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1 **Characterization of a functional V_{1B} vasopressin receptor in the male rat kidney, evidence for**
2 **crosstalk between V_{1B} and V₂ receptor signaling pathways**

3
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17
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26 **ABSTRACT**

27 Although vasopressin V_{1B} receptor (V_{1B}-R) mRNA was detected in the kidney, the precise renal
28 localization, pharmacological and physiological properties of this receptor remain unknown. Using the
29 selective V_{1B} agonist d[Leu⁴, Lys⁸]VP, either fluorescent or radioactive, we showed that V_{1B}-R is
30 mainly present in principal cells of the inner medullary collecting duct (IMCD) in the male rat kidney.
31 Protein and mRNA expression of V_{1B}-R were very low compared to V₂ receptor (V₂-R). On
32 microdissected IMCD, d[Leu⁴, Lys⁸]VP had no effect on cAMP production but induced a dose-
33 dependent and saturable [Ca²⁺]_i increase mobilization with an EC₅₀ in the nanomolar range. This effect
34 involved both intracellular calcium mobilization and extracellular calcium influx. The selective V_{1B}
35 antagonist SSR149415 strongly reduced the ability of vasopressin to increase [Ca²⁺]_i but also cAMP
36 suggesting a cooperation between V_{1B}-R and V₂-R in IMCD cells expressing both receptors. This
37 cooperation arises from a crosstalk between second messenger cascade involving protein kinase C
38 rather than receptor heterodimerization as supported by potentiation of the AVP-stimulated cAMP
39 production in HEK293 cells co-expressing the two receptor isoforms and negative results obtained by
40 bioluminescence resonance energy transfer experiments. *In vivo*, only acute administration of high
41 doses of V_{1B} agonist triggered significant diuretic effects by contrast with injection of selective V₂
42 agonist. This study brings new data on the localization and signaling pathways of V_{1B}-R in the kidney,
43 highlights a crosstalk between V_{1B}-R and V₂-R in IMCD and suggests that V_{1B}-R may counterbalance
44 in some pathophysiological conditions the antidiuretic effect triggered by V₂-R activation.

45

46 **Keywords:** Vasopressin V_{1B} and V₂ receptor, inner medullary collecting duct, calcium and cAMP
47 signaling, receptor heterodimerization, diuresis

48 INTRODUCTION

49 In mammals the nonapeptide arginine vasopressin (AVP) synthesized in the hypothalamus is
50 involved in multiple physiological effects including inhibition of diuresis, vasoconstriction of vascular
51 smooth muscle cells, stimulation of hepatic glycogenolysis and modulation of adenocorticotrophic
52 hormone (ACTH) release and in modulating social behavior in human and rodent (1-4). The effects of
53 AVP are mediated by stimulation of tissue-specific G protein-coupled receptors classified in two
54 major types, V_1 and V_2 , according to their transduction pathways. The vasopressin V_1 receptor (V_1 -R),
55 later subdivided into V_{1A} and V_{1B} subtypes, mediates signaling through Ca^{2+} and diacylglycerol (DAG)
56 transducer pathways (5, 6) whereas the vasopressin V_2 receptor (V_2 -R) signals mainly through cyclic
57 adenosine 3',5'-monophosphate production (cAMP) (7, 8).

58 In addition to this classification, another type of vasopressin receptor, called " V_2 -like" has been
59 identified in different tissues including the inner medullary collecting duct (IMCD) of the kidney (9-
60 13). The stimulation of this atypical " V_2 -like" receptor by the V_2 -R agonist 1-desamino-8-D-arginine
61 vasopressin (dDAVP) induces an increase in $[Ca^{2+}]_i$ in IMCD and this receptor isoform has been
62 proposed to be the V_{1B} -R (14). Indeed V_{1B} -R mRNA was detected in the renal medulla and protein
63 displayed pharmacological characteristics of V_2 -like receptor in cultured cells of rat IMCD similar to
64 that of human V_{1B} -R expressed in Chinese hamster ovary (CHO) cells (13, 14).

65 The IMCD plays a key role in the establishment of the corticomedullary osmolality gradient and
66 urinary concentrating mechanism. Vasopressin binding to basolateral V_2 -R promotes insertion of
67 aquaporin 2 (AQP2) into the luminal face of the CD and increases water permeability. Yet, Knepper
68 and collaborators demonstrated that vasopressin-stimulated $[Ca^{2+}]_i$ increases via V_1 -R may be
69 responsible for a negative control of water reabsorption in IMCD tubules (12). Moreover, vasopressin,
70 upon binding to V_2 -R, increases urea permeability through activation of the urea transporter UT-A1 in
71 the terminal part of IMCD, thus allowing the delivery of concentrated urea to the renal papillary
72 interstitium, a critical step in the urine concentration (15, 16). Beside these functional effects,
73 sustained elevated level of AVP concentration *in vivo* has been shown to stimulate proliferation of
74 tubular cells expressing the V_2 -R in the renal medulla (17). So far, the physiological role of V_{1B} -R in
75 the kidney, especially in IMCD, is unknown, yet to be determined. A role for V_{1B} -R in the body water

76 balance was suggested by the higher water intake and daily urine volume observed in V_{1B}-R knock-out
77 mice compared with wild-type mice (18).

78 Thus, the present work was undertaken (1) to precise the localization of the V_{1B}-R mRNA and
79 protein within the rat kidney, (2) to study on freshly microdissected IMCD the coupling of V_{1B}-R to
80 second messenger cascades (cAMP production and intracellular calcium mobilization) and (3) to
81 attempt to elucidate the physiological role of the V_{1B}-R in participating with the V₂-R to a fine
82 regulation of vasopressin action at the kidney level.

83

84

85 MATERIAL AND METHODS

86 Drugs

87 Most standard chemicals were purchased from Sigma (St Louis, MO), Roche Molecular
88 Biochemicals (Mannheim, Germany) or Merck and Co (Darmstadt, Germany) unless otherwise
89 indicated. Bromodeoxyuridine (BrdU) and phorbol-12-myristat-13-acetate (PMA) were from Sigma
90 (Lisle d'Abeau Chesne, France). Gö6976 was from Merck Chemistry (Fontenay-sous-Bois, France).
91 Arginine vasopressin (AVP) and 1-desamino-8-D-arginine vasopressin (dDAVP) were from Bachem
92 (Bubendorf, Switzerland). SSR49059, SSR121463B, SSR126768A, SSR149415 were kindly provided
93 by Sanofi (Sanofi, Toulouse, France). [³H]AVP (60-80 Ci/mmol) and [³H]adenine (27 Ci/mmol) was
94 obtained from Perkin-Elmer Life Sciences (Courtaboeuf, France). Lipofectamine2000 was from
95 Invitrogen. cAMP measurements were performed with the Gs Dynamic kit from Cisbio Bioassays
96 (Codolet, France). d(CH₂)₅[Tyr(Me)²]AVP ("Manning compound"), d[Leu⁴, Lys⁸]VP and (d[Leu⁴,
97 Lys(Alexa647)⁸]VP were synthesized and kindly provided by Dr M. Manning (College of Medicine,
98 Toledo, USA); [³H]d[Leu⁴, Lys⁸]VP (87.8 Ci/mmol) was synthesized by Dr C. Tomboly (Biological
99 Research Center, Szeged, Hungary) and characterized in our group ((19) and this study). AQP2 rabbit
100 polyclonal antibody (Cat SC-9882, lot 1295) was from Santa Cruz Biotechnologies (Dallas, USA).
101 The mouse monoclonal anti-BrdU antibody (BU33-CatB8434) was from Sigma (L'isle d'Abeau,
102 France). The Cy3-labeled secondary goat anti-rabbit antibody (Cat 711-160-132) and the Cy3-labeled

103 secondary donkey anti-mouse antibody (Cat 715-167-003) were from Jackson ImmunoResearch
104 (Interchim, Monluçon, France).

105

106 **Animals**

107 Male Sprague Dawley rats (200-250g) (Janvier, Le Genest-St-Isle, France) were housed in light
108 (12h dark and 12h light) and temperature (21°C) controlled rooms and had free access to standard dry
109 food and tap water. All procedures in this study were conform to the animal welfare guidelines of the
110 European Community and were approved by the local ethical committee (authorizations n° 34.128 for
111 experimentation).

112

113 **Binding assay on plasma membranes**

114 Membrane preparation and incubation with radioligands such as [³H]AVP or [³H]d[Leu⁴,Lys⁸]VP
115 were performed as previously described (19). Briefly, rats were sacrificed by cervical dislocation,
116 decapitated and kidney were quickly removed and placed in ice-cold homogenization buffer. Crude
117 plasma membranes were prepared from inner medulla (IM) and used immediately for binding
118 experiments. For saturation binding experiments, 10-100 µg membrane protein were incubated for 60
119 min at 37°C in 200 µL of a medium containing 50 mmol/L Tris-HCl (pH 7.4), 3 mmol/L MgCl₂, 1
120 mg/mL Bovine Serum Albumin (BSA), 0.01 mg/mL leupeptin and increasing concentrations (0.5 to
121 15 nmol/L) of tritiated hormone with (non-specific binding) or without (total binding) 0.1 µmol/L of
122 unlabeled AVP. For competition experiments, membrane proteins were incubated as described above
123 with 1 nmol/L of [³H]AVP or [³H]d[Leu⁴, Lys⁸]VP and increasing amounts of V_{1B}-R antagonist
124 SSR149415. Plasma membrane-associated radioactivity was collected by filtration through GF/C
125 filters and counted. Specific binding was calculated in each condition as the difference between total
126 and non-specific binding. The data were analyzed by GraphPad Prism™ (graphPad software Inc, San
127 Diego, CA, USA). K_d and B_{max} were deduced from Scatchard experiments and K_i from competition
128 experiments.

