Pancreatic vasopressin V$_{1b}$ receptors: characterization in In-R1-G9 cells and localization in human pancreas

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Vasopressin (AVP) receptors present in In-R1-G9 cells, a hamster glucagon-secreting α-pancreatic cell line, were characterized using SSR-149415, a selective nonpeptide V$_{1b}$ receptor antagonist, and reference AVP compounds. Binding experiments, using $^3$H]AVP as a ligand, identified a single population of high-affinity binding sites. SSR-149415 competitively inhibited this binding and exhibited nanomolar and stereospecific affinity for these sites. The affinity of various AVP/oxytocin ligands confirmed a V$_{1b}$ binding profile. In functional studies, AVP was a potent stimulant in inducing intracellular Ca$^{2+}$ increase, glucagon secretion, and cell proliferation. These effects were fully antagonized by SSR-149415 with a nanomolar potency, whereas its diastereoisomer as well as two selective V$_{1a}$ and V$_2$ receptor antagonists were much less potent. Additionally, the order of potency of AVP agonists and antagonists was in agreement with V$_{1b}$-mediated effects. By RT-PCR, we confirmed the presence of V$_{1b}$ receptor mRNA in both In-R1-G9 cells and in human pancreas. The distribution pattern of V$_{1b}$ receptors investigated in human pancreas by immunohistochemistry showed strong labeling in islets of Langerhans, and colocalization studies indicated that this receptor was expressed in α-glucagon, β-insulin, and somatostatin pancreatic cells. Thus, in In-R1-G9 cells, AVP mediates intracellular Ca$^{2+}$ increase, glucagon secretion, and cell proliferation by activating V$_{1b}$ receptors, and these effects are potently antagonized by SSR-149415. Moreover, the presence of V$_{1b}$ receptors also found in human Langerhans islets could suggest hormonal control of AVP in human pancreas.

arginine vasopressin V$_{1b}$ receptors; glucagon; SSR-149415

**VASOPRESSIN (AVP)** exerts a number of central and peripheral functions in mammals. Among them, it controls water excretion by the kidney, vascular smooth muscle cell 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 pancreas and corticotropin release by the adenohypophysis). In concert with oxytocin (OT), a structurally related neurohypophysial nonapeptide, AVP also controls several behavioral and memory processes (4, 9). These actions are mediated by specific G protein-coupled receptors, named V$_{1a}$, V$_{1b}$, V$_2$, and OT, which have been cloned and characterized in animals and in humans. The V$_2$ receptor is positively coupled to adenyl cyclase, whereas the others are linked to phospholipase C activation and intracellular Ca$^{2+}$ mobilization (29). The V$_{1b}$ (or V$_3$) receptor has been cloned in mice, rats, and humans (7, 14, 20, 27, 30). This receptor is involved mainly in the stimulating effect of AVP on corticotropin secretion in the pituitary, and its role in stress and emotional situations is now well documented (1). As recently demonstrated by RT-PCR, in situ hybridization, or immunohistochemistry, the V$_{1b}$ receptor gene/protein is expressed not only in anterior pituitary but also in brain and peripheral tissues such as the pancreas, adrenals, small intestine, and kidney and in various tumoral cells (small-cell lung cancer and corticotropin-secreting tumors), clearly suggesting that AVP may exert novel actions by acting on extrapituitary V$_{1b}$ receptors (7, 8, 14, 16, 20, 27, 30). Although species differences have been shown between rat and human tissues in the distribution of V$_{1b}$ receptor, V$_{1b}$ mRNA showed constant expression in mouse, rat, and human pancreas. It is important to underline that AVP itself, together with OT, is found in rat and human pancreas, suggesting a physiological control in this organ (2). Functional studies performed in several models by use of perfused pancreas and various rodent endocrine pancreatic islets or cell lines (HIT-T15, In-R1-G9, and RINm5F) have shown that both AVP and OT receptors may be involved in insulin and glucagon secretion. More recently, it was suggested that AVP could mediate insulin and glucagon release via activation of V$_{1b}$ receptors (10, 13, 18, 32, 33). Of note, the absence of specific AVP V$_{1b}$ radioligands or antagonists and the low expression level of these receptors in peripheral organs have severely hampered the characterization of extrapituitary V$_{1b}$ receptors.

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In the present study, we investigated the effects of the selective and orally active V₁b receptor antagonist recently described, SSR-149415 (25), on the In-R1-G9 cell line, a model of glucagon-secreting α-pancreatic cells. For the first time, binding studies were performed in these cells with [³H]AVP and various reference peptide and nonpeptide ligands, including SSR-149415 and its low V₁b affinity diasteroisomer (SR-149424) to provide characterization of AVP receptors present In-R1-G9 cells. In functional studies performed in these cells, AVP induces intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) increase, glucagon secretion, and cell proliferation. These effects were fully antagonized by SSR-149415 with a nanomolar potency, whereas its diasteroisomer as well as two selective V₁a and V₂ receptor antagonists were much less potent. The order of potency of AVP agonists and antagonists was in agreement with V₁b-mediated effects. The presence of V₁b receptor mRNA in these cells and in human pancreas was also confirmed by RT-PCR. Finally, using an immunohistochemical approach, we studied the distribution of V₁b receptors in human pancreas and demonstrated by double immunostaining that the V₁b receptor was expressed in the different cell types of islets of Langerhans. All together, these data suggest a potential hormonal control for AVP on endocrine pancreas via V₁b receptor activation.

