Biological characterization of rodent and human vasopressin V₁b receptors using SSR-149415, a nonpeptide V₁b receptor ligand

Claudine Serradeil-Le Gal,1 Danièle Raufaste,1 Sylvain Derick,1 Jörg Blankenstein,2 John Allen,2 Brigitte Pouzet,1 Marc Pascal,1 Jean Wagnon,3 and Maria Angeles Ventura4,5

1Exploratory Research Department, Sanofi-Aventis Recherche et Développement, Toulouse Cedex; 2Isotope Chemistry and Metabolite Synthesis, Sanofi-Aventis Recherche et Développement, Chilly Mazarin Cedex; 3Oncology Department, Sanofi-Aventis Recherche et Développement, Montpellier Cedex; 4Institut Cochin, Département d’Endocrinologie Metabolisme et Cancer, Université Paris Descartes, CNRS (UMR 8104), Paris; and 5Institut National de la Santé et de la Recherche Médicale, Unité 567, Paris, France

Submitted 26 January 2007; accepted in final form 17 May 2007

Serradeil-Le Gal C, Raufaste D, Derick S, Blankenstein J, Allen J, Pouzet B, Pascal M, Wagnon J, Ventura MA. Biological characterization of rodent and human vasopressin V₁b receptors using SSR-149415, a nonpeptide V₁b receptor ligand. Am J Physiol Regul Integr Comp Physiol 293: R938–R949, 2007. First published May 23, 2007; doi:10.1152/ajpregu.00062.2007.—[3H]SSR-149415 is the first tritiated nonpeptide vasopressin V₁b receptor (V₁bR) antagonist ligand. It was used for studying rodent (mouse, rat, hamster) and human V₁bR from native or recombinant origin. Moreover, a close comparison between the human and the mouse V₁bR was performed using SSR-149415/[3H]SSR-149415 in binding and functional studies in vitro. [3H]SSR-149415 binding was time-dependent, reversible, and saturable. Scatchard plot analysis gave a single class of high-affinity binding sites with apparent equilibrium dissociation constant (Kₐ) ~1 nM and maximum binding density (Bₘₐₓ) values from 7,000 to 300,000 sites/cell according to the cell line. In competition experiments, [3H]SSR-149415 binding was stereospecific and dose-dependently displaced by reference peptide and nonpeptide arginine vasopressin (AVP)/OT ligands following a V₁b rank order of affinity: SSR-149415 = AVP > dCha > dPen > dPal > dDavp > SSR-126768A > SR-49059 > SSR-149424 > OT > SR-121463B. Species differences between human, rat, mouse, and hamster V₁bR were observed. Autoradiography studies with [3H]SSR-149415 on rat and human pituitary showed intense specific labeling confined to corticotroph cells and absence of labeling in the other tissues examined. SSR-149415 potently and stereospecifically antagonized the AVP-induced inositol phosphate production and intracellular Ca²⁺ increase (EC₅₀ from 1.83 to 3.05 nM) in recombiant cell lines expressing either the mouse or the human V₁bR. AVP (10⁻⁷ M) exposure of ATR20 cells expressing mouse or human EGFP-tagged V₁bR induced their rapid internalization. Preincubation with 10⁻⁶ M SSR-149415 counteracted the internalization process. Moreover, recycling of internalized receptors was observed upon 10⁻⁶ M SSR-149415 treatment. Thus SSR-149415/[3H]SSR-149415 are unique tools for studying animal and human V₁bR.

vasopressin; V₁b receptor; rat; hamster; mouse; human
in rodents and exerts marked antidepressant-like activity in several predictive animal models (39, 16). This molecule has been now titrated, and the present study provides a detailed biochemical characterization of \(^{[3]H}\)SSR-149415, the first nonpeptide antagonist radioligand available for labeling V\(_{1b}\)R. It was used for characterizing rodent (mouse, rat, and hamster) and human V\(_{1b}\)R, of native or recombinant origin, in binding and autoradiography studies. Moreover, using SSR-149415/\(^{[3]H}\)SSR-149415, it was possible, for the first time, to extensively characterize the mouse V\(_{1b}\)R stably expressed in murine AT20 pituitary cells in comparison with the human one in terms of binding properties, inositol phosphate (IP) production, Ca\(^{2+}\) mobilization, and receptor internalization.

**METHODS**

**Chemicals.** The nonpeptide molecules SR-49059 (38), SR-121463 (34), SSR-149415 (39), a diastereomeric isomer of SSR-149415, SR-149424 (12S, 4R)-1-(35)-5-chloro-1-[2,4-dimethoxyphenylsulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl)-4-hydroxy-N,N-dimethyl-2-pyrrolidine carboxamide, and SSR-126768A (37) were synthesized at sanofi-aventis recherche & développement. All of them were initially dissolved in DMSO at a concentration of 10 mM and then diluted in the appropriate test solvent. AVP, oxytocin, desaminoo-\(\alpha\)-Arg\(\beta\)-vasopressin (dDAVP), [deaminocys,5,3-(Pyridyl)-Ala\(^{2}\)-Arg\(^{1}\)]vasopressin (dPal), [deaminopentamidine-O-Me-Tyr, Arg\(\beta\)vasopressin (dPen), arginine, phenylalanine, somatostatin, forskolin, and bacitracin were obtained from Sigma Aldrich (L’Ile d’Abeau Chesnes, France). Fura 2-AM and Pluronic F-127 were from Molecular Probes (Ozyme, Saint Quentin en Yvelines, France). The expression vector pcDNA3 was from Invitrogen (SSR- Pontoise, France), and p-EGFP-N1 was from Clontech (Ozyme, Saint Quentin en Yvelines, France). Enzymes for molecular cloning were from New England Biolabs (Ozyme, Saint Quentin en Yvelines, France). Oligonucleotides were synthesized by Invitrogen. All cell culture reagents were obtained from Boehringer Mannheim (Meylan, France) except for DMEM-F-12 from Sigma Aldrich. Nu-serum from BD Biosciences (Ozyme, Saint Quentin en Yvelines, France), and Fetalco2 (Fraction IV, no. 41150-7, from fetal bovine serum, obtained from Biochrom, Munich, Germany) were from Boehringer Mannheim (Meylan, France). Fetalco2 was used instead of fetal bovine serum because the latter contains retinoids that might affect cell growth.

