Toward Efficient Drug Screening by Homogeneous Assays Based on the Development of New Fluorescent Vasopressin and Oxytocin Receptor Ligands

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A series of fluorescent ligands designed for vasopressin and oxytocin G protein-coupled receptors was synthesized and characterized to develop fluorescence polarization or homogeneous time−resolved fluorescence (HTRF) binding assays. These ligands, labeled with europium pyridine-bis-bipyridine cryptate or with Alexa 488,546,647 selectively bound to the vasopressin V_{1a} and oxytocin receptors with high affinities and exhibited antagonistic properties. The affinities of several unlabeled ligands determined by our homogeneous assays on membrane preparations or on intact cells into 96- and 384-well plate formats were similar to those determined by usual radioligand binding methods. Compared to other binding assays, the polarization and HTRF binding assays are nonradiaoactive, therefore safer to perform, yet very sensitive and homogeneous, therefore easier and faster to automate. These methods are thus suitable for efficient drug high-throughput screening procedures and can easily be applied to other G protein-coupled receptor models.

Introduction

G protein-coupled receptors (GPCR[a]) constitute the major target for the pharmaceutical industry, representing more than 30% of the drug screening targets.[1,2] Ligand screening procedures associate many different steps and development, the primary ones being receptor ligand binding[3] and receptor signaling assays (stimulation or inhibition of second messenger production, reporter gene approaches).[1] The estimation of the receptor binding affinity for each newly generated derivative ligand remains one of the most challenging aspects of drug design, and this step is generally based on radioactive binding assays. Besides the ecologic and health hazard limitations, radioactive screening performed on a GPCR target generally requires the separation of the bound ligand fraction from the free one. This essential step is performed by filtration or by centrifugation. Similarly, the fluorescence intensity binding assays need the same separation steps. More recently, scintillation proximity assay, a homogeneous technology, has been developed and precludes the separation of the two fractions but requires immobilization of ligands or membranes on beads.[4]

By contrast, fluorescent assays based on polarization or on fluorescence resonance energy transfer (FRET) are much simpler since they can be achieved without any separation of the bound and free ligand fractions. The development of these techniques in the past decade has stemmed first from the elaboration of numerous sensitive microplate readers and second from the synthesis of new fluorescent probes. Some of them such as the Alexa series exhibit greater photostabilities and enhanced fluorescence emissions than those of the classical fluoroprobes such as fluorescein or rhodamine. These properties increase the sensitivities of the assays, facilitating the development of fluorescent based assays. Other fluoroprobes such as lanthanide complexes have also been developed because of their long fluorescence lifetimes which allow specific signals to be separated from nonspecific signals and make these fluoroprobes convenient for homogeneous time-resolved fluorescence (HTRF) approaches.[1,2] These two factors make possible a high-troughput screening (HTS) based on fluorescence techniques.

Polarization assays are based on the difference in the mobility of fluoroprobes, depending on whether the ligands to which they are linked are bound or not to the receptors. This technique has been described as fast, sensitive, and inexpensive[5] and therefore as a method of choice for HTS[6] although it has been applied to a limited number of GPCRs.[7−10]

A few assays based on the FRET technology have also been developed. The FRET signal occurs between a green fluorescent protein variant GPCR chimera and a fluorescent ligand[11] or a quencher linked to a ligand.[12] However, the labeling of receptors with large molecules such as GFP variants can result in the

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[a] Abbreviations: AVP, arginine vasopressin, H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; OT, oxytocin, H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2; HO-LVA, linear vasopressin antagonist, 4-HOPhCH_2CO-D-Tyr-(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; PVA, phenylpropionic linear vasopressin antagonist, 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2; [Lys[8](Alexa 488/546/547)]PVA, 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(Alexa 488/546/547)-NH_2; [Lys[8](Eu⊂PBBP)]PVA, 4-HOPh-$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(Eu⊂PBBP)-NH_2; SR49059, ((2*S*)-1-[(2*R*,3*S*)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzenesulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide); CHO, Chinese hamster ovary cells; Cos-7, transformed African green monkey kidney fibroblast cells; HTRF, homogeneous time-resolved fluorescence; FRET, fluorescence resonance energy transfer; HTS, high-throughput screening; GPCR, G protein-coupled receptor; PBBP, pyridine-bis-bipyridine cryptate; RP-HPLC, reverse phase-high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; HA, hemagluttinin; EDTA, acid ethylenediaminetetracetic acid; IP, inositol phosphate; HRP, horse radish peroxidase; K_i, inhibition constant.

