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Design, Synthesis, and Pharmacological Characterization of Fluorescent Peptides for Imaging Human V1b Vasopressin or Oxytocin Receptors

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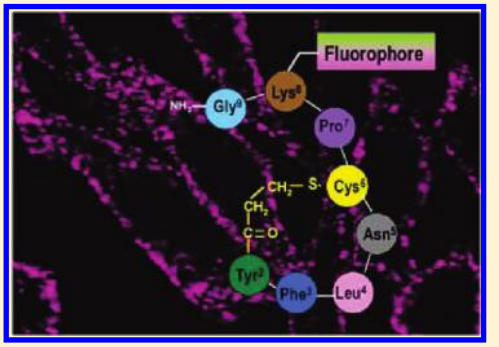
ABSTRACT: Among the four known vasopressin and oxytocin receptors, the specific localization of the V1b isoform is poorly described because of the lack of selective pharmacological tools. In an attempt to address this need, we decided to design, synthesize, and characterize fluorescent selective V1b analogues. Starting with the selective V1b agonist [deamino-Cys[1],Leu[4],Lys[8]]vasopressin (d[Leu[4],Lys[8]]VP) synthesized earlier, we added blue, green, or red fluorophores to the lysine residue at position 8 either directly or by the use of linkers of different lengths. Among the nine analogues synthesized, two exhibited very promising properties. These are d[Leu[4],Lys(Alexa 647)[8]]VP (**3**) and d[Leu[4],Lys(11-aminoundecanoyl-Alexa 647)[8]]VP (**9**). They remained full V1b agonists with nanomolar affinity and specifically decorated the plasma membrane of CHO cells stably transfected with the human V1b receptor. These new selective fluorescent peptides will allow the cellular localization of V1b or OT receptor isoforms in native tissues.



INTRODUCTION

Vasopressin (VP) and the VP receptor family including V1a, V1b, V2, and the related oxytocin (OT) receptor are involved in many different physiological functions. Among them, the V1b receptor is well-known for the activation of adrenocorticotropin hormone (ACTH) secretion in the pituitary, thus strongly participating, with corticotrophin releasing hormone (CRF), in the activation of the pituitary/corticotrope axis in stress and anxiety.[1] Vasopressin also regulates adrenal function by mediating catecholamines secretion[2] and induces pancreatic insulin secretion via V1b receptors.[3] Besides this neuroendocrine effect, V1b receptors have been suspected to be localized centrally and to participate in cognitive and behavioral functions involved in rewarding, cognition, and sociality (see ref 4 for review).

Although several publications describe the vasopressin V1a, V2, and OT receptor distribution by using autoradiography[5−7] or immunodetection[8] techniques, the lack of selective radiolabeled VP analogues or of receptor antibodies has hindered progress in the detection of V1b receptor distribution in native tissues. Results obtained by molecular approaches such as polymerase chain reaction after reverse transcription (RT-PCR)[9,10] or messenger ribonucleic acid (mRNA) detection by in situ hybridization,[11−13] although more accurate, did not provide clear information regarding the brain regions detected by immunostaining. This was partially due to the fact that the probes shared common sequences with V1a and OT receptors.[9]

Fluorescent tools have been synthesized for other receptors of the VP/OT family.[14−16] However, although good fluorescent V1a and OT ligands have been produced, to date, no good fluorescent specific ligand is available to selectively detect central and peripheral V1b receptors. Here we describe the synthesis of nine new fluorescent analogues for the human V1b receptor (hV1b-R). Two of them are promising new tools for the detection of V1b or OT receptors in native tissue. In our previous work,[17,18,3,19] by replacing the glutamine[4] of the natural arginine vasopressin (AVP) with a cyclohexylalanine or a leucine, by replacing the arginine[8] with a lysine, and by removing the NH_2 of the cysteine[1] to increase stability toward aminopeptidases, we produced analogues showing an increased selectivity for the rat V1b receptor (rV1b-R). Namely, the [deamino-Cys[1],4-leucine,8-lysine]vasopressin (d[Leu[4],Lys[8]]VP) analogue, previously synthesized in the du Vigneaud laboratory,[20] was found to be selective for the rV1b-R[3,19] and, to a lesser extent, for the hV1b-R. It conserved a nanomolar affinity for these receptor isoforms.[18] By studying the structure/activity of the ligand/V1b receptor complex, we have also been able to propose a model of the ligand−receptor interaction.[21] This model predicts that the first six amino acids would be located into a "binding pocket" inside the membrane, whereas the residues at positions 7, 8, and 9

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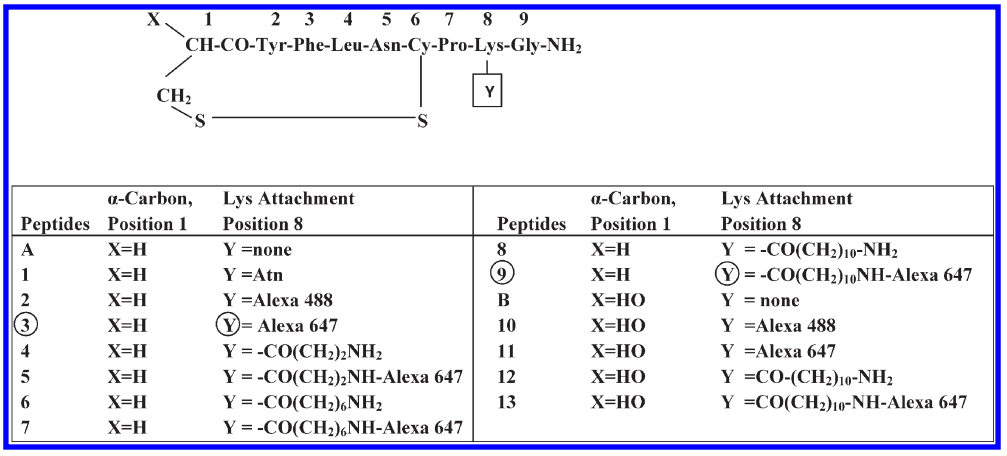

Peptides	α-Carbon, Position 1	Lys Attachment Position 8	Peptides	α-Carbon, Position 1	Lys Attachment Position 8
A	X=H	Y =none	8	X=H	Y = -CO(CH$_2$)$_{10}$-NH$_2$
1	X=H	Y =Atn	(9)	X=H	(Y) = -CO(CH$_2$)$_{10}$NH-Alexa 647
2	X=H	Y =Alexa 488	B	X=HO	Y = none
(3)	X=H	(Y)= Alexa 647	10	X=HO	Y =Alexa 488
4	X=H	Y = -CO(CH$_2$)$_2$NH$_2$	11	X=HO	Y =Alexa 647
5	X=H	Y = -CO(CH$_2$)$_2$NH-Alexa 647	12	X=HO	Y =CO-(CH$_2$)$_{10}$-NH$_2$
6	X=H	Y = -CO(CH$_2$)$_6$NH$_2$	13	X=HO	Y =CO(CH$_2$)$_{10}$-NH-Alexa 647
7	X=H	Y = -CO(CH$_2$)$_6$NH-Alexa 647			

Figure 1. General structure of fluorescent peptides (**1**−**3**, **5**, **7**, **9**−**11**, **13**) and parent peptides (**A**, **4**, **6**, **8**, **B**, **12**). X represents the modification on Cys^1 by either H atom or OH group, and Y represents the types of linker and fluorophore attached at position Lys^8 of the parent peptides d[Leu^4,Lys^8]VP (**A**) and HO^1[Leu^4,Lys^8]VP (**B**).

Table 1. Physicochemical Properties of Fluorescent Peptides 1−3, 5, 7, 9−11, 13, Parent Peptides A and B, and Intermediates 4, 6, 8, 12

			TLC, R_f[b]							
compd	peptide	yield,[a] %	a	b	c	d	HPLC[c] t_R, min	formula	MW (calcd)	MW (MS) (found)
A	d[Leu^4,Lys^8]VP[d]	60.6	0.35	0.37	0.30	0.34	12.4	$C_{47}H_{67}O_{11}N_{11}S_2$	1026.3	1026.4
1	d[Leu^4,Lys(Atn)^8]VP[e]	24.3	0.44	0.55	0.66	0.47	13.6	$C_{54}H_{72}O_{12}N_{12}S_2$	1145.4	1145.8
2	d[Leu^4,Lys(Alexa 488)^8]VP	74.4	0.20	0.14	0.07	0.13	11.8	$C_{68}H_{77}O_{21}N_{13}S_4$	1540.7	1540.5
3	d[Leu^4,Lys(Alexa 647)^8]VP	43.1	0.08	0.22	0.03	0.04	10.1			
4	d[Leu^4,Lys(β-Ala)^8]VP[e]	67.9	0.21	0.40	0.12	0.48	15.0	$C_{50}H_{72}O_{12}N_{12}S_2$	1097.3	1097.7
5	d[Leu^4,Lys(β-Ala-Alexa 647)^8]VP	64.4	0.17	0.29	0.02	0.12	10.1			
6	d[Leu^4,Lys(Aha)^8]VP[e]	36.5	0.25	0.23	0.30	0.38	13.1	$C_{54}H_{80}O_{12}N_{12}S_2$	1153.5	1153.4
7	d[Leu^4,Lys(Aha-Alexa 647)^8]VP	35.0	0.11	0.02	0.03	0.06	10.5			
8	d[Leu^4,Lys(Aud)^8]VP[e]	41.9	0.40	0.51	0.53	0.53	14.4	$C_{58}H_{88}O_{12}N_{12}S_2$	1209.5	1209.9
9	d[Leu^4,Lys(Aud-Alexa 647)^8]VP	55.9	0.18	0.26	0.12	0.17	11.7			
B	[HO^1][Leu^4,Lys^8]VP	54.2	0.26	0.22	0.18	0.38	11.1	$C_{47}H_{67}O_{12}N_{11}S_2$	1042.3	1042.6
10	[HO^1][Leu^4,Lys(Alexa 488)^8]VP	39.8	0.17	0.25	0.13	0.22	11.3	$C_{68}H_{77}O_{22}N_{13}S_4$	1556.7	1556.6
11	[HO^1][Leu^4,Lys(Alexa 647)^8]VP	49.6	0.16	0.28	0.03	0.14	11.0			
12	[HO^1][Leu^4,Lys(Aud)^8]VP[e]	54.4	0.38	0.51	0.53	0.50	12.7	$C_{58}H_{88}O_{13}N_{12}S_2$	1225.6	1226.0
13	[HO^1][Leu^4,Lys(Aud-Alexa 647)^8]VP	55.1	0.14	0.28	0.09	0.15	11.2			

[a] Yields are based on the amount of peptide used in the reaction and are uncorrected for acetic acid, TFA, and water content. Yields of peptides **3**, **5**, **7**, **9**, **11**, and **13** are approximated and based on the MW (∼1250) of Alexa 647 given by Invitrogen/Molecular Probes (pending patent). [b] Solvent systems and conditions are given in Experimental Section. [c] All peptides were at least 95% pure. For elution, a linear gradient 90:10 to 30:70 (0.05% aqueous TFA/0.05% TFA in CH_3CN) over 30 min with flow rate 1.0 mL/min was applied. [d] d[Leu^4,Lys^8]VP (A) was resynthesized as previously described.[3,19] For original synthesis see ref 20. Abbreviations of position 8 substituents and linkers are as follows: Atn (anthraniloyl), β-Ala (β-alanine), Aha (7-aminoheptanoic acid), and Aud (11-aminoundecanoyl).

would be outside. Thus, the Lys^8 residue provides an epsilon NH_2 group which could be used for further additions. Accordingly, we have taken advantage of the stability and good selectivity of d[Leu^4,Lys^8]VP[3,18] to introduce fluorophores on the side chain of the Lys^8 residue to create fluorescent tools that would conserve the pharmacology of d[Leu^4,Lys^8]VP, resist degradation, and selectively decorate with an excellent resolution the plasma membrane of CHO cells expressing V1b and/or OT receptors.

