Fluorescent Pseudo-Peptide Linear Vasopressin Antagonists: Design, Synthesis, and Applications[†,||]

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Received August 17, 1998

Fluoresceinyl and rhodamyl groups have been coupled by an amide link to side-chain amino groups at positions 1, 6, and 8 of pseudo-peptide linear vasopressin antagonists (Manning et al. *Int. J. Pept. Protein Res.* **1992**, *40*, 261−267) through different positions on the fluorophore, to give tetraethylrhodamyl-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**2**), 4-HOPh$(CH_2)_2$-CO-DTyr(Me)-Phe-Gln-Asn-Lys(5-carboxyfluoresceinyl)-Pro-Arg-NH_2 (**4**), 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Lys(5- or 6-carboxytetramethylrhodamyl)-Pro-Arg-NH_2 (**5**, **6**), 4-HOPh-$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5- or 6- carboxyfluoresceinyl)-NH_2 (**8**, **9**), and 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5- or 6- carboxytetramethylrhodamyl)-NH_2 (**10**, **11**). The closer to the C-terminus the fluorophore, the higher the affinities of the fluorescent derivatives for the human vasopressin V_{1a} receptor transfected in CHO cells. The compound **10** has a K_i of 70 pM, as determined by competition experiments with [^{125}I]-4-HOPhCH_2CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2. It showed a good selectivity for human V_{1a} receptor versus human OT (K_i = 1.2 nM), human vasopressin V_{1b} ($K_i \approx$ 27 nM), and human vasopressin V_2 (K_i > 5000 nM) receptor subtypes. All fluorescent analogues were antagonists as shown by the inhibition of vasopressin induced inositol phosphate accumulation. These fluorescent ligands are efficient for labeling cells expressing the human V_{1a} receptor subtype, as shown by flow cytofluorometric experiments or fluorescence microscopy. They are also appropriate tools for structural analysis of the vasopressin receptors by fluorescence.

Introduction

Vasopressin and oxytocin are two nonapeptides which participate in the regulation of numerous physiological functions. Among them are blood pressure and water reabsorption for vasopressin and contraction of uterine myocytes during parturition and mammary gland myocytes during lactation for oxytocin. To date, three vasopressin receptor subtypes and one oxytocin receptor subtype (OT) have been characterized.[1] The vasopressin V_{1a} and V_{1b} receptors and oxytocin receptor are coupled to phospholipase C, while vasopressin V_2 receptor activates adenylyl cyclase. All have been cloned in various species and belong to the G protein-coupled receptor superfamily.

Because of the diversity of the neurohypophysial hormone receptors and of the numerous analogues which have been synthesized, the vasopressin and oxytocin system constitutes a good model to study ligand−receptor interactions. The structural bases of their ligand recognition have been investigated using both selective ligands and site-directed mutagenesis.[2] Photoactivated ligands have been used also to map binding domains in the human V_{1a} vasopressin receptors[3] and in the bovine V_2 vasopressin receptor.[4]

In the past years, the sensitivity of detection of fluorescence has been improved, and consequently fluorescent ligands have been developed more broadly.[5] Fluorescent molecules are interesting substitutes for radiolabeled ligands since affinity measurement can be performed by flow cytometry. Moreover, the dynamic process can be addressed at a cellular level and at a molecular level. Also, with the development of confocal microscopy, the trafficking of fluorescent molecules into cells can be analyzed very precisely.[6] Fluorescence recovery after photobleaching can also be used to study the diffusion of molecules in a tissue or in one cell.[7] Fluorescence correlation spectroscopy allows the detection of the interaction between one receptor molecule

[†] A preliminary report of this work was presented at the 1997 World Congress of Neurohypophysial Hormones, Montreal, Canada.

[||] Abbreviations according to the IUPAC−IUB Commission (*Eur. J. Biochem.* **1984,** *138*, 9−37) and IUPHAR (*Trends Pharmacol. Sci.* **1998**) are used. Peptide abbreviations are given in Table 1. The others are AVP, arginine vasopressin, H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; BOP, (1*H*-benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; BroP, tris(dimethylamino)bromophosphonium hexafluorophosphate; CHO-V_{1ah}, Chinese hamster ovary cells expressing human V_{1a} receptors; HO-LVA, 4-HOPhCH_2CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; [^{125}I]HO-LVA, 4-HO,3-[^{125}I]PhCH_2CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; LVA, PhCH_2CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2; LVP, H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2; OT, oxytocin receptor; OTA, oxytocin antagonist, $(CH_2)_5$C(S)-CH_2CO-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH_2; PyBOP, (1*H*-benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; PyBroP, tripyrrolidinobromophosphonium hexafluorophosphate; V_{1a}, vascular vasopressin receptor; V_{1b}, pituitary vasopressin receptor; V_2, renal vasopressin receptor; VT, vasotocin, H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys-Gly-NH_2. Structures of fluorescent groups are shown in Figure 1: 5C-Flu, 5-carboxyfluoresceinyl; 6C-Flu, 6-carboxyfluoresceinyl; 5C-Rhm, 5-carboxytetramethylrhodamyl; 6C-Rhm, 6-carboxytetramethylrhodamyl; Rho, tetraethylrhodamyl.

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10.1021/jm9804782 CCC: $18.00
Published on Web 03/12/1999

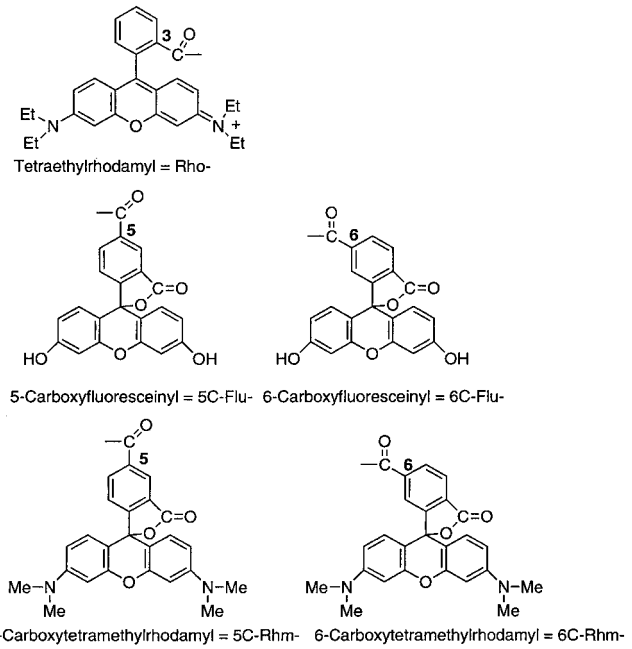

Figure 1. Structures of the fluorophores.

and one ligand molecule.[8] The process of binding of a molecule to its receptor can be studied since very transient complexes can be detected using a fluorescent ligand in stopped-flow mixing experiments.[9] Structural information for ligand−receptor interactions can also be obtained by fluorescence quenching,[10] polarization,[11] or resonance energy-transfer experiments.[12]

Up to now the spectrofluorimetric approaches mentioned above have not been used for studying interactions between vasopressin antagonists and the vasopressin V_{1a} receptor. We have developed and characterized a series of fluorescent ligands which differ by the position of the residue to which the fluorophore is linked. These ligands are highly potent and selective antagonists for the human V_{1a} receptor. The labeling of CHO cells or membranes expressing human vasopressin V_{1a} receptor has been performed.