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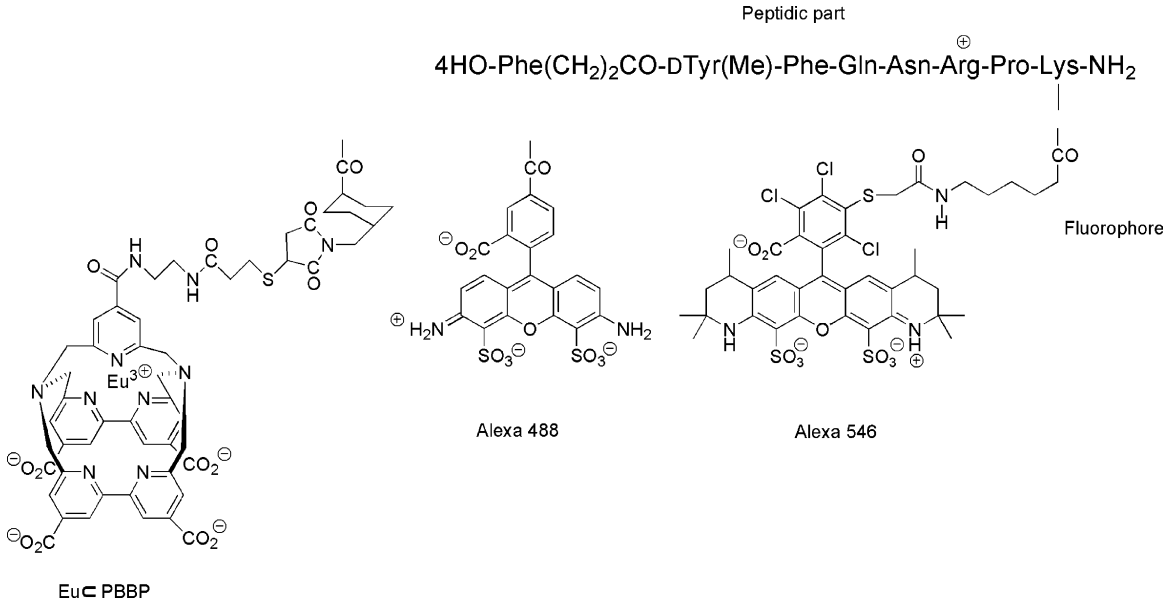

Figure 1. Structures of the fluorophores Eu⊂PBBP, Alexa 488, and Alexa 546. The structure of Alexa 647 has not been published.

decrease in the receptor expression, at least for some GPCR such as the vasopressin and the oxytocin receptors. Moreover, the energy transfer between fusion proteins results from the protein interactions from the whole cell and not of a specific subcellular cell compartment. To settle these matters, the receptors must be tagged with small epitopes specifically detected by fluorophore-conjugated antibodies. More recently, technologies based on time-resolved FRET between fluorescein and rhodamine or lanthanide and fluorophores such as Cyanine 5, Alexa 647, or XL 665[13,14] have been developed. They have been used to detect protein dimerization[15−17] or enzymatic activities but never to our knowledge used to perform HTS of drugs for GPCR.

Previous series of fluorescein- or rhodamine-conjugated peptides[18−24] have been developed for the vasopressin and oxytocin receptors but because of the low quantum yield and the low photostability of the fluorophores, these fluorescent analogues were not useful for HTS tests. In the present study, we have developed lanthanide or Alexa-labeled analogues which keep high affinities for the vasopressin V_{1a} and oxytocin receptors and which exhibit excellent fluorescence properties. These fluorescent peptides are convenient to perform HTS using polarization or FRET measurements.

Results

Design of Fluorescent Analogues. Linear vasopressin antagonists have been reported to exhibit high affinities for the vasopressin V_{1a} receptor.[19,25] We chose to anchor the fluorophores on the linear antagonist [Lys^8] PVA as previously described.[19] This pseudopeptide contains a hydroxyl-phenylpropionyl group at the N-terminus and exhibits a very high affinity for the vasopressin V_{1a} and oxytocin receptors. Moreover, the presence of a lysine residue in position 8 permits the addition of a bulky fluorescent group without losing the high affinities for vasopressin V_{1a} and oxytocin receptors.[19] Computational modeling[26] has suggested that when vasopressin or peptidic analogues anchor to the binding pocket, the lysine residue is oriented toward the extracellular medium. The fluorophore is linked to the peptidic backbone using a short spacer, avoiding an excessive rotation of the fluorophore which would have prevented the measurement of any modification in polarization intensity.

