V₁ₐ receptor, P37288; V₁ₐ receptor, P47901; V₇ receptor, P30518; and OTR, P30559) and aligned to the sequence of bovine rhodopsin (accession number P02699) using the ClustalW multiple alignment program (28). A slow pairwise alignment using BLOSUM matrix series (29) and a gap opening penalty of 15.0 were chosen for aligning the amino acid sequences to the sequence of bovine rhodopsin. Because the disulfide bridge occurring between the third transmembrane segment (TM III) and the second extracellular loop (E II) in the structure of bovine rhodopsin is conserved in all AVP/OT receptors, we manually adjusted the alignment of E II to align the corresponding cysteines.

² C. Tahtauvi, M. N. Balestré, P. Klotz, D. Rognan, C. Barberis, B. Mouillac, and M. Hibert, manuscript in preparation.
Site-directed Labeling of the V1a Vasopressin Receptor

**Chemical structure of the ligands.** The structure of the natural hormone AVP is compared with that of the cyclic peptide V1a antagonist d(CH2)5[Tyr(Me)2]AVP, the linear peptide V1aR antagonist 125I-labeled HO-LVA, and the nonpeptide V1aR antagonist SR49059.

![Chemical structure of the ligands](Image)

**RESULTS**

AVP and OT antagonists have been widely used as pharmacological tools to study the physiological and pathophysiological roles of AVP and OT, to determine tissue localization, and to define the precise pharmacological binding profiles of the different receptor subtypes. Currently, much effort is focused on the development of AVP and OT antagonists for a potential therapeutic use. As illustrated in Fig. 1, the different V1aR antagonists fall into three major classes: 1) cyclic peptide antagonists related to the parent AVP structure, like d(CH2)5[Tyr(Me)2]AVP; 2) linear peptide antagonists that retain little resemblance to the natural hormone, such as HO-LVA; and 3) nonpeptide antagonists, like SR49059, derived from screening and subsequent optimization studies. As stated in the introduction, the SR49059 nonpeptide ligand is the most potent and selective orally active V1aR antagonist described so far. Understanding how SR49059 binds to the V1aR should facilitate the rational design of new potent and selective therapeutic agents useful in the treatment of arterial hypertension and congestive heart failure. To investigate the SR49059-V1aR binding interactions at a molecular level, we generated a three-
The sequence of the three human AVP receptors and that of the OT receptor were first aligned to that of bovine rhodopsin in a consistent manner. As seen in Fig. 2, class I GPCR-specific fingerprints (41) are all aligned at key positions of each of the seven transmembrane domains. Because the two cysteine residues forming a disulfide bridge between TM III and E II in the rhodopsin x-ray crystal structure (25) are conserved in the V1aR, we postulated the existence of the same disulfide linkage in the target receptor, although no experimental data yet support this hypothesis. Taking into account the alignment described on Fig. 2 and the x-ray crystal structure of bovine rhodopsin, a three-dimensional model of the V1aR was constructed as detailed under “Experimental Procedures.” The model is suitable for an automated docking of the selective antagonist SR49059 and is compatible with most experimental data derived from studies undertaken to map the V1a antagonist-binding sites (21–24, 42, 43).

As seen in Fig. 3, the dimethoxyphenyl group of SR49059 could be extensively buried among hydrophobic and aromatic amino acids (Phe225, Tyr300, Trp304, Phe307, and Phe308) of transmembrane segments V and VI. Mutation of some of these residues is detrimental to antagonist binding, particularly those of Phe307 and Phe308 (21). The nearby indoline moiety could also develop hydrophobic contacts to TM III, V and VI, but the corresponding subsite is partly hydrophilic. Two glutamine residues (Gln185 and Gln311) known to map the SR49059 binding site (17, 21) are in close proximity to the antagonist. Interestingly, the Lys128 side chain that has previously been shown to play a key role in SR49059 binding to the V1a receptor does not contribute by the polar nitrogen atom but by its apolar carbon atoms (Fig. 3A). Similar interactions have also been evidenced by x-ray diffraction analysis of major histocompatibility complex-peptide complexes (44). The present model also accounts for the selectivity of this compound toward the human V1a receptor. Hence, the two residues (Thr333 and Ala334) located on top of TM VII and previously reported to control V1a/V2 binding selectivity (21) directly interact with SR49059 in the present model.