Results

Design and Synthesis of Fluorescent Analogues. The fluorescent probes were obtained by addition of fluorophores on Manning's pseudo-peptide (containing a phenacyl group as N-terminal part) linear antagonists,[13,14] instead of the previously used desaminolysine vasopressin (dLVP)[15] for the following reasons. They have higher affinities for the V_{1a} receptor. Their hydrophilic residues (Arg^8, Arg^6, Gln^4, Asn^5) are able to counterbalance the hydrophobicity introduced by the fluoresceinyl and rhodamyl groups. They are more stable than disulfide-bridged molecules, especially at low concentrations. They are not subject to S-quenching of fluorescence as described for Met residues[16] and for disulfide bridge-containing ligands.[17]

A fluorophore was added at the N-terminal position of the previously described linear vasopressin (LVA)[13] to give **2**. This ligand contains an iodinatable Tyr at position 9. We chose tetraethylrhodamyl (Rho-, Figure 1) to mimic the aromatic part of the pseudo-peptidic molecule and to fit with a hypothetic hydrophobic pocket of the receptor.[18] Fluorophores were also introduced on residues 6 and 8 of the pseudo-peptidic backbone. For this purpose we chose phenylpropionyl linear vasopressin antagonist (PVA) as template because the phenylpropionyl photoactivatable derivative has a slightly better affinity for vasopressin V_{1a} receptor than the phenylacetyl ligand.[18] To add the fluorophore at various positions on the ligand backbone, Arg^6 and Arg^8 were replaced successively by Lys (Table 1). Tetramethylrhodamyl (Rhm) and fluoresceinyl (Flu) were selected and introduced because of their superior fluorescent properties. We used an amide link instead of the thiocarbamyl link[19] because it is more stable and gives higher quantum yields.[20] Because of their different hydrophilicities (5% CH_3CN in RP-HPLC elution, 6C- being more hydrophilic), 5C- and 6C-Rhm-OH (Figure 1) were both used after purification. The 5C- and 6C-derivatives have different spectral properties. The two isomers were assayed separately.

We synthesized the fluorescent ligands in 10−20-mg scale for a careful characterization (mass spectroscopy, weighing, molecular extinction coefficient determination). It is well-known that pre-formed hydroxysuccinimide active esters of fluorophores hydrolyze on storage.[20] Therefore, in the present study, hydroxybenzotriazole active esters were synthesized in situ with PyBOP. The pseudo-peptide templates were synthesized on a *p*-methylbenzhydrylamine resin, using PyBOP or BOP as coupling reagent, *tert*-butyloxycarbonyl as temporary protecting group, and HF as final deprotecting reagent. The peptides were RP-HPLC purified and lyophilized. Although fluorophores can be linked directly on the peptidyl-resin,[21] they were introduced on the purified pseudo-peptide, to avoid photobleaching and carbocation additions to their aromatic part. The structure of the peptide allows this procedure because it has no other acylatable group other than the ϵ-NH_2 of Lys. When using the BOP reagent family in anhydrous medium, a low molar excess of fluorophore was needed as shown by RP-HPLC monitoring, using simultaneously 214- and 254-nm absorbances and fluorescence emission, according to the added fluorophore (Figure 2). For the coupling of the 3-carboxylate of Rho-OH, the association of PyBroP and (dimethylamino)pyridine was required.[22] The structures of the different peptides are shown in Table 1. All compounds were characterized by mass spectroscopy (fast atom bombardment positive mode). The peptides were quantified by weighing, as lyophilized CF_3CO_2H salts, and frequently checked by analytical HPLC. Absorption spectrums were recorded in aqueous solution, at pH 7.4, and molar extinction coefficients (ϵ) were determined in PBS (pH 7.4) at peak wavelength (λ_{max}) (Table 1).

Affinities for Vasopressin and Oxytocin Receptors. The affinities of the fluorescent ligands for human V_{1a} receptor subtype were determined by competition experiments. Displacement curves of [^{125}I]HO-LVA by the different Rhm fluorescent ligands (**5**, **6**, **10**, **11**) are presented in Figure 3a. K_i values of the ligands for the human V_{1a} receptor subtype are reported in Table 1. Compounds **8**−**11**, which have a fluorophore on aminoacyl 8, showed very high affinities ($K_i \approx 100$ pM) while **4**−**6**, supporting the fluorophore on aminoacyl 6, had lower affinities ($K_i \approx 5$ nM). The nature of the fluorophore (Flu- or Rhm-) and the position of the link

Table 1. Characterization and Properties of the Synthesized Compounds

structure	% CH_3CN elution	$\epsilon_{214}/\epsilon_{254}$	formula	M + 1 found	λ_{max} (nm PBS)	$\epsilon_{\lambda max}$ (10^3 M^{-1} cm^{-1})	K_i (nM)	compd no.	abbreviation
H-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2[18]	25	37	$C_{54}H_{77}N_{17}O_{12}$	1156				**1**	
tetraethylrhodamyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2	40	0.5	$C_{82}H_{107}N_{19}O_{14}$	1581			>500	**2**	
4-HOPhCH_2CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2[23]	28.5	35					0.08		HO-LVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-**Lys**-Pro-Arg-NH_2	29.5	39	$C_{54}H_{76}N_{14}O_{12}$	1113				**3**	[Lys^6]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-**Lys(5C-Flu)**-Pro-Arg-NH_2	37	4.8	$C_{75}H_{86}N_{14}O_{18}$	1472	496	42	5.6 ± 0.3	**4**	[Lys^6(5C-Flu)]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-**Lys(5C-Rhm)**-Pro-Arg-NH_2	39	2.1	$C_{79}H_{96}N_{16}O_{16}$	1526	554	70	1.3 ± 0.2	**5**	[Lys^6(5C-Rhm)]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-**Lys(6C-Rhm)**-Pro-Arg-NH_2	36.5	2.3	$C_{79}H_{96}N_{16}O_{16}$	1526	558		3.3 ± 1.0	**6**	[Lys^6(6C-Rhm)]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-**Lys**-NH_2	29.5	38	$C_{54}H_{76}N_{14}O_{12}$	1113				**7**	[Lys^8]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-**Lys(5C-Flu)**-NH_2	37	4.8	$C_{75}H_{86}N_{14}O_{18}$	1472	496	45	0.17 ± 0.04	**8**	[Lys^8(5C-Flu)]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-**Lys(6C-Flu)**-NH_2	37	3.5	$C_{75}H_{86}N_{14}O_{18}$	1472	496	46	0.23 ± 0.05	**9**	[Lys^8(6C-Flu)]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-**Lys(5C-Rhm)**-NH_2	38.7	2.1	$C_{79}H_{96}N_{16}O_{16}$	1526	554	68	0.07 ± 0.01	**10**	[Lys^8(5C-Rhm)]PVA
4-HOPh$(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-**Lys(6C-Rhm)**-NH_2	37	1.4	$C_{79}H_{96}N_{16}O_{16}$	1526	558	70	0.17 ± 0.03	**11**	[Lys^8(6C-Rhm)]PVA