Various Alexa fluorophores were covalently linked to the vasopressin linear antagonist. The Alexa 488, Alexa 546 (Figure 1), and Alexa 647 fluorophores exhibit spectral properties analogous to those of fluorescein, Cy3 and Cy5, respectively. However, Alexa 488 has been reported to be more photostable than fluorescein and the quantum yield of Alexa 546 to be 2.7 higher than that of Cy3.[27] Finally, Alexa 647 has been reported to be twice less sensitive to photobleaching and brighter than Cy5 whatever the test used.[28] When available, the tetrafluorophenyl ester (Alexa 488) was preferred to the succinimidyl ester (Alexa 546 and Alexa 647) because it appears to be more resistant to hydrolysis during its storage. Moreover, as illustrated in Figure 1, we also selected the europium pyridine-bis-bipyridine cryptate (Eu⊂PBBP) as a fluorescent probe for its long fluorescent decay (lifetime greater than 0.5 ms).[29]

Affinities for Vasopressin and Oxytocin Receptors. The affinities of the fluorescent ligands for the human vasopressin V_{1a}, V_{1b}, V_2, and oxytocin receptors were determined by competition experiments with [^{3}H]AVP on CHO cell membranes expressing the various receptors. The [Lys^8(Alexa 488)]PVA compound exhibits a very high affinity ($K_i = 0.09 \pm 0.05$ nM) for the V_{1a} receptor while the other compounds show affinities in the nanomolar range (Table 1). We investigated the selectivities of these compounds. As illustrated in Figure 2a, the compound [Lys^8(Alexa 488)]PVA also exhibits good affinities for oxytocin receptor ($K_i = 1.04 \pm 0.07$ nM) receptors and low affinities for the V_{1b} ($K_i > 0.1$ μM) and V_2 ($K_i > 1$ μM) receptors. The [Lys^8(Eu⊂PBBP)]PVA exhibits good affinities for the V_{1a} ($K_i = 1.54 \pm 0.65$ nM) and oxytocin ($K_i = 0.64 \pm 0.1$ nM) receptors and low affinities for the V_{1b} ($K_i > 1$ μM) and V_2 ($K_i > 1$ μM) receptors (Figure 2b). The Lys^8(Alexa 546)]-PVA and Lys^8(Alexa 647)]PVA compounds also exhibit dissociation constants in the nanomolar range for the vasopressin V_{1a} and oxytocin receptors, and dissociation constants greater than 1 μM for V_{1b} and V_2 receptors (Table 1).

Antagonist Effects of the Fluorescent Ligands. Because of their high affinities for vasopressin V_{1a} and oxytocin receptors, agonistic and antagonistic properties of the fluorescent ligands were evaluated. All the fluorescent analogues at a concentration up to 1 μM were devoid of any agonistic properties since they did not induce any accumulation of inositol phosphates (data not shown). On the contrary, they have been

Table 1. Determination of the Inhibition Constants of the Fluorescent Ligands for the Vasopressin and Oxytocin Receptors[a]

ligand	K_i (nM)			
	V_{1a}	V_{1b}	V_2	oxytocin
AVP	0.7 ± 0.17[b]	0.49 ± 0.06[c]	1.48 ± 0.08[d]	1.65 ± 0.49[e]
[Lys^8(Eu⊂PBBP)]PVA	1.54 ± 0.65	>1000	>1000	0.64 ± 0.1
[Lys^8(Alexa 488)]PVA	0.09 ± 0.05	≈100	>1000	1.04 ± 0.07
[Lys^8(Alexa 546)]PVA	2.1 ± 0.16	>1000	>1000	1.95 ± 0.24
[Lys^8(Alexa 647)]PVA	3.6 ± 2	>1000	>1000	1.91 ± 0.67

[a] The inhibition constants (K_i) of AVP and fluorescent antagonists for human vasopressin V_{1a}, V_{1b}, V_2 and oxytocin receptors were determined on CHO cell membranes by competition binding assays (displacement of radioactive [^{3}H]AVP). Results are expressed as mean ± SEM of three separate experiments performed in triplicate. [b] Values from ref 31. [c] Values from ref 43. [d] Values from ref 44. [e] Values from ref 45.