**MATERIALS AND METHODS**

**Chemicals.** The nonpeptide molecules, SR-49059 (24), SR-121463 (23), SSR-149415 (25) and a diasteroisomer of SSR-149415, (SR,SR)-1-[(3S)-5-chloro-1-[(2,4-dimethoxyphenylsulfonyl)-3-[(2-methoxyphenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl]4-hydroxy-N,N-dimethyl-2-pyridylidine carboxamide (SR-149424), were synthesized at Sanofi-Synthelabo Recherche, France. All of them were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10⁻² M and then diluted in the appropriate test solvent. AVP, OT, 1-desamino-8-d-arginine vasopressin (dDAVP), [desamino-Cys₂-d-3-(pyridyl)-Ala²-Arg⁶]vasopressin (dPal), [desamino-pentacillamine-O-Me-Tyr₃,Arg₆]vasopressin (dPen), arginine, theophylline, somatostatin, forskolin, and bacitracin were from Sigma Chemical (St. Louis, MO). Fura 2-acetoxymethyl ester (fura 2-AM), pluronic F-127, and the Alexa fluor 594-labeled goat anti-rabbit IgG (Molecular Probes) were kindly provided by Dr. Kimberly A. Matthews (Veterans Affairs Medical Center, Huntington, WV). Human biopsy tissues (pancreas and liver) were collected in conformity with French national ethics rules. Immediately after excision, tissues without microscopic abnormalities or tumors were fixed in paraformaldehyde (4%) and then embedded in paraffin for immunohistochemical studies. This project was approved by the human subject review committees of Sanofi-Synthelabo Recherche and was carried out in collaboration with the Department of Pathological Anatomy of Strasbourg University Hospital (Strasbourg, France). Human pituitary sections embedded in paraffin were purchased from Clinisciences (Montrouge, France).

**Culture of hamster pancreatic In-R1-G9 cells.** In-R1-G9 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 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.

**³H]AVP binding to In-R1-G9 membranes.** Membranes from confluent In-R1-G9 cells, cultured in 175-cm² flasks, were prepared as described previously (22). Binding assays on In-R1-G9 membranes were performed in an incubation medium containing 50 mM Tris-HCl (pH 7.4), 3 mM MgSO₄, 0.1% BSA, 0.1% bacitracin, [³H]AVP (0.003–20 nM for saturation experiments or 2–3 nM for kinetic and competition studies), and increasing amounts of the tested compound. The reaction was started by the addition of In-R1-G9 membranes (~200–275 µg/assay) and lasted for 45 min at 20°C. The reaction was stopped by adding 4 ml of ice-cold buffer followed by filtration through GF/B Whatman glass microfiber filters. Filters were washed twice 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. 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 by use of the Cheng and Prusoff equation (5). Data for binding experiments [apparent equilibrium dissociation constant (Kₐ)], maximum binding density (Bₘₐₓ), IC₅₀, and Hill coefficient (nₜₐₜ) were analyzed using an iterative nonlinear regression program (15).

**Intracellular Ca²⁺ concentration measurements.** Subconfluent In-R1-G9 cells, cultured in 175-cm² flasks as described, were collected by trypsinization (0.05% trypsin, 0.02% EDTA) and centrifuged (230 g, 5 min). The cells were suspended in culture medium to a final concentration of 5 × 10⁶ cells/ml and then incubated with 5 µM fura 2-AM and 0.02% pluronic F-127 at 30°C for 20 min under continuous shaking. At the end of the incubation, cells were centrifuged (230 g, 5 min) and washed with culture medium. The cells were washed twice in Hanks’ buffer (in mM: 137 NaCl, 5 KCl, 0.45 Na₂HPO₄, 5.4 glucose, 4.2 NaHCO₃, 0.8 MgSO₄, and 10 HEPES, with 0.1 mM EGTA for the first wash only, pH 7.4). The cells were resuspended in this buffer to a final concentration of 2.7 × 10⁶ cells/ml and kept at 4°C in the dark until use. Calcium transients were measured with an SLM 8000 C spectrofluorometer after an incubation
of 4 min at 37°C (excitation at 340 and 380 nm, emission at 510 nm). Cytosolic free Ca\(^{2+}\) determination was performed according to Grynkiewicz et al. (11).