**Cell culture.** In-R1-G9 cells were cultured in RPMI-1640 medium with 10% FBS, 0.03% amphotericin, 0.2% penicillin/streptomycin, and 2 mM L-glutamine. They were grown at 37°C in a humidified atmosphere of CO\(_2\). All experiments were performed using cells from passages 22 to 36. Transfected AT20 cells were cultured in DMEM-F-12 supplemented with 7.5% Fetalco2, 7.5% Nu-serum, 0.5 mM glutamine, and 0.2 mg/ml G418 at 37°C in a 5% CO\(_2\) atmosphere. Transfected Ltk- and CHO cells were grown in 10 mM HEPES, pH 7.4, and minimal essential medium supplemented with 5% FCS serum, 8 g/l sodium bicarbonate, and 0.3 mg/ml G418 at 37°C in a humidified atmosphere containing 5% CO\(_2\). Wild-type cells were routinely grown in a similar culture medium without the selective agent G418. The culture medium was removed every other day, and cells were subcultured by treatment with 0.05% trypsin and 0.02% EDTA.

**Membrane preparation.** Membranes from In-R1-G9 and CHO cells expressing the human or the rat V\(_{1b}\)R, AT20 cells expressing the murine or the human V\(_{1b}\)R, Ltk- cells expressing the human oxytocin receptor, and CHO cells expressing human V\(_{1a}\) and V\(_{2}\) receptors were prepared as described by Serradeil-Le Gal et al. (34). Briefly, cells were harvested, washed two times in PBS without Ca\(^{2+}\) and Mg\(^{2+}\), polyanion-homogenized in lysis buffer (15 mM Tris-HCl, pH 7.5, 2 mM MgCl\(_2\), and 0.3 mM EDTA), and centrifuged at 100 g for 5 min at 4°C. Pellets were washed in 50 mM Tris-HCl, pH 7.4, and 5 mM MgCl\(_2\) (buffer A) and centrifuged at 44,000 g for 20 min at 4°C.

**Membranes were suspended in a small volume of buffer A, and protein contents were determined by the method of Bradford using BSA as a standard (5). Aliquots of membranes were used immediately or stored at −80°C.**

**Binding studies.** Binding experiments of \(^{[3]H}\)SSR-149415 or \(^{[3]H}\)AVP to cellular membranes were conducted according to Serradeil-Le Gal et al. (39). V\(_{1b}\)R binding studies were carried out in an incubation medium containing 50 mM Tris-HCl (pH 7.4), 3 mM MgSO\(_4\), 0.1% BSA, 0.1% bacitracin, \(^{[3]H}\)AVP, or \(^{[3]}H\)SSR-149415 (0.02–30 nM for saturation experiments or 2–3 nM for competition studies) and increasing amounts of the tested compound (SSR-149415 and reference peptides). The reaction was started by the addition of

AJP-Regul Integr Comp Physiol • VOL. 293 • AUGUST 2007 • www.ajpregu.org

**Biological materials.** The hamster glucagonoma In-R1-G9 cells were kindly provided by Dr. Kimberly A. Matthews (Veterans Affairs Medical Center). Mouse corticotroph At20/D16V-F2 cells (CRL-1795) were obtained from ATCC (Manassas, VA). At20 wild-type cells were transfected with 5 µg/petri dish of the plasmids pcDNA-hV3, pcDNA-mV\(_{1b}\), phV\(_{1b}\)-EGFP, or pmV\(_{1b}\)-EGFP using the Lipofectamine Plus Reagent according to the instructions of the manufacturer. Cells stably expressing wild-type receptors were selected with G418 (0.6 mg/ml). The clones were screened by \(^{[3]H}\)AVP binding and purified by the limited dilution technique. Cells stably expressing pmV\(_{1b}\)-EGFP and phV\(_{1b}\)-EGFP were selected with G418 (0.6 mg/ml), the clones were purified by fluorescence-activated cell sorting, and screened by \(^{[3]H}\)AVP binding. Ltk- cells were transfected with the cDNA coding for the human OT receptor. CHO-DHFR-cells (DXB11; CRL-9096; ATCC) were transfected with an expression vector derived from plasmid 7055 containing the cDNA encoding the human V\(_{2}\), V\(_{1a}\), or V\(_{1b}\). Stably transformed cell lines were isolated as described earlier (24, 46, 34).

Pituitary tissues, without microscopic abnormalities or tumors, and neonatal biopsies obtained from pregnant women with normal uncomplicated pregnancies who were delivered by elective caesarean section before the onset of labor (38–40 wk of pregnancy) were immediately frozen after excision for autoradiography studies. Human tissue samples were collected in conformity with French national ethical rules. This project was approved by human subject review committees and was carried out in collaboration with the Pathological Anatomy Department of the University Hospitals of Strasbourg and Dijon. The protocols for the animal studies were approved by the animal care and use committee of sanofi-aventis research and development.
membranes (~5 rat CHO;1bR, mouse or human AtT20), 35 (human CHO;1bR), and 250 (In-R1-G9) µg/assay and incubation at 25°C for 1 h. The reaction was stopped by adding 4 ml of ice-cold buffer followed by filtration through GF/B Whatman glass microfiber filters presoaked in ice-cold buffer. Filters were washed two times with 4 ml of ice-cold buffer and counted for radioactivity by liquid scintillation in a Beta Packard 1900 TR. Nonspecific binding was determined in the presence of 1 µM unlabeled AVP or SSR-149415.

