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

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THE NEUROHYPOPHYSIAL HORMONE arginine vasopressin (AVP) elicits a variety of biological effects in mammals. In particular, AVP controls water excretion by the kidney, vascular smooth muscle cells and uterine contraction, platelet aggregation, clotting factor release, liver glycogenolysis, mitogenesis, and hormonal secretion (i.e., release of aldosterone by adrenals, glucagon, and insulin in the pancreas and corticotropin release by the adenohypophysis). AVP also regulates several behavioral and memory processes (6, 20). These actions are mediated by activation of three specific seven-transmembrane G protein-coupled receptors named V₁a, V₁b, and V₂R, which have been cloned in several species and characterized in a large number of tissues (2, 23, 44). Of these three receptors, the V₁bR is the least documented and is still poorly understood. The human (8, 32), rat (23, 41), and mouse (48) isoforms have been cloned; the V₁bR is mainly involved in the stimulating effect of AVP on corticotropin secretion in the pituitary, and its role in stress and emotional situations is now established (1, 16). As shown by RT-PCR, in situ hybridization, or immunohistochemistry, the V₁bR gene/protein is expressed not only in anterior pituitary but also in the brain (23, 47) and peripheral tissues such as the pancreas (49, 12), adrenals (15), small intestine, kidney (32), various tumoral cells (small cell lung cancer and corticotropin-secreting tumors; see Ref. 9 and 26) and bronchial epithelial cells (3), clearly suggesting that AVP may exert various actions by acting on extrapituitary V₁bR. However, because of the low expression level of the V₁bR in these tissues that already express considerable amounts of V₁a and/or V₂R, major species differences in the pharmacology of existing reference AVP compounds, and the absence of totally selective and high-affinity V₁b ligands, a complete characterization of the V₁bR has been severely hampered. Very recently, the discovery of the first V₁bR agonist and antagonist gave the awaited tools needed to further investigate this receptor, namely d[cy clohexylalanine⁴]AVP (d[Cha⁴]AVP) and SSR-149415, respectively (10, 39). The former results from structural modifications of the natural hormone AVP; synthetic peptides derived from [deaminocysteine¹,arginine⁸]AVP (dAVP), modified in position 4 by various amino acid residues such as d[Cha⁴] or lysine⁴ to replace the glutamine⁴ yielded high-affinity V₁b ligands. d[Cha⁴]AVP constituted the first V₁b agonist available with excellent affinity for the human V₁bR; however, because of species differences, very common in the AVP domain, this peptide exhibits a mixed V₁a/V₂ pharmacological profile for the rat AVP isoforms (18). The second molecule, SSR-149415, is an orally active nonpeptide antagonist possessing high and selective affinity for rat and human V₁bR in vitro and in vivo. This molecule also decreases anxiety...
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 [3H]SSR-149415, the first nonpeptide antagonist radioligand available for labeling V1bR. It was used for characterizing rodent (mouse, rat, and hamster) and human V1bR, of native or recombinant origin, in binding and autoradiography studies. Moreover, using SSR-149415/ [3H]SSR-149415, it was possible, for the first time, to extensively characterize the mouse V1bR stably expressed in murine AtT20 pituitary cells in comparison with the human one in terms of binding properties, inositol phosphate (IP) production, Ca2+ mobilization, and receptor internalization.

METHODS

Chemicals. The nonpeptide molecules SR-49059 (38), SR-121463 (34), SSR-149415 (39), a diasteroisomer of SSR-149415, SR-149424 (2-methoxyphenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl-[3H]-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, desamino-[n-Arg]vasopressin (dDAVP), [deamino-Cys, d-3-(Pyridyl)-Ala]-Arg3[3H]vasopressin (dPal), [deamino-phenylalanyl-3,4,5-3H(N)]vasopressin; ([3H]AVP, 75 Ci/mmol), [D-Arg]vasopressin (dDAVP), [deamino-Cys, D-3-(Pyridyl)-Ala]-Arg3[3H]vasopressin (dPal), [deamino-phenylalanyl-3,4,5-3H(N)]vasopressin; ([3H]AVP, 75 Ci/mmol), and [3H]methyl iodide in toluene was from Amersham Biosciences (Ozyme, Saint Quentin en Yvelines, France). All other chemicals were from Prolabo (VWR, Fontenay sous Bois, France).