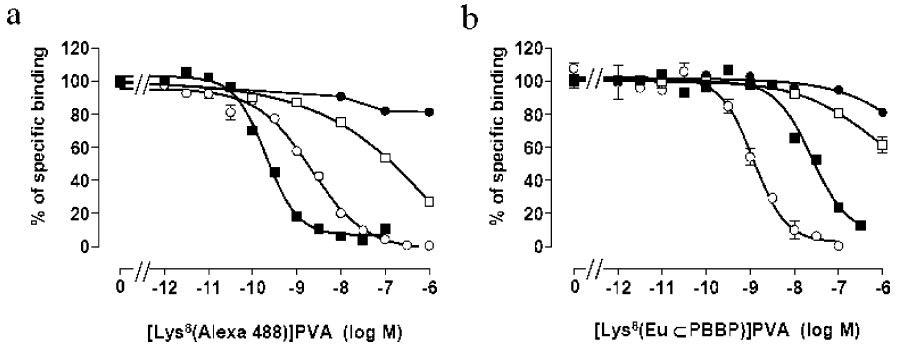

Figure 2. Competition experiments performed on membranes of CHO cells expressing human vasopressin V_{1a} (■), V_{1b} (□), V_2 (●) or oxytocin (○) receptors. Membranes were incubated in the presence of 2 nM [^{3}H]AVP and increasing concentrations of [Lys^8(Alexa 488)] PVA **(a)** or [Lys^8(Eu⊂PBBP)]PVA **(b)** (1 pM to 1 μM) for 1 h at 30 °C. Each curve is representative of at least three independent experiments performed in triplicate samples. Values are means ± SEM of triplicates. For the compound [Lys^8(Alexa 488)]PVA, the K_i values are 0.1 nM, >0.1 μM, >1 μM, and 1 nM for the V_{1a}, V_{1b}, V_2 and oxytocin receptors, respectively. Slope factors are equal to 1.2 and 0.7 for the V_{1a} and OT receptor curves, respectively. The [Lys^8(Eu⊂PBBP]PVA exhibits K_i values of 2.84 nM, >1 μM, >1 μM, and 0.83 nM for the V_{1a}, V_{1b}, V_2 and oxytocin receptors, respectively. The slope factors are equal to 1.17 and 1.06 for the V_{1a} and OT receptor curves. Black lines correspond to the best fit of the data with a sigmoidal curve considering a variable slope.

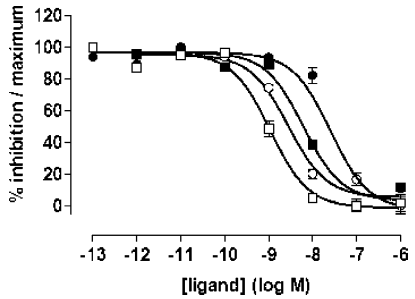

Figure 3. Antagonistic effect of fluorescent ligands on vasopressin-induced inositol phosphate accumulation. Ligand-induced accumulation of inositol phosphates in CHO cells expressing human vasopressin V_{1a} receptors measured by IP-One ELISA test. Selective ligands tested were [Lys^8(Eu⊂PBBP)]PVA (K_{inact}= 0.85 nM) (■), [Lys^8(Alexa 488)]PVA (K_{inact} = 0.15 nM) (□), [Lys^8(Alexa 546)]PVA (K_{inact} = 2.94 nM) (●), and [Lys^8(Alexa 647)]PVA (K_{inact} = 0.31 nM) (○). Each curve is representative of at least three independent experiments performed in triplicate samples. Data are means ± SEM of triplicates. The black lines correspond to the best fit obtained with a sigmoidal curve, considering a slope of 1.

shown to inhibit AVP (2 nM)-induced inositol phosphate accumulation in a dose-dependent manner. This is illustrated in Figure 3 for the V_{1a} receptor subtype stably expressed in CHO cells. The mean values of K_{inact} are in the nanomolar range (Table 2). Similar experiments were performed on oxytocin receptor: all ligands inhibited OT (50 nM)-induced inositol phosphate accumulation. The estimated K_{inact} values were also in the nanomolar range (Table 2). They were in the same range as those obtained for the V_{1a} receptor (Table 2).