Figure 2. HPLC monitoring during the BOP fluorophore coupling. One aliquot (≈1/4 μL) of the coupling mixture is injected onto the C_{18} column and eluted using a 1%/min linear CH_3CN gradient, with 0.1% (v/v) CF_3CO_2H, at 2 mL/min (profile shown by the dotted line). Fluorescence detection (530-nm excitation, 580-nm emission) shows essentially residual 5C-Rhm-OH, synthesized ligand [Lys^8(5C-Rhm)]PVA (**10**), and minor impurities. The 214-nm detection shows residual peptide [Lys^8]PVA (**7**), residual fluorophore 5C-Rhm-OH, the increasing peak of the synthesized ligand, and an unidentified impurity. The solvent Me_2SO is quickly eluted, and the HOBt peak increases with BOP consumption. The 254-nm detection shows aromatic compounds (HOBt and fluorescent groups). The evaluation of $\epsilon_{214}/\epsilon_{254}$ (Table 1) allows a simple identification of the chemical species.

between the peptide and the fluorophore (5C- or 6C-) did not significantly affect the affinities of the ligands for the human V_{1a} receptor subtype. Two ligands were selected, namely **8** and **10,** and their selectivities for the various human receptor subtypes of vasopressin and oxytocin were determined. Results of the displacement of [^{125}I]HO-LVA (for human V_{1a} and V_{1b} receptor subtypes), [^{125}I]OTA (for oxytocin receptor), and [$^3H_3$]-AVP (for human V_2 receptor subtype) by increasing concentrations of **10** are illustrated in Figure 3b and Table 2. Like the HO-LVA antagonist, the fluorescent ligands present very good affinities for human V_{1a} receptors. To a lesser extent, the two ligands also have good affinities for human oxytocin receptor (≈1 nM), while their affinities are low for human V_{1b} and V_2 receptors (>10 and >1000 nM, respectively). Their selectivity toward the V_2 subtype is good in regard to nonfluorescent peptidic[23,24] or nonpeptidic[25] antagonists.

Antagonist Properties of the Fluorescent Ligands. Inositol phosphate accumulation was evaluated by cell incubation with fluorescent ligands at a concentration of 100-fold K_i.[26] No increase in the accumulation of second messengers was detected (Figure 4a). This indicates that all the fluorescent ligands are devoid of intrinsic agonist activity. Moreover, at the same con-

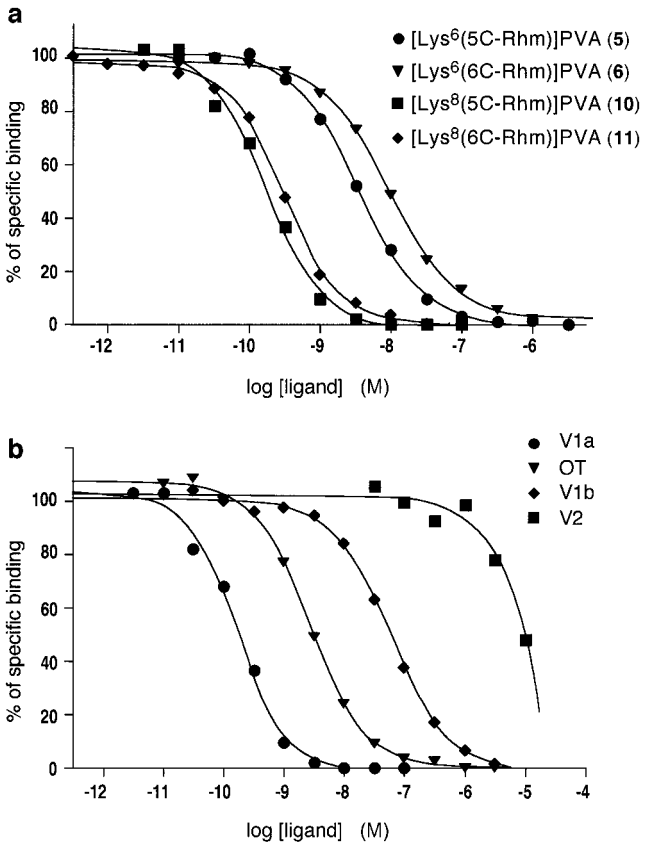

Figure 3. a. Determination of fluorescent vasopressin linear analogue affinities for the human V_{1a} receptor by concentration−displacement binding experiments. CHO-V_{1ah} cell membranes (0.5−1 μg of protein/assay) were incubated for 1 h at 30 °C in the presence of increasing concentrations of fluorescent analogue and 60 pM [^{125}I]HO-LVA. b. Determination of affinities of the fluorescent vasopressin linear analogue [Lys^8-(5C-Rhm)]PVA (**10**) for human vasopressin and oxytocin receptor subtypes by concentration−displacement binding experiments. Membranes were prepared from CHO cells expressing one human vasopressin or oxytocin receptor subtype. They were incubated for 1 h at 30 °C in the presence of increasing concentrations of fluorescent analogue and 60 pM [^{125}I]HO-LVA (for V_{1a} and V_{1b} receptor subtypes), 210 pM [^{125}I]-OTA (for OT receptor subtype), or 2 nM [$^3H_3$]AVP (for V_2 receptor subtype). Membranes were used at 0.5−1 μg of protein/assay for radioiodinated and 5−10 μg of protein/assay for tritiated ligands. Values of specific binding measured in the presence of fluorescent analogues are expressed as a fraction of the specific total binding measured in the absence of competitor. The apparent dissociation constants (K_i) for the fluorescent ligands were calculated by using the following relation: $K_i = IC_{50}/(1 + [*L]/K_d)$, where IC_{50} is the concentration of fluorescent ligand leading to 50% inhibition of specific total binding and [*L] and K_d are the concentration and dissociation constants of labeled ligand. Concentration−displacement curves are representative of three independent experiments, each performed in triplicate.

centration, fluorescent ligands completely inhibited AVP-induced inositol phosphate accumulation. The inhibition constant values (K_{inact}) were 0.18 ± 0.05 and 0.07 + 0.04 nM for **8** and **10**, respectively, when cells were incubated with 1 nM AVP and different concentrations of antagonist (Figure 4b).