Binding data analysis. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of binding of the specific binding. Inhibition constant (K_i) values were calculated from the IC_{50} values using the Cheng and Prusoff (7) equation. Data for equilibrium binding [apparent equilibrium dissociation constant (K_d)], maximum binding density (B_max), competition experiments [IC_{50}, Hill coefficient (n_H)] and kinetic constants [observed association rate constant (K_{obs}) and apparent dissociation rate constant (K_{-1})] were analyzed using an iterative nonlinear regression program (34).

 Autoradiography. Serial sections (16 µm thick) from frozen whole rat pituitaries or human pituitary samples were mounted on gelatin chrome-alum slides, rinsed to eliminate endogenous AVP, and incubated with 5 nM [3H]SSR-149415 with (nonspecific binding) or without (total binding) 1 µM unlabeled SSR-149415. After 60 min incubation at room temperature, the sections were washed three times for 10 min each in ice-cold binding buffer, dipped briefly in distilled water, and dried under a stream of cold air. Rinsed labeled sections were placed on a phosphor-imaging plate (Fuji) for 1 to 2 wk and further analyzed with a Bio-Image Analyzer (BAS 2000; Fuji) as previously described (36). Similar experiments were performed on human pregnant uterus sections using [3H]OT (2 nM), [3H]SSR-149415 (2 nM), or [3H]OVTA (0.05 nM) as ligands and 10 µM unlabeled OT for determination of nonspecific binding as previously described (37).

IP assays. IP production was determined as previously described (10). Briefly, AtT20 cells stably transfected with the mouse or human V_{1b}R were plated at 100,000 cells/well. Cells were grown for 24 h in their respective culture medium (see above) and further incubated for another 24 h in a serum- and mitosol-free medium supplemented with 1 µCi/ml-1 myo-[2-3H]inositol. Cells were then washed two times with Hanks’ buffer saline medium, incubated for 15 min in this medium supplemented with 20 mM LiCl, and further stimulated for 15 min with increasing concentrations of analogs to be tested. The reaction was stopped by adding perchloric acid (5% vol/vol). Total accumulated IP was extracted, purified on Dowex AGI-X8 anion exchange chromatography columns as previously described (9), and counted. Data were analyzed using the RS1 software (BBN Domain, Cambridge, MA). Values are expressed as means ± SD of at least three independent experiments.

Intracellular Ca^{2+} concentration measurements. Subconfluent CHO and AtT20 cells, cultured in 175-cm² flasks as described above, were collected by trypsinization (0.05% trypsin, 0.02% EDTA) and centrifuged (230 g, 5 min). At 80% confluency, cells were transfected with 4 µg aequorin plasmid expression vector using the Lipofectamine 2000 reagent (18); 24 h after transfection, medium was removed, and cells were detached using PBS/5 mM EDTA and incubated for 3 h at a concentration of 2×10^6 cells/ml in DMEM-F-12 medium without phenol red (Invitrogen) supplemented with 0.1% FCS and 5 µM coelenterazine (Molecular Probes). Cells were diluted 10-fold in the same medium and incubated for an additional 3-h period. Loaded cell suspension (100 µl) was distributed in 96-well microplates, and then cells were incubated with increasing concentrations of agonists alone or with the nonpeptide antagonists SSR-149415 and SSR-149424 15 min before adding 10 nM AVP. Light emission was recorded for 60 s using a Luminoskan microplate luminsometer (Labsystems), and the data were expressed in relative light units. After the 60-s measurement period, cells were lysed using 100 µl PBS/2.5% Triton X-100–50 mM CaCl₂, and luminescence induced corresponding to the nonmobilized cell Ca^{2+} was recorded. Each assay was carried out in triplicate. Luminescence data (peak integration) were calculated and expressed as percent of the maximal luminescence recorded (i.e., sum of sample and lysis luminescence recordings). Data were analyzed using the RS1 software (BBN Domain). Values are expressed as means ± SD of at least three independent experiments.

Time-lapse confocal videomicroscopy. AtT20 cells stably expressing the EGFP-tagged human or mouse V_{1b}R were plated in Lab-Tek chambered coverglasses (Nunc, Dominique Dutcher, Brumath, France). Later (2 days), culture medium was replaced by DMEM-F-12 devoid of phenol red and supplemented with 0.2% dialyzed BSA and 25 mM HEPES, pH 7.4. The cells were placed on the stage of a Leica TCS SP2 AOBS confocal microscope laid in a thermostated chamber at 37°C. Images were acquired every 30 s for up to 45 min in recycling experiments. Between the second and the third image, drugs were added to the cells on the stage of the microscope, the time was set to zero, and image collection continued. When required, a second treatment was added at the times indicated in the legend to Fig. 6. AVP (0.1 µM) and SSR-149415 (1 µM) were used. Each experiment was carried out at least two times, yielding similar results.

RESULTS

Tritiation of SSR-149415. For the incorporation of the tritium label, the 2,4-dimethoxyphenol positions of SSR-149415 were chosen (Fig. 1). Therefore, the dihydroxyl precursor of SSR-149415 was methylated using high-specific-activity...
methyl iodide under standard methylation conditions (5 eq of K₂CO₃, 2.2 eq of a 1:1 mixture of [³H]methyl iodide and methyl iodide) at 60°C in DMF to obtain [³H]SSR-149415 (Fig. 1). The specific activity was calculated to be 81 Ci/mmol, and, after purification by high-pressure liquid chromatography, a radiochemical purity of 99.0% was obtained.