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 CO2. All experiments were performed using cells from passages 22 to 36. Transfected AtT20 cells were cultured in DMEM-F-12 supplemented with 7.5% Fetalclone, 7.5% Nu-serum, 0.5 mM glutamine, and 0.2 mg/ml G418 at 37°C in a 5% CO2 atmosphere. Transfected Ltk- and CHO cells were grown in 10 mM HEPES, pH 7.4, and minimal essential medium supplemented with 5% FCS, 8 g/l sodium bicarbonate, and 0.3 mg/ml G418 at 37°C in a humidified atmosphere containing 5% CO2. 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 V1bR, AtT20 cells expressing the murine or the human V1bR, Ltk- cells expressing the human oxytocin receptor, and CHO cells expressing human V1a and V2 receptors were prepared as described by Serradeil-Le Gal et al. (36). Briefly, cells were harvested, washed twice in PBS without Ca2+ and Mg2+, polytron-homogenized in lysis buffer (15 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 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 MgCl2 (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. Aliquots of membranes were used immediately or stored at −80°C.

Binding studies. Binding experiments of [3H]SSR-149415 or [3H]AVP to cellular membranes were conducted according to Serradeil-Le Gal et al. (39). V1bR binding studies were carried out in an incubation medium containing 50 mM Tris-HCl (pH 7.4), 3 mM MgSO4, 0.1% BSA, 0.1% bacitracin, [3H]AVP, or [3H]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...
membranes (~5 rat CHOV1bR, mouse human AtT20, 35 human CHOV1bR, 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 SSR-149415 or SSR-149445.

**Binding data analysis.** The IC₅₀ value was defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding. Inhibition constant (Kᵢ) values were calculated from the IC₅₀ values using the Cheng and Prusoff (7) equation. Data for equilibrium binding [apparent equilibrium dissociation constant (Kᵢ)], maximum binding density (Bmax), competition experiments [IC₅₀, Hill coefficient (nH)] and kinetic constants [observed association rate constant (k(obs)) and apparent dissociation rate constant (k(app))] were analyzed using an iterative nonlinear regression program (34).

**Autoradiography.** Serial sections (16 μm thick) from frozen whole rat pituitary 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 [¹²⁵I]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 V₁bR 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 insulin-free medium supplemented with 1 μCi/ml-1 myo-[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 AG1-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 Ca²⁺ 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 equilin 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 × 10⁶ 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-149425 15 min before adding 10 nM AVP. Light emission was recorded for 60 s using a Luminoskan microplate luminometer (Labsystems), and the data were expressed in relative light units. After the 60-s measurement period, cells were lysed using 10 μl PBS/2.5% Triton X-100–50 mM CaCl₂, and luminescence induced corresponding to the nonmobilized cell Ca²⁺ 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 V₁bR were plated in Lab-Tek chambered coverglasses (Nunc, Dominque Butcher, Bruumath, France). After (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 tritium label, the 2,4-dimethoxyphenol positions of SSR-149415 (2 nM) were used. Each experiment was carried out at least two times, yielding similar results.

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**Fig. 1. Chemical process for labeling SSR-149415.** The dihydroxy precursor reacted with tritiated methylidene (C[^³H]I) in the presence of N,N-dimethyformanamide (DMF) and K₂CO₃ to yield tritiated [³H]SSR-149415 (SA: 81 Ci/mmol).
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 \( K_{\text{obs}} \) 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 \( K_d \) values of 0.031 and 0.040 min⁻¹ for human and mice V₁bR, respectively (Fig. 2). From these kinetics experiments, calculated \( K_d \) 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 to 250 nM (Fig. 3, A). Saturation isotherms were obtained by incubation of membranes of either CHO cells transfected with the human V₁bR (30 μg/ml; •) or AtT20 cells with the mouse V₁bR (5 μg/ml; ○) 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.