129 To validate the new radioactive probe [³H]d[Leu⁴, Lys⁸]VP, crude plasma membranes from rat
130 tissues such as liver or kidney naturally expressing V_{1A}-R or V₂-R respectively were challenged.
131 Membranes from transiently transfected CHO or AtT20 cells expressing rat OT-R or V_{1B}-R were also
132 used as previously described (19). Results showed that the [³H]d[Leu⁴, Lys⁸]VP exhibited a very good
133 affinity (0.36 nmol/L) for the rat V_{1B}-R and a relatively good selectivity vs the other rat vasopressin
134 receptor isoforms (K_d of 25.6 nmol/L for V_{1A}-R, 5.7 nmol/L for V₂-R and 5.6 nmol/L for OT-R). This
135 selectivity was better than the one of [³H]AVP, which displayed a similar K_d for all receptor types (1.5
136 nmol/L for V_{1A}-R, 0.7 nmol/L for V₂-R, 0.9 nmol/L for OT-R and 0.5 nmol/L for V_{1B}-R (R. Marir
137 unpublished results). Moreover, the high specific radioactivity (87.8 Ci/mmol) of [³H]d[Leu⁴, Lys⁸]VP
138 allowed detecting low amounts of V_{1B}-R binding sites.

139

140 **Labeling and imaging rat kidney slices using V_{1B}-R fluorescent analogue**

141 Rats were sacrificed, kidneys were immediately immersed in the sectioning buffer (20) and
142 sectioned in 350 µm-thick slices at 2°C in sagittal orientation. The labeling protocol was previously
143 published (21). Briefly, the slices were collected and incubated in 12-well plates first for 30 min at
144 12°C in 1ml of DMEM, 0.2 mg/ml BSA, 25 mmol/L HEPES buffer, pH 7.4 containing 250 nmol/L of
145 the Manning compound to prevent any V_{1A}-R/OT-R detection, then for 1h with 150 nmol/L d[Leu⁴,
146 Lys(Alexa647)⁸]VP and in the presence of the same V_{1A}-R /OT-R antagonist. Non-specific binding
147 was evaluated on adjacent slices by displacement in the presence of an excess (1 µmol/L) of AVP.
148 Slices were washed 3 times with 1.4 ml of PBS on ice and 4 % PFA was added for overnight fixation
149 at 4°C. After 2 new washings, antibodies were added at the relevant dilution in PBS-2 mg/ml BSA-0.2
150 % Triton (1 ml/slice) and the 12-well plate was incubated overnight at 4°C. To localize principal cells
151 of kidney tubule, a rabbit polyclonal AQP2 antibody was used at a final dilution 1/1000 (overnight at
152 4°C). The next day, after 3 washings (1.4 ml PBS), the Cy3-labeled secondary antibody goat anti-
153 rabbit (1/2000) was added in the same PBS/BSA/triton buffer and the slices were incubated for 1h at
154 room temperature with gentle rocking. After 3 washings, 0.5 µl of Hoechst in 1ml PBS was added,
155 incubated at least for 15 min and rinsed. Slices were kept in sodium azide 0.2% at 4°C before imaging.

156 Imaging was performed with a macroconfocal wide field LSI Leica microscope using a 5X air
157 objective. Slices were mounted in chambers consisting of glass slides equipped with spacers finally
158 covered with a coverslip and filled with PBS at room temperature. Three lasers were used: Diode 405
159 (Hoescht) for detecting nuclei, Helium/Neon 561 nm (Cy3) for AQP2 and Helium 633 for Alexa647-
160 labeled V_{1B}R ligand bound to receptors with the corresponding window settings for collecting
161 emissions. Overlapping was avoided by correct and exclusive adjustment of the emission windows.
162 Images (.lif) were converted by the Leica software in .tif and mounted using Photoshop.

163

164 **Microdissection of nephron segments**

165 The left kidney of male Sprague Dawley rats, 150 g body weight, was prepared for
166 microdissection of nephron as previously described (22). Pieces of collecting duct (CD) were isolated
167 under stereomicroscopic observation from the cortex (CCD), the outer (OMCD) and the inner medulla
168 (IMCD). Afferent arterioles were isolated with the glomerulus and identified according to their
169 morphology as previously described (23). Microdissected segments (3 to 13) were obtained from three
170 to eight rats for each mean experimental value.

171

172 **Quantification of mRNA expression levels by real-time RT-PCR**

173 Approximately 20 mm tubular length were used for total RNA extraction. Total RNAs were
174 extracted using Trizol LS reagent (Invitrogen, Basel, Switzerland) according to the manufacturer's
175 instructions. RNA (1 µg) was first reverse transcribed using Superscript III reverse transcriptase
176 (Invitrogen) and 250 ng of random hexamer (Amersham Biosciences Europe, Orsay, France) in a final
177 volume of 20 µl. Real-time PCR were performed using SYBR Green PCR master mix (Applied
178 Biosystems, Foster City, CA) with 1:10 of the reverse-transcription product and were carried out on
179 ABI 7500 Sequence Detector (Applied Biosystems). The sequences of the primers used (concentration
180 300 nmol/L) were: 5'CCAATGAAGATTCTACCAATGTG3' (forward) and
181 5'ATGGTGGCTCAAGGAACG3' (reverse) for *Avpr1b* mRNA, 5'GTGCCATCTGCCGCCCTAT 3'
182 (forward) and 5'CCCACTGCCATTTCCCACATC3' (reverse) for *Avpr2* mRNA and finally

183 5'ATGATTCTACCCACGGCAAG3' (forward) and CTGGAAGATGGTGATGGGTT3' (reverse) for
184 *Gapdh* mRNA used as internal control. After an initial denaturation step of 10 min at 95°C, the
185 thermal cycling conditions were 40 cycles at 95°C for 15 s and 60°C for 1 min. Each sample was
186 tested in triplicate. Data were normalized to the expression levels of GAPDH mRNA.

187

188 **Intracellular calcium concentration measurement**

189 Experiments were performed as previously described (24). Briefly, microdissected nephron
190 segments and glomerular arterioles were loaded with 5 $\mu\text{mol/L}$ Fura-2 AM at room temperature for
191 120 minutes. Each nephron segment or arteriole was continuously superfused at 37°C with either
192 medium or the solutions to be tested. Vasopressin, V_{1B} selective agonist (d[Leu⁴, Lys⁸]VP), and V_2
193 selective agonist (dDAVP) were applied for 5 min. For experiments performed in the absence of
194 external Ca^{2+} , IMCD were superfused in Ca^{2+} -free medium 2 min before adding the agonist. For
195 studies with specific antagonists, collecting ducts were superfused for 15 min before addition of the
196 agonist. The calcium response was evaluated by determining the magnitude of the response ($\Delta[\text{Ca}^{2+}]_i$)
197 corresponding to the difference between peak and basal concentrations (in nmol/L) or by determining
198 the area under the curve (AUC, in $\text{nmol}\cdot\text{sec/L}$) obtained by the integral of the Ca^{2+} signal.

199

200 **Intracellular cAMP measurement in microdissected IMCD**

201 Cyclic AMP levels were measured in microdissected IMCD by using the Gs Dynamic2 kit based
202 on homogeneous time-resolved fluorescence (HTRF) technology. The method is a competitive
203 immunoassay between native cAMP produced by the cells of the collecting duct and the cAMP
204 labeled with the dye 2. The tracer binding is visualized by a Mab anti-cAMP labeled with cryptate.
205 Results are expressed in fmoles of cAMP produced per 0.4 mm tubular length and per 10 min
206 incubation time at 37°C.

207

208 **Measurement of cAMP accumulation in HEK293 cells co-expressing V_{1B} and/or V_2**
209 **receptors**

210 HEK293 cells were seeded in 96 well-plates pre-coated with poly-ornithine at the density of 30,000
211 cells/well and transfected with V_{1B}-R and/or V₂-R plasmids using Lipofectamine 2000 according to
212 the manufacturer. DNA constructs were generated by PCR amplification using the full-length human
213 wild-type V_{1B}-R (25) and V₂-R (kindly provided by D. Devost and M. Bouvier, Montreal University,
214 Canada) as previously described. Forty-eight hours after transfection, cells were pretreated with PMA
215 (15 min, 1 μmol/L) and/or Gö6976 (30 min, 100 nmol/L), then treated for 30 min at 37°C with or
216 without AVP in the presence of phosphodiesterase inhibitor (RO201724 0.1mmol/L). The cAMP
217 produced was detected using the HTRF Gs Bioassay (Dynamic 2 kit, CisBio). Plates were read on a
218 PHERAstar FS (BMG Labtech, Champigny, France). The specific FRET signal was calculated using
219 the following equation: $\Delta F\% = 100 \times (R_{pos} - R_{neg}) / (R_{neg})$, with R_{pos} being the fluorescence ratio
220 (665/620nm) calculated in wells incubated with both donor-labeled antibody and acceptor d2, and
221 R_{neg} being the same ratio for the negative control incubated only with the donor fluorophore-labeled
222 antibody. The FRET signal ($\Delta F\%$), inversely proportional to the quantity of cAMP, was then
223 transformed in cAMP concentration using a calibration curve prepared on the same plate.