Characterization of [³H]SSR-149415 Binding to Animal and Human AVP V₁bR

Kinetics experiments. [³H]SSR-149415 binding was characterized in the various cell lines expressing either recombinant (CHO, AtT20) or constitutive (In-R1-G9) V₁bR from rodent (mouse, rat, and hamster) and human origin. Using the different cell membranes, we showed that [³H]SSR-149415 specific binding increased linearly as a function of the membrane protein concentration and then reached (or tended to reach) saturation as shown in Fig. 2 on membranes of CHO or AtT20 cells expressing either the human or the mouse V₁bR, respectively. For each membrane preparation, a protein concentration in the linear portion of the curve was chosen for subsequent binding studies.

As shown in Fig. 2, kinetics experiments showed that the specific binding of [³H]SSR-149415 to membranes from CHO or AtT20 cells expressing the human or the mouse V₁bR, respectively, was time dependent and reached equilibrium in ~60 min at 25°C. Close Kᵦ values (0.051 ± 0.016 and 0.024 ± 0.019 min⁻¹, respectively) and a time of half-maximal association ranging from 15 (CHO cells) to 30 (AtT20 cells) min were calculated from these time course binding studies. Steady-state stability was maintained at least during the 3-h observation period. [³H]SSR-149415 binding to these membranes was totally reversed by addition of 10⁻⁵ M unlabeled SSR-149415 and occurred rapidly, yielding Kᵦ values of 0.031 and 0.040 min⁻¹ for human and mice V₁bR, respectively (Fig. 2). From these kinetics experiments, calculated Kᵦ values ~2 nM were obtained. Of note, similar association/dissociation profiles were obtained using mouse or human recombinant V₁bR in CHO or AtT20 cells, respectively, and the native hamster In-R1-G9 V₁bR. After equilibrium and under standard conditions, nonspecific binding represented 5–15% of total binding, according to the preparation studied.

Saturation binding studies. Specific [³H]SSR-149415 binding to the various V₁bR-expressing membranes was saturable as observed by increasing radioligand concentrations from 0.2

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Time course of association (●) and dissociation (○) of [³H]SSR-149415 to membranes of CHO cells expressing the human V₁b receptor (V₁bR; A) and AtT20 cells expressing the mouse V₁bR (B). Incubations were carried out as described in METHODS in the presence of 2 nM [³H]SSR-149415 for various periods of time. The arrow indicates time (45 min) at which unlabeled SSR-149415 (10⁻⁵ M) was added to initiate dissociation. Inset: specific binding as a function of the protein concentration in CHO cells expressing the human V₁bR (A) and AtT20 cells expressing the mouse V₁bR (B). Results represent data from a typical experiment performed in duplicate and repeated three times without noticeable changes.

![Graph](https://via.placeholder.com/150)

**Fig. 3.** A: saturation of [³H]SSR-149415 specific binding to the human and mouse V₁bR saturation isotherms. B: Scatchard plots. Membranes from either CHO cells transfected with the human V₁bR (30 µg/ml; ○) or AtT20 cells with the mouse V₁bR (5 µg/ml; ●) were incubated for 1 h at 25°C in the presence of increasing concentrations of [³H]SSR-149415 as described in METHODS. Data are means calculated from a typical experiment performed in duplicate and repeated three times without noticeable modifications.
to 18 nM (Fig. 3A). Scatchard plot analysis obtained from saturation isotherms gave in each case linear plots consistent with the presence of a single class of high-affinity binding sites as shown in Fig. 3B using both the human and the mouse V1bR (Kd values of 1.03 ± 0.10 and 1.28 ± 0.29 nM, respectively). Whatever the species studied, [3H]SSR-149415 demonstrated constant nanomolar affinity for the V1bR (Table 1). Of note, similar binding characteristics were obtained for the human V1bR either expressed in CHO or AtT20 cells. Variable Bmax were measured ranging from 45 to 12,060 fmol/mg protein (i.e., from 7,000 to 300,000 sites/cell) according to the transfection efficiency in CHO and AtT20 cells or the natural expression of V1bR in the In-R1-G9 cell line. Comparative saturation binding experiments, performed under similar experimental conditions, using the tritiated natural hormone [3H]AVP also gave a linear Scatchard plot and nanomolar Kd values, but Bmax values were slightly lower (~10–20%) in general than those obtained with [3H]SSR-149415 (Table 1).

**Table 1. Equilibrium binding parameters (Kd and Bmax) of [3H]SSR-149415 compared with [3H]AVP for animal and human V1bR in various cell lines**

<table>
<thead>
<tr>
<th></th>
<th>[3H]SSR-149415</th>
<th>[3H]AVP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd, nM</td>
<td>Bmax, fmol/mg Protein (sites/cell)</td>
</tr>
<tr>
<td>Human (CHO)</td>
<td>1.03 ± 0.10</td>
<td>2,214 ± 79 (85,000)</td>
</tr>
<tr>
<td>Human (AtT20)</td>
<td>1.46 ± 0.44</td>
<td>3,632 ± 147 (109,000)</td>
</tr>
<tr>
<td>Rat (CHO)</td>
<td>1.45 ± 0.31</td>
<td>12,060 ± 460 (300,000)</td>
</tr>
<tr>
<td>Hamster (In-R1-G9)</td>
<td>1.57 ± 0.17</td>
<td>45 ± 5 (7,000)</td>
</tr>
<tr>
<td>Mouse (AT20)</td>
<td>1.28 ± 0.29</td>
<td>10,768 ± 432 (314,700)</td>
</tr>
</tbody>
</table>

Values are means ± SD. Data for equilibrium binding [apparent equilibrium dissociation constant (Kd) and maximum binding density (Bmax)] were measured as described in METHODS and analyzed using an iterative nonlinear regression program (34). V1bR, vasopressin type 1b (V1b) receptor; AVP, arginine vasopressin.