Glucagon release. In-R1-G9 cells were plated onto 24-well plates (Corning, Oneonta, NY) at 10\(^5\) cells/well and were grown for 4 days. The culture medium was then removed, and cells were preincubated (37°C, 5% CO\(_2\), 95% humidity) in a Krebs-Ringer bicarbonate buffer (KRB) for 15 min to determine the dose responses of AVP and various AVP/OT agonists (dPal, dDAVP, and OT), cells were preincubated with successive dilutions from 10\(^{-9}\) to 10\(^{-6}\) M in the KRB buffer for 15 min. Similar experiments were also conducted in the presence of various modulators of glucagon secretion: arginine (10\(^{-3}\) M), theophylline (10\(^{-2}\) M), and somatostatin (10\(^{-6}\) M). For the antagonism study, the compound to be tested was administered 15 min before the addition of 10\(^{-7}\) M AVP, and then the plate was incubated for 15 min. The concentration of glucagon in the medium was measured by radioimmunooassay using the glucagon RIA kit provided by Linco Research (St. Charles, MO). The titration was performed in duplicate.

Glucose consumption. In-R1-G9 cells were grown for 48 h in a 96-well plate with a clear bottom (10,000 cells/well). The cell proliferation was measured using the CellTiter 96 cell proliferation assay from Promega (Madison, WI). Cells were washed with 200 \(\mu\)L of PBS and treated with increasing concentrations of either agonist or AVP (5.10\(^{-10}\) to 10\(^{-4}\)) and increasing concentrations of antagonist compounds. After 18 h of incubation (37°C, 5% CO\(_2\), 80% humidity), 20 \(\mu\)L of dye solution were added to each well. The plate was incubated for 3 h, and absorbance was recorded at a 490-nm wavelength with a Multiskan Ex plate reader (Labsystem) (29).

\(\text{V}_{1b}\) receptor expression in In-R1-G9 cells and human pancreas. Total Chinese hamster ovary (CHO) or In-R1-G9 cell RNA was extracted using the Qiagen RNeasy kit. Human pancreas RNA was purchased from Invitrogen. cDNAs were synthesized from 5 \(\mu\)g of RNA with Superscript RT II (Invitrogen), and 1/10th of the reaction product was used for PCR amplification consisting of 35 or 45 cycles of 30-s denaturation at 95°C, 30-s annealing at 56°C, and 1-min extension at 72°C. PCR products were separated by electrophoresis in a 2% agarose gel. Both primers were designed from published human, mouse, and rat \(\text{V}_{1b}\) sequences: sense 5’-GATA GGTGTTGAGC-3’; reverse 5’-GATGGTGAA-GGCCACATTGG-3’. The expected size for the amplicon was 600 bp.

Immunohistochemistry studies. Human tissue sections (5 \(\mu\)m) mounted on electrostatically treated slides were processed for antigen retrieval, which included deparaffinization and rehydration. Briefly, the slides were immersed in the Trilogy reagent (diluted 1:20 with distilled water) and heated to 90°C in a microwave oven for 20 min. The slides were washed three times for 5 min with phosphate-buffered saline (PBS), pH 7.4. For immunohistochemical detection of \(\text{V}_{1b}\) receptor, sections were incubated with 3% H\(_2\)O\(_2\) diluted in PBS for 15 min. After a wash in PBS containing 0.05% Tween-20 to neutralize nonspecific binding sites, the sections were covered with PBS containing 5% NGS and 0.3% Triton X-100 for 20 min at room temperature and then stained and incubated for 120 min at room temperature with rabbit anti-\(\text{V}_{1b}\) receptor antibody diluted (1:150) in PBS containing 1.5% NGS and 0.1% Triton X-100 (antibody diluting buffer). Subsequently, the slides were covered with the EnVision HRP-conjugated dextran polymer coupled to goat anti-rabbit IgG antibodies for 30 min at room temperature. Staining was completed by incubation with peroxidase substrate DAB for 4 min at room temperature. Sections were counterstained with hematoxylin, a nuclear marker, and then mounted with a coverslip. For fluorescent immunohistochemistry after the HPR treatment, the sections were treated with the fluorophore tyramide amplification reagent labeled with fluorescein.

In another set of experiments, a double fluorescent immunostaining method was applied for simultaneous localization of the \(\text{V}_{1b}\) receptor with insulin, glucagon, or somatostatin. For double labeling, the same conditions were used for reaction with the \(\text{V}_{1b}\) antibody and secondary labeling with EnVision-HRP. Then the sections were incubated with the tyramide amplification reagent containing fluorescein for 4 min. In the subsequent step after intensive washing, the sections were covered for 45 min at room temperature with mouse monoclonal anti-glucagon (1:1,500), anti-insulin (1:1,500), or anti-somatostatin (1:20) antibodies. The detection was performed with goat anti-mouse antibody coupled to Alexa 594 (10 \(\mu\)g/ml diluted in PBS containing 1% NGS) for 30 min. Sections were counterstained with the nuclear marker DAPI, washed in distilled water, and mounted using Gel/Mount (Biomeda).