224

225 **Bioluminescence resonance energy transfer assay (BRET)**

226 The V_{1A}-R, V₂-R and OT-R ~~different~~ hybrid constructs were generated from human receptors
227 subcloned in PRK5 vector using the GeneEditor In Vitro Site-Directed Mutagenesis System (Promega
228 Corp, Madison, USA) as described by Terrillon et al (26) (gift from Dr Devost and M. Bouvier,
229 Montreal, Canada). The hV_{1B} and hV_{1B}-RL were respectively cloned in pRL-CMV and pcDNA3.0
230 vectors whereas CRF1-EYFP was in pEYFP-N1 (Clontech) and GABA_B2R-EGFP in pcDNA3.0
231 according to the constructions used in our previous work (25). All vectors contained similar CMV
232 promotor and SV40 polyadenylation sites for comparable expression levels. Mild levels of receptor
233 expression were chosen in these experiments to mimic physiological conditions (less than 1 pmol/mg
234 protein (25)). BRET experiments were conducted as previously described (25). Briefly, human wild
235 type hV_{1B}-R, hV_{1B}-RL and/or hV₂-EYFP receptors were transiently transfected in HEK293 cells using
236 the Jet PEI technique with a constant amount of the donor construct (hV_{1B}-RL, 1 μg of DNA), and
237 from 0.03 to 10 μg of acceptor (hCRF₁-EYFP, hV₂-EYFP or hGABA_{B2}-EYFP) DNA constructs in 96-

238 well plates, each well seeded with 20,000 cells. For BRET competition, hV₂-RLuc and hV₂-EYFP
239 were co-transfected at the same concentration of 1 µg each and wild type untagged receptors (hV_{1A}-R,
240 hV_{1B}-R or hOT-R) were added at increasing concentrations. Two days after transfection, HEK293
241 cells were washed twice in HBS medium, 5.55 mmol/L glucose and further incubated in the same
242 medium supplemented with 2.5 µM coelenterazine h substrate (Promega, France) in a total volume of
243 50 µL/well at 30°C. BRET reading was performed immediately using the Mithras LB 940 plate reader
244 (Berthod Biotechnologies, Oak Ridge, TN) allowing the integration of the short wavelength filter
245 (440-500 nm) and the long wavelength filter (510-590 nm). Background values obtained with mock-
246 transfected HEK293 cells were subtracted in total luminescence and in fluorescence measurements
247 and mean values of triplicate wells/sample were calculated. BRET signals were calculated and
248 expressed in milli BRET unit of BRET ratio.

249

250 **Urine flow measurement**

251 The effects of agonist or antagonist on diuresis were tested in separate series of Sprague Dawley
252 male rats. Rats were individually housed in metabolic cages with free access to tap water and
253 powdered food. After 3-5 days of habituation, they were divided into groups of equivalent urine
254 volume and osmolality. In experiment I, rats received an *i.p.* injection of vehicle (saline) or low (0.42
255 µg/kg) or high dose (72 µg/kg) of V_{1B}-R agonist d[Leu⁴, Lys⁸]VP, n = 5/group. In experiment II, rats
256 received by oral route either vehicle (2 ml/kg in 5% DMSO, 5% cremophor EL, 90% saline) or V_{1B}
257 antagonist SSR149415 (30 mg/kg), n = 6/group. Vehicle or drug were administrated at 10 a.m. Urine
258 was collected for 5-6 h starting just after the injection. Urine volume was determined gravimetrically,
259 assuming the density of urine was equal to unity. Urine osmolality was measured using a freezing
260 point osmometer (Roebing, Germany). Similar experiment was performed in rats pretreated with AVP
261 (400 ng/day infused *i.p.* for 5 days with Alzet minipumps) inducing an 300% increase of plasmatic
262 AVP (17) that is in physiologic range of plasma concentration of this hormone.

263

264 ***In vivo* measurement of renal proliferation upon vasopressin analogues administration**

265 Male Sprague Dawley rats were *s.c.* implanted for three days with osmotic minipumps (Alzet,
266 model 2001, Charles River, France) delivering d[Leu⁴, Lys⁸]VP (40 µg/kg.day), dDAVP (2 µg/kg.day)
267 or phosphate-buffer saline alone. *In vivo* cell proliferation studies were performed using 5-bromo-2-
268 deoxyuridine (BrdU) incorporation as previously described (17). Labeled nuclei were numbered on
269 four sections of different renal zones, and four animals per experimental group. Data were statistically
270 compared using the nonparametric test of Mann and Whitney.

271

272 **Data Analysis**

273 Results are expressed as the mean ± S.E. For experiments on [Ca²⁺]_i or cAMP, the number of
274 segments is indicated in the figure legends. Statistical differences were assessed using paired t-test
275 taking into account values obtained before the addition of the agonist (basal level) and those obtained
276 after stimulation with the agonist (peak value) or one-way ANOVA on weighted means followed by
277 Fisher's test.

278

279

280 **RESULTS**

281 **Evidence of V_{1B} mRNA and receptor binding sites in the inner medulla**

282 V_{1B} and V₂-R mRNAs were quantified by real-time RT-PCR in three parts of the collecting duct,
283 the CCD, the OMCD and the IMCD (Fig. 1A). The highest V_{1B}-R mRNA expression was found in
284 IMCD whereas a weak expression was detected in CCD and OMCD. V₂-R mRNA was present in all
285 segments and 211-fold more abundant than V_{1B}-R mRNA in IMCD.

286 Localization of V_{1B}-R protein within the rat kidney was studied using the selective fluorescent
287 V_{1B} analogue d[Leu⁴, Lys(Alexa647)⁸]VP (150 nmol/L), which exhibit a high fluorescence brightness
288 in the far red spectrum area where autofluorescence of the tissue is low (19). A discreet but
289 reproducible labeling was observed only in the inner medulla. This labeling was specific since it
290 completely disappeared when incubation with the fluorescent probe was performed in the presence of
291 an excess of unlabeled AVP (1 µmol/L) (Fig. 1B). Numerous cells exhibiting V_{1B}-R specific staining

292 (74 ± 6%, n = 30 cells from 3 distinct preparations) were also immunoreactive for the anti-AQP2
293 antibody (Fig. 1B bottom). As it is well known that AQP2 and V₂-R are expressed in the principal
294 cells of the renal collecting duct (27), this strongly suggests that V_{1B}-R was co-localized with V₂-R in
295 the IMCD cells, but the scattering of the fluorescent signal prevents to determine precisely the apical
296 or basolateral location of the receptor.

297

298 **Pharmacological characterization of V_{1B}-R in plasma membranes of inner medulla**

299 Radioligand binding assay with [³H]d[Leu⁴, Lys⁸]VP was used to quantify V_{1B}-R density in
300 plasma membranes from inner medulla. The specific binding of [³H]d[Leu⁴, Lys⁸]VP was dose-
301 dependent and saturable (Fig. 2A) as observed for [³H]AVP binding on plasma membrane from IMCD
302 (Fig. 2B). The Scatchard representation indicates the presence of a single class of binding sites
303 exhibiting a nanomolar affinity (K_d = 2.1 ± 0.3 nmol/L) and a low maximal binding capacity (B_{max} =
304 19 ± 5 fmol/mg protein) for [³H]d[Leu⁴, Lys⁸]VP. By comparison, experiments performed on the same
305 membrane preparation with [³H]AVP that binds to all vasopressin receptor isoforms gave a K_d of 0.7 ±
306 0.1 nmol/L and a B_{max} of 493 ± 62 fmol/mg protein. The binding selectivity of the radioligand
307 [³H]d[Leu⁴, Lys⁸]VP was further confirmed in heterologous displacement experiments. The V_{1B}-R
308 specific antagonist SSR149415 effectively displaced [³H]d[Leu⁴, Lys⁸]VP with a rather good affinity
309 (K_i = 29 ± 14 nmol/L), while it competed the for [³H]AVP binding sites with much lower affinity (K_i =
310 6400 ± 900 nmol/L) (Fig. 2C).