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.

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 efficacy 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).

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 diasteroisomer (SSR-149424) displayed micromolar affinities for these sites. Finally, the OT (SSR-121463A), V1a (SR-49059), and V2 (SR-121463) receptor antagonists exhibited lower or no measurable 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-

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>Cell Line</th>
<th>[3H]SSR-149415</th>
<th>[3H]AVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (CHO)</td>
<td>1.03 ± 0.10</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>Human (AtT20)</td>
<td>1.46 ± 0.44</td>
<td>0.95 ± 0.10</td>
</tr>
<tr>
<td>Rat (CHO)</td>
<td>1.45 ± 0.31</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td>Hamster (In-R1-G9)</td>
<td>1.57 ± 0.17</td>
<td>0.66 ± 0.05</td>
</tr>
<tr>
<td>Mouse (AtT20)</td>
<td>1.28 ± 0.29</td>
<td>0.67 ± 0.04</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.

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-126768A (●), SR-121463 (■). B and D: peptide compounds: AVP (○), dCha (○), dPen (○), dPal (○), dDAVP (△), OT (△). 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.
molar affinities for these sites; other reference V1\(_b\) agonists such as dPal (V1\(_b\)) and dDAVP (V1\(_a\), V2) or antagonists such as dPen (V1\(_a\), V1\(_b\)) exhibited much lower affinities for the V1\(_b\)R than the native hormone, OT, another AVP-related pituitary nonpeptide, displayed ~300-fold weaker affinity than AVP at these sites. In each case, we obtained dose-displacement curves yielding \(n_H\) about one in agreement with a competitive inhibition. Moreover, in saturation experiments with [\(^3\)H]SSR-149415 and AVP (data not shown) or [\(^3\)H]AVP and SSR-149415 (39), we obtained a competitive profile (modification of the \(K_d\) without modifying the \(B_{max}\)); this information authorizes calculating a \(K_i\) from the IC\(_{50}\) value using the equation of Cheng and Prusoff (Ref. 7 and Table 2).

It is worth noting that [\(^3\)H]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 V1\(_a\) or V2 ligands such as [\(^3\)H]SSR-49059 or [\(^3\)H]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 a V1\(_b\)R 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 existing for both peptide (dPal, dDAVP, dPen) and nonpeptide AVP compounds.

### Table 2. Inhibition of [\(^3\)H]SSR-149415 binding to mouse, hamster, rat, and human V1\(_b\)R by reference peptide and nonpeptide AVP compounds

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Mouse (AT20) (K_i), nM</th>
<th>Rat (CHO) (K_i), nM</th>
<th>Hamster (In-R1-G9) (K_i), nM</th>
<th>Human (CHO) (K_i), nM</th>
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</thead>
<tbody>
<tr>
<td>AVP</td>
<td>0.9 ± 0.4</td>
<td>1.6 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>dCha</td>
<td>5.1 ± 0.6</td>
<td>0.9 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>dPal</td>
<td>122 ± 84</td>
<td>106 ± 14</td>
<td>8.2 ± 2.3</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>dPen</td>
<td>19 ± 5</td>
<td>53 ± 6</td>
<td>8.3 ± 3.4</td>
<td>26 ± 12</td>
</tr>
<tr>
<td>dDAVP</td>
<td>192 ± 84</td>
<td>347 ± 33</td>
<td>23 ± 15</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>OT</td>
<td>494 ± 93</td>
<td>294 ± 87</td>
<td>206 ± 137</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Nonpeptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR-149415</td>
<td>1.6 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>5.1 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>SSR-149424</td>
<td>1.024 ± 20</td>
<td>1.575 ± 64</td>
<td>3.405 ± 224</td>
<td>1.320 ± 32</td>
</tr>
<tr>
<td>SSR-49059</td>
<td>158 ± 88</td>
<td>671 ± 49</td>
<td>1,135 ± 124</td>
<td>265 ± 78</td>
</tr>
<tr>
<td>SR-121463</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>SSR-126768A</td>
<td>29 ± 9</td>
<td>60 ± 24</td>
<td>113 ± 44</td>
<td>223 ± 51</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = 3–6\) experiments. dCha, [cyclohexylalanyl]AVP; dPal, [deaminocys(D-3)(Pyridyl)-Ala\(^2\)-Arg\(^8\)]vasopressin; dPen, [deaminopenicillamine-O-Me-TyrArg\(^8\)]vasopressin; dDAVP, [deaminop-Arg\(^8\)]vasopressin. Inhibition constants (\(K_i\)) were calculated according to the equation of Cheng and Prusoff (7) as described in METHODS.