**Competition binding studies.** The relative affinities of several reference peptide and nonpeptide AVP/OT ligands were studied to characterize the binding of [3H]SSR-149415 to the different V1bR-expressing preparations. As shown in Fig. 4, A and C, [3H]SSR-149145 binding to the human or the mouse V1bR was totally inhibited in a dose-dependent manner by nonpeptide ligands and unlabeled SSR-149415, giving Ki values for this latter molecule of 1.6 ± 0.2 and 1.6 ± 0.5 nM for human and mouse, respectively, consistent with Kd values obtained from Scatchard plots and kinetics experiments. Of note, SSR-149415 demonstrated stereospecificity for the V1bR since its diastereoisomer (SSR-149424) displayed micromolar affinities for these receptors. Another set of competition studies was performed using peptide ligands (Fig. 4, B and D). AVP and the selective V1b agonist ligand recently described, d[Cha4]AVP, showed nano-

![Fig. 4. Competition binding experiments of [3H]SSR-149415 to the human (A and B) and mouse (C and D) V1bR using reference nonpeptide and peptide arginine vasopressin (AVP)/OT compounds. A and C: nonpeptide compounds: SSR-149415 (○), SSR-149424 (■), SR-49059 (○), SSR-121463 (■). B and D: peptide compounds: AVP (○), d[Cha4]AVP (●), dPen (■), dPal (○). Membranes from CHO cells transfected with the human V1bR (30 μg/ml) were incubated for 1 h at 25°C in the presence of 2 nM [3H]SSR-149415 and increasing concentrations of the compound to be tested as described in METHODS. Data are means calculated from a typical experiment performed in duplicate and repeated 3 times without noticeable modifications.](http://ajpregu.physiology.org/)
molar affinities for these sites; other reference V1b agonists such as dPal (V1b) and dDAVP (V1a, V2) or antagonists such as dPen (V1a, V1b) exhibited much lower affinities for the V1bR than the native hormone, OT, another AVP-related pituitary nonapeptide, displayed ~300-fold weaker affinity than AVP at these sites. In each case, we obtained dose-displacement curves yielding nH about one in agreement with a competitive inhibition. Moreover, in saturation experiments with [3H]SSR-149415 and AVP (data not shown) or [3H]AVP and SSR-149415 (39), we obtained a competitive profile (modification totally displaced, even by high peptide concentrations, indicating a Ki without modifying the Bmax); this information authorizes calculating a Ki from the IC50 value using the equation of Cheng and Prusoff (Ref. 7 and Table 2).

It is worth noting that [3H]SSR-149415 binding was not totally displaced, even by high peptide concentrations, indicating a proportion of ~10–15% sites insensitive to AVP or peptide ligands (Fig. 4, B and D) as already reported with other nonpeptide V1a or V2 ligands such as [3H]SSR-49059 or [3H]SSR-121463, respectively (36). All the AVP/OT ligand affinities measured in the different preparations from mouse, rat, hamster, and human origin are reported in Table 2 and confirm in every case a rank order of affinity compatible with the V1bR profile. These data also highlight species differences existing for both peptide (dPal, dDAVP, dPen) and nonpeptide (SSR-126768A, SR-49059) compounds that are very common in the AVP/OT field.

**Specificity of [3H]SSR-149415**

The selectivity of SSR-149415 for the V1bR was previously demonstrated in a wide number of assays for receptors, enzymes, and ion channels (n = 100) in which lack of interaction was observed up to 10 μM (39). In the present study, using various native tissues such as rat liver or kidney constitutively expressing the V1a and the V2R, respectively, and CHO cells transfected or not with the human V1a, V2, or OTRs, we were unable to detect significant [3H]SSR-149415 specific binding under standard binding conditions using increasing membrane and [3H]SSR-149415 concentrations (data not shown). These data confirmed the higher affinity of SSR-149415 for the human/rat V1bR than for the V1a, V2, and OTR previously described in classical binding studies (39). Moreover, in our hands, Ki values of 120, 2,500, and 230 nM were found at mice V1a, V2, and OTR receptors, respectively, using [3H]AVP (V1a and V2R assays) or [125I]OVT (OTR assays) as radioligands, confirming the V1bR selectivity also in the mouse (data not shown).

**Autoradiographic studies with [3H]SSR-149415**

The V1bR is mainly localized in anterior pituitary corticotroph cells where AVP stimulates ACTH secretion and potentiates the action of corticotropin-releasing factor (CRF) on ACTH liberation (14, 30). Using [3H]SSR-149415, we examined the localization of V1bR both in rat and human pituitary sections (Fig. 5). Intense specific labeling totally displaced by 10 μM SSR-149415 (or 10 μM AVP; data not shown) was observed in human sections obtained from three different pituitary samples (Fig. 5A). An irregular patchy signal of high density was observed in corticotrophs. In the rat pituitary, a fainter specific signal was observed in the anterior pituitary, whereas a high level of nonspecific binding was observed in the central neural lobe, and no specific labeling was detected in the intermediate lobe area as previously described with [3H]AVP (45). Using [3H]AVP as a reference ligand, we obtained a similar labeling signal confined to corticotrophs on these preparations (data not shown).

In autoradiographic studies performed on reference human tissues expressing V1a (liver), V2 (kidney) (data not shown) or OTR (pregnant uterus), no specific labeling was observed with [3H]SSR-149415, in particular in the pregnant uterus, which expresses significant amounts of high-affinity OTR sites distinctly labeled by the native triated hormone [3H]OT (Fig. 6). Of note, these data do not support the SSR-149415 interaction (effect similar to that of OT itself) at human recombinant OTR recently reported by Griffante et al. (17). Additional experiments with [3H]SSR-149415 to address V1bR localization in rat brain sections failed to show clear specific labeling in CNS structures (data not shown). The limited specific activity of the tritium label associated with low levels of V1bR is probably the main difficulty that will have to be overcome to map extrapituitary V1bR, especially in the brain. An absence of specific binding was also observed in rat brain membranes using [3H]SSR-149415 as a ligand.