To validate the three-dimensional model described above, different analogues of SR49059 derivatized with sulfhydryl-reactive moieties were synthesized. These compounds containing an isothiocyanate moiety or an \( \alpha \)-chloroacetamide as chemical
reactive entities as well as their precursors are shown in Fig. 4. All of the chemical substitutions were introduced on the dimethoxyphenyl ring. Assuming a conserved binding mode for close analogues of SR49059, the isothiocyanate moiety of compound 4 (or the $\text{HNO}_2$-chloroacetamide of compound 5) should be located close enough to three residues of the V1a receptor (Phe225, Tyr300, and Phe308) to afford covalent coupling with the respective cysteine mutants (F225C, Y300C, or F308C). These three residues were chosen to maximize chances of covalent linkage for three main reasons: 1) they are close enough to the dimethoxyphenyl moiety of SR49059, 2) their side chains are almost coplanar to the dimethoxyphenyl ring of the reference antagonist, and 3) their cysteine mutants could react with synthetically accessible reactive analogues of SR49059. We thus decided to construct these receptor mutants and to verify this hypothesis. Lys128, which is not supposed to directly interact with the isothiocyanate moiety of compound 4 (or the $\alpha$-chloroacetamide of compound 5), was substituted with a cysteine residue as well as a negative control.

Affinities of the nonpeptide antagonist compounds were first

![Fig. 3. SR49059 docked into the binding cavity of the human V1a receptor.](image)

A, as described under “Experimental Procedures,” the Gold 1.2 docking program (36) was used to automatically dock the selective V1a antagonist SR49059. The best solution proposed by Gold is shown (fitness score of 62.70). The residues are numbered according to their position in the primary sequence. The numbers in parentheses indicate the transmembrane helix to which the current residue belongs. Trp204, Ala205, and Phe207 belong to the extracellular loop 2. Carbon atoms of the SR49059 compound and of the V1a receptor are displayed by cyan and white sticks, respectively. B, schematic view of the interaction model. All of the residues potentially interacting with the different parts of the nonpeptide SR49059 are shown. Numbering of the residues and of the transmembrane helices is equivalent to that used in A.

![Fig. 4. Chemical structure of the analogues derived from SR49059.](image)

Compounds 1–3 are inactive and cannot create a covalent bond with a sulphydryl moiety of a cysteine residue. Compounds 4 (isothiocyanate moiety) and 5 ($\alpha$-chloroacetamide moiety) are sulphydryl reactive. Based on the three-dimensional model of the SR49059 docked into the V1aR, these two analogues could bind covalently to cysteine residues introduced in the receptor-binding pocket, assuming that close contact and optimal orientation can occur.
Table I

Affinities of SR49059 and its analogues for the human V1a receptors

|            | Wild-type V1aR | F225C V1aR | F308C V1aR | K128C V1aR |
|------------|----------------|------------|------------|------------|
| Ki         |                |            |            |            |
| SR49059    | 2.8 ± 0.3      | 11.2 ± 2.8 | 85.5 ± 9   | 232.3 ± 69.5 |
| Compound 1 | 582 ± 79       | ND         | ND         | ND         |
| Compound 2 | 97.6 ± 9.1     | 540 ± 180  | 1450 ± 557 | >5000      |
| Compound 3 | 15.2 ± 1.2     | 98 ± 19    | 411 ± 75   | 925 ± 113  |
| Compound 4 | 6.3 ± 1.7      | 25 ± 8.3\(^*\) | 192 ± 20.5\(^*\) | 876 ± 105\(^*\) |
| Compound 5 | 163.3 ± 22     | 53.4 ± 13\(^*\) | 574 ± 164\(^*\) | 830 ± 176\(^*\) |

\(^*\) Apparent Ki values instead of true Ki values.