Rationale for Fluorescent Selective V1b Synthesis. We used d[Leu^4,Lys^8]VP (peptide **A** in Figure 1 and Tables 1, 2, and 3) as the parent molecule for designing fluorescent V1b analogues, since we had previously shown that this peptide is a V1b selective agonist for both rat and human VP/OT receptors.[3,18,19] First, we carried out the addition of a hydroxyl (OH) group at the N terminal position of d[Leu^4,Lys^8]VP to give [1-L-(−)-2-hydroxy-3-thiopropanoic acid,4-leucine,8-lysine]vasopressin ([HO^1][Leu^4,Lys^8]VP) (peptide **B** in Tables 1 and 2). We had shown previously that this modification was helpful in the design of fluorescent agonists for the hOT-R.[16,22] We observed that the OH modification at position 1 did not affect the ligand's affinity but strongly increased its selectivity for the hV1b-R (see Table 2). We thus also synthesized a short series of OH-modified fluorescent compounds. We selected different

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Table 2. Binding Properties of Fluorescent Vasopressin Analogues for Human Vasopressin and Oxytocin Receptors[a]

		affinity K_i for [^{3}H]AVP (nM)				hV_{1b}-R selectivity index (SI)		
analogue	peptide	hV_{1a}-R	hV_{1b}-R	hV_2-R	hOT-R	V_{1a}/V_{1b}	V_2/V_{1b}	OT/V_{1b}
AVP	AVP[b]	1.1 ± 0.1	0.68 ± 0.01	1.2 ± 0.2	1.7 ± 0.5	1.6	1.8	2.5
A	d[Leu^4,Lys^8]VP[b]	69 ± 16	0.52 ± 0.07	6714 ± 806	29 ± 6	133	12912	56
1	d[Leu^4,Lys(Atn)^8]VP	246 ± 53	0.65 ± 0.22	4918 ± 1447	21 ± 5	378	7566	32
2	d[Leu^4,Lys(Alexa488)^8]VP	258 ± 36	1.2 ± 0.2	>100000	30 ± 9	215	>83333	25
3	d[Leu^4,Lys(Alexa647)^8]VP	12,033 ± 1903	165 ± 16	>100000	36 ± 7.5	73	>606	0.2
4	d[Leu^4,Lys(β-Ala)^8]VP	215 ± 50	1,3 ± 0,5	5200 ± 190	12.6 ± 2.1	165	4000	9.7
5	d[Leu^4,Lys(β-Ala-Alexa647)^8]VP	8011 ± 2035	65 ± 11	>100000	62 ± 9	123	>1538	1
6	d[Leu^4,Lys(Aha) ^{8}]VP	266 ± 28	0.61 ± 0.05	22010 ± 3310	22 ± 7	436	36082	36
7	d[Leu^4, Lys(Aha−Alexa647)^8]VP	2158 ± 369	74 ± 6	70705 ± 17325	121 ± 16	29	955	1.6
8	d[Leu^4,Lys(Aud)^8]VP	384 ± 93	3.7 ± 1.5	31100 ± 7900	210 ± 67	104	8405	57
9	d[Leu^4,Lys(Aud-Alexa647)^8]VP	1930 ± 326	13.0 ± 3.9	>100000	86 ± 27	148	>7692	6.6
B	HO[Leu^4, Lys^8]VP	879 ± 123	1.7 ± 0.5	1558 ± 137	179 ± 20	517	934	105
10	HO[Leu^4,Lys(Alexa488)^8]VP	55300 ± 11250	149 ± 26	>100000	963 ± 197	371	>336	671
11	HO[Leu^4,Lys(Alexa647)^8]VP	29300 ± 5700	1799 ± 255	>100000	324 ± 29	16	>56	0.2
12	HO[Leu^4,Lys(Aud)^8]VP	2790 ± 381	8.4 ± 2.7	45000 ± 13400	3742 ± 713	332	5357	445
13	HO[Leu^4,Lys(Aud-Alexa647)^8]VP	>100000	668 ± 89	>100000	3415 ± 965	>150	>150	5.1

[a] Binding assays were performed on plasma membranes derived from CHO cells stably expressing the different VP/OT receptors as described in the Experimental Section. K_i values are the mean ± SEM of at least three independent experiments, each performed in triplicate. For each analogue, the hV_{1b}-R SI was calculated as follows: SI = (K_i analogue for hV_x-R)/(K_i analogue for hV_{1b}-R), where hV_x-R is hV_{1a}-R, hV_2-R, or hOT-R. Abbreviations of the linkers are as follows: β-Ala (β-alanine, 4A), Aha (7-aminoheptanoic acid, 8A), and Aud (11-aminoundecanoyl, 12A). Atn is for anthraniloyl. [b] Data from reference.[3]

Table 3. Functional Properties of Some Fluorescent Analogues on Phospholipase C Activity in CHO Cells Stably Expressing Human V1b or OT Receptors[a]

		CHO hV1b-R		CHO hOT-R	
analogue	peptide	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
AVP[b]		2.0 ± 0.6	100	8.3 ± 2.2	27.5 ± 1.8
OT		24.5 ± 2.6	31 ± 1	10.8 ± 1.3	100
A	d[Leu^4,Lys^8]-VP[b]	1.5 ± 0.4	99 ± 6	nd	nd
1	d[Leu^4,Lys(Atn)^8]-VP	2.48 ± 2.1	108 ± 8.5	nd	nd
2	d[Leu^4,Lys(Alexa 488)^8]-VP	6.93 ± 4	93.1 ± 4.6	nd	nd
3	d[Leu^4,Lys(Alexa 647)^8]-VP	401 ± 14	112 ± 9	70.6 ± 19	73.4 ± 5.2
7	d[Leu^4,Lys(Aha-Alexa 647)^8]-VP	267 ± 44	106.7 ± 4.3	nd	nd
9	d[Leu^4,Lys(Aud-Alexa 647)^8]-VP	11.4 ± 2.6	102 ± 2	172 ± 22	69.7 ± 3.05

[a] Phospholipase C stimulation was performed on CHO cells stably expressing hV1b-R or hOT-R. For each analogue, EC_{50} values and E_{max} were calculated as described in Experimental Section. Results are the mean ± SEM of at least three independent experiments, each performed in triplicate. [b] Data from ref 3.

fluorophores to attach to the d[Leu^4,Lys^8]VP (peptide **A**, Figure 1). First, we chose the anthraniloyl (Atn) group, a small fluorescent molecule with MW = 97 Da and a quantum yield (R) of 0.358, used for the design of analogue **1** and highly sensitive to microenvironmental changes.[23] We have also selected the Alexa fluorophores (Molecular Probes) for their brightness and their resistance to photobleaching.[24] We have used Alexa Fluor 488 (Alexa 488) (MW = 643; quantum yield R = 0.60; molar extinction coefficient ε = 71 000) and Alexa Fluor 647 (Alexa 647) (MW ≈ 1250; R not determined, ε = 239 000), the latter being one of the brightest fluorescent molecules reported so far.[25] Moreover, Alexa fluorophores are charged molecules, soluble in water, and not prone to create molecular stacking as the tetramethylrhodamine is (which they are derived from). The aliphatic ω-aminocarboxylic acids, 3-aminopropionic acid (β-Ala), 7-aminohexanoic acid (Aha), and 11-aminoundecanoic acid (Aud) were selected as linkers of various sizes to optimize the interaction of the fluorescent ligand to the V1b receptor. Their spacer arms consist of 4, 8, and 12 atoms, respectively, and will be referred to as 4A, 8A, and 12A further in the text. The structures of the fluorescent peptides and of their parent analogues, designed according to the above rationale, are given in Figure 1 and Table 1.

RESULTS

Binding Pharmacological Properties of Fluorescent Analogues. We first characterized the binding properties of each

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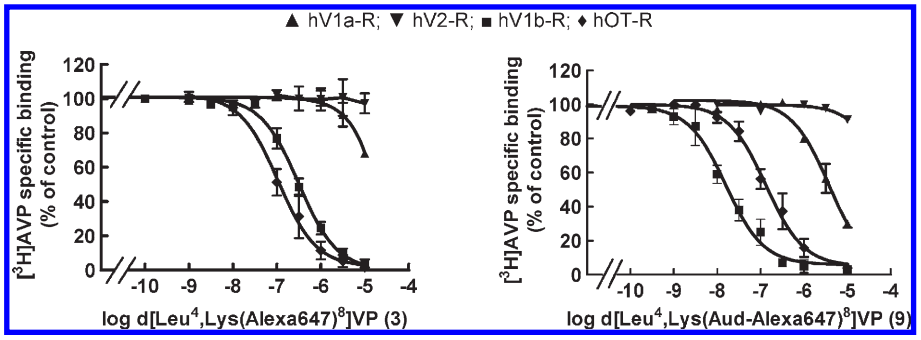

Figure 2. Binding pharmacological profiles of d[Leu^4,Lys(Alexa 647)^8]VP (analogue **3**) and d[Leu^4,Lys(Aud-Alexa 647)^8]VP analogue **9** for human vasopressin and oxytocin receptors stably expressed in CHO cells lines. The binding properties of analogues **3** and **9** were determined by competition experiments on plasma membranes prepared from CHO cells stably expressing hV1a-R, hV2-R, hV1b-R, or hOT-R. Plasma membranes expressing the different VP/OT receptor isoforms were incubated for 1 h at 30 °C with ~1 nM ^{3}[H]AVP in the presence or absence (control, C) of increasing concentrations of unlabeled analogue **3** or **9**. Nonspecific and total binding were determined respectively with and without 1 μM of either AVP or OT, depending on the nature of the receptor studied. Specific binding calculated as the difference between total and nonspecific binding is expressed as percent of the corresponding control specific binding and is the mean ± SEM of at least three independent experiments each performed in triplicate.

analogue and compared their affinities to those of AVP or of the corresponding parent peptide **A** or peptide **B** taken as references (Figure 2, Table 2). Attaching the Atn fluorophore directly to the d[Leu^4,Lys^8]VP (**A**) to give d[Leu^4,Lys(Atn)^8]VP (analogue **1**) did not affect the affinity for the hV1b-R (0.65 vs 0.52 nM) and poorly changed the selectivity versus the hV1a, hV2, and hOT receptors. Thus, since there was no major decrease in affinity or selectivity, we did not perform further modifications of analogue **1**.