311

312 **Effect of d[Leu⁴, Lys⁸]VP on intracellular calcium mobilization along the collecting duct**

313 The [Ca²⁺]_i response to the V_{1B} selective agonist d[Leu⁴, Lys⁸]VP was tested in CCD, OMCD and
314 IMCD. In good agreement with the distribution of V_{1B}-R mRNA, 10 nmol/L d[Leu⁴, Lys⁸]VP elicited
315 significant [Ca²⁺]_i response only in the IMCD (Fig. 3A) and not in OMCD (Fig. 3B) or in CCD (Fig.
316 3C) whereas AVP induced a strong [Ca²⁺]_i response in all segments. The calcium response induced by
317 d[Leu⁴, Lys⁸]VP in the IMCD was rapid and reversible (Fig. 3A). This effect was dose-dependent,
318 discreet but robust, with an EC₅₀ of 19 ± 6 nmol/L (Fig. 3D). The increase in [Ca²⁺]_i was maximal with
319 100 nmol/L of d[Leu⁴, Lys⁸]VP with an E_{max} of 108 ± 22 nmol/L (n = 8). The calcium response

320 induced by AVP in this nephron segment was not affected by a first application of 100 nmol/L V_{1B}
321 agonist ($\Delta[Ca^{2+}]_i$: 343 ± 57 (n=8) and 339 ± 45 nmol/L (n=14) without and with pre-application,
322 respectively).

323 As the V_2 -R present in IMCD also elicits $[Ca^{2+}]_i$ increases (24), we compared the calcium
324 response induced by d[Leu⁴, Lys⁸]VP to that induced by the selective V_2 agonist dDAVP, and to the
325 natural hormone AVP respectively. Both selective agonists (V_{1B} and V_2 respectively) exhibited a
326 saturable dose-dependent response (Fig. 3D). The dDAVP was more efficient at low dose that is in
327 good agreement with its higher binding affinity for the V_2 -R. Interestingly, both the V_{1B} and V_2
328 agonists induced the same maximal response, 2.5 times lower than that obtained with the natural
329 hormone AVP (100 nmol/L) (Fig. 3E). We also investigated whether the responses induced by infra
330 maximal doses of d[Leu⁴, Lys⁸]VP (10 nmol/L) or dDAVP (1 nmol/L) were additive. As shown on
331 Fig. 3F, the simultaneous application of the two agonists at these concentrations elicited additive
332 effects.

333 To evaluate the relative importance of the intracellular Ca^{2+} release and/or external Ca^{2+} influx
334 induced by d[Leu⁴, Lys⁸]VP, we have compared the results obtained in the presence or absence of
335 external Ca^{2+} . As illustrated in Fig. 4A, the peak phase induced by 10 nmol/L d[Leu⁴, Lys⁸]VP was
336 attenuated and returned quickly to basal level in the absence of external Ca^{2+} . By comparing the
337 integral of the calcium responses obtained with and without 2 mmol/L of external Ca^{2+} , it was possible
338 to calculate the relative part of the intracellular calcium mobilization and calcium influx. As shown in
339 Fig. 4B, activating the V_{1B} receptors mainly triggered Ca^{2+} influx (about 70% of the global calcium
340 response) rather than the release from intracellular pools. By contrast, an opposite profile was obtained
341 using dDAVP where influx and calcium release participated equally to the global calcium response
342 (Fig. 4 C and 4D).

343

344 **Specificity of the $[Ca^{2+}]_i$ response induced by d[Leu⁴, Lys⁸]VP in IMCD**

345 To confirm the respective contribution of V_{1B} and V_2 -R to the AVP-stimulated Ca^{2+} mobilization,
346 we performed experiments with selective AVP and oxytocin receptor antagonists. The effect of 10
347 nmol/L d[Leu⁴, Lys⁸]VP on the $[Ca^{2+}]_i$ responses was totally abolished by a 15 min pretreatment with

348 100 nmol/L of the selective V_{1B} antagonist SSR149415 and could be restored after washing (Fig. 5A),
349 whereas this antagonist had no effect on $[Ca^{2+}]_i$ response induced by the V_2 agonist dDAVP (Fig. 5B).
350 Pretreatment with the specific V_2 antagonist SSR121463B totally blocked the $[Ca^{2+}]_i$ response to
351 dDAVP whereas carbachol, used as a control for cell viability, induced a clear calcium response (Fig.
352 5C).

353 Considering the effect of V_{1B} specific agonist $d[Leu^4, Lys^8]VP$ in the IMCD, pretreatments with
354 specific V_2 , V_{1A} , or OT antagonists (10 nmol/L SSR121463B, 100 nmol/L SSR49059 or 100 nmol/L
355 SSR126768A respectively) did not significantly affect the $[Ca^{2+}]_i$ response to $d[Leu^4, Lys^8]VP$ (basal:
356 73 ± 19 nmol/L ($n = 4$) vs 38 ± 5 nmol/L ($n = 6$), or vs 45 ± 3 nmol/L, ($n = 3$), or vs 52 ± 6 nmol/L (n
357 $= 14$) respectively (Fig. 5D-5F). This indicates that V_2 -R, V_{1A} -R or OT-R do not contribute to the
358 $d[Leu^4, Lys^8]VP$ -induced Ca^{2+} activation.

359 The pharmacological selectivity of the $d[Leu^4, Lys^8]VP$ was further verified on two other freshly
360 microdissected renal structures using the calcium mobilization assay. In glomerular afferent arterioles
361 known to express only V_{1A} -R localized on vascular smooth muscle cells (28), 10 nmol/L $d[Leu^4,$
362 $Lys^8]VP$ did not induce any significant response (Fig. 5G). By contrast, calcium response induced by
363 AVP was fully antagonized by pretreatment with the selective V_{1A} antagonist SSR49059 (Fig. 5H). In
364 OMCD that expresses the V_2 -R and V_{1A} -R (1) but not V_{1B} -R, $d[Leu^4, Lys^8]VP$ had no significant
365 effect on $[Ca^{2+}]_i$ whereas 10 nmol/L dDAVP induced a marked response (Fig. 5I). This effect of
366 dDAVP was fully abolished by pretreatment with 10 nmol/L of the specific V_2 antagonist
367 SSR121463B. The cell viability of the structures was verified by subsequent application of angiotensin
368 II (AngII) (Fig. 5J) that promoted the expected Ca^{2+} mobilization. These data demonstrate the
369 specificity of the V_{1B} and V_2 agonists both used at 10 nmol/L in this study.

370

371 **Effect of $d[Leu^4, Lys^8]VP$ on cAMP accumulation in IMCD**

372 As illustrated on Fig. 6A, $d[Leu^4, Lys^8]VP$ did not significantly stimulate cAMP production even
373 at the concentration of 100 nmol/L, a value allowing an almost complete occupation of V_{1B} -R (fig 2A).

374 In contrast, saturating doses of dDAVP (10 nmol/L) or AVP (50 nmol/L) induced a robust and
375 significant cAMP production (3.4-fold and 6.4-fold stimulation over basal level, respectively).

376 By combining 10 nmol/L d[Leu⁴, Lys⁸]VP (a dose inducing 45% of maximal V_{1B}-R occupancy
377 and ineffective to stimulate cAMP) with 0.1 nmol/L dDAVP (a dose inducing 26% of maximal V₂-R
378 occupancy (29), we observed a weak increase in the dDAVP-induced cAMP production but this effect
379 remained not significant (Fig. 6B).

380

381 **Effect of SSR149415 on cAMP production and intracellular calcium mobilization induced** 382 **by AVP in IMCD**

383 To elucidate the potential role of V_{1B}-R on the overall response of the natural hormone AVP, we
384 also evaluated the effect of a selective V_{1B} antagonist on signaling responses induced by AVP. As
385 shown on Fig. 7A and 7B, 100 nmol/L of SSR149415, which had no effect on basal level of [Ca²⁺]_i
386 and cAMP, reduced by 38% and 72% the maximal AVP-stimulated effect on [Ca²⁺]_i and cAMP
387 production respectively. It suggests a possible synergism between V_{1B}-R and V₂-R during AVP
388 stimulation since V_{1B}-R activation by d[Leu⁴, Lys⁸]VP did not increase cAMP level by itself (Fig. 6A).
389 As controls, we performed similar experiments using 10 nmol/L of dDAVP. Preincubation with 100
390 nmol/L SSR149415 had no effect either on dDAVP-stimulated [Ca²⁺]_i increase (Fig. 5B) or on
391 dDAVP-stimulated cAMP production (111 ± 17 fmol/0.4 mm in the absence *vs* 99 ± 17 fmol/0.4 mm
392 in the presence of V_{1B} antagonist, n = 4-5 independent determinations).

393

394 **V_{1B} and V₂-R synergism evaluated in HEK293 cells**

395 To decipher which mechanism was involved in V_{1B}-R/V₂-R synergism, we performed
396 experiments on HEK293 cells on which we may manipulate the relative amounts of hV_{1B} and hV₂-R
397 by transfection. We decided to use the human V_{1B}-R and V₂-R since i) the tagged versions of these
398 two human VP receptors have been previously generated and pharmacologically validated in the lab
399 (25) and ii) as compared to the rat VP receptors, the hV_{1B}-R and hV₂-R exhibit very similar
400 pharmacological properties and high sequence homologies in the intracellular loops that are involved
401 in G protein-coupling and to second messengers production (30).