Slides were analyzed by transmission or fluorescence microscopy using a Leica microscope (Leitz DMREB) equipped with a charge-coupled device camera. Micrographs were made using the MetaMorph 4.6r6 image analysis system.


**RESULTS**

Characterization of \([^3H]\text{AVP}\) binding to In-R1-G9 cells. Cultured In-R1-G9 cells growing in an RPMI 1640 medium with 10% fetal calf serum exhibited a typical spherical morphology and tended to form aggregates, as previously described (28). Preliminary binding experiments to In-R1-G9 cell membranes have shown that specific binding for \([^3H]\text{AVP}\) was obtained as a function of the protein content in the assay. \([^3H]\text{AVP}\) binding at 20°C was time dependent, rapidly reached an apparent equilibrium (in ~30 min), and was stable for up to 3 h (not shown). At equilibrium, under standard operating conditions, nonspecific binding represented from 30 to 40% of total binding. In competition binding experiments, the relative affinities of several reference peptide and nonpeptide AVP/OT compounds were studied to further characterize AVP receptors expressed in In-R1-G9 cells. As shown in Fig. 1A, \([^3H]\text{AVP}\) binding was totally and dose-dependently...
inhibited by the natural hormone AVP, and by the selective V1b receptor antagonist, SSR-149415, with subnanomolar affinities (Ki = 0.20 ± 0.11 and 0.81 ± 0.38 nM, respectively). Of note, SSR-149415 demonstrated stereospecific affinity for these sites, since its diastereoisomer (so-called SR-149424) displayed much less (1,000-fold) affinity than SSR-149415. Reference V1b agonists such as dPal (V1b) and dDAVP (V1b, V2) or antagonist dPen (V1a, V1b) exhibited significant affinity for the receptor labeled with [3H]AVP in In-R1-G9 cells (K_i = 17 ± 11, 24 ± 9, and 11 ± 3 nM, respectively). Finally, oxytocin and the two selective nonpeptide V1a (SR-49059) and V2 (SR-121463) ligands displayed much lower or no measurable affinity for these receptors (K_i = 68 ± 28 and 128 ± 32 nM, and K_i > 10 μM, respectively). All these observations confirmed a rank order of affinity compatible with a V1b receptor-binding profile (29).

Dose-response curves for all the compounds tested gave a linear Hill plot and pseudo-Hill coefficient (n_H) near unity (not shown). Analysis of data from saturation binding experiments performed in In-R1-G9 membranes in the absence (control) or presence of SSR-149415 (1.25, 2.5, or 5 nM) indicated that [3H]AVP specific binding was saturable. Scatchard representation for control gave a linear plot consistent with the presence of a single class of high-affinity binding sites having an apparent K_d of 0.27 ± 0.07 nM and a B_max of 25 ± 9 fmol/mg protein (n = 4), which corresponds to an approximative number of 4,000 sites/cell. Scatchard plots presented in Fig. 1B also indicated that SSR-149415 inhibited [3H]AVP binding in a competitive manner, since in the presence of various concentrations of this molecule the control K_d value decreased dose dependently, whereas the B_max was not modified. The K_i value calculated from Scatchard plots (1.61 ± 0.17 nM) was consistent with the K_i value obtained according to the Cheng and Prusoff equation (5) in competition experiments (0.91 ± 0.38 nM; Table 1). It should be noted that competitive interaction has been previously observed for SSR-149415 at human V1b receptors expressed in CHO cells with a similar nanomolar K_i value (25).

Table 1. Binding affinity, [Ca^{2+}], response, mitogenic activity, and effect on glucagon release of several AVP/OT agonists and antagonists in In-R1-G9 cells

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>K_i (nM)</th>
<th>EC_{50} (nM)</th>
<th>EC_{50} (nM)</th>
<th>EC_{50} (nM)</th>
</tr>
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<tbody>
<tr>
<td>AVP</td>
<td>0.20 ± 0.11</td>
<td>24 ± 20</td>
<td>5.2 ± 1.4</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>OT</td>
<td>68 ± 28</td>
<td>303 ± 196</td>
<td>564 ± 27</td>
<td>69 ± 68</td>
</tr>
<tr>
<td>dPal</td>
<td>18 ± 11</td>
<td>196 ± 52</td>
<td>108 ± 55</td>
<td>47 ± 23</td>
</tr>
<tr>
<td>dDAVP</td>
<td>24 ± 10</td>
<td>121 ± 26</td>
<td>181 ± 33</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>SR-49059</td>
<td>11 ± 3</td>
<td>60 ± 11</td>
<td>20 ± 16</td>
<td>60 ± 23</td>
</tr>
<tr>
<td>SR-121463</td>
<td>128 ± 32</td>
<td>132 ± 39</td>
<td>198 ± 162</td>
<td>411 ± 486</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Agonists</th>
<th>K_i (nM)</th>
<th>[Ca^{2+}]</th>
<th>Glucagon Release</th>
<th>Cell Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVP</td>
<td>0.81 ± 0.38</td>
<td>0.66 ± 0.47</td>
<td>1.2 ± 1.1</td>
<td>0.71 ± 0.41</td>
</tr>
<tr>
<td>OT</td>
<td>787 ± 493</td>
<td>1,185 ± 936</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>dPen</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