Chinese hamster ovary cells or COS-7 cells were electroporated with a pRK5 vector containing a cDNA coding either for wild-type V1a, mutant F225C, mutant F308C, or mutant K128C, and membranes were prepared as described under “Experimental Procedures.” Depending on the mutant, the cells were treated overnight with 5 mM sodium butyrate before harvesting. For wild-type, F225C, and F308C with affinities (apparent or true Ki) for the different compounds were determined in competition binding assays by displacement of \([^{3}H]AVP\) used at 1–2 nM and with 10–20 \(\mu\)g of membrane proteins/assay. The Ki value of \([^{3}H]AVP\) for the V1a receptor expressed in CHO cells has been previously published and is 0.67 ± 0.17 nM (22). For F225C and K128C mutants, because affinity for \([^{3}H]AVP\) cannot be measured, affinities (apparent or true Ki) for the different compounds were determined in competition binding assays by displacement of \([^{125}I]HO-LVA\) used at 100–500 pM and with 1–3 \(\mu\)g (for the wild-type V1a) or 10–20 \(\mu\)g of membrane proteins/assay. Depending on the radioligand, nonspecific binding was determined in the presence of an excess of HO-LVA (400 nM) or AVP (10 nM). The data were analyzed as described under “Experimental Procedures.” All of the values in this table are expressed as the means ± S.E. calculated from three independent determinations. ND, not determined.

Determined for the V1a receptor and compared with those of the parent ligand SR49059. As seen in Table I, all of the chemical substitutions led to decreases in affinity, with the SR49059 being the most potent. Interestingly, the two sulfydryl-modifying compounds 4 and 5 still displayed a relative high affinity (Ki) for the receptors and because affinity of an irreversible covalent binding assay, only apparent affinities are usually determined. These two parameters were equivalent to those measured from the wild-type-bound SR49059 coordinates nor the GOLD docking score (63.52 for wild type versus 60.49 for the F225C mutant).

In the next step, potential irreversible binding of the sulfhydryl-reactive analogues of SR49059 was verified on the different cysteine mutants of the human V1a receptor. To facilitate the covalent chemical modification of the receptor mutants, compound 4 (or compound 5) in combination with the F225C mutant receptor and at a much lesser extent with F308C and K128C mutants, thus representing a particularly favorable situation for applying the site-directed irreversible labeling approach.

In the case of each receptor mutant, affinities for the SR49059 and for its analogues were all diminished (Table 1). For the F225C V1aR mutant, the Ki values for SR49059 and compounds 2–5 are decreased three to six times when compared with that obtained with the wild-type receptor, leading to concentrations still in the 10–100 nM range. The moderate decrease in the binding affinity of SR49059 for the F225C V1a receptor mutant can be easily explained from the current molecular model. Hence, Phe\(^{225}\) is only one of the eight amino acids delimiting a strong hydrophobic pocket interacting with the dimethoxyphenyl moiety of SR49059 (Fig. 3B). The single point mutation does not significantly modify the polarity and the physicochemical property of that subsite. Docking SR49059 into the three-dimensional model of F225C mutant alters neither the binding mode (root mean square deviation of 0.25 Å from the wild-type-bound SR49059 coordinates) nor the GOLD docking score (63.52 for wild type versus 60.49 for the F225C mutant). For F308C V1aR and K128C V1aR mutants, the affinities are much lower, being in the 100–1000 nM range. Like for photoactivatable ligands (22, 23), which can be covalently bound to their receptors upon UV irradiation, the affinity for sulfhydryl-modifying affinity ligands is expected to be high to favor such covalent chemical reactions. Taking into account the previous results, using compound 4 (or compound 5) in combination with the F225C mutant receptor and at a much lesser extent with F308C and K128C mutants, thus represented a particularly favorable situation for applying the site-directed irreversible labeling approach.