402 First, we tested the hypothesis of a crosstalk between hV_{1B}-R and hV₂-R. As illustrated in Fig.
403 8A, co-expression of increasing amounts of hV_{1B}-R cDNA with a constant amount of hV₂-R cDNA
404 synergized the V₂-cAMP response in a dose-dependent manner. Maximal potentiation (around 30%)
405 was obtained for an hV_{1B}/hV₂-R ratio of 10. Interestingly this potentiation was observed only on
406 doubled transfected cells and was completely blocked by a pre-incubation with the V_{1B} antagonist
407 SSR149415 (Fig. 8B). As a control, we verified on singled hV₂-R transfected cells that SSR149415
408 did not modify the AVP-stimulated cAMP dose-response (Fig. 8C). To further analyze which
409 mechanism triggers the hV₂-R cAMP potentiation, we examined the effect of PMA, a potent PKC
410 activator. PMA significantly potentiated the AVP-stimulated cAMP accumulation both in hV₂-R
411 transfected cells and in hV_{1B}/hV₂-R doubled transfected cells (Fig. 8D and 8E). Such effect was
412 blunted by the PKC inhibitor Gö6976 (Fig. 8F). All these data suggest that AVP which stimulates
413 PKC *via* the hV_{1B}-R activation, may potentiated the AVP/cAMP response triggers by the hV₂-R
414 stimulation.

415 We have previously shown that the hV_{1B}-R and hCRF1-R act in synergism involving crosstalk at
416 the level of second messengers and also receptor heterodimerization (25). In the present study, we
417 verified if hV₂/hV_{1B} receptors heterodimerization may also participates to the V₂/V_{1B} second
418 messenger crosstalk. Indeed, receptor-tagged versions (hV_{1B}-RL) and (hV₂-EYFP) expressed in
419 HEK293 cells led to similar cAMP results as the non-tagged vasopressin receptors (data not shown).
420 HEK293 cells were then co-transfected with hV_{1B}-RL and hV₂-YFP receptors and physical
421 interactions were challenged by direct BRET. As previously described (25), in the presence of hV_{1B}-
422 RL as a donor, increasing concentrations of hCRF₁-YFP as acceptor lead to a saturable curve
423 indicating energy transfer (Fig. 9A). Transfection of hV₂-EYFP in the presence of hV_{1B}-RL showed a
424 linear, non-saturable curve of BRET, typical of that observed when no dimerization occurs as it was
425 also observed for hV_{1B}-RL/hGABA_{B2}-EYFP (Fig. 9A). The lack of hV_{1B}/hV₂-R heterodimerization
426 was confirmed by BRET competition experiments (Fig. 9B) since adding increasing amounts of DNA
427 encoding untagged wild type hV_{1B}-R to pre-existing hV₂-RL/hV₂-EYFP association was ineffective to
428 interrupt BRET signal, on the contrary to hV_{1A}-R or hOT-R known to heterodimerize with hV₂-R (26).

429 Such results indicate that the potentiation of cAMP response induced by AVP in hV₂/hV_{1B} co-
430 transfected HEK293 cells could not account for V₂/V_{1B} vasopressin receptor heterodimerization.

431

432 **Effect of selective V_{1B} agonist and antagonist on urinary flow rate**

433 As shown on Table 1, acute administration of V_{1B} agonist d[Leu⁴, Lys⁸]VP at low dose (0.42 µg
434 /kg) did not induced significant change in urine flow rate and urine osmolality. In contrast, a higher
435 dose (72 µg /kg) induced a marked increase in urine flow rate and decrease in urine osmolality in V_{1B}
436 agonist group compared to control group. V_{1B} antagonist SSR149415 (30 mg/kg) did not significantly
437 modify diuresis neither in rats in basal conditions (2.9 ± 0.3 vs 3.1 ± 0.4 ml/6hr in vehicle and V_{1B}
438 antagonist groups, respectively), nor in rats with high urine concentrating activity (pretreated for 5
439 days with Alzet minipumps delivering 400 ng/day AVP) (0.40 ± 0.14 vs 0.30 ± 0.10 mL/6hr in vehicle
440 and V_{1B} antagonist groups, respectively).

441

442 ***In vivo* effect of VP agonists on rat kidney cell proliferation**

443 Higher cell proliferation assayed by BrdU incorporation was detected in the medullary zones than
444 in cortex in control rats and s.c. treatment with 2 µg/kg.day dDAVP strongly increased proliferation
445 (near 4 fold) in each renal zone, as previously described (17). By contrast, treatment with 40 µg/kg.day
446 of d[Leu⁴, Lys⁸]VP did not significantly affected the rate of cell proliferation in each renal zone, as
447 compared to saline treatment (Table 2).

448

449

450 **DISCUSSION**

451 *Originality and new data*

452 The presence of the gene encoding the V_{1B}-R within the renal medulla had been previously
453 mentioned (14, 31, 32). For the first time, we identified and characterized the V_{1B}-R in IMCD using
454 original ligands such as selective V_{1B} fluorescent or radioactive agonists, and antagonist. Equilibrium
455 binding studies revealed a single high affinity V_{1B}-R binding sites that was present at low density.
456 Functional studies showed that the agonist-induced stimulation of the V_{1B}-R activates only the

457 IP3/calcium signaling pathway and not the cAMP production in native IMCD. Furthermore, our
458 results demonstrate a crosstalk between the V_{1B} and V_2 -R signaling pathways upon AVP stimulation in
459 freshly dissected rat IMCD.

460

461 *Localization and pharmacological characterization of the kidney V_{1B} -R*

462 V_{1B} -R mRNA was previously detected roughly in the rat medulla (14) but the specific cells
463 expressing the V_{1B} -R in this renal zone had not been investigated so far. Moreover, the absence of
464 specific antibodies or labeled agonist hampered the determination of precise receptor V_{1B} -R protein
465 localization along the nephron. Autoradiographic studies with tritiated SSR149415 failed to detect
466 V_{1B} -R in human kidney, probably because of the weak specific activity of the tritiated ligand and of
467 the low expression of V_{1B} -R in this organ (33). By using RT-PCR performed on microdissected
468 nephron segments and by using the fluorescent selective V_{1B} peptide d[Leu⁴, Lys(Alexa647)⁸]VP, we
469 have shown that V_{1B} mRNA and protein are predominantly expressed in IMCD and colocalize with
470 AQP2 and V_2 -R.

471 The use of tritiated form of d[Leu⁴, Lys⁸]VP corroborated the presence of V_{1B} -R within the inner
472 medulla. The pharmacological properties of the selective binding sites detected in the inner medulla fit
473 well with those of a V_{1B} -R, exhibiting a subnanomolar affinity for d[Leu⁴, Lys⁸]VP and an affinity in
474 the nanomolar range for the selective V_{1B} -R antagonist SSR149415 (30). However, the V_{1B} -R
475 expression is very low (19 fmol/mg protein) as compared to those of V_2 -R in the rat inner medulla
476 (493 fmol/mg protein and (34)) or with V_{1B} -R in the rat pituitary (150-300 fmol/mg protein) (31).

477

478 *Calcium and cAMP signaling pathways associated to V_{1B} -R in the IMCD*

479 To better preserve the coupling properties of the receptor and to analyze mechanisms that really
480 occurred *in vivo* our experiments were performed on freshly microdissected IMCD preserving the
481 structural integrity of the collecting duct. Moreover, to be sure that we selectively activate the V_{1B} -R,
482 which is co-expressed at low density with the V_2 -R in this structure, we carefully verified the
483 pharmacologic specificity of the analogues since we previously have shown that dDAVP is considered
484 as a mixed V_2/V_{1B} agonist depending on the dose used (30). Our signaling studies showed that the

485 stimulation of V_{1B} -R by d[Leu⁴, Lys⁸]VP is directly coupled to an increase in intracellular calcium
486 mobilization. This effect is dose-dependent and saturable with a nanomolar affinity constant. In the
487 absence of extracellular calcium, the persistence of spike of $[Ca^{2+}]_i$ response to d[Leu⁴, Lys⁸]VP
488 indicated that the initial increase in $[Ca^{2+}]_i$ originated from the mobilization of intracellular pools. The
489 role of extracellular calcium entry was evidenced in calcium free-medium by a significant reduction of
490 the peak response and the steady-state sustained increase in $[Ca^{2+}]_i$. Thus, V_{1B} -R-induced elevation of
491 $[Ca^{2+}]_i$ in IMCD is dependent on both the intracellular and the extracellular Ca^{2+} pools, similarly to
492 V_{1A} -R in primary cultures of rat adrenal glomerulosa cells both coupled to the PLC pathway and to
493 voltage calcium channels (35).

494 By contrast, we observed that V_{1B} -R activation by d[Leu⁴, Lys⁸]VP even at a high concentration
495 did not increase cAMP production. Such results are in agreement with earlier studies showing that the
496 V_{1B} -R present in the rat pituitary is not coupled to adenylate cyclase (36). A discrete activation of both
497 cAMP and calcium pathways was only detected in transfected cell lines stably expressing a very high
498 level of V_{1B} -R (37) when compared to the native tissues analyzed in the present study. If V_{1B} -R
499 stimulation does not stimulate cAMP accumulation *per se* when using a selective V_{1B} agonist (Fig. 6),
500 it plays an important role in modulating AVP-stimulated cAMP response. This is clearly evidenced by
501 Fig. 7 showing that SSR149415 strongly inhibited AVP-stimulated cAMP production. This implies
502 that the AVP-stimulated cAMP response is due to both V_2 -R and V_{1B} -R activation.