Fluorescent Labeling of CHO Cells Expressing Human V_{1a} Receptor. The ligands were tested for the labeling of CHO cells, stably transfected with the human V_{1a} receptor (CHO-V_{1ah} cells). **10** was convenient

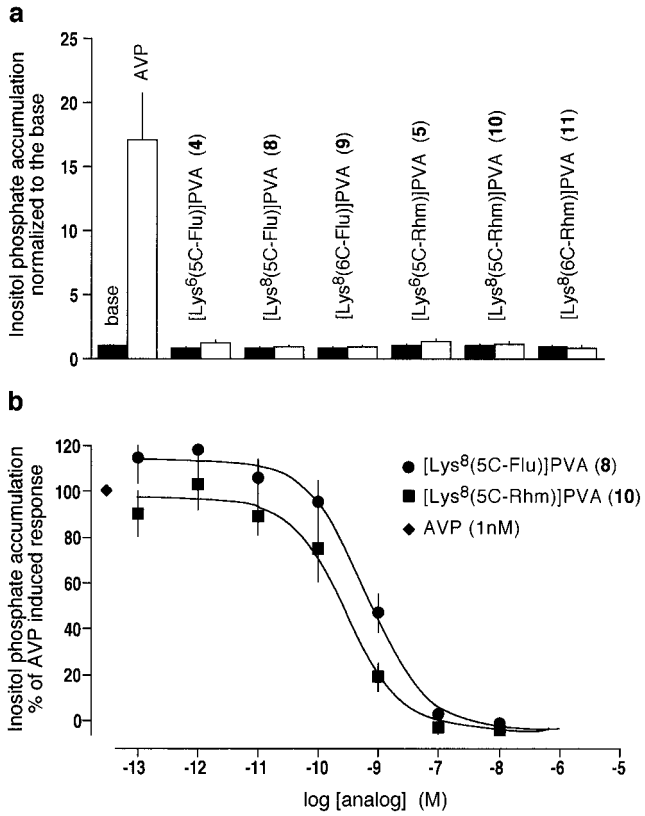

Figure 4. Determination of the agonist and antagonist properties of the fluorescent ligands. *myo*-[^{3}H]Inositol-prelabeled CHO-V_{1ah} cells were preincubated for 10 min at 37 °C in PBS:LiCl medium. a. Fluorescent ligands were added at 1 μM (**4**), 100 nM (**8**, **9**, **11**), 500 nM (**5**), and 50 nM (**10**) and, 10 min later, with (open bars) or without (solid bars) 10 nM AVP. Total inositol phosphates were determined. The values are normalized to the cells' response in control conditions. All values are expressed as the mean ± SE, calculated from five independent determinations. b. Determination of the inhibition constants of the fluorescent vasopressin linear analogues [Lys^8-(5C-Flu)]PVA (**8**) and [Lys^8(5C-Rhm)]PVA (**10**). Fluorescent ligands were added at the various concentrations, and 10 min later, cells were stimulated with 1 nM AVP. Total inositol phosphates were determined. The values are normalized to response of AVP-stimulated cells in the absence of fluorescent ligand. All values are expressed as the mean ± SE calculated from five, for **8**, or three, for **10**, independent determinations. The apparent inactivation constants were calculated as: $K_{inact} = IC_{50}/(1 + [AVP]/K_{act})$, where IC_{50} is the concentration of fluorescent ligand leading to 50% inhibition of specific total binding, [AVP] = 1 nM, and K_{act} is the concentration of AVP inducing half-maximal stimulation of total inositol phosphate accumulation (K_{act} = 0.32 nM).

for epifluorescence microscopy analysis because the photobleaching is reduced with this fluorophore. CHO-V_{1ah} cells were incubated with 0.35 nM **10** and with or without 1 μM HO-LVA, for 1 h at 37 °C. A clear labeling of cells was observed as shown in Figure 5a. Panels 1 and 2 illustrate labeling of cells at different magnifications. In both panels, cytoplasmic membranes are labeled but further experiments in confocal microscopy are needed to conclude that the labeling is restricted to this area. Panel 1 shows that most of the cells were labeled but labeling intensity varied from one cell to the other as in cytofluorometric experiment (see below). The nonspecific labeling, obtained with an excess of HO-LVA, was very low (panel 4). Panel 3 shows the same cells observed in transmitted light.

Table 2. Affinities of the Lys[8]-Tagged Fluorescent Probes for Oxytocin and Vasopressin Receptor Subtypes[a]

compound	receptor subtype			
	V_{1a}	V_{1b}	V_2	OT
[Lys[8](5C-Flu)]PVA (**8**)	0.17 ± 0.04	142 ± 30	9200 ± 500	1.6 ± 0.3
[Lys[8](5C-Rhm)PVA (**10**)	0.07 ± 0.01	27 ± 5	7500 ± 2500	1.2 ± 0.2
HO-LVA[23]	0.08	5.9	137	2.7
SR 49059[25]	1.0	129	119	33
LVA[24]	0.8	9.4	282	1.1
OTA	3.9	10229	929	0.18
AVP	1.7	1.1	1.1	1.65

[a] The inhibition constants (K_i in nM) of [Lys[8](5C-Flu)]PVA (**8**) and [Lys[8](5C-Rhm)]PVA (**10**) were determined by displacement experiments using membranes of CHO cells expressing one subtype of human receptor and the convenient radiolabeled ligand: [^{125}I]HO-LVA for V_{1A} and V_{1b} receptors, [^{125}I]OTA for oxytocin receptor, and [$^3H_3$]AVP for V_2 receptor. All values are expressed as the mean ± SE, calculated from three independent determinations.

The compound **8** was used in flow cytofluorometric experiments (Figure 5b). CHO-V_{1ah} cells were incubated for 1 h at 37 °C with 1 nM **8** and with (nonspecific labeling, dotted line) or without (total labeling, solid line) 0.1 μM HO-LVA. The total labeling histogram presents a biphasic curve with a shoulder corresponding to the nonspecific labeling of CHO-V_{1ah} cells, while the peak corresponds to the positively labeled cells. This indicates that around 12% of the cells were not labeled.

Fluorescent Labeling of the Receptors in Membranes. Three aliquots of CHO-V_{1ah} cell membranes were incubated alone, with the fluorescent ligand, without or with an excess of nonfluorescent antagonist. They were washed and measured for fluorescence emission (Figure 5c,d). The difference of emission between nonspecific labeling (trace N) and total labeling (trace T) presents a maximum at $\lambda = 580$ nm, at which the accessibility of the fluorophore could be tested by collisional quenching of fluorescence. Using the same excess in concentration in regard to the inhibition constant ($5 \times K_i$), the nonspecific binding for **10** is particularly low (Figure 5c) and it is higher for **5** (Figure 5d).

Discussion

We have designed and characterized a series of fluorescent vasopressin antagonists for studying ligand−receptor interactions. While several authors have attached fluorophores on the disulfide-bridged peptides deaminolysine vasopressin (dLVP) and its antagonist derivative d$(CH_2)_5$[DTyr(Et)[2],Val[4]]LVP,[19,27,28] we chose to add fluorophores to linear vasopressin antagonists which have very high affinities for the V_{1a} vasopressin receptor subtype and are more stable molecules than disulfide-bridged compounds. The substitution of Lys for Arg in position 6 or 8 allows the coupling of fluorophores. Moreover the linkage of a fluorophore may only slightly affect the characteristics of the ligand. Indeed, fluorescent analogues have high affinities for vasopressin V_{1a} receptor subtype contrary to fluorescent analogues previously described.[19,27,28] Despite the size of the fluorophore, the affinities of **8** and **10** are comparable to the affinities of the parent peptide. It clearly appears from Table 2 that analogues with the fluorophore in position 8 (**8**−**11**) have higher affinities for the human vasopressin V_{1a} receptor than analogues with the fluorophore in position 6 (**4**−**6**), since the K_i values of the Lys[6] analogues are at least 20-fold greater. The position of the link between the fluorophore and the pseudo-peptide (5C- or 6C-) affects slightly the affinities of the ligand for the receptor (Table 1). The 5C- derivatives generally have lower K_i but are more lipophilic (Table 1, % CH_3CN elution).