For analogue **2**, d[Leu^4,Lys(Alexa 488)^8]VP, with Alexa 488 replacing Atn, the affinity for the hV1b receptor was still very good, with a K_i of 1.2 nM compared to 0.52 nM for the parent analogue **A**. Its selectivities toward the hV1a-R and hV2-R were excellent (Table 2). For analogue **3**, d[Leu^4,Lys(Alexa 647)^8]VP, the affinity for the hV1b-R dropped dramatically to 165 nM (Table 2 and Figure 2), probably because of the relative size of the fluorescent moiety. The Alexa 647 fluorophore is indeed about twice as big as Alexa 488, thus conferring a MW of ~2200 to analogue **3** vs 1500 for analogue **2**. In spite of its loss of affinity, analogue **3** retained good selectivity for the hV1b versus the hV1a and hV2 receptors. Moreover, both fluorescent V1b analogues **2** and **3** exhibited a good affinity for the hOT-R: 30 nM for analogue **2** (Alexa 488) and 36 nM for analogue **3** (Alexa 647). Introducing an OH group at position 1 of analogue **A** to give **B** ([HO][Leu^4,Lys^8]VP) reduced the affinity of this analogue for the hOT-R (K_i of 179 nM for analogue **B** vs 29 nM for analogue **A**). Thus, we synthesized analogues of **B** coupled to Alexa 488 (analogue **10**) and to Alexa 647 (analogue **11**). Introducing an Alexa 488 or Alexa 647 to **B** led to decreases of the affinities for the hOT-R of the resultant fluorescent peptides **10** and **11**. For the Alexa 488 analogue **10**, the K_i for hOT-R was 963 nM compared with 30 nM for analogue **2**. The Alexa 647 derivative (analogue **11**) exhibited a K_i for hOT-R of 324 nM compared with 36 nM for analogue **3**. However, the affinities of peptides **10** and **11** for hV1b-R also dropped dramatically (from 1.2 to 149 nM for the Alexa 488 analogues (**2** and **10**) and from 165 to 1799 nM for the Alexa 647 analogues (**3** and **11**).

In order to improve the affinity of the Alexa 647 analogues for the hV1b-R, we introduced linkers of different lengths. We first verified that attaching linkers to the reference analogue d[Leu^4,Lys^8]VP (**A**) (Table 1) maintained the affinity of the nonfluorescent analogues for the hV1b-R within the nanomolar range (Figure 3 and Table 2). Adding a 4A-spacer (3-aminopropionyl) βAla to d[Leu^4,Lys(Alexa647)^8]VP analogue **3** to give d[Leu^4,Lys(β-Ala-Alexa 647)^8]VP (analogue **5**) improved its affinity (65 nM for analogue **5** compared with 165 nM for analogue **3**) but did not improve its selectivity for hV1b versus hOT receptors (selectivity index of 1 for analogue **5** vs 0.2 for analogue **3**). Increasing the length of the spacer to eight atoms with a 7-aminohexanoyl (Aha) linker to give d[Leu^4,Lys(Aha-Alexa 647)^8]VP (analogue **7**) modestly improved the affinity and selectivity of analogue **7**. Further increasing the length of the spacer to 12 atoms with an 11-aminoundecanoyl (Aud) linker to give d[Leu^4,Lys(Aud-Alexa 647)^8]VP (analogue **9**) significantly improved the affinity and selectivity of analogue **9**. The K_i was 13 nM for analogue **9** compared to 165 nM for analogue **3**. This improvement might result from the fact that the high MW fluorescent moiety is now more distant from the core peptide (**A**) and does not hamper the ligand−receptor interaction as much (Figure 3). The selectivity was also much better (SI of 6.6 compared to 0.2, Table 2 and Figure 2). In order to try to gain more V1b-R selectivity vs OT-R, an OH-version of analogue **9** (HO[Leu^4,Lys(Aud-Alexa 647)^8]VP, analogue **13**) was also synthesized. The affinity severely dropped (from 13 to 668 nM) without any improvement in its selectivity. We thus decided to forego using HO[Leu^4,Lys^8]VP (peptide **B**) for any further designs of fluorescent V1b ligands.

Functional Properties of Fluorescent Peptides: Capacity To Stimulate Phospholipase C. As both the hV1b and hOT receptors are directly coupled to phospholipase C (PLC),[26] we measured the ability of each fluorescent analogue to activate PLC in stably transfected CHO cells. First we looked at the capacity of the most promising analogues to stimulate PLC in a CHO hV1b-R cell line. As illustrated in Figure 4 and summarized in Table 3, with the exception of OT, which behaved as a partial agonist of the hV1b-R (31% of maximal VP-stimulated PLC activation), all analogues tested were full agonists with analogue **3** having a higher EC_{50}. The Atn analogue (**1**) and the Alexa 488 analogue (**2**) showed EC_{50} in the same range as analogue **A** and AVP (Table 3). The Alexa 647 analogues **3**, **7**, and **9**, although full agonists, showed EC_{50} values of 401 nM for analogue **3** (Alexa 647 directly attached to analogue **A**) and 267 nM for analogue **7** (having the 8A, Aha linker). Only the 12A Aud-peptide (analogue **9**) displayed a good EC_{50} of 11.4 nM for hV1b-R coupling. Second, as the Alexa 647 compounds

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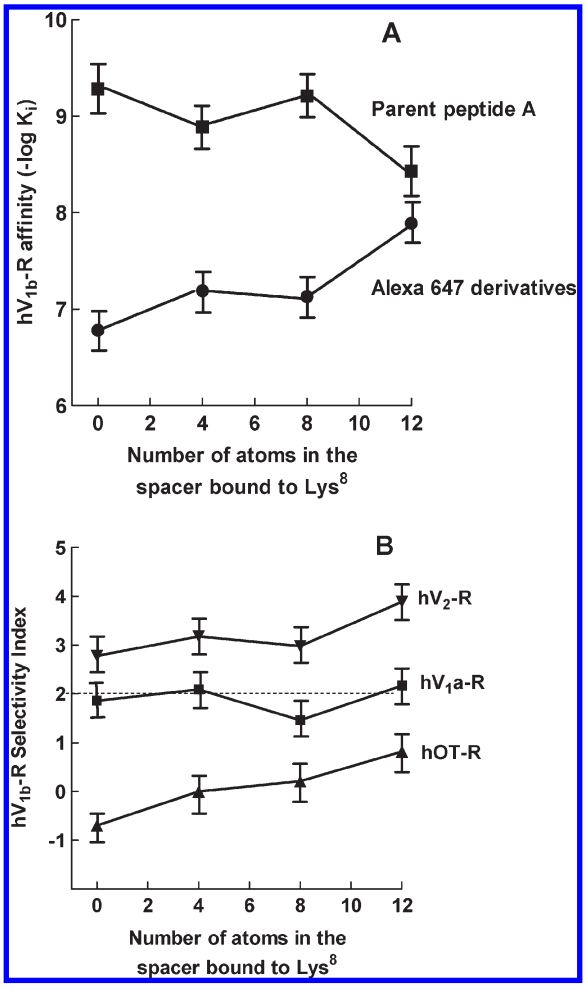

Figure 3. Influence of the size of the linker between Alexa 647 and peptide **A** on its V1b affinity and selectivity for human vasopressin and oxytocin receptors. (A) The V1b affinity of parent analogues **A**, **4**, **6**, and **8** and that of their corresponding peptides with Alexa 647 attached to linkers of different lengths (analogues **3**, **5**, **7**, **9**) are plotted as the numbers of atoms in the spacer bound to Lys^8. Values are from Table 2. (B) The hV1b-R selectivity index of analogues described above is plotted as the number of atoms of the spacer bound to Lys^8. Values are from Table 2 with V1b selectivity index calculated as $\log[K_i(\text{hVx-R})/K_i(\text{hV1b-R})]$, where Vx is hV2-R, hV1a-R, or hOT-R.

displayed some affinity for the hOT-R too, we also compared their capacities to activate PLC in a CHO hOT-R cell line. We observed that, compared to OT, the natural ligand AVP was a partial agonist (27.5% of maximal OT-stimulated PLC activation). Analogues **3** and **9** also displayed partial agonism, with ~70% of PLC activation compared to the one induced by the natural agonist OT. In general, the respective EC_{50} of all analogues for either the hV1b-R or the hOT-R remained well correlated with their binding properties (compare Tables 2 and 3).

Labeling Properties of Fluorescent Peptides Evaluated by Spectrofluorimetry and Cell Imaging. The Atn analogue **1**, d[Leu^4,$Lys(Atn)^8$]VP, was designed to provide a fluorescent V1b selective ligand with blue emission that could be a possible partner of an Alexa 488 analogue to record experimental fluorescence resonance energy transfer (FRET) between V1b homodimers. Since UV confocal microscopes are not easily available, the Atn analogue **1** cannot be tested by imaging but by spectrofluorimetry of cell suspensions in cuvettes. Living CHO cells, stably expressing hV1b receptors, were exposed to analogue **1** for 1 h at 12 °C, washed, and evaluated for the fluorescence emitted at the wavelength (λ) of 412 nm by spectrofluorimetry. Complete excitation and emission spectra were established previously, and the value of 412 nm was found to be the λ_{max} of experimental emission for analogue **1** whereas the λ_{max} of excitation was confirmed to be 343 nm (data not shown). As shown in Figure 5 (A), analogue **1** can display a saturable fluorescent binding in Dulbecco's modified Eagles medium/bovine serum albumin/4-2-hydroxylethyl-l-piperazineethanesulfonic acid (DMEM/BSA/HEPES) buffer with a fluorescence (EC_{50}) of 1.7 nM, a value very close to the one obtained by binding experiments performed on membrane preparations derived from CHO cells stably expressing hV1b-R (0.65 nM). This binding (analogue **1** used at the saturating concentration of 10 nM) can be completely suppressed with an excess of the nonfluorescent analogue **A** (1 μM) demonstrating its specificity (Figure 5A).