503

504 *Crosstalk between V_{1B} -R and V_2 -R signaling pathways*

505 It is clear that a balance between intracellular cAMP and calcium concentrations represents the
506 key factor for regulating water permeability of the principal cells of the renal tubule (9, 10). Molecular
507 studies earlier performed on this structure or presented in this article allow to better understand how
508 the intricacy between the two second messenger pathways associated to V_2 -R and V_{1B} -R stimulation
509 may account for the complex physiological actions of AVP on IMCD.

510 As V_{1B} -R and V_2 -R are co-expressed in the same IMCD cells and as AVP has the same affinity
511 for both receptors (30), it is tempting to speculate that, in this segment, both V_{1B} and V_2 receptors are
512 involved in the physiological action of AVP. Indeed, we showed that each receptor stimulated by its

513 selective agonist increases $[Ca^{2+}]_i$ and that these respective responses are independent since additive
514 when infra maximal doses of dDAVP or d[Leu⁴, Lys⁸]VP were used. Such results are in agreement
515 with the partial inhibitory effect of SSR149415 on the AVP-stimulated $[Ca^{2+}]_i$ increase acting only on
516 the V_{1B} sensitive part of the global calcium response. By contrast, the cooperation between the two
517 receptor isoforms concerns the cAMP pathways since while the V_{1B} agonist d[Leu⁴, Lys⁸]VP cannot
518 trigger *per se* cAMP production, the selective V_{1B} antagonist SSR149415 strongly reduced the AVP-
519 stimulated cAMP production (Fig. 7).

520 Two hypotheses may be proposed to explain these observations. The first one is the
521 heterodimerization of V₂-R and V_{1B}-R leading to a receptor complex exhibiting novel coupling
522 properties. Thus, V_{1A}, V₂ and oxytocin receptors have been previously shown to constitute both homo-
523 and heterodimers (26). In HEK293 cells co-transfected with hV_{1B} and hCRF1 receptors, which exhibit
524 the same type of coupling than those of V_{1B}-R and V₂-R (Gq and Gs respectively), receptors
525 heterodimerization induced a synergistic non-canonical coupling (25). However, direct BRET and
526 competition BRET assays performed in the present study did not support a dimerization V_{1B}-R and V₂-
527 R. Obviously, such experiments were performed on human but not on rat vasopressin receptors. Yet,
528 as previously discussed in this paper, the human and rat V_{1B}-R and V₂-R exhibit very strong structural
529 and functional similarities. Moreover, as shown in this study, the V_{1B} potentiating effect on AVP-
530 stimulated cAMP accumulation was observed both on freshly dissected rat IMCD and on HEK289
531 cells doubly transfected with hV₂-R and hV_{1B}-R (Fig. 7 and 8).

532 The second hypothesis would be a crosstalk between second messenger cascades triggered by a
533 concomitant V₂-R and V_{1B}-R activation. Many arguments favored such explanation. Thus, the
534 comparison of the maximal cAMP accumulation obtained on crude plasma membranes derived from
535 the inner medulla with those obtained on freshly dissected IMCD is very interesting. On crude plasma
536 membrane from rat inner medulla, the specific V₂ agonist dDAVP induced a maximal cAMP
537 production quite similar (88%) to that obtained with AVP (29). By contrast, on intact principal cells
538 from freshly microdissected IMCD, this ratio is only of 53% (this study). This discrepancy probably
539 arises from the ability of AVP to induce increase both in intracellular calcium concentration and in

540 DAG production in microdissected IMCD where the cellular integrity is maintained whereas in the
541 membrane assay, the calcium and DAG-potentiating effect is suppressed.

542

543 A non-exhaustive review of literature also comforts such crosstalk hypothesis since in rat IMCD,
544 the importance of the calcium influx and/or intracellular calcium mobilization from internal stores on
545 adenylate cyclases (AC) or phosphodiesterase (PDE) activities able to regulate the intracellular cAMP
546 content is well documented. In this tissue cAMP generation is controlled by different AC exhibiting
547 opposite sensitivity to calcium. It is now well established that the AC6 isoform is expressed all along
548 the collecting duct together with the AC5 only present in the OMCD and CCD. Both isoforms are
549 functionally active since an increase of calcium influx inhibited cAMP accumulation (38). More
550 recently, a functional calcium/calmodulin sensitive AC3 blocked by specific calmodulin inhibitors has
551 also been discovered (39). Distinct phosphodiesterases responsible for the hydrolysis of cAMP are
552 also expressed in the renal collecting duct and play an important role. If PDE1, PDE3 and PDE4 are
553 present in the renal collecting duct (40), PDE4 is more particularly involved in regulating AVP-
554 stimulated cAMP degradation (41). Yet, as PDE 1 is activated by a calcium/calmodulin process (42),
555 the increase of intracellular calcium induced both by V_2 and V_{1B} receptor activation in the IMCD (see
556 Fig. 3) may also participate to a feedback of cAMP production triggered by V_2 receptor activation.
557 Similarly, protein kinase A activated by cAMP was shown to stimulate PDE4 activities leading thus to
558 another negative feedback of the cAMP pathway (43). Intracellular calcium increase due to V_{1B} -R
559 activation may also have a negative feedback on cAMP accumulation driven by V_2 -R activation
560 indirectly via a stimulation of prostaglandin E2 synthesis known to inhibit cAMP production via a
561 complex mechanism involving an increase of PDE expression (44).

562 The role of PKC activation induced upon V_{1B} -R activation may also explain the crosstalk between
563 the V_2 -R and V_{1B} -R. Indeed, studies performed by Ishikawa and collaborators on rat renal papillary
564 collecting tubule cells in culture showed that PMA, a PKC activator like DAG, can modulate basal
565 cAMP production (45). We tried to reproduce these experiments in our freshly microdissected IMCD
566 but clearly this tissue does not represent an appropriate model for studying crosstalk mechanisms due
567 to the limited amounts of tissues available and the relative higher dispersion of cAMP data compared

568 to those obtained on cell culture. We thus performed such experiments on hV_{1B}-R /hV₂-R doubly
569 transfected HEK293 cells. We showed on this heterologous cellular model that PMA potentiates the
570 hV₂ cAMP accumulation induced by AVP. Moreover, the use of Gö6958, a PKC inhibitor blunts the
571 cAMP potentiating effect of hV_{1B}-R activation by hV₂ response (Fig. 8F). Altogether these data
572 strongly suggest the role of PKC in the fine tune regulation of hV₂-R regulated cAMP accumulation.

573 Our data obtained both on a heterologous cellular model and on freshly dissected IMCD tubules
574 clearly show that both positive or negative regulation loops contribute to the global response to AVP
575 stimulation. However, it is difficult to precisely define the level of intricacy between the calcium and
576 the cAMP pathways inside the principal cells of the IMCD since i) our cAMP measurements
577 concerned cells lysates and calcium signal is obtained on whole IMCD fragments and ii) the two
578 second messenger responses exhibit different spatial and temporal patterns in living cells due namely
579 to different subcellular distribution of the enzymes involved in second messenger generation like AC,
580 PDE, protein kinases, calcium channels. Only a spatial and temporal controlled second messenger
581 production performed on IMCD principal cells would allow a better comprehension of precise
582 mechanisms by which vasopressin second messenger cascades at the cellular level.

583

584 *Physiological and pathophysiological roles of V_{1B}-R in the kidney*

585 Together with previous studies performed on freshly-dissected collecting ducts or primary
586 cultures from IMCD from rat male kidney, our data allow a better understanding of the complex
587 mechanisms by which vasopressin regulate water reabsorption by interacting both with the well
588 characterized renal V₂-R but also with other vasopressin/oxytocin receptors also expressed in this
589 organ like the V_{1B} isoform extensively characterized in this study.

590 Vasopressin at very low doses (0.1 nmol/L) strongly stimulates the cAMP production (46). At
591 this concentration, a small [Ca²⁺]_i response is described. This dual effect is clearly mediated by the V₂-
592 R since mimicked by the selective V₂ agonist dDAVP and blocked by a selective V₂ antagonist (Fig.
593 5J and (46, 47)). In this context, the respective role of cAMP and [Ca²⁺]_i increase in regulating renal
594 water permeability may be better understood. First, the interaction of AVP with the V₂-R stimulates
595 the AC activity *via* a Gs stimulatory process (7) and leads to cAMP accumulation. Such increase

596 stimulates the activity of protein kinase A which then phosphorylates AQP2, a step necessary to
597 induce its translocation to the apical membrane of the principal collecting tubule cell and responsible
598 for increasing osmotic water permeability. If cAMP represents the main intracellular messenger
599 triggering the hormonal effect, it has been elegantly demonstrated that the small increase of $[Ca^{2+}]_i$ is
600 also important since IMCD preincubation with an intracellular calcium chelator inhibits the AVP-
601 stimulated $[Ca^{2+}]_i$ increase and the osmotic water permeability. Moreover, this $[Ca^{2+}]_i$ increase acts
602 downstream the AC stimulation since intracellular calcium chelator does not inhibit AVP-stimulated
603 cAMP production and permeant cAMP analogues have been described to mimic small $[Ca^{2+}]_i$ increase
604 (47). Such calcium increase originates from Ryanodine sensitive intracellular stores activated by
605 cAMP and not from intracellular inositol 1,4,5-trisphosphate (IP3) sensitive store (48). The authors
606 showed that this calcium increase favored AQP2 translocation to the apical membrane via a
607 calmodulin kinase dependent process facilitating the osmotic water permeability (47).