Values are means ± SE, in nM, of 3–9 independent determinations. AVP, arginine vasopressin; OT, oxytocin; see text for definitions of other chemicals. Binding assays, intracellular free Ca^{2+} concentration ([Ca^{2+}]), measurements, and proliferation assays were performed as described in MATERIALS AND METHODS. Inhibition constants (K_i) were determined from competition experiments calculated according to equation of Cheng and Prusoff (5).
Effect of SSR-149415 and AVP analogs on \([\text{Ca}^{2+}]_i\) increase in In-R1-G9 cells. As measured by fura 2 fluorescence, AVP induced a dose-dependent increase in \([\text{Ca}^{2+}]_i\) in In-R1-G9 cells, with a dose required for a half-maximal response (EC50) of 24 ± 20 nM (n = 7). Reference peptides tested, dPal, dDAVP, and OT, exhibited an agonist profile in these cells, although they were ~10-fold less potent than AVP in stimulating \([\text{Ca}^{2+}]_i\) elevation (EC50 = 196 ± 52, 121 ± 26, and 303 ± 196 nM, respectively). However, dDAVP showed only a partial agonistic activity in these cells, with a maximal effect 2.5-fold lower than that of AVP or other agonists (Fig. 2A). This observation is consistent with previous results showing that dDAVP acted as a partial agonist on total IP production in COS-1 cells transfected with rat V1b receptors, whereas a full agonistic activity was observed in human V1b receptor-expressing cells (21). Among the antagonists tested, SSR-149415 strongly and dose-dependently inhibited 100 nM AVP-induced \([\text{Ca}^{2+}]_i\) increase (Ki of 0.66 ± 0.47; n = 3). This effect was stereospecific, as demonstrated by the lower activity of its diastereoisomer SSR-149424 in this model (Ki = 1,185 ± 936 nM; n = 4). Under similar operating conditions, the mixed V1b/V1a antagonist dPen and the V1a receptor antagonist SR-49059 were less effective (Ki = 60 ± 11 and 128 ± 40 nM, respectively; n = 3; Fig. 2B). Finally, the V2 receptor blocker SR-121463 failed to antagonize \([\text{Ca}^{2+}]_i\) elevation stimulated by AVP (Table 1). None of the antagonists tested exhibited agonistic effects when tested alone up to 1 μM.

Effect of SSR-149415 and several AVP agonists and antagonists on glucagon release in In-R1-G9 cells. AVP induced dose-dependent secretion of glucagon in In-R1-G9 cells with an EC50 value of 5.2 ± 1.4 nM (n = 5), similar to that previously described by Yibchok-anun et al. (31). The reference AVP/OT peptide agonists tested (dPal, dDAVP, and OT) were much less potent than AVP, and their order of potency was in agreement with the affinity found for the V1b receptor identified in binding experiments (Table 1 and Fig. 3A). Interest-

Fig. 3. Effects of different AVP peptide and nonpeptide compounds on glucagon release by In-R1-G9 cells. A: dose-response curves of agonist peptides AVP (●), dPal (○), dDAVP (●), and OT (○). In-R1-G9 cells were incubated with increasing concentrations of agonist for 15 min. B: effects of SSR-149415 (●), dPen (■), and SR-49059 (○) on 100 nM AVP-induced glucagon release. Cells were preincubated with antagonists for 15 min before addition of 100 nM AVP for 15 min, as described in MATERIALS AND METHODS. Data are means of 3–6 experiments.
ingly, Dunning et al. (10) reported a significant effect of AVP, and to a lesser extent OT and dDAVP, on glucagon release in the perfused rat pancreas, in agreement with these in vitro data. To complete the functional identification of the AVP receptor involved in glucagon release, several specific V₁b (SSR-149415), V₁a (SR-49059), and V₂ (SR-121463) receptor antagonists were used to counteract AVP effects. SSR-149415 exerted powerful antagonism on 100 nM AVP-induced glucagon secretion (Kᵢ = 1.2 ± 1 nM), whereas SR-49059 was >100-fold less potent and SR-121463 was devoid of any effect up to 1 µM (Table 1 and Fig. 3B). Once again, we observed stereospecificity in the effect of SSR-149415 in antagonizing AVP-evoked glucagon production in In-R1-G9 cells (Kᵢ of SR-149424 >1 µM). In another set of experiments, the effects of AVP were compared with those of well-known modulators of glucagon secretion. Amino acids, such as arginine, and agents that increase the intracellular cAMP concentration promote the liberation of the hyperglycemic hormone glucagon (19). As shown in Fig. 4, 100 nM AVP induced an effect comparable to 10 nM arginine on glucagon secretion (153 and 154% vs. basal value, respectively). As expected, theophylline, a nonspecific phosphodiesterase inhibitor known to increase intracellular cAMP production in In-R1-G9 cells, also stimulated glucagon secretion (175% vs. basal value). When associated with one of these agents (arginine or theophylline), AVP (100 nM) tends to enhance its action on glucagon secretion (170 and 200%, respectively). Of note, SSR-149415 was devoid of any inhibitory effect on basal and glucagon production evoked by the various stimulators except AVP (Fig. 4), demonstrating specific interaction at AVP V₁b receptors. Finally, somatostatin, an inhibitor of pancreatic secretion, had no significant effect on basal or AVP-stimulated In-R1-G9 glucagon production under standard operating conditions. However, a significant inhibitory action of somatostatin was achieved on AVP-induced glucagon production after a longer preincubation time (50% inhibition at 2 h; not shown).