Fluorescent derivatives of the V1b agonist d[Leu^4,Lys^8]VP (A) were designed to provide tools for imaging the V1b-R on cells in culture. To verify this point, confocal imaging was conducted on living CHO stable cell lines, expressing either one of the four human receptors, i.e., V1a-R, V1b-R, V2-R, or OT-R. Only CHO cells expressing the hV1b-R and the hOT-R could be labeled. The CHO hV1a-R and the CHO hV2-R remained unlabeled, even at the concentration of 500 nM of either Alexa 488 or Alexa 647 analogues (data not shown). In CHO hV1b-R, the binding appeared to be patchy and mainly localized at the plasma membrane (Figure 5B and Figure 6 right panels). This "patchy" effect was not due to internalization, since the experiments were conducted at 12 °C, a temperature below the chain-melting transition temperature of the membrane lipids (16 °C). This aspect was also observed after one night of incubation at 4 °C, a condition where internalization cannot take place either (data not shown). Moreover, the distribution of the fluorescent labeling with either Alexa 488 or Alexa 647 compounds was found to be similar and was completely suppressed in the presence of 1 μM analogue **A** (Figure 5B).

Figure 6 further shows the ability of the fluorescent analogues **3** and **9** to selectively label hV1b or hOT receptors at the plasma membrane of stably transfected CHO cells. Plasma membrane labeling observed on CHO hV1b-R cells incubated with 250 nM of analogue **3** (a mixed OT/V1b agonist; see Table 2) is totally suppressed by co-incubation with 100 nM analogue **A** but only very partially when similar experiments were performed on CHO hOT-R cells (Figure 6, top rows). The complete displacement of analogue **3** from CHO hOT-R was obtained only with 500 nM or more of **A**, in agreement with its respective affinities for hV1b-R (0.52 nM) compared to hOT-R (29 nM).[3] As expected, [1-β-mercapto-β,β-cyclopentamethylenepropionyl,2-0-methyl tyrosine,8-arginine]vasopressin (Manning compound) (500 nM), a mixed OT/V1a antagonist,[26,27a,27b] totally displaced analogue **3** from CHO hOT-R cells but not from CHO hV1b-R cells. Similarly, total displacement of analogue **3** from CHO hOT-R cells was also obtained with a specific non-peptide OT antagonist, 4-chloro-3-[(3*R*)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-*N*-ethyl-*N*-(3-pyridylmethyl)benzamide, hydrochloride (**14**) (SR126768A)[28] (100 nM), and this displacement was not seen on CHO hV1b-R cells (not shown). Analogue **3** labeling was not displaced by

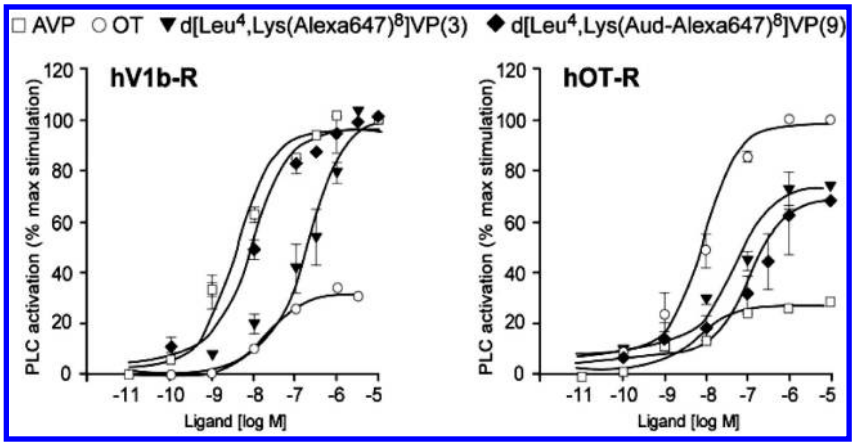

Figure 4. Functional properties of d[Leu^4,Lys(Alexa 647)^8]VP (analogue **3**) and d[Leu^4,Lys(Aud-Alexa 647)^8]VP (analogue **9**) on phospholipase C activity in CHO cells stably expressing the human V1b and OT receptors. Left: CHO cells stably expressing the hV_{1b}-R were incubated with or without (control) increasing amounts of fluorescent or parent peptides. Total inositol phosphate (InsP) that accumulated was measured as described in Experimental Section and expressed as % of maximal AVP stimulation (1 μM). Data are the mean ± SEM of three independent experiments, each performed in triplicate. Right: Similar experiments were performed with CHO cells stably expressing the hOT-R. Total inositol phosphate that accumulated was measured as described in Experimental Section and expressed as the % of maximal OT stimulation (1 μM). Data are the mean ± SEM of three independent experiments, each performed in triplicate.

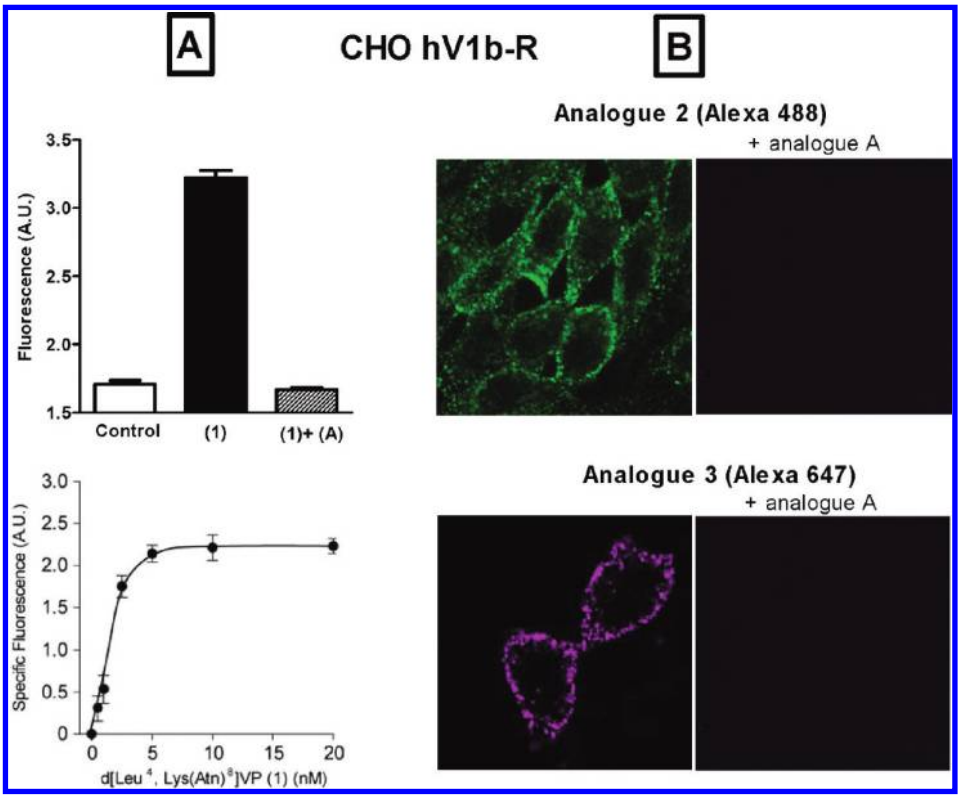

Figure 5. Fluorescent properties of d[Leu^4,Lys(Atn)^8]VP (analogue **1**), d[Leu^4,Lys(Alexa 488)^8]VP (analogue **2**), and d[Leu^4,Lys(Alexa 647)^8]VP (analogue **3**) on CHO cells stably expressing the human V1b receptor. (A) Histogram (top): Cells in suspension were incubated for 1 h at 12 °C with 10 nM analogue **1** in the absence (dark bar) or in the presence (hatched bar) of 1 μM d[Leu^4,Lys^8]VP (analogue **A**) and compared to control cells incubated without fluorescent ligand (white bar). Cells were washed three times with cold PBS and measured for fluorescent emission at 412 nm in cuvettes of spectrofluorimetry. Saturation curve (bottom): Aliquots of cells were incubated with increasing concentrations of analogue **1** for 1 h at 12 °C, washed three times, and counted for fluorescence emission at 412 nm. The endogenous fluorescence at 412 nm of control cells, not exposed to fluorescent ligand, was deduced for each concentration. (B) CHO hV1b-R cells grown on coverslips were incubated in the presence of analogue **2** (50 nM) or of analogue **3** (250 nM) for 1 h at 12 °C in the absence or in the presence of 1 μM d[Leu^4,Lys^8]VP (analogue **A**). Confocal imaging was performed with Zeiss LSM510 Meta microscope using a 63× (NA 1.4) objective with an argon laser excitation at 488 nm and a BP 505−530 nm emission filter for analogue **2** (green) and with a helium/neon laser excitation at 633 nm with a LP650 for emission for analogue **3** (purple).

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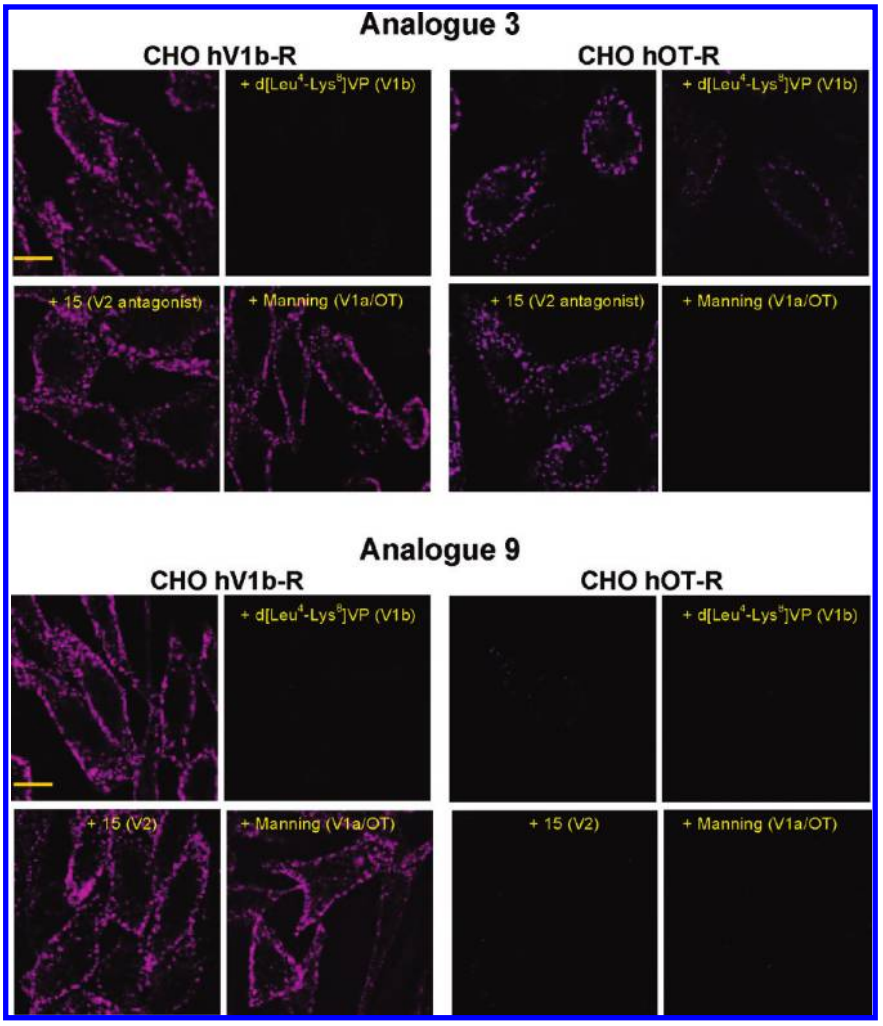