Polarization Experiments. Numerous vasopressin and oxytocin fluorescent ligands have already been synthesized[18−24] but none of them has been used to perform polarization experiments. To estimate the ratio-specific signal/nonspecific signal, we

Table 2. Inhibition of Agonist-Induced Inositol Monophosphate ($InsP_1$) Accumulation on CHO Cells Expressing the V_{1a} or OT Receptors[a]

ligand	K_{inact} (nM)	
	V_{1a}	OT receptor
[Lys^8]PVA	0.17 ± 0.06	0.82 ± 0.25
[Lys^8(Eu⊂PBBP)]PVA	2.2 ± 0.9	2.7 ± 1.1
[Lys^8(Alexa 488)]PVA	0.28 ± 0.13	0.17 ± 0.05
[Lys^8(Alexa 546)]PVA	4.55 ± 0.87	0.96 ± 0.47
[Lys^8(Alexa 647)]PVA	0.63 ± 0.12	0.66 ± 0.28

[a] The inactivation constants (K_{inact}) of [Lys8]PVA, [Lys8(Eu⊂PBBP)]PVA, [Lys^8(Alexa 488)]PVA, [Lys^8(Alexa 546)]PVA and [Lys^8(Alexa 647)]PVA were measured in CHO cell lines expressing either vasopressin V_{1a} or oxytocin receptor by IP-One ELISA or radioactive inositol phosphate assays. These values are the mean ± SEM of three separate experiments performed in triplicate.

incubated membranes of CHO cells expressing V_{1a} receptor with increasing concentrations of [Lys^8(Alexa 488)]PVA, [Lys^8-(Alexa 546)]PVA, or [Lys^8(Alexa 647)]PVA corresponding to $1 \times K_i$, $2 \times K_i$, and $5 \times K_i$ respectively, in the presence or in the absence of an excess of vasopressin (1 μM). This ratio is at a maximum for [Lys^8(Alexa 488)]PVA at a concentration of $5 \times K_i$ ($r = 0.5 \pm 0.01$) (Figure 4a). The ratio is less than 0.2 for the other conditions. Therefore, we carried out competition experiments in 96-well plate by polarization measurement with [Lys^8(Alexa 488)]PVA at a concentration of $5 \times K_i$. Membranes of CHO cells expressing V_{1a} receptors were incubated in the presence of [Lys^8(Alexa 488)]PVA (0.5 nM) and increasing concentrations of AVP (1 pM to 1 μM). Variations of polarization intensity are illustrated in Figure 4b as a function of AVP concentration. The inhibition constant was 0.26 ± 0.02 nM (Table 3). Similar experiments were performed with two antagonists of vasopressin for the V_{1a} receptor: the linear vasopressin antagonist, HO-LVA,[19] and the nonpeptidic antagonist, SR 49059[30] (Figure 4b). The polarization signal reached