While fluorescent derivatives of dLVP are nonselective,[27,28] the two compounds (**8**, **10**) for which it has been investigated are very selective for vasopressin human V_{1a} receptor versus other human vasopressin and oxytocin receptor subtypes. Surprisingly, **8** and **10** are even much more selective than HO-LVA. Addition of fluorophore induces a slight decrease of the K_i for vasopressin V_{1a} receptor since it severely decreases affinities for the V_{1b} and V_2 receptors. This suggests that the steric hindrance due to the fluorophore is perhaps more critical for vasopressin V_{1b} and V_2 receptors than for the V_{1a} receptor.

The antagonist properties of the ligands for the human V_{1a} vasopressin receptor are not affected by the addition of a fluorophore. None of the fluorescent ligands have agonistic properties even at high concentrations. Moreover all these ligands are full antagonists since vasopressin-induced inositol phosphate accumulation is completely inhibited by the different fluorescent ligands. The inhibition constants of **8** and **10** are 0.18 ± 0.05 and 0.07 ± 0.04 nM, respectively.

Because of their high affinities for the human V_{1a} vasopressin receptor, these ligands are well-adapted to fluorescent techniques. The ligands **8** and **10** are efficient for labeling vasopressin receptor in epifluorescence microscopy, while **8** can easily be used for flow cytofluorometric studies. The low concentration needed to label cells reduces the nonspecific fluorescence. Contrary to Lutz et al.,[15] we chose to start synthesis of fluorescent compounds with pure isomer of C-Rhm and C-Flu to obtain chemically well-defined fluorescent peptides. All our Rhm ligands have high fluorescence intensity and reduced photobleaching and are well-adapted to structural analysis. The dissociation of the ligands from the receptor is very slow, as has been observed for other linear vasopressin antagonists.[23,29] This property could be very useful for further quenching studies.[10]

In conclusion, this series of fluorescent ligands will be helpful tools to investigate structural bases of ligand recognition by vasopressin receptors and possibly oxytocin receptors. The presence of fluorophores at various positions on the pseudo-peptide, the high affinities for human vasopressin V_{1a} and oxytocin receptors, and the low nonspecific binding render these ligands valuable for quenching experiments and anisotropy measurements and hence to define accessibility and mobility

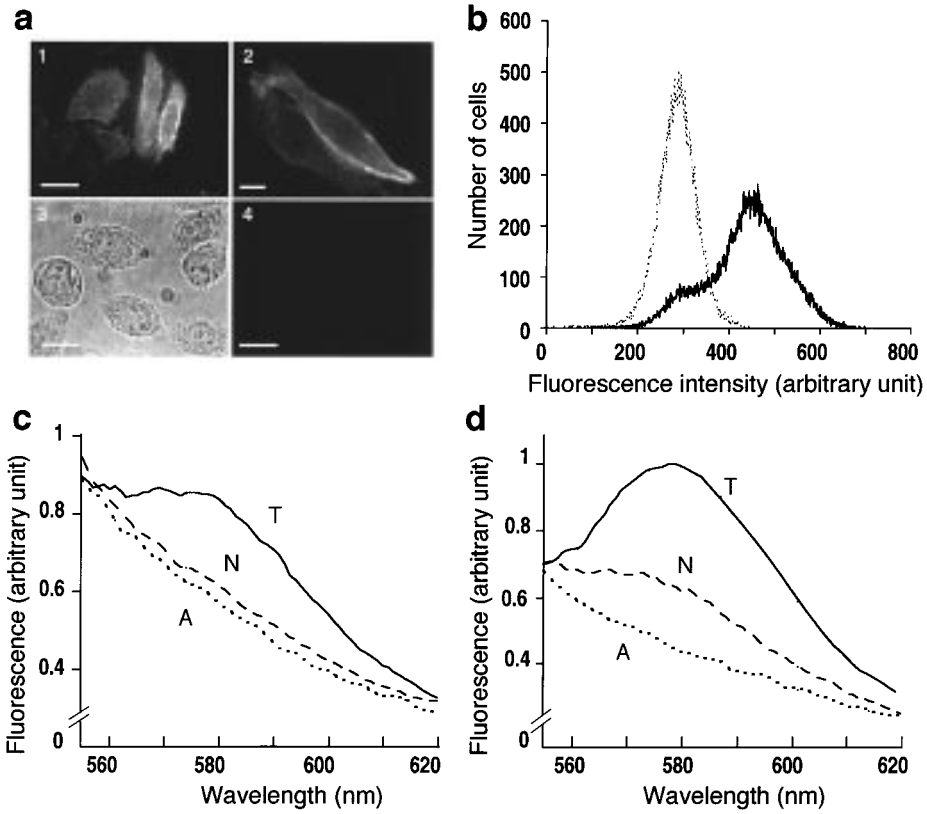

Figure 5. a. Labeling of CHO-V_{1ah} cells with 0.35 nM [Lys^8(5C-Rhm)]PVA (**10**). CHO-V_{1ah} cells were incubated with 0.35 nM **10** and without (panels 1 and 2) or with (panel 4) 1 μM HO-LVA for 1 h at 37 °C and were observed on epifluorescence microscope. Panels 1 and 2 show total labeling of cells at different magnifications. Panel 4 shows nonspecific labeling, and panel 3 corresponds to the same cells as panel 4, observed in transmitted light (bar: 15 μm). b. Flow cytometric analysis of CHO-V_{1ah} cells labeled with [Lys^8(5C-Flu)]PVA (**8**). Cells were incubated 1 h at 37 °C with 1 nM **8** and with (solid line) or without (dotted line) 0.1 μM HO-LVA. Cells were washed twice and analyzed in flow cytometry. c and d. Spectrofluorimetric determination of the fluorescence bound to receptors in CHO-V_{1ah} cell membranes labeled with the Rhm fluorophore in position 8 (c) and position 6 (d) on the peptide backbone. Membranes (130 μg, containing 5.2 pmol of [^{125}I]HO-LVA binding sites) were incubated alone (autofluorescence of the membranes, trace A), with the fluorescent ligand, supplemented (nonspecific binding, trace N) or not (total binding, trace T) with 0.5 μM nonfluorescent peptide [Lys^8]PVA (**7**), in PBS:Mg^{2+}:BSA (30 mL), 1 h at 30 °C, cold-washed, and resuspended in PBS:Mg^{2+} (500 μL) for the measure, under stirring (530-nm excitation wavelength). c. Labeling with 0.5 nM [Lys^8(5C-Rhm)]PVA (**10**). d. Labeling with 5 nM [Lys^6(5C-Rhm)]PVA (**5**).

of the fluorophore when the ligand binds to the receptor. Finally, **8** and **10**, which present the highest affinities for the human vasopressin V_{1a} receptor subtype, can be used in cellular and subcellular analysis and colocalization with other markers in fluorescence microscopy. **8** is adapted to flow cytofluorometry in cell analysis and cell sorting. It is an interesting substitute to iodinatable ligands for screening new molecules acting on human vasopressin receptors.