**Effect of SSR-149415 and AVP analogs on In-R1-G9 cell proliferation.** As shown in Table 1, AVP promoted a strong proliferative effect in In-R1-G9 cells, and this effect was dose dependent (EC₅₀ = 0.082 ± 0.022 nM; n = 14). Interestingly, Thibonnier et al. (29) also reported a mitogenic effect of AVP, using CHO cells transfected with the human V₁b receptors. dPen and OT induced In-R1-G9 cell proliferation with EC₅₀ values of 47 ± 22 (n = 3) and 69 ± 68 nM (n = 5) (Table 1). Surprisingly, dDAVP, a well-known V₂/V₁b agonist, had no agonist effect on cell proliferation. To determine the AVP receptor subtype involved in this stimulation, various reference peptide and specific nonpeptide antagonists were used. SSR-149415 counteracted 0.5 nM AVP-induced cell proliferation with the highest potency (Kᵢ = 0.71 ± 0.41 nM; n = 4), and its effect was stereospecific, since SSR-149424 failed to block the mitogenic effect of AVP up to 3 µM. dPen was much less potent and antagonized the AVP effect with a Kᵢ of 60 ± 35 nM (n = 3). The V₁a receptor antagonist SR-49059 had a weaker potency in blocking cell proliferation (Kᵢ = 411 ± 286 nM; n = 3), and the V₂ receptor antagonist SR-121463 failed to inhibit this effect, even at high concentrations (up to 3 µM).

**Expression of V₁b mRNA in In-R1-G9 cells and in human pancreas.** By use of specific primers, V₁b receptor RNA expression was studied by PCR analysis in In-R1-G9 cells and human pancreas. A single band of 600 bp was generated from both cell types, in agreement with results obtained with CHO cells expressing human or rat V₁b receptors as a positive control. In human pancreas, signal intensity was low after 35-cycle amplification but was easily detectable 10 cycles later (Fig. 5). Amplification of the DNA sequence resulting from genomic contamination of RNA samples can be ruled out, because the primers were designed on two different exons, separated in the human genome by a 5,414-bp intron. Moreover, negative controls were used where RT was omitted during cDNA synthesis. Similar results were obtained with 3 other primer pairs (data not shown).
Localization of V₁b receptors in human pancreas by immunohistochemistry. In preliminary control experiments performed to validate the anti-V₁b receptor antibody and to check its specificity, immunolabeling was observed only in CHO cells expressing the V₁b receptor but not in wild-type CHO nor in cells transfected with V₁a or V₂ receptor cDNA (not shown). Further validation of the antibody was performed using reference normal human tissues. As expected, intense staining was observed in human anterior pituitary sections used as a positive control tissue, whereas in liver, an enriched V₁a receptor preparation, no labeling was detected (negative control) (Fig. 6, A and B). Under similar operating conditions, strong staining intensity was observed in human pancreas, confined to the endocrine cells of islets of Langerhans (Fig. 6C). Of note, some weak and patchy staining was occasionally observed in exocrine pancreas. Immunoabsorption of the primary V₁b receptor antibody with the rat immunogenic peptide sequence, used to raise the antibody, and with the corresponding human sequence peptide, abolished the immunosignal in the tissue sections of human pancreas (Fig. 6D) and pituitary (not shown). Equally, replacement of the primary antibody with buffer eliminated the presence of the immunosignal in both human pancreas and pituitary (not shown), in agreement with the specificity of labeling observed in pancreas and hypophysis.

Double immunofluorescent detection of V₁b receptors in islets of Langerhans with glucagon, insulin, or somatostatin was performed to further identify the cellular localization of the V₁b receptor in human endocrine pancreas. As shown in Fig. 7, these experiments revealed colocalization of the immunofluorescent signals for V₁b receptor and for each one of these hormones (glucagon, insulin, or somatostatin); the colocalization was evidenced by the yellow/orange color resulting from the superimposition of the green (anti-V₁b receptor antibody) and red (glucagon, insulin, or somatostatin antibody) colors (Fig. 7, C, F, and I, respectively). These experiments revealed the expression of the V₁b receptor protein in a significant number of glucagon- (Fig. 7B), insulin- (Fig. 7E), and somatostatin- (Fig. 7H) producing cells. Similar results were obtained with seven different human pancreas samples.