**Effect of SSR-149415 on AVP-induced IP production in mouse and human V1bR recombinant cell lines.** Activation of the V1bR by AVP has been associated with phospholipase C stimulation, producing diacylglycerol and inositol triphosphate, which in turn increase intracellular Ca2+ concentration ([Ca2+]i; see Ref. 44).

In recombinant AtT20 cells expressing either the mouse or human V1bR, AVP induced dose-dependent IP accumulation with nanomolar EC50 values (1.83 ± 0.68 and 1.87 ± 0.86 nM, respectively), whereas OT was much less potent (EC50 values of 510 ± 293 and 144 ± 114 nM, respectively; Table 3). As shown in Fig. 7A, SSR-149415 dose-dependently and totally antagonized 10 nM AVP-induced IP production with IC50 values of 12.3 ± 8.4 and 11.00 ± 9.8 nM for the mouse and human V1bR, respectively. Of note, this effect was stereose-
Effect of SSR-149415 on AVP-induced intracellular Ca\(^{2+}\) mobilization in mouse and human V\(_{1b}\)R recombinant cell lines. AVP exposure of the mouse and human recombinant V\(_{1b}\)R cell lines induced a dose-dependent increase in \([\text{Ca}^{2+}]_{i}\) by activating V\(_{1b}\)R with EC\(_{50}\) values ranging from 2.13 ± 0.90 to 3.05 ± 1.32 nM. Under similar operating conditions, the AVP-related hormone OT exhibited an agonist profile, although it was much less potent than AVP (at least >54-fold; Table 4). SSR-149415 competed as a potent antagonist in inhibiting AVP-evoked intracellular \([\text{Ca}^{2+}]_{i}\) elevation in a dose-dependent manner, yielding nanomolar IC\(_{50}\) values for the mouse and human recombinant V\(_{1b}\)R (Fig. 7B). This effect was stereospecific, since SSR-149415’s diasteroisomer, SSR-149424, was much less efficient (about 3 orders of magnitude) and gave IC\(_{50}\) in the micromolar range (Table 4).

Effect of SSR-149415 on AVP-induced V\(_{1b}\)R internalization in AtT20 cells. The functional effect of SSR-149415 on AVP-induced internalization was investigated by time-lapse confocal videomicroscopy using AtT20 cells stably expressing either mouse or human EGFP-tagged V\(_{1b}\)R. The EGFP tag did not alter the affinity of the hV\(_{1b}\)R or the mV\(_{1b}\)R, since the \(K_d\) measured in membrane preparations using \[^3H\]AVP as a ligand was in the range of that of the wild type, 0.23 vs. 0.95 and 1.16 vs. 0.67 nM, respectively. As shown in Fig. 8A, SSR-149415 (10\(^{-6}\) M) by itself had no effect on receptor traffic, but totally prevented the internalization induced by 10\(^{-7}\) M AVP (Fig. 8B). Moreover, SSR-149415 (10\(^{-6}\) M) treatment allowed observation of the recycling to the cell membrane of V\(_{1b}\)R previously internalized by AVP pretreatment (Fig. 8C). Similar results were obtained whatever the species considered.

DISCUSSION

Despite a large panoply of vasopressin analogs designed to target each AVP receptor subtype and thus to determine specificity and function, until recently, the characterization of the V\(_{1b}\)R was severely hampered by the lack of specific high-affinity ligands directed toward this receptor. The present work provides a detailed biochemical characterization of \[^3H\]SSR-149415, the first antagonist radioligand available so far for labeling rodent and human V\(_{1b}\)R. Our data indicate that, in binding studies, \[^3H\]SSR-149415 displays high affinity and specificity for the mouse, hamster, rat and human V\(_{1b}\)R and constitutes a suitable probe for mapping these receptors. Additionally, using SSR-149415/[^3H]SSR-149415, the mouse V\(_{1b}\)R stably expressed in murine AtT20 pituitary cells was, for the first time, extensively characterized in comparison with the human one in terms of binding properties and signaling as measured by IP production, \([\text{Ca}^{2+}]_{i}\) mobilization, and receptor internalization.

Radioligand binding experiments using \[^3H\]SSR-149415 and a variety of reference AVP/OT receptor agonists and antagonists showed that \[^3H\]SSR-149415 presented similar properties at V\(_{1b}\)R sites from mouse, hamster, rat, and human origin. In each case, \[^3H\]SSR-149415 binding was time and protein concentration dependent, reversible stereospecific, and saturable. A single high-affinity population of binding sites in the nanomolar range was identified in agreement with \(K_i\) values previously obtained with SSR-149415 for the rat, bovine, and human pituitary V\(_{1b}\) isoform (39). In contrast to previous results reported in the mouse (28), the affinity of SSR-149415 was also found excellent (i.e., nanomolar) for the murine V\(_{1b}\)R with a selectivity ratio >50-fold between the mouse V\(_{1b}\) and the other AVP/OT receptors.
SSR-149415 $K_d$ and $K_i$ values obtained from saturation and competition experiments, respectively, are always nanomolar and highly similar except on In-R1-G9 membranes where a slight difference exists by the two methods ($K_d$ value of 1.57 vs. $K_i$ of 5.1 nM; Tables 1 and 2). In fact this natural cell line expresses a very low level of native V$_{1b}$R (45 fmol/mg protein) which could induce a variability in our results in comparison with the higher number (>100-fold) of recombinant V$_{1b}$R expressed in CHO or AtT20 cells. A certain variability in affinity as a function of cell culture passages could also explain this small difference. Functional experiments performed on the mouse V$_{1b}$R in murine AtT20 pituitary cells confirmed the strong interaction with the murine V$_{1b}$R, since SSR-149415 antagonized both AVP-induced IP production and $[\text{Ca}^{2+}]_i$ increase with nanomolar efficiency. Interestingly, binding data also showed that SSR-149415 had an affinity similar to that of the natural hormone AVP at the V$_{1b}$R and underlined the absence of species differences for the molecule. This constant high affinity is a remarkable property in a field where commonly peptide or nonpeptide ligands targeting AVP/OT receptors can lose totally, or partially, their affinity and selectivity or have their functional pharmacological properties modified depending on the species (29, 33, 42, 18). As an illustration, the variable binding affinity of some peptide (dPal, dDAVP) and nonpeptide (SSR-126768A, SR-49059) compounds is supportive of species-dependent V$_{1b}$R affinity in our study (Table 2).

