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

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ABSTRACT

Although vasopressin V_{1B} receptor (V_{1B} -R) mRNA was detected in the kidney, the precise renal localization, pharmacological and physiological properties of this receptor remain unknown. Using the selective V_{1B} agonist d[Leu⁴, Lys⁸]VP, either fluorescent or radioactive, we showed that V_{1B} -R is mainly present in principal cells of the inner medullary collecting duct (IMCD) in the male rat kidney. Protein and mRNA expression of V_{1B} -R were very low compared to V_2 receptor (V_2 -R). On microdissected IMCD, d[Leu⁴, Lys⁸]VP had no effect on cAMP production but induced a dose-dependent and saturable $[Ca^{2+}]_i$ increase mobilization with an EC_{50} in the nanomolar range. This effect involved both intracellular calcium mobilization and extracellular calcium influx. The selective V_{1B} antagonist SSR149415 strongly reduced the ability of vasopressin to increase $[Ca^{2+}]_i$ but also cAMP suggesting a cooperation between V_{1B} -R and V_2 -R in IMCD cells expressing both receptors. This cooperation arises from a crosstalk between second messenger cascade involving protein kinase C rather than receptor heterodimerization as supported by potentiation of the AVP-stimulated cAMP production in HEK293 cells co-expressing the two receptor isoforms and negative results obtained by bioluminescence resonance energy transfer experiments. *In vivo*, only acute administration of high doses of V_{1B} agonist triggered significant diuretic effects by contrast with injection of selective V_2 agonist. This study brings new data on the localization and signaling pathways of V_{1B} -R in the kidney, highlights a crosstalk between V_{1B} -R and V_2 -R in IMCD and suggests that V_{1B} -R may counterbalance in some pathophysiological conditions the antidiuretic effect triggered by V_2 -R activation.

Keywords: Vasopressin V_{1B} and V_2 receptor, inner medullary collecting duct, calcium and cAMP signaling, receptor heterodimerization, diuresis

INTRODUCTION

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

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

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

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

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

MATERIAL AND METHODS

Drugs

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

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

Animals

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

Binding assay on plasma membranes

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

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

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

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

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

Microdissection of nephron segments

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

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

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

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

Intracellular calcium concentration measurement

Experiments were performed as previously described (24). Briefly, microdissected nephron segments and glomerular arterioles were loaded with 5 µmol/L Fura-2 AM at room temperature for 120 minutes. Each nephron segment or arteriole was continuously superfused at 37°C with either medium or the solutions to be tested. Vasopressin, V_{1B} selective agonist (d[Leu⁴, Lys⁸]VP), and V₂ selective agonist (dDAVP) were applied for 5 min. For experiments performed in the absence of external Ca²⁺, IMCD were superfused in Ca²⁺-free medium 2 min before adding the agonist. For studies with specific antagonists, collecting ducts were superfused for 15 min before addition of the agonist. The calcium response was evaluated by determining the magnitude of the response ($\Delta[Ca^{2+}]_i$) corresponding to the difference between peak and basal concentrations (in nmol/L) or by determining the area under the curve (AUC, in nmol.sec/L) obtained by the integral of the Ca²⁺ signal.

Intracellular cAMP measurement in microdissected IMCD

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

Measurement of cAMP accumulation in HEK293 cells co-expressing V_{1B} and/or V₂ receptors

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

Bioluminescence resonance energy transfer assay (BRET)

The V_{1A}-R, V₂-R and OT-R ~~different~~ hybrid constructs were generated from human receptors subcloned in PRK5 vector using the GeneEditor In Vitro Site-Directed Mutagenesis System (Promega Corp, Madison, USA) as described by Terrillon et al (26) (gift from Dr Devost and M. Bouvier, Montreal, Canada). The hV_{1B} and hV_{1B}-RL were respectively cloned in pRL-CMV and pcDNA3.0 vectors whereas CRF1-EYFP was in pEYFP-N1 (Clontech) and GABAB₂R-EGFP in pcDNA3.0 according to the constructions used in our previous work (25). All vectors contained similar CMV promotor and SV40 polyadenylation sites for comparable expression levels. Mild levels of receptor expression were chosen in these experiments to mimick physiological conditions (less than 1 pmol/mg protein (25)). BRET experiments were conducted as previously described (25). Briefly, human wild type hV_{1B}-R, hV_{1B}-RL and/or hV₂-EYFP receptors were transiently transfected in HEK293 cells using the Jet PEI technique with a constant amount of the donor construct (hV_{1B}-RL, 1 µg of DNA), and from 0.03 to 10 µg of acceptor (hCRF₁-EYFP, hV₂-EYFP or hGABA_{B2}-EYFP) DNA constructs in 96-