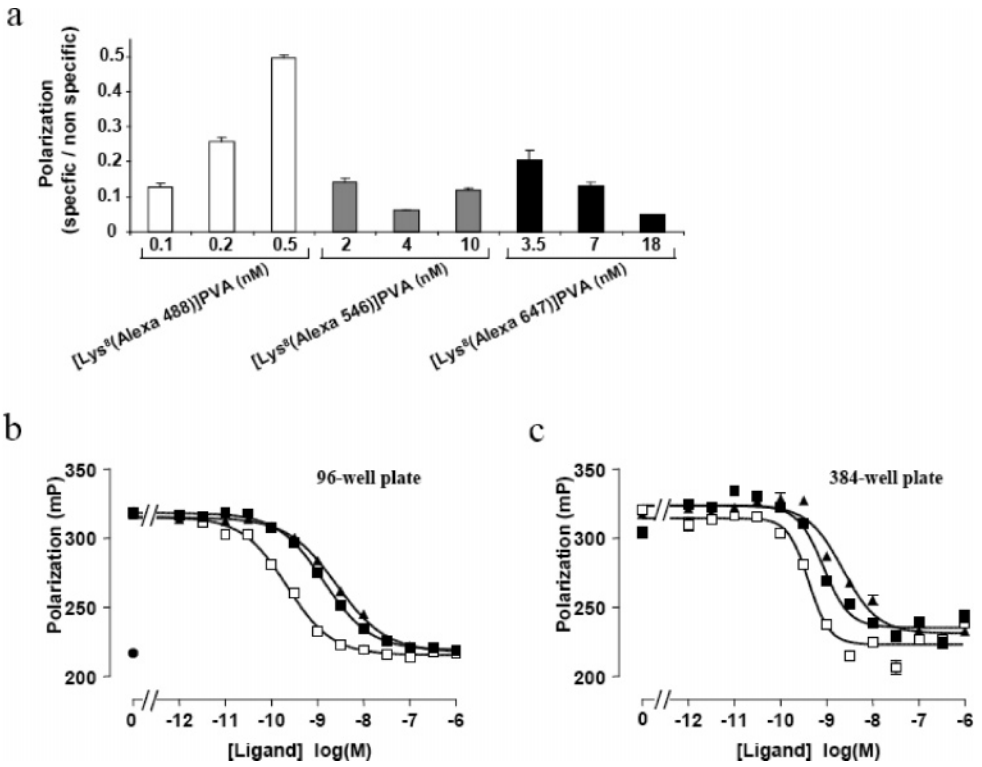

Figure 4. Polarization binding assays performed on CHO-expressing vasopressin V_{1a} receptor. (a) Determination of the specific/nonspecific ratio measured in polarization with the Alexa-labeled ligand series. Membranes were incubated 2 h at room temperature with the labeled ligands at the concentration mentioned (which corresponded to 1, 2, and 5 times K_i), in the presence or the absence of an excess of vasopressin (1 μM). The specific/nonspecific signal ratio was plotted as a function of the ligand concentration. (b) Determination of the affinity constants by polarization experiments. Competition experiments were performed with [Lys^8(Alexa 488)]PVA and AVP (■), HO-LVA (□), or SR 49059 (▲). Membranes expressing the human vasopressin V_{1a} receptor were incubated for 2 h at room temperature with [Lys^8(Alexa 488)]PVA (0.5 nM) and increasing concentrations of competitor ranging from 1 pM to 1 μM, before the polarization value was determined. ●: Membranes of CHO cells which do not expressed vasopressin receptors are incubated with [Lys^8(Alexa 488)]PVA (0.5 nM). Competition experiments were performed in 96-well plate in a final volume of 200 μL and with membranes at a concentration of 10−15 μg protein /assay. The K_i values are 0.21 nM, 0.03 nM, and 0.4 nM for AVP, HO-LVA, and SR 49059, respectively. (c) Competition experiments were performed in a 384-well plate in a final volume of 50 μL with membranes at a concentration 3−5 μg protein/assay. The K_i values are 0.13 nM, 0.06 nM, and 0.35 nM for AVP (■), HO-LVA (□), and SR 49059 (▲), respectively. Data were analyzed with Graphpad Prism software. The polarization curves are representative of at least three independent experiments each performed in triplicate. Values are means ± SEM of triplicates.

Table 3. Determination of the Inhibition Constants of AVP, HO-LVA, and SR 49059 by Competition Experiments Using Radioactivity, Polarization, and HTRF-FRET Binding Methods[a]

	K_i (nM)					
		fluorescence				
		polarization		HTRF-FRET		
ligand	radioactivity	96-well plate membranes	384-well plate membranes	96-well plate cells	96-well plate membranes	384-well plate cells
AVP	0.7 ± 0.17[b]	0.26 ± 0.02	0.35 ± 0.09	1.35 ± 0.51	1.53 ± 0.35	3.95 ± 1.73
HO-LVA	0.27[c]	0.036 ± 0.005	0.032 ± 0.009	0.28 ± 0.06	0.21 ± 0.14	0.21 ± 0.08
SR49059	0.62 ± 0.12[d]	0.7 ± 0.05	1.48 ± 0.3	0.85 ± 0.08	0.19 ± 0.14	0.52 ± 0.15

[a] The inhibition constants (K_i) of AVP, HO-LVA and SR49059 for the human vasopressin V_{1a} receptor expressed on CHO or Cos-7 cells were determined by competition experiments using radioactive and fluorescent (polarization and HTRF-FRET) binding methods as described in Experimental Section. Data shown are the mean ± SEM of at least three separate experiments performed in triplicate. [b] Values from ref 31. [c] Values from ref 33. [d] Values from ref 32.

for high concentrations of competitors was of the same amplitude for the three ligands (Figure 4b) and of the amplitude of the polarization signal obtained with CHO membrane preparations which did not express vasopressin V_{1a} receptor (Figure 4b, closed circle). These data underscore the specificity of the signal. The K_i values are 0.036 ± 0.005 nM and 0.7 ± 0.05 nM, respectively (Table 3). Similar results were obtained when experiments were performed in a 384-well plate (Figure 4c): the K_i values are 0.35 ± 0.09 nM, 0.032 ± 0.009 nM, and 1.48 ± 0.3 nM for AVP, HO-LVA, and SR 49059, respectively (Table 3).