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COS cells were transfected with a pRK5 vector containing the cDNA for wild-type V1α (20 μg of carrier DNA and 0.075 μg of specific DNA for 10^7 cells), mutant F225C (20 μg of carrier DNA and 4 μg of specific DNA for 10^7 cells), mutant F308C (20 μg of carrier DNA and 2 μg of specific DNA for 10^7 cells), or mutant K128C (20 μg of carrier DNA and 2 μg of specific DNA for 10^7 cells). The cells were cultured and, depending on the mutant, were treated overnight with 5 mM sodium butyrate before harvesting. The membranes were prepared as described under "Experimental Procedures." After calculating the protein content of the samples, the membranes were treated with vehicle (DMSO) or 10^-5 M of analogue 4 (isothiocyanate moiety) for 30 min in binding buffer (10^-4 M for the wild-type V1α receptor). The membranes were washed, and the protein content was again measured. The different batches were used for radiolabeled binding studies with [125I]HO-LVA (50–800 pM for the mutants; 20–400 pM for the wild-type V1α) or [3H]AVP (0.1–20 nM). Nonspecific binding was determined in the presence of an excess of HO-LVA (400 nM) or AVP (10 μM), respectively. Scatchard representations of the residual binding of [125I]HO-LVA (wild-type V1α mutant F225C, and K128C) or [3H]AVP (mutant F308C) in the saturation studies were analyzed to determine the Kd and Bmax values. The values are expressed as the means ± S.E. calculated from several independent determinations from different batches of electroporated COS cells. The numbers of independent determinations are given in parentheses. NM, not measurable (a residual specific binding could be detected (corresponding to 10–30% of the control condition), but Kd and Bmax values could not be accurately calculated).

The Kd and Bmax values were 0.15 ± 0.02 nM and 0.15 ± 0.016 pmol/mg of membrane proteins, respectively, some residual binding was measured (10–30%), but we were unable to calculate the Kd and Bmax parameters in this condition (Table II). This result suggested an irreversible and almost complete binding of analogue 4. The results obtained with F225C mutant V1α receptor were very encouraging and led us to check whether the effect of the sulfydryl-reactive analogue was dose-dependent. The membranes expressing the F225C mutant were treated with different concentrations of analogue 4 and tested for [125I]HO-LVA residual binding. As illustrated in Fig. 5, a dose-dependent effect of the nonpeptide antagonist was unambiguously measured. At 100 nM, the SR49059 analogue was almost without effect because residual binding of [125I]HO-LVA represented 94.2 ± 2.9% of that of the control condition (membranes treated with vehicle only). At 1 μM, the residual binding was decreased, being 44.7 ± 6.9%, whereas at 10 μM, binding of the radioligand only represented 26.6 ± 3.4%. Concentrations of compound 4 required to irreversibly inactivate more than 50% of receptors are higher than measured Kd affinity values, suggesting that the covalent chemical reaction between the two partners is slower than their association/dissociation. One should also remember that, as explained above, because affinity of an irreversible ligand cannot be determined reliably in a conventional competition binding assay, only an apparent Kd value (25 nM here for the F225C mutant) instead of a true Kd value was measured for this nonpeptide ligand. The apparent affinity determined in this way may actually represent both reversible and irreversible binding. Thus, simultaneous covalent and competitive binding should overestimate the apparent Kd for the mutant receptors. Consequently, the true Kd value for this compound could be much higher, in the 100 nM range or even worse.

The specificity of the irreversible binding reaction using the wild-type V1α receptor and the nonreactive analogue 2 (which is also the precursor of analogue 4) as two negative controls was then investigated. To be able to quantify residual binding (Kd and Bmax values) for [125I]HO-LVA after treatment of the membranes, we used analogue 4 at 1 μM. This concentration has been demonstrated to inhibit approximately 50% of the radiolabeled HO-LVA ligand binding (Fig. 5). The membranes expressing either the wild type or the F225C mutant were treated with vehicle, analogue 2 (1 μM), or analogue 4 (1 μM). Representative Scatchard plots of the residual binding of [125I]HO-LVA are illustrated in Fig. 6. As expected for the wild-type receptor, analogues 2 and 4 were without effect on the binding of [125I]HO-LVA, and Scatchard representations were almost superimposable with that of the vehicle condition (Kd were

**Table II**

| Vehicle (Me2SO) | Analogue 4 (R = NCS) |
|----------------|----------------------|
| Kd (nM) | Bmax (pmol/mg protein) | Kd (nM) | Bmax (pmol/mg protein) |
| Wild-type V1α | 0.13 ± 0.06 (3) | 1.06 ± 0.08 (3) | 0.16 ± 0.06 (3) | 0.96 ± 0.02 (3) |
| F225C V1α | 0.15 ± 0.02 (6) | 0.15 ± 0.02 (6) | NM | NM |
| F308C V1α | 0.86 ± 0.23 (4) | 2.37 ± 0.33 (4) | 1.33 ± 0.31 (4) | 2.53 ± 0.47 (4) |
| K128C V1α R | 0.59 ± 0.1 (2) | 0.33 ± 0.11 (2) | 0.5 ± 0.1 (2) | 0.37 ± 0.15 (2) |

* In the case of mutant K128C, the experiments have been done only two times.