Experimental Section

Chemicals. Protected amino acids, PyBOP, and *p*-methylbenzhydrylamine resin were from Bachem and Neosystem. PyBroP was synthesized in our laboratory. Solid-phase peptide synthesis was performed on a manual device as previously described[30] using analytical grade solvents. Peptides were deprotected with HF from Matheson, using a Kel-F apparatus from the Protein Research Foundation (Japan). C-Rhm-OH and C-Flu-OH were obtained as mixtures of 5 and 6 derivatives by Research Organics (Coger, France) and purified by RP-HPLC before use. Alternately, the 5C- derivatives were obtained as pure isomers (Research Organics and Molecular Probes). UV spectroscopic grade DMF and Me_2SO were used for their coupling (anhydrous, in this case) and 10^{-3} M solutions of the probes. Radioiodinated ligands (2200 Ci/mmol) were prepared as previously described.[23,31] [$^3H_3$]AVP was from New England Nuclear and was purified before use on neurophysin column at ≈50 Ci/mmol.

Analytical HPLC. The monitoring was performed on a 4- × 250-mm C_{18} end-capped column (5-μm particle size, 100 Å porosity; Lichrospher, Merck) using a dual wavelength 214- and 254-nm UV detection (photometers 441 and 440, from Waters) and a fluorimetric detection (RF-10 AXL spectrofluorometric detector from Shimadzu), with pertinent excitation and emission wavelengths (530 and 580 nm, for Rhm), recorded on a two-channel analogic apparatus (Kipp and Zonen). Linear 1%/min CH_3CN:CF_3CO_2H (0.1% v/v) in H_2O:CF_3CO_2H (0.1% v/v) gradients were generally performed in high-pressure mode, at 2 mL/min by two pumps (LC-9A, from Shimadzu) using a static mixing chamber. The void volume of the system is $v_0 = 1.7$ mL, and the values given as analytical HPLC data are the percent CH_3CN composition of the eluent in the detector cell at the time of the UV detection (corrected for the v_0).

Semipreparative HPLC. The purifications were performed alternately on two C_{18} columns: one of 22 × 500 mm, 10-μm particle size, 80 Å porosity (Whatman Partisil ODS 3 Magnum 20) with a void volume $v_0 = 110$ mL and the other of 22 × 250 mm, 10-μm particle size, 300 Å porosity (218TP1022 from Vydac) with a $v_0 = 50$ mL, all protected by a precolumn, in high-pressure gradient mode generated by two pumps (model 400 from Applied Biosystems) and a controller (738A, from Applied Biosystems), at 10 mL/min. The detection was performed at 214 nm by a spectrophotometric detector (738A,

2.1-mm optical length) and at 254 nm by a photometer (440, from Waters) and an analogical dual trace recorder (Houston Instruments). The products were collected in peak mode coupled to the 214-nm monitoring by an automated fraction collector (202, from Gilson), flash-frozen in liquid nitrogen, and lyophilized.

Synthesis of the Peptidic Parts. 4-HOPhCH$_2$CO-D-Tyr-(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH$_2$ (HO-LVA), H-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$ (**1**), 4-HOPh(CH$_2$)$_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Lys-Pro-Arg-NH$_2$ (**3**), and 4-HOPh(CH$_2$)$_2$-CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH$_2$ (**7**) were obtained as CF_3CO_2H salts by PyBOP, *tert*-butyloxycarbonyl synthesis, with a manual device, as previously described,[30] with HF deprotection and RP-HPLC purification using CF_3CO_2H as counterion.

Typical Coupling of a Fluorophore. To 11.7 μmol of **3** (15.7 mg) were added 12 μmol of RP-HPLC purified 5C-Rhm-OH, 12 μmol of BOP (5.3 mg) or PyBOP, and 5 μL of (iPr$_2$)-EtN, in 400 μL of anhydrous UV grade Me$_2$SO. The mixture was kept in the dark. Aliquots (≈1/4 μL) were injected for the HPLC monitoring (Figure 2).[32] Eventually, BOP or rhodamine was added in order to convert a maximum of peptide **7** into fluorescent derivative. In some cases a complete reaction could not be obtained and impurities appeared, but the yields were generally good. The coupling mixture was acidified with CF_3CO_2H and injected onto the semipreparative RP-HPLC sytem, and the formed fluorescent peak was split and screened for purity. The fractions were selected according to purity and not yield, flash-frozen, and lyophilized to give the purple antagonist (9.1 mg, 47% yield).

UV Spectroscopy. Absorption spectra were recorded on a digital spectrophotometer (DU 640, from Beckman), in 1-cm quartz cells (Suprasil from Hellma), using 10 μM solutions in PBS, starting from 10^{-3} M solutions in spectroscopic grade DMF, obtained by the weighing of the CF_3CO_2H salts (**4**, **8**, **9** are calculated for 1 CF_3CO_2H and **5**, **6**, **10**, **11** for 2 CF_3CO_2H).

Cell Culture. CHO cell line expressing human vasopressin V_{1a} receptor subtype was a gift from Dr. Mouillac. Cells were maintained in culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4 mM Gln, and 500 units/mL penicillin and streptomycin in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Membrane Preparations. Depending on the experiments to be conducted, cells were treated overnight with 5 mM sodium butyrate to increase receptor expression.[33] The pharmacological properties of the receptor were not modified by this treatment. Culture dishes of CHO cells expressing human vasopressin V_{1a} or V_{1b} or V_2 receptors or human oxytocin receptors were washed twice in PBS without Ca^{2+} and Mg^{2+}. Lysis buffer (15 mM Tris:HCl, 2 mM $MgCl_2$, 0.3 mM EDTA, pH 7.4) was added. Cells were scraped, Polytron homogenized, and centrifuged at 100g for 5 min at 4 °C. Supernatants were recovered and centrifuged at 44000g for 20 min at 4 °C. Pellets were suspended in buffer A (50 mM Tris:HCl, 5 mM $MgCl_2$, pH 7.4) and centrifuged at 44000g for 20 min at 4 °C. Membranes were resuspended in an appropriate volume of buffer A and protein content was determined. Membranes were used immediately or stored in liquid nitrogen.