Similar competition experiments were carried out with the [Lys^8(Alexa 546)]PVA and [Lys^8(Alexa 647)]PVA in 96-well plates, although the ratios "specific signal/nonspecific signal" were low. K_i values could not be estimated with good precision since variations in fluorescence polarization intensity were too small (data not shown).

Homogeneous Time-Resolved Fluorescence Experiments. Europium pyridine-bis-bipyridine cryptate (Eu⊂PBBP) exhibits interesting fluorescent properties such as a long fluorescent lifetime and is then suitable as a donor for time-resolved fluorescent experiments. Moreover, combined with the use of Alexa 647 or Cyanine 5 fluorescent probes as acceptors, it is convenient to perform HTRF experiments.[15−17]

We developed a HTRF competition binding assay based on a FRET signal between fluorophores carried by the ligand and by an antibody against a tagged receptor as illustrated in Figure

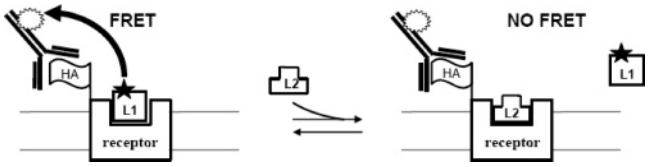

Figure 5. Time-resolved FRET competition experiment principle. In the absence of any competitor, a FRET signal between an europium pyridine-bis-bipyridine cryptate (★) labeled ligand L_1 and an Alexa 647 (✹) labeled monoclonal antibody against the N-terminal HA-tagged receptor is recorded. Increasing ligand L_2 concentration induces a decrease in the FRET signal.

5. The proximity of the two molecules when they bind to the receptor leads to a FRET signal, and the displacement of the fluorescent ligand from the receptor by the addition of an unlabeled competitor induces a decrease in this signal.

In a first series of experiments, membranes were incubated in the presence of [^{3}H]AVP and in the absence or the presence of anti-hemagluttinin (HA) antibodies labeled with Eu⊂PBBP or Alexa 647. No significant variation in the maximal binding was observed (Figure 6a) in the presence of antibodies. In order to see the amplitude and the stability of the FRET signal, Cos-7 cells which transiently expressed HA-tagged V_{1a} receptor were incubated at 4 °C with [Lys^8(Eu⊂PBBP)]PVA and with anti-HA monoclonal antibodies labeled with Alexa 647. The FRET signal measured after various times of incubation reached a plateau after an 8-h incubation (Figure 6b). This duration corresponds to the incubation time needed at 4 °C to get the antibody binding[17] and the ligand binding at the equilibrium. A competition assay was then developed on Cos-7 cells expressing HA-V_{1a} receptor. Cells were incubated with [Lys^8-(Eu⊂PBBP]PVA and anti-HA monoclonal antibodies labeled with Alexa 647, and with increasing concentrations of vasopressin (1 pM to 1 μM) as competitor. The experiments were performed at 4 °C to avoid any receptor internalization consecutive to ligand binding. The FRET signal measured at various times of incubation: 1, 2, 4, 8, and 20 h (Figure 6c) reached a plateau after an incubation of 8 h. After 20 h of incubation, the inhibition constant (K_i) of vasopressin measured in such conditions is 0.56 nM. Similar experiments were performed with two antagonists of vasopressin for the V_{1a} receptor: HO-LVA and SR 49059 (Figure 6d). The K_i values obtained were 0.24 nM and 0.65 nM, respectively. Mean values of K_i for AVP, HO-LVA, and SR 49059 were 1.35 ± 0.51 nM, 0.28 ± 0.06 nM, and 0.85 ± 0.08 nM, respectively (Table 3).

Competition experiments were also carried out on membrane preparations in the same experimental conditions. After a 20-h incubation time, the K_i value was 1.13 nM for AVP (Figure 7a). In order to test the sensitivity of the assay, competition experiments based on the same principle were also performed in a 384-well plate and similar results were obtained (Figure 7a). The K_i value was 1.3 nM for AVP. Mean values of K_i have been reported in Table 3. Finally, competition experiments have been executed with the opposite labeling: cells were incubated with [Lys^8(Alexa 647)]PVA and anti-HA monoclonal antibodies labeled with the fluorescent Eu⊂PBBP probe with increasing concentration of AVP (1 pM to 1 μM). Similar results were obtained, AVP exhibits K_i of 0.4 nM (Figure 7b).