DISCUSSION

Both AVP and OT are found in rat and human pancreas (2), and functional studies performed in several models using perfused pancreas and various rodent endocrine pancreatic islets or cell lines (HIT-T15,
In-R1-G9 and RINm5F) have shown that AVP and OT receptors may be involved in insulin and glucagon secretion. More recently, it was suggested that AVP could mediate insulin and glucagon release via activation of V1b receptors (10, 13, 18, 32, 33). In the present study, AVP receptors present in In-R1-G9 cells, a hamster glucagon-secreting α-pancreatic cell line, were characterized by using SSR-149415, a selective non-peptide V1b receptor antagonist, its low-affinity diastereoisomer (SR-149424), and various reference AVP/OT ligands. We demonstrated that the V1b receptor, extensively characterized in binding studies, is involved in AVP-induced [Ca^{2+}]_i increase and glucagon secretion and also mediates In-R1-G9 cell proliferation. By RT-PCR, we showed the presence of V1b receptor mRNA in these cells and in human pancreas. Finally, the distribution of the V1b receptors in human pancreas was also addressed. Using double-fluorescence immunohistochemistry, we observed specific cellular localization for the V1b receptor protein in glucagon, insulin, and somatostatin cells of islets of Langerhans, suggesting a potential role for AVP in the regulation of

Fig. 7. Colocalization study of V1b receptor immunoreactivity in islets of Langerhans in human pancreas by double immunofluorescence with anti-glucagon, anti-insulin, or anti-somatostatin antibodies. Reactivity with the primary anti-V1b antibody was traced with the tyramide amplification reagent containing fluorescein (green) and anti-glucagon, anti-insulin, or anti-somatostatin with Alexa 594 (red). Note corresponding distribution of V1b (A, D, G, green) and glucagon (B), insulin (E), or somatostatin (H) in 1 islet of Langerhans. In C, F, and I, colocalization is recognized by a brighter green to orange-yellow color (see white arrowheads for illustration). Blue represents nuclei stained with 4',6-diamidino-2-phenylindole (DAPI), a nuclear marker. Bar, 10 μm. Results shown here represent 1 of 7 similar experiments.
endocrine pancreas functions via V1b receptor activation.

[3H]AVP binding, performed for the first time on In-R1-G9 cells, was time dependent and showed high affinity (Kd = 0.27 ± 0.07 nM), specificity, and saturability (25 fmol/mg protein, i.e., ~4,000 sites/cell). In competition studies using different peptides with various selectivities for the AVP/OT receptors and selective nonpeptide V1a (SR-49059), V1b (SSR-149415), and V2 (SR-121463) receptor antagonists, we observed a potency order consistent with a typical V1b receptor profile (Table 1). The Kd/Ks values obtained are in good agreement with those reported for the rat and human pituitary V1b receptor (7, 14, 20, 27, 29). From a quantitative point of view, Kd values in In-R1-G9 cells are closer to rat than to human V1b receptors expressed in CHO cells. Of note, OT exhibited a 20- to 50-fold higher affinity for hamster In-R1-G9 and rat V1b receptors than for the human ones. This may reflect species differences very common in the AVP/OT family (17). It is important to underline that SSR-149415, a high-affinity V1b receptor antagonist for rat, bovine, and human (25), also behaves as a potent competitive antagonist at pancreatic V1b receptors in hamster In-R1-G9 cells.