Binding Experiments. Affinities of the fluorescent ligands for the various human receptors were determined from competition experiments using 50−70 pM [^{125}I]HO-LVA antagonist (for V_{1a} vasopressin receptor), 1 nM [^{125}I]HO-LVA antagonist (for V_{1b} vasopressin receptor), 200−220 pM [^{125}I]OTA antagonist (for oxytocin receptor), and 2 nM [$^3H_3$]AVP (for V_2 vasopressin receptor) as radioligands. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. Concentrations of fluorescent ligand varied from 1 pM to 10 μM. As previously described,[23] binding assays were performed at 30 °C, for 1 h, equilibrium being reached after 30 min. The ligand binding data were analyzed with the computer program Ligand, and the inhibition constants (K_i) were determined with a nonlinear least-squares regression from at least three assays performed each in triplicate.

Inositol Phosphate Assays. Inositol phosphate accumulation was determined as previously described.[26] Briefly, CHO-V_{1ah} cells were labeled for 24 h with *myo*-[2-^{3}H]inositol (10−20 Ci/mmol; DuPont New England Nuclear) at a final concentration of 1 μCi/mL in a serum- and inositol-free medium. Then cells were incubated for 1 h in a serum- and inositol-free medium, washed twice in PBS, and incubated with 10 mM LiCl for 10 min. Cells were then incubated with antagonists at various concentrations for 10 min and stimulated with 1 nM AVP for 15 min. The reaction was stopped with perchloric acid, inositol phosphates were extracted on an anion-exchange column (Dowex AG1 × 8, formate form, 200−400 mesh; Bio-Rad) and collected, and their radioactivity was determined by scintillation counting. K_{inact} values were determined from three different dose−response curves, performed in triplicate, and correspond to the concentration of antagonist leading to the half-inhibition of the stimulation induced with AVP at a concentration equal to the K_{act}. It was calculated as $K_{inact} = IC_{50}/(1 + [AVP]/K_{act})$, with [AVP] = 1 nM and K_{act} = 0.35 nM.[3]

Fluorescent Labeling of CHO Cells Expressing the Human V_{1a} Vasopressin Receptor. Cells (CHO-V_{1ah} cells, 300 000−800 000 receptors/cell) were washed twice with culture medium without fetal calf serum and incubated for 1 h at 37 °C with fluorescent ligand at a concentration of 5 × K_i. Then cells were washed three times, fixed with 4% paraformaldehyde for 30 min, washed twice with 0.1 M glycine solution, and quickly rinsed with PBS. Cells were observed on a Zeiss axiovert 2 microscope equipped for epifluorescence (excitation range, 450−495 nm; barrier filter, 515 nm) using 60× oil immersion objective.

Cytofluorimetric Analysis of Fluorescent Cells. EDTA-treated cells (1 × 10^6) were centrifuged at 500g for 10 min, washed with PBS, incubated in 25 mL of PBS containing 1 nM [Lys^8(5C-Flu)]PVA (**8**) with (nonspecific binding, trace N) or without (total binding, trace T) 0.1 μM HO-LVA. After 1 h, cells were washed with ice-cold PBS and analyzed on a FACScan apparatus (Becton-Dickinson). The cell number was plotted against the log of the mean fluorescence intensity. For each condition, 10^4 cells were analyzed.

Fluorescent Labeling of CHO-V_{1ah} Cell Membranes. CHO cell membranes expressing the human V_{1a} receptor (40.7 pmol/mg) were incubated (5.5 pmol/assay) with 0.5 nM [Lys^8-(5C-Rhm)]PVA (**10**) without (total binding, T) or with (nonspecific binding, N) 0.5 μM [Lys^8]PVA (**7**) or without any ligand (autofluorescence of the membranes, A), in PBS (30 mL) added with 5 mM Mg^{2+} and 1 mg/mL BSA, for 1 h at 30 °C. They were centrifuged (100000g) for 30 min at 4 °C, washed with the same buffer without BSA, centrifuged (100000g, 4 °C), and resuspended in the same medium for the measure, performed at 20 °C, under magnetic stirring, using 530-nm excitation and 550−640-nm emission.[10] The fluorescence emission spectra were recorded on a Photon Technology International modular fluorimeter, equipped with a 75 W xenon lamp. Excitation and emission slits were 5- and 10-nm band-pass, respectively. Results were recorded and analyzed with Felix software.

Acknowledgment. This work was supported by INSERM and PCV97-123 from Action Incitative Physique et Chimie du Vivant CNRS. Thanks are due to Dr. B. Mouillac for providing the CHO-V_{1ah} cell line, L. Guillou, S. Ponthieux, and S. Redon for peptide synthesis, Dr. Duperray for technical assistance during flow cytofluorometry experiments, and M. Passama and L. Charvet for the figures. We are most thankful to Dr. P. Jouin for constant interest.

References

(1) Jard, S.; Elands, J.; Schmidt, A.; Barberis, C. Vasopressin and oxytocin receptors: an overview. In *Progress in Endocrinology*; Imura, H., Shizume, K., Eds.; Elsevier: Amsterdam, 1988; pp 1183−1189.