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

Urine flow measurement

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

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

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

Data Analysis

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

RESULTS

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

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

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

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

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

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

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

The [Ca^{2+}]_i response to the V_{1B} selective agonist d[Leu⁴, Lys⁸]VP was tested in CCD, OMCD and IMCD. In good agreement with the distribution of V_{1B}-R mRNA, 10 nmol/L d[Leu⁴, Lys⁸]VP elicited significant [Ca^{2+}]_i response only in the IMCD (Fig. 3A) and not in OMCD (Fig. 3B) or in CCD (Fig. 3C) whereas AVP induced a strong [Ca^{2+}]_i response in all segments. The calcium response induced by d[Leu⁴, Lys⁸]VP in the IMCD was rapid and reversible (Fig. 3A). This effect was dose-dependent, discreet but robust, with an EC₅₀ of 19 ± 6 nmol/L (Fig. 3D). The increase in [Ca^{2+}]_i was maximal with 100 nmol/L of d[Leu⁴, Lys⁸]VP with an E_{max} of 108 ± 22 nmol/L (n = 8). The calcium response

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

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

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

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

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

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

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

The pharmacological selectivity of the $d[Leu^4, Lys^8]VP$ was further verified on two other freshly microdissected renal structures using the calcium mobilization assay. In glomerular afferent arterioles known to express only V_{1A} -R localized on vascular smooth muscle cells (28), 10 nmol/L $d[Leu^4, Lys^8]VP$ did not induce any significant response (Fig. 5G). By contrast, calcium response induced by AVP was fully antagonized by pretreatment with the selective V_{1A} antagonist SSR49059 (Fig. 5H). In 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 effect on $[Ca^{2+}]_i$ whereas 10 nmol/L dDAVP induced a marked response (Fig. 5I). This effect of dDAVP was fully abolished by pretreatment with 10 nmol/L of the specific V_2 antagonist SSR121463B. The cell viability of the structures was verified by subsequent application of angiotensin II (AngII) (Fig. 5J) that promoted the expected Ca^{2+} mobilization. These data demonstrate the specificity of the V_{1B} and V_2 agonists both used at 10 nmol/L in this study.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

Originality and new data

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

For higher AVP concentrations (10 nmol/L and above), AVP still stimulates cAMP production to the same level than that obtained with 0.1 nmol/L (Fig. 3 and (46)). Using such high AVP concentrations, we and other (9-11) observed the development of a robust and reproducible calcium signal. No saturation of the AVP calcium response was observed even at 10 or 100 nmol/L (Fig. 3 and (11) suggesting an EC_{50} higher than 20 nmol/L. On freshly microdissected rat IMCD it was also shown that at supra-nanomolar concentration, hormones mobilizing the calcium would inhibit osmotic water permeability (12). The authors suggest that such an important calcium increase may counterbalance the osmotic effect induced by low vasopressin concentration that mainly stimulates cAMP production via the V_2 -R activation. Such hypothesis may explain i) the bell-shape dose-response curve obtained *in vivo* by measuring the antidiuresis upon AVP injection in rat (49), ii) the classical antidiuretic saturation dose-response curve obtained with dDAVP known to strongly stimulates cAMP production and more weakly maximal $[Ca^{2+}]_i$ increase compared to AVP (this study and (49)). In the light of these results, we propose that high vasopressin concentrations able to induce a calcium response *via* V_{1B} -R activation may counterbalance the well-known antidiuretic effects induced by AVP interaction with the V_2 -R. Nevertheless, this effect is probably weak according to the 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 µg/kg) were too weak to alter notably the antidiuretic V₂-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 µg/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

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

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

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DISCLOSURES

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

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FIGURE LEGENDS

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

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

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

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

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

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

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

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

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

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

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

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

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

A and C. Representative traces of intracellular Ca^{2+} obtained either with d[Leu⁴, Lys⁸]VP or dDAVP in the presence or absence of extracellular calcium.

B and D. Histograms for Ca^{2+} release and Ca^{2+} influx elicited by d[Leu⁴, Lys⁸]VP or dDAVP. Results were calculated from traces as illustrated in panels A and C respectively. Each result represents the integral of the Ca^{2+} signal calculated from 5 independent determinations, * $p < 0.05$.

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

Representative recordings of $[Ca^{2+}]_i$ levels in IMCD elicited by (A) 15-min pretreatment with 100 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

Table 1: Effect of V_{1B}-R agonist d[Leu⁴, Lys⁸]VP on urine volume, urine osmolality and urinary osmolar excretion during the 5hrs after the compound administration.

	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*

Results are means ± SE, n= 5/group, * p<0.05, *** p<0.001 vs corresponding vehicle group

Table 2: Effect of 3 days treatment with V_{1B}-R agonist d[Leu⁴, Lys⁸]VP or V₂-R agonist dDAVP on renal cell proliferation

	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***

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