In terms of biological function, the pituitary V$_{1b}$R is mainly involved in the stimulating effect of AVP on ACTH secretion as a direct ACTH secretagogue or in combination with CRF in several species, including humans, and thus controls the stress response (14, 30, 13, 11). It was shown that in vivo SSR-149415 directly interacted at rat pituitary V$_{1b}$R in inhibiting elevated ACTH secretion by various stimulants such as hormones (AVP, AVP + CRF) and physical stress (restraint stress, dehydration; see Ref. 39). Interestingly, this molecule attenuates anxiety and stress-related behaviors and exerts marked antidepressant-like activity in rodents (16).
phosphate (IP) measurements were performed as described in METHODS. For troph cells both in human and, to a lesser extent, in rat anterior density specific signal confined to ACTH-secreting cortico-
formed in duplicate (IP) or triplicate (Ca\(^{2+}\)) of 10 nM AVP. Data are means calculated from a typical experiment per-
V1bR using in situ autoradiography. We obtained a high-
Ca\(^{2+}\) mobilization (\(\text{EC}_{50}\)) and Ca\(^{2+}\) production (\(\text{EC}_{50}\)) or the mouse (\(\text{IC}_{50}\)) in AtT20 cells expressing either the human or human V1bR
Table 3. Effect of SSR-149415 on AVP-induced IP production in recombinant AtT20 cells expressing either the mouse or human V1bR

<table>
<thead>
<tr>
<th></th>
<th>EC(<em>{50}) or IC(</em>{50}), nM</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonists (EC(_{50}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>1.83±0.68</td>
<td>1.87±0.86</td>
<td></td>
</tr>
<tr>
<td>OT</td>
<td>510±293</td>
<td>144±114</td>
<td></td>
</tr>
<tr>
<td>Antagonists (IC(_{50}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR-149415</td>
<td>12.3±8.4</td>
<td>11.00±9.8</td>
<td></td>
</tr>
<tr>
<td>SSR-149424</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of 3–4 independent determinations. Total inositol phosphate (IP) measurements were performed as described in METHODS. For antagonists, IC\(_{50}\) values were measured in the presence of 10 nM AVP.

[\(^{3}\)H]SSR-149415, we addressed the pituitary localization of V1bR using in situ autoradiography. We obtained a high-
density specific signal confined to ACTH-secreting cortico-
troph cells both in human and, to a lesser extent, in rat anterior pituitary sections, validating [\(^{3}\)H]SSR-149415 as a new V1b radioligand. However, despite literature data reporting the presence of rat brain V1bR mRNA or protein, we failed to evidence specific V1bR binding sites on rat brain membranes or sections using in situ autoradiography. Recently, we confirmed the antidepressant-like effects of SSR-149415 in the forced-swimming test following specific intracerebroventricular injec-
tions in the lateral septum, giving further convincing evidence of the presence of V1bR in the rat central nervous system (CNS) and showing a central component in the mechanism of action of our compound (40). In fact, the low expression levels of V1bR in only some specific brain areas or nuclei, associated with the well-known low specific activity of tritium, are major drawbacks for labeling these receptors in the brain, and one should expect better results with a V1b radiodiodinated ligand. Indeed, discrete expression of V1bR is a common feature either in extrapituitary tissues (pancreas, kidney, adrenals . . .) or in some cell lines constitutively expressing these receptors (small-cell lung cancer, bronchial epithelial, or pancreatic cells . . .). As an illustration, the natural glucagon-secreting pancreatic cell line In-R1-G9 studied in the present work exhibited low maximal binding capacity (45 fmol/mg protein, 7,000 sites/cell; Table 1).

It is worth noting the higher number of binding sites generally detected with [\(^{3}\)H]SSR-149415 in comparison with [\(^{3}\)H]AVP in the different cellular membranes in this study (Table 1). This property has already been reported with other selective V1a (i.e., [\(^{3}\)H]SR-49059) or V2 (i.e., [\(^{3}\)H]SR-121463) receptor antagonist radioligand (35, 36). In fact all of these molecules are hydrophobic radioligands that can bind AVP receptors either from right side-out or right side-in vesicles generated during membrane preparation, whereas [\(^{3}\)H]AVP, as an hydrophylic peptide, can only target externally accessible V1a, V1b, or V2 receptor sites. These nonpeptide molecules belong to a class of membrane-permeant antagonists called “pharmacological chaperones” that are able to interact with both cell surface and intracellular receptors and even, as demonstrated for SR-121463, to rescue natural misfolded endoplasmic reticulum V1R mutants responsible for nephrogenic diabetes insipidus (25). To date, V1bR overexpression has been reported in some corticotrophin-secreting tumors (9), but no natural mutation of the V1bR has been associated with diseases. However, an endoplasmic reticulum-retained V1bR mutant was generated by site-directed mutagenesis, and, in such a situation, SSR-149415 behaves as a pharmacological chaperone and rescues maturation by promoting correct cell-surface expression and the signaling function of the artificial mutant (31). Natural mutations may also exist for the V1bR but may be more difficult to detect than V2R ones associated with obvious clinical signs such as dramatic diuresis.