Functional characterization of the V1b receptors identified on In-R1-G9 cells was further addressed in several in vitro models. Earlier cellular events provoked by the occupancy of V1b receptors by AVP include activation of phospholipase C and protein kinase C, inositol 1,4,5-trisphosphate production, and the mobilization of intracellular free Ca2+, mainly via Gq/11 protein recruitment. In α-cells of the endocrine pancreas, it has been shown that Ca2+ is a key signal for AVP to trigger glucagon secretion; of note, Ca2+ transients have been studied extensively in In-R1-G9 cells (19, 31). Recently, in CHO cells transfected with the human V1b receptors, other intracellular pathways have also been described (e.g., cAMP production, stimulation of DNA synthesis, and MAP kinase activation), clearly depending on the level of the V1b receptor expression (29). Thus we explored these functions of AVP and V1b receptors in In-R1-G9 cells. Taken together, a generally good correlation was observed between AVP and OT analog binding to In-R1-G9 membranes and the physiological responses elicited on [Ca2+]i increase, glucagon release, and In-R1-G9 cell proliferation (Table 1). In addition, the use of potent and selective V1a, V1b, and V2 blockers confirmed the involvement of V1b receptors in these effects; whatever the model, SSR-149415 has a powerful effect in antagonizing AVP functional effects, whereas the V1a antagonist SR-49059 was much less effective and the V2 blocker SR-121463 was devoid of any effect in counteracting AVP stimulation. Moreover, SSR-149415 exerted a marked stereospecific inhibition on both binding and AVP responses, another argument for supporting a specific V1b-mediated action. In the different tests, AVP induced dose-dependent stimulation (EC50 values from 0.08 to 18 nM; Table 1). The least efficacy was obtained on AVP-induced [Ca2+]i increase (EC50 = 18 ± 7 nM), suggesting that a threshold occupancy of V1b receptors is required for full [Ca2+]i mobilization. Other explanations involving the operating conditions specific to the test could also explain the difference between affinity and stimulation of [Ca2+]i efficacy. A striking finding in this work is the subnanomolar efficacy of AVP in stimulating cell growth, a property described for the first time in pancreatic In-R1-G9 cells. AVP has been associated with cell proliferation and mitogenicity in various tissues and cell lines. Of note, in CHO cells transfected with the human V1b receptors, Thibonnier et al. (29) reported stimulation of DNA synthesis and MAP kinase activation clearly depending on the level of V1b receptor expression. The presence of V1b receptors has been also reported in some small-cell lung cancer tumors (16), and the V1b (V3) receptor gene is overexpressed in corticotropin-secreting tumors (8). Thus a potential role of V1b receptors in pancreatic cell growth or during pancreas development needs to be explored further.

According to the literature, the role and the mechanism of action of AVP and OT in the pancreas is a matter of debate; the two hormones AVP and OT and their receptors are found in the pancreas, and OT (like AVP) has also been associated with glucagon and insulin release in the perfused rat pancreas model. It has been suggested that pancreatic β-cells may possess AVP (V1-like/V1b) receptors or that OT receptors could cross-react with pancreatic AVP receptors. Recent data showed that, in physiological situations, AVP and OT induced glucagon secretion in the perfused rat pancreas through activation of V1b and OT receptors, respectively (32). Experiments by fluorescent labeling with OT and AVP and [3H]OT in rat pancreas sections also support the presence of specific OT receptors in glucagon-producing α-cells of islets of Langerhans in rats (26). In the present work, by combining binding with selective ligands, various functional studies using a specific blocker, and a molecular approach with specific primers directed against the V1b receptor cDNA, we demonstrated that the V1b receptor is present in hamster In-R1-G9 α-cells and is responsible for Ca2+ transients, cell proliferation, and glucagon secretion and that OT has a much lower efficacy than AVP on these cells. Thus the OT effects observed in In-R1-G9 are mediated by V1b receptor, and not by OT receptor, activation.

Obviously, the absence of human pancreatic endocrine cell lines available for cell culture is a major drawback in evaluating the role of V1b receptors in pancreatic functions in humans, even though we confirmed in this study the presence of V1b mRNA in both animal and human pancreas. Therefore, to complete this work, we addressed the expression pattern of the V1b receptors in normal human pancreas by use of an immunohistochemistry approach. Colocalization was evidenced in a number of glucagon, insulin, and somatostatin cells, indicating a wide and specific distribution of the V1b receptor in endocrine pancreas. Among various regulatory hormones, insulin and glucagon are the primary agents responsible for controlling glucose.
levels in the blood. Diabetes is a polygenic disease characterized by insulin deficiency and/or insensitivity, with elevated glucose plasma levels. Of note, the existence of type 1 or type 2 diabetes has been correlated with high plasma levels of glucagon, which contributes to the hyperglycemia of diabetes by inappropriately stimulating hepatic glucose secretion (6).

Moreover, in this disease, the glucagon effect is no longer counterbalanced by the opposing action of insulin. Several studies have also reported that plasma AVP is elevated in patients with type 1 or 2 diabetes as well as in various animal models (experimental or genetic) of diabetes, even if this AVP increase is poorly understood (3). On the basis of this latter observation and the results of the present report, one can speculate that, in the diabetic state, AVP could be involved (at least in part) in the abnormal glucagon secretion by stimulating V1b receptors present in pancreatic a-cells. Interestingly, glucagon receptor antagonists, which act downstream on the AVP/V1b receptor pathway, dropped plasma glucose levels in diabetic rats (6).

In conclusion, the pancreatic receptors involved in intracellular Ca\(^{2+}\) increase, cell proliferation, and glucagon release in In-R1-G9 cells fulfill all the criteria of V1b AVP receptors. These receptors are functional and display high affinity for AVP. All these effects are blocked by a selective V1b receptor antagonist such as SSR-149415. The present work also provides new insight into the expression pattern of V1b receptors in human pancreas and indicates a wide distribution of the V1b receptor protein in cells of islets of Langerhans, suggesting a potential control of AVP on endocrine pancreas.

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