608 For higher AVP concentrations (10 nmol/L and above), AVP still stimulates cAMP production to
609 the same level than that obtained with 0.1 nmol/L (Fig. 3 and (46)). Using such high AVP
610 concentrations, we and other (9-11) observed the development of a robust and reproducible calcium
611 signal. No saturation of the AVP calcium response was observed even at 10 or 100 nmol/L (Fig. 3 and
612 (11) suggesting an EC_{50} higher than 20 nmol/L. On freshly microdissected rat IMCD it was also
613 shown that at supra-nanomolar concentration, hormones mobilizing the calcium would inhibit osmotic
614 water permeability (12). The authors suggest that such an important calcium increase may
615 counterbalance the osmotic effect induced by low vasopressin concentration that mainly stimulates
616 cAMP production via the V_2 -R activation. Such hypothesis may explain i) the bell-shape dose-
617 response curve obtained *in vivo* by measuring the antidiuresis upon AVP injection in rat (49), ii) the
618 classical antidiuretic saturation dose-response curve obtained with dDAVP known to strongly
619 stimulates cAMP production and more weakly maximal $[Ca^{2+}]_i$ increase compared to AVP (this study
620 and (49)). In the light of these results, we propose that high vasopressin concentrations able to induce
621 a calcium response *via* V_{1B} -R activation may counterbalance the well-known antidiuretic effects
622 induced by AVP interaction with the V_2 -R. Nevertheless, this effect is probably weak according to the
623 low density of V_{1B} -R that we measured as compared to that of V_2 -R.

624

625 Such interpretation may also explain the intriguing effects that we observed *in vivo* on diuresis
626 with V_{1B} agonist. In normo-hydrated rats with relatively low concentration of AVP, the changes in
627 intracellular calcium concentration induced by low doses of V_{1B} -R agonist d(Leu⁴, Lys⁸)VP (0.42
628 $\mu\text{g}/\text{kg}$) were too weak to alter notably the antidiuretic V_2 -R effect. Accordingly, previous studies have
629 shown in rats under normal conditions that acute administration of 0.05 nmol/100g, d(Leu⁴, Lys⁸)VP,
630 a similar dosage as we used, did not modify urinary flow rate or electrolyte excretion (50, 51). At high
631 doses (72 $\mu\text{g}/\text{kg}$) d(Leu⁴, Lys⁸)VP probably induced a maximal calcium response known to modulate
632 osmotic water permeability and thus a significant effect on diuresis (Table 1). However, a central
633 effect of high dose of d(Leu⁴, Lys⁸)VP could not be excluded in this condition since activation of the
634 pituitary V_{1B} -R leads to an increase in CRF and ACTH production (52), consequently an increase in
635 corticosteroid secretion (53), and secondarily an increase in water intake and diuresis ((54).

636 Analogously to the absence of diuretic effect of low doses of d(Leu⁴, Lys⁸)VP, SSR149415 did
637 not change urine flow rate in normal rats. Indeed, in basal conditions, the circulating level of AVP is
638 too low to stimulate the V_{1B} -R, Consequently SSR149415 could not prevent AVP binding on V_{1B} -R
639 and is inefficient on diuresis. As expected, SSR149415 treatment also did not change urine flow rate in
640 homozygous Brattleboro rats devoid of AVP (C. Serradeil-Le Gal, personal communication).
641 Noticeably, the antidiuretic effect of SR49059, a selective V_{1A} -R antagonist which prevents AVP-
642 stimulated intracellular calcium mobilization, has been highlighted only in rats with high plasma AVP
643 concentration (49).

644 Noteworthy previous studies reported a daily higher urine volume in mice lacking V_{1B} -R when
645 compared with control mice (18). The apparent discrepancy with our results on the acute diuretic
646 effects of V_{1B} -R stimulation may be explained either by species differences in V_{1B} -R density and/or in
647 renal pattern or, more probably, by the complex interactions between receptor signaling and
648 compensatory mechanisms that occur in the life-long absence of one component of the vasopressin
649 system.

650

651 Overall, our results indicate that under physiological conditions plasmatic concentration of AVP
652 mainly stimulates the V₂-R activation, thus producing cAMP mainly responsible for osmotic water
653 permeability. For higher plasmatic concentrations AVP may both activate the V₂-R, the V_{1A}-R
654 expressed in the OMCD (55) and the V₂-R, the V_{1B}-R and the OT-R co-expressed in the IMCD (this
655 study and (56)) since AVP is an agonist of nanomolar affinity for all these VP/OT receptor isoforms
656 (30). This concerted activation of kidney VP/OT receptors tightly coupled to [Ca²⁺]_i may
657 counterbalance the antidiuretic effect of AVP triggered by V₂-R activation. Obviously, the densities of
658 each of these receptors are low as compared to those of the V₂-R but, by acting all together in different
659 kidney regions where the V₂-R is expressed, they may produce a fine tune regulation of the
660 vasopressin antidiuretic activity in some pathophysiological conditions in which the plasmatic AVP
661 concentration is largely increased like in SIADH syndrome or severe dehydration.

662

663 In conclusion, this study clearly demonstrated the presence of low amounts of functional V_{1B}-R
664 in the IMCD co-expressed with V₂-R and AQP2 in the same principal cells. These data contribute to
665 our understanding of how V_{1B}-R may cooperate with V₂-R to finely regulate the cAMP and calcium
666 signaling pathways in a native biological model.

667

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677

678 **DISCLOSURES**

679 No conflicts of interest, financial or otherwise, are declared by the authors.

680

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- 835

836 **FIGURE LEGENDS**

837 **Figure 1: Distribution of mRNA and V_{1B} receptor within the kidney**

838 A. Expression of V_{1B}-R and V₂-R mRNA in the cortical (CCD,) outer medullary (OMCD) and inner
839 medullary collecting duct (IMCD). Results are expressed as the relative value from 2 mm tubular
840 length normalized with the housekeeping gene GAPDH. Each histogram represents the mean data ±
841 SE, in CCD and OMCD (n = 3 rats), IMCD (n = 6 rats). *** p<0.0001 vs CCD and OMCD.

842 B. Colocalization of V_{1B}-R and V₂-R in the inner medulla. Top: Representative confocal images of rat
843 kidney slices incubated with the fluorescent V_{1B} analogue d[Leu⁴(Lys-Alexa647)⁸]VP (purple) or
844 AQP2 antibody (red) in the presence (non-specific binding) or in the absence (total binding) of 1
845 μmol/L of unlabeled AVP (scale bar = 100 μm). Bottom: zoomed images of inner medulla (scale bar =
846 25 μm), showing that V_{1B}-R and AQP2 are colocalized in the same cells (merge, arrows).

847

848 **Figure 2: Pharmacological characterization of rat V_{1B}-R in the kidney using a selective tritiated**
849 **probe**

850 **A and B:** Crude plasma membranes from the inner medulla of rat kidney were incubated 1h at 37°C
851 with either increasing amounts of (A) [³H]-d[Leu⁴, Lys⁸]VP (100 μg protein /assay) or (B) [³H]-AVP
852 (10 μg protein/assay), with or without 100 nmol/L of unlabeled AVP. Specific binding was calculated
853 in each experimental condition and Scatchard representation of dose-dependent binding curves
854 illustrated. Results are the mean ± SE of 3 independent experiments, each in triplicate.

855 **C.** Binding displacement of [³H]-AVP or [³H]-d[Leu⁴, Lys⁸]VP with the selective V_{1B} antagonist
856 SSR149415. Experiments were performed as described in panels A and B either with 0.5 nmol/L [³H]-
857 AVP or 1.5 nmol/L [³H]-d[Leu⁴, Lys⁸]VP and with or without (control) increasing amounts of
858 SSR149415. Specific binding calculated in each experimental condition are expressed as percent of
859 control specific binding and are the mean ± SE of 3 distinct experiments each performed in triplicate.

860

861 **Figure 3: Effect of vasopressin agonists on intracellular calcium mobilization along the collecting**
862 **duct**

863 **A-C.** Representative recordings (traces) of $[Ca^{2+}]_i$ levels elicited by 10 nmol/L d[Leu⁴, Lys⁸]VP and as
864 controls 10 to 100 nmol/L AVP in the inner medulla (IMCD), the outer medulla (OMCD) and the
865 cortex (CCD) of the collecting duct.

866 **D.** Dose-dependency curve of d[Leu⁴, Lys⁸]VP and dDAVP-induced $[Ca^{2+}]_i$ responses in IMCD. Each
867 point represents the mean \pm SE of at least 3 independent determinations.