Under standard binding conditions using increasing membrane and [\(^3\)H]SSR-149415 concentrations (data not shown). These data confirmed the higher affinity of SSR-149415 for the human/rat V1\(_b\)R than for the V1\(_a\), V2, and OTR previously described in classical binding studies (39). Moreover, in our hands, \(K_i\) values of 120, 2,500, and 230 nM were found at mice V1\(_a\), V2, and OTR receptors, respectively, using [\(^3\)H]AVP (V1\(_a\) and V2R assays) or [\(^12\)I]OVTA (OTR assays) as radioligands, confirming the V1\(_b\)R selectivity also in the mouse (data not shown).

** Autoradiographic studies with [\(^3\)H]SSR-149415.** The V1\(_b\)R 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 [\(^3\)H]SSR-149415, we examined the localization of V1\(_b\)R both in rat and human pituitary sections (Fig. 5). Intense specific labeling totally displaced by 10 \(\mu\)M SSR-149415 (or 10 \(\mu\)M 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 obtained in the central neural lobe, and no specific labeling was detected in the intermediate lobe area as previously described with [\(^3\)H]AVP (45). Using [\(^3\)H]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 V1\(_a\) (liver), V2 (kidney) (data not shown) or OTR (pregnant uterus), no specific labeling was observed with [\(^3\)H]SSR-149415, in particular in the pregnant uterus, which expresses significant amounts of high-affinity OTR sites distinctly labeled by the native tritiated hormone [\(^3\)H]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 [\(^3\)H]SSR-149415 to address V1\(_b\)R 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 V1\(_b\)R is probably the main difficulty that will have to be overcome to map extrapituitary V1\(_b\)R, especially in the brain. An absence of specific binding was also observed in rat brain membranes using [\(^3\)H]SSR-149415 as a ligand.

**Effect of SSR-149415 on AVP-induced IP production in mouse and human V1\(_b\)R recombinant cell lines.** Activation of the V1\(_b\)R by AVP has been associated with phospholipase C stimulation, producing diacylglycerol and inositol triphosphate, which in turn increase intracellular \(Ca^{2+}\) concentration ([\(Ca^{2+}\)]i; see Ref. 44).

In recombinant AT20 cells expressing either the mouse or human V1\(_b\)R, AVP induced dose-dependent IP accumulation with nanomolar EC\(_{50}\) values (1.83 ± 0.68 and 1.87 ± 0.86 nM, respectively), whereas OT was much less potent (EC\(_{50}\) 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 IC\(_{50}\) values of 12.3 ± 8.4 and 11.00 ± 9.8 nM for the mouse and human V1\(_b\)R, respectively. Of note, this effect was stereose-
specific, since SSR-149415’s diasteroisomer, SSR-149424, was almost inactive (IC50 values >1 μM; Table 3).