For the K128C V1αR, the experiments have been done only two times. The specificity of the irreversible binding reaction using the wild-type V1α receptor and the nonreactive analogue 2 (which is also the precursor of analogue 4) as two negative controls was then investigated. To be able to quantify residual binding (Kd and Bmax values) for [125I]HO-LVA after treatment of the membranes, we used analogue 4 at 1 μM. This concentration has been demonstrated to inhibit approximately 50% of the radiolabeled HO-LVA ligand binding (Fig. 5). The membranes expressing either the wild type or the F225C mutant were treated with vehicle, analogue 2 (1 μM), or analogue 4 (1 μM). Representative Scatchard plots of the residual binding of [125I]HO-LVA are illustrated in Fig. 6. As expected for the wild-type receptor, analogues 2 and 4 were without effect on the binding of [125I]HO-LVA, and Scatchard representations were almost superimposable with that of the vehicle condition (Kd were
sulfydryl-reactive analogue 4 significantly decreased the number of the membranes expressing the F225C mutant with 0.94 pmol/mg of membrane protein). On the contrary, treatment either the mutant F225C receptor (20 nM analogue 2, and 10 µM analogue 4, respectively; corresponding max values were 1.05, 1.18, and 1.26 M for vehicle, analogue 2, and analogue 4, respectively. Pretreatment of the membranes expressing either the wild-type V1a receptor; specificity of the inhibition effect of the isothiocyanate compound 4 (M) and 0.075 M analogue 2 was without effect on mutant F225C in a dose-dependent manner. Unfortunately, these results could not be reproduced with a high concentration (10 µM) of analogue 5, which carries an α-chloroacetamide as a sulfydryl-reactive group, whereas analogue 4 carries an isothiocyanate moiety (data not shown).

DISCUSSION

Conventional loss-of-function mutagenesis has not always produced definitive answers for the identification of ligand-binding sites of G protein-coupled receptors. Indeed, site-directed mutagenesis experiments must be questioned regarding the observed effect is due to a disruption of a direct interaction of the mutated amino acid with the ligand or to an allosterically induced effect. In the present study, we have used an extension of the substituted cysteine accessibility method, which is based on the irreversible chemical modification of a receptor cysteine mutant with an affinity-selective sulfydryl-reacting ligand. This method provides unambiguous results regarding ligand-receptor interactions after identification of the labeled amino acid residue. The application of this strategy to the definition of the nonpeptide antagonist-binding site of the human V1a receptor allowed us to create an irreversible covalent bond between the isothiocyanate analogue of the SR49059 compound and the F225C mutant receptor. Because such a chemical bond formation is only possible if a close contact (less than 3 Å) exists between reactive elements created both on the ligand and the receptor, our results suggest that residue Phe225, which is located in transmembrane domain V of the receptor, probably directly interacts with the nonpeptide antagonist compound. The three-dimensional model of the SR49059 docked onto the human V1a receptor proposed in this work was thus supported and validated by the results obtained through construction of site-directed cysteine mutants and subsequent irreversible binding with a sulfydryl-reactive analogue of the nonpeptide antagonist. A close structural analogue having an α-chloroacetamide instead of an isothiocyanate moiety as a sulfydryl-reactive group could not irreversibly label the F225C mutant V1a receptor, suggesting that an optimal orientation of the sulfydryl group of the cysteine residue and the electrophilic moiety of the reactive ligand is necessary to afford such a covalent bond. Moreover, the α-chloroacetamide group is much more bulky than an isothiocyanate moiety, a steric parameter that could be crucial as well.