(2) Mouillac, B.; Chini, B.; Balestre, M. N.; Elands, J.; Trumpp-Kallmeyer, S.; Hoflack, J.; Hibert, M.; Jard, S.; Barberis, C. The binding site of neuropeptide vasopressin V1a receptor. Evidence for a major localization within transmembrane regions. *J. Biol. Chem.* **1995**, *270*, 25771−25777.
(3) Phalipou, S.; Cotte, N.; Carnazzi, E.; Seyer, R.; Mahé, E.; Jard, S.; Barberis, C.; Mouillac, B. Mapping peptide-binding domains of the human V1a vasopressin receptor with a photoactivatable linear peptide antagonist. *J. Biol. Chem.* **1997**, *272*, 26536−26544.
(4) Kojro, E.; Eich, P.; Gimpl, G.; Fahrenholz, F. Direct identification of an extracellular agonist binding site in the renal V2 vasopressin receptor. *Biochemistry* **1993**, *32*, 13537−13544.
(5) MacGrath, J. C.; Arribas, S.; Daly, C. J. Fluorescent ligands for the study of receptors. *Trends Pharmacol. Sci.* **1996**, *17*, 393−399.
(6) Hein, L.; Meinel, L.; Pratt, E.; Dzau, V. J.; Kobilka, B. K. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol. Endocrinol.* **1997**, *11*, 1266−1277.
(7) Zagyansky, Y. A.; Jard, S. Does lectin-receptor complex formation produce zones of restricted mobility within the membrane? *Nature* **1979**, *280*, 591−593.
(8) Sterrer, S.; Henco, K. Fluorescence correlation spectroscopy (FCS) − a highly sensitive method to analyse drug/target interactions. *J. Recept. Signal Transd. Res.* **1997**, *17*, 511−520.
(9) Raines, D. E.; Krishnan, N. S. Transient low-affinity agonist binding to *Torpedo* postsynaptic membranes resolved by using sequential mixing stopped-flow fluorescence spectroscopy. *Biochemistry* **1998**, *37*, 956−964.
(10) Turcatti, G.; Vogel, H.; Chollet, A. Probing the binding domain of the NK2 receptor with fluorescent ligands: evidence that heptapeptide agonists and antagonists bind differently. *Biochemistry* **1995**, *34*, 3972−3980.
(11) Tota, M. R.; Daniel, S.; Sirotina, A.; Mazina, K. E.; Frong, T. M.; Longmore, J.; Strader, C. D. Characterization of a fluorescent substance P analogue. *Biochemistry* **1994**, *33*, 13079−13086.
(12) Turcatti, G.; Nemeth, K.; Edgerton, M.; Knowles, J.; Vogel, H.; Chollet, A. Fluorescent labeling of NK2 receptor at specific sites in vivo and fluorescence energy transfer analysis of NK2 ligand−receptor complexes. *Recept. Channels* **1997**, *5*, 201−207.
(13) Manning, M.; Kolodziejczyk, A. S.; Kolodziejczyk, A. M.; Stoev, S.; Klis, W. A.; Wo, N. C.; Sawyer, W. H. Highly potent and selective Tyr-NH_2^9-containing linear V1 antagonists and D-Tyr^2-containing linear V2 agonists: potential radioiodinated ligand for vasopressin receptors. In *Peptides 1990*; Giralt, E., Andreu, D., Eds.; Escom Science Publishers B.V.: Leiden, 1991; pp 665−667.
(14) Manning, M.; Bankowski, K.; Barberis, C.; Jard, S.; Elands, J.; Chan, W. Y. Novel approach to the design of synthetic radioiodinated linear V1a receptor antagonists of vasopressin. *Int. J. Pept. Protein Res.* **1992**, *40*, 261−267.
(15) Lutz, W. H.; Londowski, J. M.; Kumar, R. The synthesis and biological activity of four novel fluorescent vasopressin analogues. *J. Biol. Chem.* **1990**, *265*, 4657−4663.
(16) Yuan, T.; Weljie, A. M.; Vogel, H. J. Tryptophan fluorescence quenching by methionine and selenomethionine residues of calmodulin: orientation of peptide and protein binding. *Biochemistry* **1998**, *37*, 3187−3195.
(17) Szmacinski, H.; Wiczk, W.; Fishman, M. N.; Eis, P. S.; Lakowicz, J. R.; Johnson, M. L. Distance distributions from the tyrosyl to disulfide residues in the oxytocin and [Arg^8]-vasopressin measured using frequency-domain fluorescence resonance energy transfer. *Eur. Biophys. J.* **1996**, *24*, 185−193.
(18) Carnazzi, E.; Aumelas, A.; Barberis, C.; Guillon, G.; Seyer, R. A new series of photoactivatable and iodinatable linear vasopressin antagonists. *J. Med. Chem.* **1994**, *37*, 1841−1849.
(19) Guillon, G.; Barbeau, D.; Neugebauer, W.; Guay, S.; Bilodeau, L.; Balestre, M. N.; Gallo-Payet, N.; Escher, E. Fluorescent peptide hormones: development of high affinity vasopressin analogues. *Peptides* **1992**, *13*, 7−11.
(20) Haugland, R. P. In *Handbook of fluorescent probes and research chemicals*; Spence, M. T. Z., Ed.; Eugene, 1996.
(21) Faure, M.-P.; Gaudreau, P.; Shaw, I.; Cashman, N. R.; Beaudet, A. Synthesis of a biologically active fluorescent probe for labeling neurotensin receptors. *J. Histochem. Cytochem.* **1994**, *42*, 755−763.
(22) Frérot, E.; Coste, J.; Pantaloni, A.; Dufour, M. N.; Jouin, P. PyBOP and PyBroP: two reagents for the difficult coupling of the α,α-dialkyl amino acid, Aib. *Tetrahedron* **1991**, *47*, 259−270.
(23) Barberis, C.; Balestre, M.-N.; Jard, S.; Tribollet, E.; Arsenijevic, Y.; Dreifuss, J.-J.; Bankowski, K.; Manning, M.; Chan, W. Y.; Schlosser, S. S.; Holsboer, F.; Elands, J. Characterization of a novel linear radioiodinated vasopressin antagonist: an excellent radioligand for vasopressin V1a receptors. *Neuroendocrinology* **1995**, *62*, 135−146.
(24) Schmidt, A.; Audigier, S.; Barberis, C.; Jard, S.; Manning, M.; Kolodziejczyk, A. S.; Sawyer, W. H. A radioiodinated linear vasopressin antagonist: a ligand with high affinity and specificity for V1a receptors. *FEBS Lett.* **1991**, *282*, 77−81.
(25) Serradeil-Le Gal, C.; Wagnon, J.; Garcia, C.; Lacour, P.; Guiraudou, P.; Christophe, B.; Villanova, G.; Nisato, D.; Maffrand, J. P.; Le Fur, G.; Guillon, G.; Cantau, B.; Barberis, C.; Trueba, M.; Ala, Y.; Jard, S. Biochemical and pharmacological properties of SR 49059, a new potent, non-peptide antagonist of rat and human vasopressin V1a receptors. *J. Clin. Invest.* **1993**, *92*, 224.
(26) Kirk, C. J.; Guillon, G.; Balestre, M.-N.; Jard, S. Stimulation, by vasopressin and other agonists, of inositol-lipid breakdown and inositol phosphate accumulation in WRK1 cells. *Biochem. J.* **1986**, *240*, 197−204.
(27) Lutz, W.; Londowki, J. M.; Sanders, M.; Salisbury, J.; Kumar, R. A vasopressin analogue that binds but does not activate V1 or V2 vasopressin receptors is not internalized into cells that express V1 or V2 receptors. *J. Biol. Chem.* **1992**, *267*, 1109−1115.
(28) Jans, D. A.; Peters, R.; Zsigo, J.; Fahrenholz, F. The adenylate cyclase-coupled vasopressin V2-receptor is highly laterally mobile in membranes of LLC−PK1 renal epithelial cells at physiological temperature. *EMBO J.* **1989**, *8*, 2481−2488.
(29) Strakova, Z.; Kumar, A.; Watson, A. J.; Soloff, M. S. A new linear V1a vasopressin antagonist and its use in characterizing receptor/G protein interactions. *Mol. Pharmacol.* **1997**, *51*, 217−224.
(30) Coste, J.; Le-Nguyen, D.; Castro, B. PyBOP: a new peptide coupling reagent devoid of toxic byproduct. *Tetrahedron Lett.* **1990**, *31*, 205−208.
(31) Elands, J.; Barberis, C.; Jard, S.; Tribollet, E.; Dreifuss, J. J.; Bankowski, K.; Manning, M.; Sawyer, W. H. ^{125}I-labeled d$(CH_2)_5$-[$Tyr(Me)^2$,Thr^4, Tyr-NH_2^9]OVT: a selective oxytocin receptor ligand. *Eur. J. Pharmacol.* **1987**, *147*, 197−207.
(32) Seyer, R.; Aumelas, A. Synthesis of biotinylated and photoreactive probes for angiotensin receptors. *J. Chem. Soc., Perkin Trans. 1* **1990**, 3289−3299.
(33) Kassis, S.; Henneberry, R. C.; Fishman, P. H. Induction of catecholamine-responsive adenylate cyclase in HeLa cells by sodium butyrate. *J. Biol. Chem.* **1984**, *259*, 4910−4916.

JM9804782