Effect of SSR-149415 on AVP-induced intracellular Ca2+ mobilization in mouse and human V1bR recombinant cell lines. AVP exposure of the mouse and human recombinant V1bR cell lines induced a dose-dependent increase in [Ca2+]i by activating V1bR with EC50 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 Ca2+ elevation in a dose-dependent manner, yielding nanomolar IC50 values for the mouse and human recombinant V1bR (Fig. 7B). This effect was stereospecific, since SSR-149415’s diasteroisomer, SSR-149424, was much less efficient (about 3 orders of magnitude) and gave IC50 in the micromolar range (Table 4).

Effect of SSR-149415 on AVP-induced V1bR 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 V1bR. The EGFP tag did not alter the affinity of the hV1bR or the mV1bR, since the Kd 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 V1bR 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 V1bR 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 V1bR. Our data indicate that, in binding studies, [3H]SSR-149415 presented similar properties at V1bR 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 Ki values previously obtained with SSR-149415 for the rat, bovine, and human pituitary V1b 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 V1bR with a selectivity ratio >50-fold between the mouse V1b and the other AVP/OT receptors. In the different preparations,
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 V1bR (45 fmol/mg protein) which could induce a variability in our results in comparison with the higher number (>100-fold) of recombinant V1bR 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 V1bR in murine AtT20 pituitary cells confirmed the strong interaction with the murine V1bR, 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 V1bR 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 V1bR affinity in our study (Table 2).

In terms of biological function, the pituitary V1bR 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 V1bR 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). Using

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Fig. 6. Autoradiograms with $[^3\text{H}]$OT, $[^3\text{H}]$SSR-149415, and $[^12\text{C}]$OVTA in human pregnant myometrium sections. Binding of $[^12\text{C}]$OVTA (A), $[^3\text{H}]$OT (B), and $[^3\text{H}]$SSR-149415 (C) in the absence (total binding, TB) and in the presence (nonspecific binding, NS) of 10 μM OT. Serial sections (16 μm thick) were incubated with 2 nM $[^3\text{H}]$OT or $[^3\text{H}]$SSR-149415 and 0.05 nM $[^12\text{C}]$OVTA as described in METHODS and exposed onto a phosphor-imaging plate.
[3H]SSR-149415, we addressed the pituitary localization of \( \text{V}_{1\beta} \)R using in situ autoradiography. We obtained a high-density specific signal confined to ACTH-secreting corticotroph cells both in human and, to a lesser extent, in rat anterior pituitary sections, validating [3H]SSR-149415 as a new \( \text{V}_{1\beta} \)R antagonist. We obtained a high number of binding sites specifically detected with [3H]SSR-149415 in comparison with [3H]AVP, which 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 [3H]SSR-149415 in comparison with [3H]AVP in the different cellular membranes in this study (Table 1). This property has already been reported with other selective \( \text{V}_{1a} \) (i.e., [3H]SR-49059) or \( \text{V}_{2} \) (i.e., [3H]SR-121463) receptor antagonist radioligand (35, 36). In fact all of these molecules are hydrophilic radioligands that can bind AVP receptors either from right side-out or right side-in vesicles generated during membrane preparation, whereas [3H]AVP, as an hydrophylic peptide, can only target externally accessible \( \text{V}_{1a}, \text{V}_{1\beta}, \text{or V}_{2} \)R 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 \( \text{V}_{2} \)R mutants responsible for nephrogenic diabetes insipidus (25). To date, \( \text{V}_{1\beta} \)R overexpression has been reported in some corticotrophin-secreting tumors (9), but no natural mutation of the \( \text{V}_{1\beta} \)R has been associated with diseases. However, an endoplasmic reticulum-retained \( \text{V}_{1\beta} \)R 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 \( \text{V}_{1\beta} \)R but may be more difficult to detect than \( \text{V}_{2} \)R ones associated with obvious clinical signs such as dramatic diuresis.

The selectivity of SSR-149415 was confirmed in binding studies using [3H]SSR-149415 in various preparations expressing different \( \text{V}_{1\beta} \)R splice variants.