The selectivity of SSR-149415 was confirmed in binding studies using [\(^{3}\)H]SSR-149415 in various preparations express-

Table 4. Effect of SSR-149415 on AVP-induced [Ca\(^{2+}\)]\(_{i}\) increase in cell lines expressing the mouse or human V1bR

<table>
<thead>
<tr>
<th></th>
<th>EC(<em>{50}) or IC(</em>{50}), nM</th>
<th>Mouse (AtT20)</th>
<th>Human (AtT20)</th>
<th>Human (CHO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonists (EC(_{50}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>3.05±1.32</td>
<td>2.13±0.90</td>
<td>2.40±0.20</td>
<td></td>
</tr>
<tr>
<td>OT</td>
<td>165±52</td>
<td>214±78</td>
<td>162±24</td>
<td></td>
</tr>
<tr>
<td>Antagonists (IC(_{50}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR-149415</td>
<td>1.58±0.98</td>
<td>3.00±0.98</td>
<td>3.71±0.23</td>
<td></td>
</tr>
<tr>
<td>SSR-149424</td>
<td>1.447±735</td>
<td>2.348±240</td>
<td>1.723±203</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of 3–7 independent determinations. Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) measurements were performed as described in METHODS. For antagonists, IC\(_{50}\) values were measured in the presence of 10 nM AVP.
ing recombinant or native human V1a, V2, and OTR in which no significant specific binding was detected. Interestingly, in autoradiographic experiments performed on reference human tissues expressing V1a (liver), V2 (kidney), or OTR (pregnant uterus), no specific labeling was observed with \[ ^3\text{H}\]SSR-149415, in particular in the pregnant uterus, that expresses significant amounts of high-affinity OTR sites, distinctly labeled by the native tritiated hormone \[ ^3\text{H}\]OT or the peptide antagonist \[ ^{125}\text{I}\]OVTA (Fig. 6). Of note, these data did not support the reported interaction of SSR-149415 (almost similar to that of OT itself) at human recombinant OTR (17). The discrepancy could be due to different experimental conditions (ligand, source of receptors . . .). However, the properties of the OTR could also be modified in recombinant cells and vary as a function of the OTR number or environment. That is why we choose to use in our study pregnant tissue samples expressing the native OTR to address the intrinsic affinity of SSR-149415 and its tritiated form under physiological and native conditions. It is well known that the OTR in pregnant tissue exhibited exacerbated affinity for the natural hormone and could exist as homo/heterodimers in particular with the V1aR also expressed in the uterus (43). Additionally, membrane lipidic/cholesterol environment also strikingly modifies OTR affin-

---

Fig. 8. Functional effect of SSR-149415 on internalization and recycling of the human V1bR expressed in AtT20 cells following AVP stimulation. AtT20 cells stably expressing the epidermal growth factor protein (EGFP)-tagged human or mouse V1bR were grown in Lab-Tec chambered cover glasses. Images were acquired every 30 s using a confocal microscope. After 2 control images (time 0), the cells were treated as follows. A: SSR-149415 (1 μM) was added. B: upper cases: AVP (0.1 μM) was added; lower cases: SSR-149415 (1 μM) was added, followed 3 min later by AVP (0.1 μM). C: upper cases: AVP (0.1 μM) was added; lower cases: AVP (0.1 μM) was added, followed 10 min later by SSR-149415 (1 μM). The time elapsed since the beginning of the first treatment is indicated at top of each image.
ity (21) and could explain these different results obtained under in vitro binding and ex vivo autoradiographic conditions.

Finally, functional characterization of SSR-149415 at the V1R was performed on earlier cellular events, upstream of corticotropin release, such as AVP activation of IP production, intracellular [Ca2+]i mobilization, and receptor internalization. SSR-149415 presents a full, stereospecific antagonist profile at mouse and human V1R on AVP-induced IP accumulation and [Ca2+]i elevation with nanomolar efficiency, and the much lower potency of SSR-149415’s diasteroisomer was consistent with data from binding studies in these cells. SSR-149415 inhibition was dose dependent. No agonistic effects were observed when tested alone. Pure antagonist properties of SSR-149415 inhibition was dose dependent. No agonistic effects were observed when tested alone. Pure antagonist properties were further confirmed on the basis of our confocal microscopy data performed on AtT20 cells stably transfected with the V1R coding sequence. Importantly, SSR-149415 (1 μM) was able to restore to the membrane-bound V1R previously internalized by AVP by interrupting the endocytosis process initiated by AVP in AtT20 cells and favored the observation of receptor recycling to the membrane as shown at time 40 min in our experiments (Fig. 8C).

Of the four AVP/OT receptor subtypes cloned so far, the V1R had been poorly characterized because of its limited and scarce expression, even in the pituitary, and the lack of V1R agonist or antagonist ligands. Using [3H]SSR-149415, we have performed an extensive characterization of rodent (mouse, rat, hamster) and human V1R in various cell lines and in pituitary tissue. [3H]SSR-149415 binds to animal and human pituitary V1R with potent affinity and exhibits a full antagonist profile in inhibiting key steps of AVP signaling (IP production, Ca2+ increase, and V1R internalization). Thus the orally active V1R antagonist SSR-149415 and its tritiated form constitute unique tools for further exploring the biological role and functions of V1R.

ACKNOWLEDGMENTS

We are greatly indebted to M. C. Gendron from the Service de Cytométrie of the Institut Jacques Monod, Paris, for the sorting of epidermal growth factor protein-tagged cells, to F. Letourneur from the Sequencing facility, and to M. Bardou from the Faculté de Medecine Dijon, France, for expert assistance. We acknowledge A. J. Patachini for critical reading of the manuscript and M. Laborde for skillful secretarial assistance.

REFERENCES