868 **E.** Comparison of maximal $[Ca^{2+}]_i$ levels elicited by maximal doses of the V_{1B} agonist (d[Leu⁴,
869 Lys⁸]VP), of the selective V₂ agonist (dDAVP) or by 10 or 100 nmol/L of AVP in IMCD. Each bar
870 represents the mean \pm SE of at least 7 independent determinations. *** p< 0.001 compared to 100
871 nmol/L AVP.

872 **F.** Additive effects of d[Leu⁴, Lys⁸]VP and dDAVP on $[Ca^{2+}]_i$ levels in IMCD. Experiments were
873 performed with non-saturating concentrations of agonist (10 nmol/L for d[Leu⁴, Lys⁸]VP and 1
874 nmol/L of dDAVP). Each bar represents the mean \pm SE of at least 7 independent determinations. *
875 p<0.05 compared to d[Leu⁴, Lys⁸]VP + dDAVP.

876

877 **Figure 4: Influence of extracellular calcium concentration on intracellular calcium mobilization**
878 **induced by d[Leu⁴, Lys⁸]VP or dDAVP in IMCD**

879 Experiments using 10 nmol/L d[Leu⁴, Lys⁸]VP or dDAVP were performed in the absence or presence
880 of 2 mmol/L extracellular calcium.

881 **A** and **C.** Representative traces of intracellular Ca²⁺ obtained either with d[Leu⁴, Lys⁸]VP or dDAVP
882 in the presence or absence of extracellular calcium.

883 **B** and **D.** Histograms for Ca²⁺ release and Ca²⁺ influx elicited by d[Leu⁴, Lys⁸]VP or dDAVP. Results
884 were calculated from traces as illustrated in panels A and C respectively. Each result represents the
885 integral of the Ca²⁺ signal calculated from 5 independent determinations, * p<0.05.

886

887 **Figure 5: Pharmacology of the $[Ca^{2+}]_i$ responses induced by d[Leu⁴, Lys⁸]VP in various kidney**
888 **structures**

889 Representative recordings of $[Ca^{2+}]_i$ levels in IMCD elicited by (A) 15-min pretreatment with 100
890 nmol/L SSR149415 followed by successive applications of 10 and 100 nmol/L d[Leu⁴, Lys⁸]VP, (B)

891 10^{-8} mol/L dDAVP after 15-min pretreatment in the presence of 100 nmol/L SSR149415, (C) 15-min
892 pretreatment with 10 nmol/L SSR121463B followed by successive applications 10 nmol/L dDAVP or
893 10^{-4} mol/L carbachol to assess the viability of IMCD, (D) 10 nmol/L d[Leu⁴, Lys⁸]VP after 15-min
894 pretreatment with 10 nmol/L SSR121463B, (E) 10 nmol/L d[Leu⁴, Lys⁸]VP after 15-min pretreatment
895 with 100 nmol/L SSR49059, (F) 10 nmol/L d[Leu⁴, Lys⁸]VP after 15-min pretreatment with 100
896 nmol/L SSR126708A .

897 Representative recordings of [Ca²⁺]_i levels in glomerular afferent arteriole elicited by (G) 5-min
898 incubation with 10 nmol/L d[Leu⁴, Lys⁸]VP followed by a 5-min incubation with 3 nmol/L AVP, (H)
899 5-min pretreatment with 100 nmol/L SSR49059 followed by the successive applications of 3 nmol/L
900 AVP and 0.1 mol/L carbachol.

901 Representative recordings of [Ca²⁺]_i levels in OMCD elicited by (I) 5-min incubation with 10 nmol/L
902 d[Leu⁴, Lys⁸]VP followed by a 5-min incubation with 10 nmol/L dDAVP, (J) 5-min pretreatment with
903 10 nmol/L of the V₂ antagonist SSR121463B followed by the successive applications of 10 nmol/L
904 dDAVP, then 100 nmol/L Angiotensin II.

905

906 **Figure 6: Effect of d[Leu⁴, Lys⁸]VP on intracellular cAMP accumulation in IMCD**

907 **A.** Comparative effect of d[Leu⁴, Lys⁸]VP, dDAVP and AVP on cAMP accumulation. IMCD were
908 incubated with each vasopressin agonist. n = 5-13 independent determinations.

909 **B.** Additive effect of infra maximal doses of d[Leu⁴, Lys⁸]VP (10 nmol/L) and dDAVP (0.1 nmol/L)
910 on cAMP accumulation. n = 5-6 independent determinations.

911 Each bar represents the mean ± SE of at least 5 determinations. *** p<0.001; ** p<0.002 vs basal
912 value.

913

914 **Figure 7: Effect of SSR149415 on intracellular calcium mobilization and cAMP production** 915 **induced by AVP in IMCD**

916 Effect of the V_{1B} antagonist SSR149415 alone, AVP alone or in combination on (A) intracellular
917 calcium accumulation or (B) cAMP production in the IMCD. Each bar represents the mean ± SE of at
918 least 5 independent determinations. * p<0.05; *** p<0.01 vs basal or SSR149415; # p<0.05 vs AVP.

919

920 **Figure 8: Second messenger synergism between the hV₂ and hV_{1B} receptors in HEK293 cells**

921 Functional synergism between the hV_{1B}-R and hV₂-R was challenged by measuring cAMP
922 accumulation induced by AVP. HEK293 cells were doubly transfected with 0.5 ng hV₂-R and
923 increasing amounts of hV_{1B}-R (0 to 10 ng) (panel **A**); with 1 ng hV₂-R alone or co-transfected with 5
924 ng hV_{1B}-R (panels **B** to **F**). The accumulated cAMP was determined in the presence of 10 nmol/L
925 AVP (panels **A**, **B**, **E**, **F**) or increasing amounts of AVP (panel **C**, **D**) for a 30 min incubation period at
926 37°C. In panels **B** and **C**, a 15 min preincubation with or without 100 nmol/L SSR149415 (control)
927 was performed before AVP addition. In panels **D** and **E**, a 15 min pre-incubation without (control) or
928 with 1 micromole/L PMA was performed before AVP addition. In panel **F**, a 15 min pre-incubation
929 without (control) or with Gö6976 was performed before AVP addition. In each condition, cAMP was
930 measured and expressed as % of maximal AVP response over basal obtained for hV₂-R singly
931 transfected cells. Values are the mean ± SE of at least 3 distinct experiments each performed in
932 triplicate. * p<0.05, ** p<0.01, *** p<0.001 vs control.

933

934 **Figure 9: Challenging V_{1B}-R/V₂-R heterodimerization**

935 The hV_{1B}/hV₂ receptor interaction was challenged by BRET saturation (A) or BRET competition (B)
936 experiments. HEK293 cells were co-transfected with plasmids encoding tagged or wild type (wt)
937 receptors and BRET was measured. For direct interaction (BRET saturation), the donor hV_{1B}-RLuc
938 was incubated in the presence of increasing concentrations of acceptor, namely hCRF₁-EYFP, hV₂-
939 EYFP or hGABA_{B2}-EYFP and the “BRET net” was measured in each condition. For BRET
940 competition, a constant amount of hV₂-RLuc/hV₂-EYFP known to give a positive BRET signal of
941 homodimerization (26) was incubated with increasing concentrations of plasmids encoding different
942 unlabeled acceptors *i.e.* wild type hV_{1B}-R, hV₁-R or hOT-R. The potential decreases of “BRET net” as
943 a function of the dose of unlabeled acceptor were then measured. Values are the mean ± SE of at least
944 3 independent experiments, each performed in triplicate.

945

946 Table 1: Effect of V_{1B}-R agonist d[Leu⁴, Lys⁸]VP on urine volume, urine osmolality and urinary
 947 osmolar excretion during the 5hrs after the compound administration.
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	Volume ml	Osmolality mosm/kg H ₂ O	Osmolar excretion μosm
vehicle	1.9 ± 0.3	2235 ± 93	4094 ± 602
l[Leu ⁴ , Lys ⁸]VP 0.42 μg/kg	2.7 ± 0.6	1705 ± 272	4133 ± 385
vehicle	1.2 ± 0.2	2150 ± 88	2448 ± 241
d[Leu ⁴ , Lys ⁸]VP 72 μg/kg	6.9 ± 0.7***	534 ± 10***	3596 ± 124*

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 950 Results are means ± SE, n= 5/group, * p<0.05, *** p<0.001 vs corresponding vehicle group
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 957 Table 2: Effect of 3 days treatment with V_{1B}-R agonist d[Leu⁴, Lys⁸]VP or V₂-R agonist dDAVP on
 958 renal cell proliferation
 959

	Control	d[Leu ⁴ , Lys ⁸]VP 40 μg/kg.day	dDAVP 2 μg/kg.day
Cortex	15 ± 4	26 ± 8	79 ± 8 *
Outer Medulla	37 ± 3	60 ± 15	210 ± 15*
Inner medulla	68 ± 7	90 ± 8	307 ± 23***

960
 961 Results are means ± SE of the number of BrdU-labeled nuclei counted within a 500 μm side-squared
 962 field centered on the different kidney regions on four sections per animal and four animals per group.
 963 * p<0.05, *** p<0.001 vs respective zone in control group
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