Figure 6. Displacement of d[Leu^4,Lys(Alexa 647)^8]VP (analogue **3**) and of d[Leu^4,Lys(Aud-Alexa 647)^8]VP (analogue **9**) binding with nonfluorescent ligands on CHO hV1b-R and hOT-R stable cell lines. CHO cells on coverslips were incubated in the presence of analogue **3** (250 nM) or of analogue **9** (150 nM) for 1 h at 12 °C in the absence or in the presence of 100 nM selective V1b agonist d[Leu^4,Lys^8]VP (analogue **A**) or of 1 μM non-peptide AVP V2 antagonist **15**[29] or of 500 nM V1a/OT peptide antagonist Manning compound.[27a] Confocal imaging was performed with Zeiss LSM510 Meta microscope using a 63× (NA 1.4) with laser excitation at 633 nm and LP650 for emission. The bar represents 10 μm (zoom 4×). Images are representative of three independent experiments.

the selective non-peptide AVP V2 antagonist (1-[4-*N*-*tert*-butylcarbamoyl)-2-methoxybenzenesulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]indol-2-one, phosphate monohydrate (**15**) (SR121463)[29] (1 μM), neither from CHO hV1b-R nor from CHO hOT-R cells.

The labeling of hV1b-R CHO cells with 150 nM analogue **9** (a selective V1b agonist; see Table 2) was intense and completely displaced by 100 nM **A** but neither by 500 nM Manning compound nor by 1 μM **15** (Figure 6, bottom row left). When similar experiments were performed on CHO hOT-R cells, the labeling was very low and both **A** (V1b selective) and Manning compound (OT/V1a peptide antagonist) totally displaced analogue **9** labeling (Figure 6, bottom row right). However, this labeling was not displaced by the non-peptide AVP V2 antagonist **15** (1 μM) neither from CHO hV1b-R nor from CHO hOT-R cells as previously shown for analogue **3**.

In order to fully characterize the imaging properties of the two analogues **3** and **9**, we have established saturation curves of each analogue in the conditions of living cells (DMEM/BSA/HEPES) on CHO cells expressing either V1b-R or OT-R. The membrane fluorescence was quantified with a laser scanning microscope (LSM) browser after confocal imaging using helium/neon laser 633 nm for detecting Alexa 647 analogues (Figure 7). Analogue **9**, which displays a better affinity for the hV1b-R as compared to analogue **3** (as measured by competition studies on membrane preparations with ^{3}[H]-AVP, see Table 2), also showed a better capacity to bind membrane V1b-R sites on living cells at low concentration (affinity of the fluorescent ligand of 14.23 ± 2.19 nM for analogue **9** compared to 172.3 ± 19 nM for analogue **3**, Figure 7 left panel). On CHO hOT-R cells, the affinities of the two analogues were quite similar, with a slight preference for analogue **3** (109 ± 9.7 nM) compared to analogue **9** (162 ± 17 nM) (Figure 7, right panel), reflecting the results obtained in the displacement studies performed with ^{3}[H]AVP (Table 2).

DISCUSSION AND CONCLUSION

Receptors of the vasopressin and OT family are important in the regulation of the stress processes (review in ref 30). Centrally, the V1b receptors have been involved in stress and especially in learning and memory processes. Important data have been

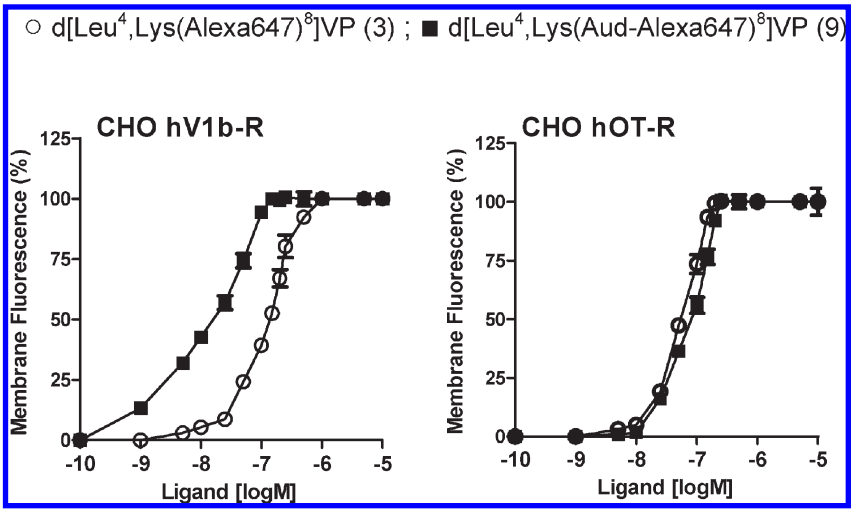

Figure 7. Fluorescent binding properties of d[Leu^4,Lys(Alexa 647)^8]VP (analogue **3**) and d[Leu^4,Lys(Aud-Alexa 647)^8]VP (analogue **9**) on CHO cells stably expressing the human V1b and OT receptors. CHO hV1b-R cells on coverslips were incubated in the presence of increasing concentrations of analogue **3** or of analogue **9** for 1 h at 12 °C, washed 3 times with PBS, and fixed with 4% PFA. Confocal imaging was performed with a Zeiss LSM510 Meta microscope using a 63× objective with a helium/neon laser excitation at 633 nm with a LP650 for emission. Membrane fluorescence was evaluated on 30−40 cells for each concentration of analogue by LSM Browser and ImageJ using the 1−250 fluorescent unit dynamics. After deduction of the values of control unlabeled lines 5−7 cells, saturation curves were constructed with the mean of three distinct experiments and expressed as % of the maximal labeling.

obtained by the use of knock-out (KO) animals, but after a period of cloning and pharmacological characterization in the past decade, it became necessary to elucidate the distribution of these receptors to better understand their central functions in vivo.

To do so, tracers are necessary and the radiolabeled natural hormones were the first candidates to detect receptors in situ. However, because of the close affinity between AVP and OT for the OT receptors,[26,31] it is very difficult to discriminate between their receptor sites. Other studies were conducted using receptor antibodies, but since no good antibody against the V1b-R is available, the information about V1b receptor localization remains uncertain. Moreover, data obtained by in situ hybridization experiments were not always reliable, because of the partial similarities between the VP and OT receptor probes,[9,10] and reflect mRNA levels encoding the receptor and not the expression of the receptor itself.

Agonists and antagonists for different classes of receptors have been produced, but still, all were not completely selective, especially toward the V1b-R.[27b] A selective V1b agonist was produced by replacement in AVP of the glutamine residue in position 4 by a leucine[18] and made highly resistant to degradation by deamination at the Cys^1 position. The subsequent replacement of Arg^8 by a Lys^8, leading to the V1b agonist **A**,[3,19,20] did not modify its V1b selectivity. This allowed the covalent addition of a fluorophore to the free ε NH_2 group of the Lys residue at position 8 in **A**, thus making possible the synthesis of new fluorescent V1b agonists.

Taking advantage of the progress in confocal microscopy and in the production of new fluorophores, we elected to design and synthesize the fluorescent V1b analogues reported here. We selected three different colors for complementary purposes. The Atn, successfully attached to the μ opioid receptor agonist DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2 with Dmt = 2′,6′-dimethyltyrosine),[32,33] is a good reporter of environment[23] displaying a relatively small size. Thus, we confirm here that the Atn fluorophore does not prevent d[Leu^4,Lys(Atn)^8]VP (**1**) from binding to the V1b-R with a similar affinity as the parent peptide **A** (0.65 vs 0.52 nM). It may be a good compound to probe ligand binding interactions with V1b receptors and to confirm the data about the vasopressin V1b binding pocket we have obtained by modeling.[21] It may also be a good donor to a green acceptor in FRET experiments for identification of V1b-R homodimers in vivo. Thus, although other receptors of the vasopressin/oxytocin family are known to be expressed as homodimers (OT-R/OT-R)[34,35] or heterodimers (V1a-R/V2-R),[36,37] nothing is known about V1b-R monomers or dimers. Only heterodimers of V1b-R with corticotrophin-releasing hormone receptor type 1 (CRHR1) have been described.[38] We have also chosen the "Alexa family" of fluorophores (Molecular Probes) that develop very good brightness and good resistance to degradation and to photobleaching, compared to Cyanines **3** and **5**, for example,[24,39] and that are relatively easy to attach to peptides. The Alexa 488 was selected because it is excited in the 488 nm range, a wavelength very common on any fluorescent microscope, including the electrophysiological settings, where it can be an excellent tool to reveal cells to be recorded (data not shown). It was also a good acceptor for energy transfer with Atn (good overlapping of the emission of Atn/excitation of Alexa 488 spectra) so that we could conduct studies on V1b-R homodimers in natural tissues later. Alexa 647 was also selected because it provides an extremely bright signal (ε = 239 000) compared to other Alexa fluorophores including Alexa 488 (ε = 165 000), a quality that will be essential for detecting low levels of receptors in tissue slices. As Alexa 647 excitation is in the far red, cell autofluorescence is also very low.