Taking into account the result of affinity-directed irreversible binding, the model is in agreement with previous experimental results. First, we have already demonstrated that the SR49059 could be in contact with residues Thr333 and Ala334 located at the top of transmembrane domain VII (21). Based on a gain-of-function mutagenesis strategy, these two amino acids were shown to control the V1a/V2 receptor subtype binding selectivity of the nonpeptide antagonist. This result and the one described in this study make the three-dimensional model of the SR49059-V1a receptor complex much more reliable. In addition, based on the three-dimensional model, Lys128 (TM III), Gin145 (TM IV), Trp204, Phe207, and Phe208 (TM VI) could also participate in the binding site of the nonpeptide ligand, an hypothesis compatible with previous site-directed mutagenesis results. Indeed, mutation of these residues was detrimental to the antagonist binding (17–19, 21–24). In conclusion, SR49059 seems to bind deeply in the transmembrane region of the human V1a receptor, surrounded by a hydrophobic/aromatic cage. The involvement of Phe207 confirmed the importance of aromatic residues in the binding of all classes of V1a receptor-specific antagonists, both cyclic and linear peptides as well as nonpeptides (21–24).
The binding sites for SR49059 and AVP only partially overlap. This overlap is constituted with polar residues such as Lys$^{225}$ and Gin$^{205}$ (21, 24) and to a certain degree Gin$^{211}$ and Gin$^{111}$ (17). On the contrary, although AVP and agonists interact with extracellular residues of the receptor such as Tyr$^{225}$ in the extracellular loop I, which is responsible for receptor subtype selectivity (19), or residue Arg$^{24}$ in the N-terminal part (20), SR49059 does not. In addition, Phe$^{225}$ does not play any role in the binding of agonists (21), but the homologous residue Ile$^{224}$, adjacent to Phe$^{225}$, controls the rat/human V1a receptor with a sulfydryl-reactive affinity ligand has been applied very effectively (25).

However, incorporating such a cysteine mutant to be labeled by the residue present at the equivalent position in the V1a receptor (F225). Moreover, transmembrane V, in which Phe$^{225}$ is located, seems to play an important role for other nonpeptide $V_1_a$ antagonists. It has been experimentally demonstrated that Ile$^{225}$, adjacent to Phe$^{225}$, controls the rat/human V1a receptor selectivity toward the OPRC1268 nonpeptide antagonist binding (42). Based on molecular modeling hypotheses, transmembrane domain V could also interact with the nonpeptide YM087, a potent $V_2/V_3$ antagonist (43). Finally, it is now commonly accepted that Phe$^{225}$, like other conserved hydrophobic/ aromatic residues in the transmembrane domains of all GPCRs, participates into an intramolecular network of interactions that maintains the receptors as a constrained/inactive conformation (46). SR49059, while interacting with Phe$^{225}$, could reinforce this network of intramolecular interactions, thus behaving as an antagonist.

Cysteine scanning has been widely used to allow the incorporation of chemical probes into membrane receptors (26). However, incorporating such a cysteine mutant to be labeled with a sulfydryl-reactive affinity ligand has been applied very few times, for example, in the case of the $gamma$-aminobutyric acid, type A receptor with noncompetitive channel blockers (47) or in the case of the human $delta$-adrenergic receptor (30). For this GPCR, chloroethylcaine, an alkyllating derivative of the $alpha$-adrenergic agonist clonidine, bound irreversibly to the receptor by forming a covalent bond with the sulfydryl side chain of a cysteine residue exposed in the binding cavity, leading to inactivation of the receptor. Using cysteine mutants as chemical sensors for interacting with sulfydryl-reactive affinity ligands is a powerful strategy to investigate ligand-receptor interactions. This approach is complementary to molecular modeling, site-directed mutagenesis and photoaffinity labeling. Like photoaffinity labeling, this approach results in unambiguous identification of residues directly involved in the binding of the ligands and in the precise definition of the binding pockets of GPCRs. Interestingly, this approach does not require the use of a radiolabeled ligand, the overproduction of the target protein, and its tedious sequencing to locate the labeled residue. However, this strategy necessitates a reliable three-dimensional model of the hypothetical ligand-receptor interactions and the introduction of a mutation in the receptor, which could itself be detrimental to the overall structure of the protein.

AVP and OT receptors represent a logical target for drug development, and the strategy we described in the present paper would be of high interest for defining the binding sites of nonpeptide antagonists and agonists selective to the vasopressin V$_2$ and V$_1_b$ receptors or to the OTR. Such characterization should facilitate the rational design of potent and selective therapeutic molecules useful in many physiopathological conditions.

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