### Table 3. Effect of SSR-149415 on AVP-induced IP production in recombinant AtT20 cells expressing either the mouse or human \( \text{V}_{1\beta} \)R

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

Values are means ± SD of 3–7 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.

Fig. 7. Inhibition curves of SSR-149415 on AVP-induced inositol phosphate (IP) production (A) and Ca\(^{2+}\) mobilization (B) in AtT20 cells expressing either the human (○) or the mouse (●) recombinant \( \text{V}_{1\beta} \)R. Total IP and intracellular Ca\(^{2+}\) measurements were performed as described in METHODS in the presence of 10 nM AVP. Data are means calculated from a typical experiment performed in duplicate (IP) or triplicate (Ca\(^{2+}\)) and repeated 3–5 times without noticeable modifications.

### Table 4. Effect of SSR-149415 on AVP-induced [Ca\(^{2+}\)]\(_{i}\) increase in cell lines expressing the mouse or human \( \text{V}_{1\beta} \)R

<table>
<thead>
<tr>
<th>Agonists (EC(_{50}))</th>
<th>Mouse (AtT20)</th>
<th>Human (AtT20)</th>
<th>Human (CHO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVP</td>
<td>3.05±1.32</td>
<td>2.13±0.90</td>
<td>2.40±0.20</td>
</tr>
<tr>
<td>OT</td>
<td>165±52</td>
<td>214±78</td>
<td>162±24</td>
</tr>
<tr>
<td>Antagonists (IC(_{50}))</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>
</tr>
<tr>
<td>SSR-149424</td>
<td>1,447±753</td>
<td>2,348±240</td>
<td>1,723±203</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 [3H]SSR-149415, in particular in the pregnant uterus, that expresses significant amounts of high-affinity OTR sites, distinctly labeled by the native tritiated hormone [3H]OT or the peptide antagonist [125I]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-
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 V1bR was performed on earlier cellular events, upstream of corticotropin release, such as AVP activation of IP production, intracellular [Ca\(^{2+}\)] mobilization, and receptor internalization. SSR-149415 presents a full, stereospecific antagonist profile at mouse and human V1bR on AVP-induced IP accumulation and [Ca\(^{2+}\)] elevation with nanomolar efficiency, and the much lower potency of SSR-149415’s diastereoisomer 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 for this molecule were further confirmed on the basis of our confocal microscopy data performed on AtT20 cells stably expressing the EGFP-tagged human or mouse V1bR. SSR-149415 (1 μM) had no effect by itself on V1bR traffic but blocked V1bR internalization following AVP stimulation (Fig. 8, A and B). Interestingly, SSR-149415 (1 μM) was able to restore to the membrane V1bR previously internalized by AVP by interrupting the endocytosis process (Fig. 8C). The V1bR is a class A-type receptor undergoing dynamic endocytosis and rapid recycling (30 min) upon agonist stimulation (Ventura, a class A-type receptor undergoing dynamic endocytosis and confocal microscopy data performed on AtT20 cells stably for this molecule were further confirmed on the basis of our 149415 inhibition was dose dependent. No agonistic effects consistent with data from binding studies in these cells. SSR-149415 binds to animal and human pituitary tissue. [3H]SSR-149415 binds to animal and human pituitary V1bR antagonist SSR-149415 and its tritiated form constitute unique tools for further exploring the biological role and unique expression, even in the pituitary, and the lack of V1bR antagonist or agonist ligands. Using [3H]SSR-149415, we have performed an extensive characterization of rodent (mice, rat, hamster) and human V1bR in various cell lines and in pituitary tissue. [3H]SSR-149415 binds to animal and human pituitary V1bR with potent affinity and exhibits a full antagonist profile in inhibiting key steps of AVP signaling (IP production, Ca\(^{2+}\) increase, and V1bR internalization). Thus the orally active V1bR antagonist SSR-149415 and its tritiated form constitute unique tools for further exploring the biological role and functions of V1bR.

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