The pharmacological properties (binding, coupling to PLC) were determined for the 13 analogues and compared with the parent peptide d[Leu^4,Lys^8]VP (analogue **A**) and to the two natural peptides AVP and OT (Table 2). First it appears that all analogues conserved a very good selectivity for V1b-R vs V1a-R and V2-R. Directly attaching the fluorophore reduced slightly the selectivity, from 56-fold in d[Leu^4,Lys^8]VP (analogue **A**) to

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32-fold (analogue **1**, Atn) and to 25-fold in analogue **2** (Alexa 488). The selectivity was totally inverted for analogue **3** (Alexa 647), becoming 5 times better for the OT-R compared to the V1b-R. As a possible explanation, it has been previously proposed that in the OT molecule, compared to AVP, the neutral Leu^8 is an important amino acid to keep OT-R selectivity, while this position has to be positively charged to interact with vasopressin receptors.[40,41] As the Lys^8 of d[Leu^4,Lys^8]VP (**A**) (that has replaced the Arg^8 naturally present in AVP) is now neutralized by attaching a bulky residue on the free ε NH_2, it may explain the modification of V1b-R toward OT-R selectivity. It is also possible that the bulky fluorescent molecule (especially big in the case of Alexa 647) prevents the 1−6 sequence of the peptide **A** to fit into the binding pocket situated at the top of TM5 and TM7 as described in our V1b-R model.[21,42] To overcome this problem, we added linkers of different sizes, 4A (βAla), 8A (Aha), and 12A (Aud). These linkers, comprising carbon chains of different lengths, all have a C-terminal NH_2 group to which a fluorophore could be covalently attached. We observed that, as shown in Figure 3A, the longer the linker, the more the affinity of the Alexa 647 analogue for the V1b-R becomes closer to that of the parent peptide **A**. This affinity reaches 13 nM for analogue **9**, compared to 0.52 nM for **A** and compared to 3.7 nM observed for analogue **8**, which corresponds to an analogue of **A** simply connected to the Aud linker with no fluorophore. The selectivity toward the hV1b-R also increased. Thus ,analogue **3** (Alexa 647 without a linker) is more selective for the hOT-R compared to the hV1b-R (36 nM vs 165 nM). With the Aud linker (analogue **9**), it became more selective for the hV1b-R (13 nM) compared to the hOT-R (86 nM). Thus, we totally inverted the compound selectivity (Figure 3B). The selectivity for the hV1a-R and the hV2-R was not strongly modified, and both analogues **3** and **9** remained low-affinity agonists for these two receptor isoforms (over 2000-fold).

We also noticed that replacing one of the hydrogens on the α carbon at position 1 by a hydroxyl group in peptide **A** to give peptide **B** enhanced the hV1bR/hOTR selectivity. Thus, for analogue **B**, the affinity for the hV1b-R was conserved (1.7 nM vs 0.52 nM), whereas the hV1bR/hOTR selectivity was doubled (V1b-R SI of 105 instead of 56). We thus synthesized OH-substituted versions of the analogues coupled to Alexa 488 or Alexa 647 (peptides **10**−**13**). Unfortunately, although the affinity for the hOT-R remained weak, the affinity for the hV1b-R also dropped (to 149 nM for the Alexa 488 analogue (**10**) and to 1799 nM for the Alexa 647 analogue (**11**). Adding a 12A linker (analogue **13**) did not improve the affinities, neither for the hOT-R (3415 nM) nor for the hV1b-R (668 nM). So we did not pursue this track any further.

To fully characterize the analogues, functional coupling was evaluated for the most promising compounds (Table 3). The activation of PLC was measured for the parent peptide **A** and the fluorophore-coupled analogues Atn (analogue **1**), Alexa 488 (analogue **2**), and Alexa 647 (analogue **3**) and with linkers (analogues **7** and **9**). The activity was compared to that of the corresponding native peptides AVP and OT in two different stable cell lines: one expressing hV1b-R, the other hOT-R. First, it appears that the EC_{50} values were in the same range compared to the K_i values. All analogues (original **A**, Atn-, Alexa 488-, or Alexa 647-coupled analogues) were full agonists at the hV1b-R compared to the natural peptide AVP (Table 3). Only OT displayed partial agonism. The two Alexa 647 analogues **3** and **9** were also tested on CHO hOT-R, and both displayed partial agonism, with ∼70% of activity compared to 100% observed with OT. AVP was also a partial agonist at hOT-R with an E_{max} of only 27.5%. It is well-known that the OT-R can couple to G_q (PLC) and also to G_i,[40,41] which is not the case for the V1b-R. These data confirm that OT can be a partial agonist for AVP receptors and, on the contrary, AVP can be a partial agonist for the OT-R. Since it is known that there is a functional antagonism between AVP and OT, the first (AVP) being released during the stress and the second (OT) being present in nursing and calming behaviors, each acting at its own receptors, the partial agonism shown here could play a role in this functional antagonism by competing for the opposite site and preventing the "opposite effect" hormone from interacting with its own receptor, thus reinforcing the physiological effects.

The membrane hV1b receptors are labeled as patches. This patchy effect could reveal domains of the membrane where the receptors are concentrated. However, it does not seem to be due to the fact that the ligand is an agonist, since it also happened with other peptides from the vasopressin family including the V1a antagonist Rhm^8-PVA (4OH-Ph$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-(5-carboxytetramethylrhodamyl)-NH_2) on rat hepatocytes cells,[15] V1a/OT derivatives attached to Rhm (tetramethylrhodamyl)[22] and with other ligands such as Alexa 488-opioid dermorphin and deltorphin[43] on CHO cells. However, this aspect was similar to the one observed on living cells before paraformaldehyde (PFA) fixation (not shown) and we preferred this technique to the one using antibodies, where fixation and Triton permeation are necessary for receptor detection and may give distortions. Thus, we depict here functional receptors capable of PLC activation and of internalization (not shown).

In this study, in contrast to studies performed only on membranes, we have validated the capacity of the fluorescent ligands **3** and **9** to label directly specific living cells and we have verified that the pharmacological and functional data fit well with the imaging data. Often, many new compounds lack full characterization on all VP and OT receptor isoforms, and because they display only a partial specificity (V1b SI < 50), they could induce nonspecific labeling of several OT/AVP receptors.[27b] From our data, analogues **3** and **9** seem really promising for detecting human V1b or OT receptors. d[Leu^4,Lys^8(Aud-Alexa 647)]VP analogue **9** seems more selective for the hV1b-R but also presents some affinity for hOT-R. Utilized at 150 nM and in the presence of 100 nM OT-R selective OT antagonist **14** (or of the Manning compound), it provides a very selective labeling of the hV1b-R. d[Leu^4,Lys^8(Alexa 647)]VP (analogue **3**) presents a mixed affinity for hV1b and hOT receptors and can also be used in the presence of 100 nM of the non-peptide OT antagonist **14** or of the Manning compound to selectively label V1b sites. It can also be used to label OT sites in the presence of the selective non-peptide AVP V1b antagonist (2*S*,4*R*)-1-[5-chloro-1-[2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1*H*-indol-3yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamine (**16**) (SR149415).[44] Thus, the labeling of analogue **3** on CHO hOT-R cells was only completely suppressed with high concentrations of analogue **A**, more than 500 nM or with the non-peptide V1b antagonist **16** at a concentration higher than 500 nM (not shown) at which it can also compete with hOT-R as described.[44]

To conclude, by inserting different fluorophores at position 8 in d[Leu^4,Lys^8]VP (**A**), we generated two new promising fluorescent analogues d[Leu^4,Lys^8(Alexa 647)]VP (**3**) and d[Leu^4,Lys^8(Aud-Alexa 647)]VP (**9**) capable of selectively

labeling hV1b-R or hOT-R expressed on CHO cells. We completely characterized their conditions of utilization. In natural conditions on nonfixed tissue, these fluorescent peptides **3** and **9**, having the Alexa 647 fluorophore attached to the lysine residue at position 8, will allow the detection of very low levels of V1b or OT binding sites on native tissues in the CNS, with almost no background, and will permit, for the first time, the cartography of the V1b receptor within the brain.

EXPERIMENTAL SECTION

Materials. All reagents used were analytical grade. Most standard chemicals were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany) unless otherwise indicated. AVP and OT were from Bachem (Bubendorf, Switzerland) and [^{3}H]AVP (44 Ci/mmol) and myo-[2-^{3}H]inositol (20 Ci/mmol) from PerkinElmer (Courtabœuf, France). Fetal calf serum and polyornithine came from Sigma (Saint-Quentin Fallavier, France). Dulbecco's modified Eagle medium (DMEM) and penicillin−streptomycin were purchased from Invitrogen (Cergy Pontoise, France). Inositol-free DMEM came from ICN Biochemicals (Orsay, France). Dowex AG1-X8 formate form 200−400 mesh was purchased from Bio-Rad (Munich, Germany). All peptides and fluorescent derivatives listed in Table 1 and Manning compound[27a] were synthesized in the laboratory of Dr. M. Manning (College of Medicine, University of Toledo, OH, U.S.) as described below. The non-peptide OT antagonist **14**,[28] the non-peptide AVP V2 antagonist **15**,[29] and the non-peptide AVP V1b antagonist **16**[44] were from Sanofi-Synthelabo (Toulouse, France).

L-(−)-2-Hydroxy-3-methoxybenzylthiopropanoic acid (HO-Mpr-(Mob) was synthesized as previously described for HO-Mpr(Bzl).[45] The Mpr(Meb) and Boc-11-aminoundecanoic acid were from Chem-Impex International (Wood Dale, IL). The N^{α}-Boc protected amino acids were purchased from Bachem (Torrance, CA) and Chem-Impex International (Wood Dale, IL). The Boc-Gly-resin was from Calbiochem-Novabiochem Corp. (San Diego, CA). The Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Alexa Fluor 488 5-TFP) (5-isomer), and Alexa Fluor 647 carboxylic acid succinimidyl ester were from Invitrogen/Molecular Probes, Inc. (Eugene, OR). The *N*-carboxyanthranilic anhydride (isatoic anhydride) and Boc-β-alanine (Boc-3-aminopropionic acid) were from Sigma-Aldrich, Inc. (St. Louis, MO). The Boc-7-aminoheptanoic acid (BOC-Aha) was from Bachem (Torrance, CA). Peptide d[Leu^4,Lys^8]VP[20] (**A**) was resynthesized as previously described.[3,19] Peptide [HO^1]Leu^4,Lys^8]VP (**B**) was synthesized specifically for this study by the Merrifield solid-phase method[46,47] with modifications previously described[48,49,3,19] as shown in Solid-Phase Synthesis Procedures below. TLC was run on precoated silica gel plates (60F-254, 3 Merck) with the following solvent systems: (a) 1-butanol/AcOH/H_2O (upper phase) (4:1:5); (b) 1-butanol/AcOH/H_2O (4:1:2); (c) 1-butanol/AcOH/H_2O/pyridine (15:3:3:10); (d) 1-butanol/AcOH/H_2O (2:1:1). Loads of 10−15 μg each were applied, and chromatograms were developed at a minimal length of 10 cm. For detection, a combination of monitoring on a UV lamp (model UVGL-58, UVP Inc., San Gabriel, CA) and the chlorine gas procedure for the KI-starch reagent[47] was used. Analytical HPLC was performed on a Hitachi D-7000 HPLC system under the following conditions: 90:10 to 30:70 0.05% aqueous TFA/0.05% TFA in MeCN, linear gradient over 30 min at 1.0 mL/min (λ = 254 or 214 nm) on a Vydac 218TP54 C18 column (Grace Vydac, Hesperia, CA). For semipreparative HPLC the same apparatus and gradient were used over 30 min at 5.0 mL/min (λ = 214 nm) on a Vydac 218TP510 C18 column. All peptides were at least 95% pure. Mass spectra (MS) were done by the Tuft's Core Facility, Department of Physiology (Boston, MA) on a MALDI-TOF Voyager mass spectrometer (Perspective Biosystems/Applied Biosystems) using dihydrobenzoic acid as the matrix.

Solid-Phase Synthesis Procedures. The schematic structures of d[Leu^4,Lys^8]VP (**A**) and parent molecules including all fluorescent analogues are illustrated in Figure 1. Their syntheses will be described below.

*HO-Mpr(Mob)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Mob)-Pro-Lys(2ClZ)-Gly-NH$_2$ (Protected Precursor for Peptide **B** (PrB)).* Boc-Gly-resin (0.7 g, 0.5 mmol) was subjected to eight cycles of deprotection, neutralization, and coupling with Boc-Lys(2ClZ), Boc-Pro, Boc-Cys(Mob), Boc-Asn-ONp, Boc-Leu, Boc-Phe, Boc-Tyr(Bzl), and HO-Mpr(Mob), respectively. A 1 M HCl/AcOH mixture was used in all the deprotection steps.[46] Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. All coupling reactions (except when Boc-Asn was involved) were performed by the DCC/HOBt procedure[50] in CH_2Cl_2/DMF (9:1, v/v). Boc-asparagine was coupled as its nitrophenyl ester[51] in DMF. The resulting protected peptidyl resin was cleaved by ammonolysis in methanol.[52] The protected peptide was extracted with hot DMF (30 mL), and the product was precipitated by the addition of hot water (~300 mL). After cooling, the product was collected, dried in vacuo over P_2O_5, and reprecipitated from DMF (30 mL) and ether (~200 mL). Collection and drying in vacuo over P_2O_5 gave the required protected nonapeptide amide (PrB) 0.53 g 68.5% yield.** Mp 112−114 °C. TLC, R_f (solvent system): 0.73 (a); 0.78 (b); 0.70 (c); 0.77 (d).

*[HO^1][Leu^4,Lys^8]VP (Free Peptide **B**).* The Na/liquid NH_3 procedure[53] with the modifications previously described[5,48,49,54] was used for the deprotection of the protected precursor PrB. A solution of PrB in sodium-dried ammonia (~400 mL) was treated at the boiling point and with stirring with sodium from a stick of metal contained in a small-bore glass tube until a light-blue color persisted in the solution for about 30 s. NH_4Cl was added to discharge the color. Reoxidation of the resulting deblocked disulfydryl peptide (**B**) was performed with potassium ferricyanide ($K_3[Fe(CN)_6]$) using the modified reverse procedure[55] as follows. The peptide residue was dissolved in 25 mL of 50% AcOH, and the solution was diluted with 50 mL of H_2O. The peptide solution was added dropwise with stirring over a period of 15−30 min to a 600 mL aqueous solution that contained 20 mL of a 0.01 M solution of $K_3[Fe(CN)_6]$. Meanwhile, the pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. Following oxidation, the free peptide (**B**) was isolated and purified as follows. After acidification with AcOH to pH 4.5 and stirring for 20 min with an anion exchange resin (Bio-Rad, AG 3 × 4, Cl^- form, 5 g damp weight), the suspension was slowly filtered and washed with 0.2 M AcOH (3 × 30 mL). The combined filtrate and washings were lyophilized. The resulting powder was desalted on a Sephadex G-15 column (110 cm × 2.7 cm), eluting with aqueous AcOH (50%), with a flow rate of 5 mL/h.[56] The eluate was fractionated and monitored for absorbance at 254 nm. The fractions making up the major peak were checked by TLC, pooled, and lyophilized. The residue was further subjected to gel filtration on Sephadex LH-20 using a 100 cm × 1.5 cm column, eluting with aqueous AcOH (2 M) with a flow rate of 4 mL/min. The peptide was eluted in a single peak (absorbance at 254 nm). After lyophilization of the pertinent fractions, the collected peptide was subjected to a final semipreparative HPLC purification to give 54.9 mg (54.2%) of desired [HO^1]Leu^4, Lys^8]VP (**B**). TLC, R_f (solvent system): 0.26 (a); 0.22 (b); 0.18 (c); 0.38 (d). HPLC, t_R: 11.1 min. MW (calculated): 1042.3. MW (found by MS): 1042.6.

Fluorescent Analogue Synthesis

Peptide Labeling with Anthraniloyl (Atn) Fluorophore (Peptide 1, Table 1). We adapted the synthesis of Boc-Dap(Atn)[23] and Boc-Lys(Atn)[32] for the preparation of d[Leu^4,Lys(Atn)^8]VP (peptide **1**) as follows: To a stirred solution of 12.5 mg (12.2 μM) of d[Leu^4, Lys^8]VP (**A**) and 1.3 mg (12.2 μM) of dry Na_2CO_3 in water/acetonitrile (1:1 v/v, 150 μL), isatoic anhydride, 2.38 mg (14.6 μM) in 100 μL of acetonitrile, was added. The mixture was stirred overnight at room temperature at pH ~8 and with TLC monitoring. The mixture was

acidified to pH 2 with 5% aqueous $KHSO_4$ and was subjected to a two-step purification procedure, first on a Sephadex G-15 column with 25% acetic acid as eluent and finally by semipreparative HPLC to give the desired peptide **1.**

General Procedure for Peptide Labeling with Alexa 488 and Alexa 647 Fluorophores

Fluorescent Peptides with Alexa Fluorophore Directly Attached at Position 8 of Parent Peptide d[Leu^4,Lys^8]VP (A) and [HO^1][Leu^4, Lys^8]VP (B) (Peptides 2, 3, 10, and 11 (Table 1)). The preparation of the fluorescent peptides **2**, **3**, **10**, and **11** was performed as described by Albizu and co-workers[57] with some modifications as follows: 1.2 μM (in excess) parent peptide d[Leu^4,Lys^8]VP (**A**) or [HO^1][Leu^4,Lys^8]VP (**B**) was dissolved in 100 μL of anhydrous DMF and mixed with 5 μL of DIPEA. The solution was added to 1 mg of the appropriate fluorophore. For fluorescent peptides **2** and **3** the parent peptide was d[Leu^4,Lys^8]VP (**A**). For fluorescent peptides **10** and **11** the parent peptide was [HO^1][Leu^4,Lys^8]VP (**B**). The fluorophores were as follows: for peptides **2** and **10** Alexa 488 tetrafluorophenyl ester (5-carboxy pure isomer) was used. For peptides **3** and **11**, Alexa 647 succinimidyl ester was utilized. The reaction mixture was whirled using a vortex for 1 h at room temperature in the dark with pH, TLC, and HPLC monitoring. After the reaction was over, the mixture was acidified (6 μL of TFA) and evaporated in vacuo. The fluorescent peptide was isolated by semipreparative HPLC and lyophilized. Yields of **2**, **3**, **10**, and **11** are given in Table 1.

Fluorescent Peptides with the Alexa 647 Fluorophore Attached at Position 8 of Parent Peptide d[Leu^4, Lys^8]VP (A) by Means of Spacer (Peptides 5, 7, 9, and 13 (Table 1)). All peptides **5**, **7**, **9**, and **13** were prepared by a general two-step procedure as follows. First, the spacer-containing peptides d[Leu^4,Lys(β-Ala)^8]VP (**4**), d[Leu^4,Lys(Aha)^8]VP (**6**), d[Leu^4,Lys(Aud)^8]VP (**8**), and [HO^1][Leu^4,Lys(Aud)^8]VP (**12**) (Table 1) were synthesized as described below. Next, the Alexa 647 fluorophore was attached to peptides **4**, **6**, **8**, and **12** as described above to give the desired peptides **5**, **7**, **9**, and **13**.

Preparation of d[Leu^4,Lys(Aha)^8]VP (6). 7.35 mg (30 μM) of Boc-7 aminoheptanoic acid (Boc-Aha), 7.35 mg (30 μM) of DCC, and 4.1 mg (30 μM) of HOBt were dissolved in ~200 μL of dry DMF, and the mixture was stirred for 30 min. Next, 23.6 mg (23 μM) of d-[Leu^{4}Lys^8]VP (**A**) as a solution in ~150 μL of dry DMF and neutralized in advance with DIPEA was added, and the reaction mixture was incubated overnight at room temperature with continuous stirring and pH ~8.5 monitoring. The desired pH was maintained using DIPEA. After the reaction was over (TLC and HPLC monitoring), the reaction mixture was separated on a Sephadex G-15 column using 25% AcOH as an eluent. The fraction containing the peptide was collected and lyophilized. The Boc-protecting group of the peptide was removed by treatment with 50% TFA in CH_2Cl_2 for 30 min at room temperature. The reaction mixture was evaporated by a flow of N_2, and the resulting residue was subjected to a final purification by semipreparative HPLC to give the spacer-containing peptide d[Leu^4,Lys(Aha)^8]VP (**6**) with a yield of 9.7 mg (36.5%) (Table 1). Spacer-containing peptides d[Leu^4, Lys(β-Ala)^8]VP (**4**) and d[Leu^4,Lys(Aud)^8]VP (**8**) were prepared in the same manner as peptide (**6**), using the parent peptide d[Leu^4, Lys^8]VP (**A**) and Boc-β-Ala and Boc-Aud, respectively. Peptide [HO^1]Leu^4,Lys(Aud)^8]VP (**12**) was prepared by the same approach, utilizing Boc-Aud and the parent peptide [HO^1][Leu^4,Lys^8]VP (**B**). Yields of **4**, **8**, and **12** are given in Table 1.

Synthesis of d[Leu^4,Lys(Aha-Alexa 647)^{8}VP (7). 1.38 mg (1.2 μM) (in excess) of peptide d[Leu^4,Lys(Aha)^8]VP (**6**) was dissolved in 100 μL of anhydrous DMF, mixed with 5 μL of DIPEA. The solution was added to 1 mg of Alexa 647 succinimidyl ester, and the reaction mixture was whirled using vortex for 1 h at room temperature in the dark at pH ~8 with TLC and HPLC monitoring. After the reaction was over, the mixture was acidified (6 μL TFA) and evaporated in vacuo. The fluorescent peptide d[Leu^4,Lys(Aha-Alexa 647)^8]VP (**7**) was isolated by semipreparative HPLC and lyophilized. Yield 1 mg (~35.0%) (Table 1).

Peptides d[Leu^4,Lys(β-Ala)^8]VP (**4**), d[Leu^4,Lys(Aud)^8]VP (**8**), and [HO^1][Leu^4,Lys(Aud)^8]VP (**12**) were labeled utilizing Alexa 647 succinimidyl ester as described above for peptide **7** to give peptides d[Leu^4,Lys(β-Ala-Alexa 647)^8]VP (**5**), d[Leu^4,Lys(Aud-Alexa 647)^8]VP (**9**), and [HO^1][Leu^4,Lys(Aud-Alexa 647)^8]VP (**13**). Yields of **5**, **9**, and **13** are given in Table 1.

The structures of peptides **1**, **2**, **4**, **6**, **8**, **10**, and **12** were confirmed by MALDI-TOF mass spectrometry (MS). The structures of the Alexa 647 containing peptides **3**, **5**, **7**, **9**, **11**, and **13** were not confirmed by mass spectrometry, since the structure of the Alexa 647 fluorophore has not been revealed by Invitrogen/Molecular Probes because of a pending patent application. The analytical and some other physicochemical data of the fluorescent peptides **1**−**3**, **5**, **7**, **9**−**11**, **13** and of the intermediates **4**, **6**, **8**, **12** are presented in Table 1.

Membrane Preparation. Four different Chinese hamster ovary (CHO) cell lines stably expressing the human V1a, V1b, V2, or OT receptors were washed twice in phosphate buffered saline (PBS) without $CaCl_2$ and $MgCl_2$, harvested in lysis buffer (15 mM Tris-HCl, pH 7.4; 2 mM $MgCl_2$; 0.3 mM ethylene diaminetetraacetic acid (EDTA), Polytron homogenized, and centrifuged at 44000*g* for 20 min at 4 °C as previously described.[3] Pellets were washed in 50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$ and centrifuged at 44000*g* for 20 min at 4 °C. Membranes were resuspended in a small volume of the same buffer. Protein concentration was determined by the method of Bradford (Bio-Rad protein assay kit) using bovine serum albumin (BSA) as a standard, and membranes were stored in liquid nitrogen.

Binding Assays. Membrane incubations with [^{3}H]AVP were performed as previously described.[3] Briefly, 10−20 μg of membrane proteins were incubated for 60 min at 30 °C in a medium containing 50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 1 mg/mL BSA, 0.01 mg/mL leupeptine, and 1 nM ^{3}[H]AVP in the presence (nonspecific binding) or in the absence (total binding) of 1 μM unlabeled AVP in a final volume of 200 μL. For K_i determinations, increasing amounts of the unlabeled analogue to be tested were added to the incubation medium to a fixed concentration of tritiated ligand. The membrane-associated radioactivity was collected by filtration through GF/C filters and counted. Specific binding was calculated as the difference between total and nonspecific binding values and expressed as percent of the specific binding determined without unlabeled analogue.

Phospholipase C Assay. Inositol phosphate (InsP) accumulation was determined as described previously.[3] CHO cells were seeded at 100 000 cells per well in 24-well plates and grown for 24 h in their regular culture medium, then incubated for another 24 h period in serum- and inositol-free medium supplemented with 2 μCi/ml myo-[2-^{3}H]inositol. Cells were then washed twice with HBS, incubated for 15 min in HBS supplemented with 20 mM LiCl, and further stimulated for 15 min at 37 °C in the same buffer with increasing concentrations of vasopressin analogues. The reaction was stopped by perchloric acid (5% vol/vol). Total InsPs was extracted, purified on Dowex AG1-X8 anion exchange chromatography column, and counted.

Cell Labeling and Imaging. CHO cells, seeded on 12 mm glass coverslips precoated with polyornithine, were incubated in DMEM, BSA 0.2 mg/mL, HEPES 25 mM, pH 7.4, for 1 h at 12 °C with fluorescent analogues in the absence or in the presence of nonfluorescent VP analogues. In the latter case, a 30 min preincubation of the cells in the same medium containing only these ligands was performed. After three washes with cold PBS, the cells were finally fixed in 4% PFA at 4 °C overnight and mounted with mowiol. The fluorescent cells were imaged using a confocal microscope Zeiss LSM510 Meta equipped with an Axiovert200 M microscope. The objective 63× (NA 1.4) for oil immersion was used. For detection of Alexa 488-labeled cells, the

excitation was performed with an argon laser at λ = 488 nm. The green emission was collected using a BP 505−530 nm emission filter. For detection of Alexa 647, helium/neon laser 633 nm was used. The emission was collected with a LP650 nm filter. Images were captured in multitracking mode to avoid channel cross-talk, and the settings were established on unlabeled cells used as negative control. For fluorescence quantification, the Zeiss LSM Browser was used as previously described[58] with 30−40 cells evaluated for each fluorescent binding condition.

Alternatively, the binding of the Atn-labeled peptide (analogue **1**) was evaluated by cuvette spectrofluorimetry. Cells were detached with PBS, 10 mM EDTA and incubated with the fluorescent analogue in DMEM, 0.2 mg/mL BSA, 25 mM HEPES, pH 7.4, at 12 °C as previously described. After three cold washes with PBS and gentle centrifugation (900*g*, 4 min), the cells were resuspended in PBS and placed in a cuvette at 4 °C. The fluorescence was measured using λ = 343 nm excitation and λ = 412 nm emission in a Photon Technology International 810 spectrometer equipped with a UV lamp. These values were chosen after full excitation and emission curves had been established with soluble Atn-analogue **1**. The specific detection was calculated by deducing the fluorescence emission measured in CHO hV1b-R cells incubated with an excess of nonfluorescent ligand.

Data Analysis. All data were were analyzed using Prism (GraphPad Software, Inc., San Diego, CA). For the radioligand binding data, the inhibitory dissociation constant (K_i) for unlabeled vasopressin analogues was calculated from binding competition experiments according to the Cheng and Prusoff equation: $K_i = IC_{50}(1 + [L]/K_d)$, where IC_{50} is the concentration of unlabeled analogue leading to half-maximal inhibition of specific binding, [L] the concentration of the radioligand present in the assay, and K_d its affinity for the receptor studied. The respective K_d values of ^{3}[H]AVP for hV1a-R (1.1 nM), hV1b-R (0.68 nM), hV2-R (1.2 nM), and hOT-R (1.7 nM) had been previously determined by Pena et al.[3] and are listed in Table 2 (line 1). For the PLC assay, results are expressed as the mean ± SEM of the number of distinct experiments indicated (n). Statistical analysis of the data was performed using the one-way analysis of variance test.

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ABBREVIATIONS USED

AcOH, acetic acid; ACTH, adrenocorticotropin hormone; β-Ala, 3-aminopropionyl; Alexa 488, Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (5-isomer), and the related acyl group; Alexa 647, Alexa Fluor 647 carboxylic acid succinimidyl ester and the related acyl group (structure unknown, pending patent application); Aha, 7-aminohexanoyl; Atn, anthraniloyl; Aud, 11-aminoundecanoyl; AVP, arginine vasopressin; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; cAMP, cyclic adenosine monophosphate; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CHO hV1a-R, CHO hV1b-R, CHO hV2-R, and CHO hOT-R, CHO cells stably expressing human vasopressin V1a, V1b, V2, and oxytocin receptors, respectively; CRH (or CRF), corticotrophin releasing hormone; CRHR1, corticotrophin-releasing hormone receptor type 1; 2CLZ, 2-chlorobenzyloxycarbonyl; DALDA, H-Dmt-D-Arg-Phe-Lys-NH_2, Dmt, 2′,6′-dimethyltyrosine; Dap, L-2,3-diaminopropionic acid; DCC, dicyclohexylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; d-[Leu^4,Lys^8]VP, [deamino-Cys^1,4-leucine,8-lysine]vasopressin; DMF, dimethylformamide; DMEM, Dulbecco's modified Eagle's medium; ε, molar extinction coefficient; EC_{50} or K_{act}, concentration of agonist leading to half-maximal activity; E_{max}, maximal efficiency; EDTA, ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; ESMS, electron spray mass spectrometry; Et_3N, triethylamine; FRET, fluorescence resonance energy transfer; Gi, G-protein type i; Go, G-protein type o; HOBt, 1-hydroxybenzotriazole; HBS, HEPES buffer saline; HO^1[Leu^4,Lys^8]VP, [1-L-(−)-2-hydroxy-3-thiopropanoic acid,4-leucine,8-lysine]vasopressin; HO-Mpr, L-(−)-2-hydroxy-3-thiopropanoyl; HPLC, high-performance liquid chromatography; InsPs, total inositol phosphates; K_d, affinity at concentration of ligand leading to half-maximal specific binding deduced from saturation experiments; K_i, inhibitory dissociation constant at concentration of peptide leading to half-maximal specific binding deduced from competition experiments; KO, knock-out animals; λ, wavelength; MeCN, acetonitrile; LSM, laser scanning microscope; mowiol, mounting medium for fluorescence microscopy; mRNA, messenger ribonucleic acid; Manning compound, [1-β-mercapto-β,β-cyclopentamethylenepropionyl,2-*O*-methyltyrosine,8-arginine]vasopressin (VP V1a/OT antagonist); Mob, *p*-methoxybenzyl; Mpr, 3-mercaptopropionyl; ONp, *p*-nitrophenyl ester; OT, oxytocin; OT-R, oxytocin receptor; PBS, phosphate buffered saline; PFA, paraformaldehyde; PLC, phospholipase C; Rhm, tetramethylrhodamyl; Rhm^8-PVA, 4-HO-Ph$(CH_2)_2$-CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5-carboxytetramethylrhodamyl]-NH_2; D-Tyr(Me), *O*-methyl-D-tyrosine; RT-PCR, polymerase chain reaction after reverse transcription; SEM, standard error of the mean; SI, selectivity index; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; VP, vasopressin; V1a-R, V1b-R, and V2-R, vasopressin receptor isoforms V1a (vascular), V1b (pituitary), and V2 (renal), respectively

ADDITIONAL NOTE

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1989**, *180*, A9−A11) and

IUPHAR (*Trends Pharmacol. Sci.* **2001**). All amino acids are in the L-configuration unless otherwise noted.

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