Discussion

We synthesized fluorescent ligands exhibiting high affinities and good fluorescence properties to develop sensitive homogeneous screening tests. Due to its linear structure and the presence of a lysyl in position 8, the linear antagonist [Lys^8]-PVA was a suitable peptide to develop fluorescent ligands. As mentioned above, the Alexa fluorophore series and europium pyridine-bis-bipyridine cryptate fluorophore appeared to be convenient to develop sensitive assays. Although all the fluorescent analogues have kept good affinities for the vasopressin V_{1a} and oxytocin receptors, it is noteworthy that the [Lys^8(Alexa 488)]PVA surprisingly exhibits an affinity for the V_{1a} receptor at least 10 times greater than the others. This very high affinity is then receptor specific, suggesting an adequacy between the structure of the fluorophore-labeled ligand and the binding pocket of the receptor. The size of the fluorophore is probably an important parameter: indeed the size of Alexa 488 is smaller than that of Alexa 546. Unfortunately, the structure of Alexa 647 was not published, and one can only speculate about its size from the molecular mass of [Lys^8(Alexa 647)]-PVA which is greater than that of [Lys^8(Alexa 488)]PVA and a little bit smaller than that of [Lys^8(Alexa 546)]PVA. It suggests that Alexa 647 is more bulky than Alexa 488. All the ligands keep antagonistic properties.

Surprisingly [Lys^8(Alexa 488)]PVA is the only ligand which can be used to perform polarization experiments. Indeed [Lys^8-(Alexa 546)]PVA and [Lys^8(Alexa 647)]PVA exhibit low specific polarization signals whatever the concentration used ($1 \times K_i$, $2 \times K_i$ or $5 \times K_i$). It is difficult to identify the reason for such low specific signals. Because [Lys^8(Alexa 546)]PVA displays high hydrophobicity properties (elution at 46% MeCN), we suspected a high nonspecific binding resulting from the adsorption of the peptide on the cell surface and consequently in an important nonspecific polarization signal. However this hypothesis is not valid for [Lys^8(Alexa 647)]PVA since its hydrophobicity is not that different from the one of [Lys^8(Alexa 488)]PVA. It is also noteworthy that due to their lower affinities, [Lys^8(Alexa 546)]PVA and [Lys^8(Alexa 647)]PVA were used at a higher concentration than [Lys^8(Alexa 488)]PVA. However, decreasing the concentration of [Lys^8(Alexa 546)]PVA and [Lys^8(Alexa 647)]PVA under the K_i to perform polarization experiments would result in the decrease in receptor occupancy and in a loss in the sensitivity of the method.

By contrast to the polarization assay, using [Lys^8(Alexa 647)]-PVA in the same range of concentration in the HTRF assay did not prevent the obtention of a significant FRET signal. Indeed FRET sets only if fluorophores are in a close vicinity. Nonspecific binding or unbound fluorescent ligands do not result in a FRET signal or in a signal of small amplitude which hardly affects total FRET signal intensity. This probably explains why a significant HTRF signal has been measured with [Lys^8(Alexa 647)]PVA while a specific polarization signal was not significant.

In order to validate the polarization and HTRF assays, we performed competition experiments for the V_{1a} receptor and compared the estimated inhibition constant (K_i) values to those obtained by radioactive binding assays (Table 3). No discrepancies between the K_i values have been observed for vasopressin and SR 49059: vasopressin has K_i values of 0.7 ± 0.17 nM, when determined by the radioactive binding method,[31] 0.26 ± 0.02 nM by the polarization assay, and 1.53 ± 0.35 nM by the HTRF assay. Similarly, all the values of K_i for SR 49059 are in the same range: 0.62 ± 0.12 nM by radioligand binding,[32] 0.7 ± 0.05 nM by polarization and 0.19 ± 0.14 nM by HTRF assay. By contrast, a small discrepancy in K_i values is observable for HO-LVA: K_i value determined by polarization (0.036 ± 0.005 nM) is about 7 times lower than those determined by radioligand binding (0.27 nM)[33] and HTRF (0.21 ± 0.14 nM). Although no clear explanations could be provided, it should be

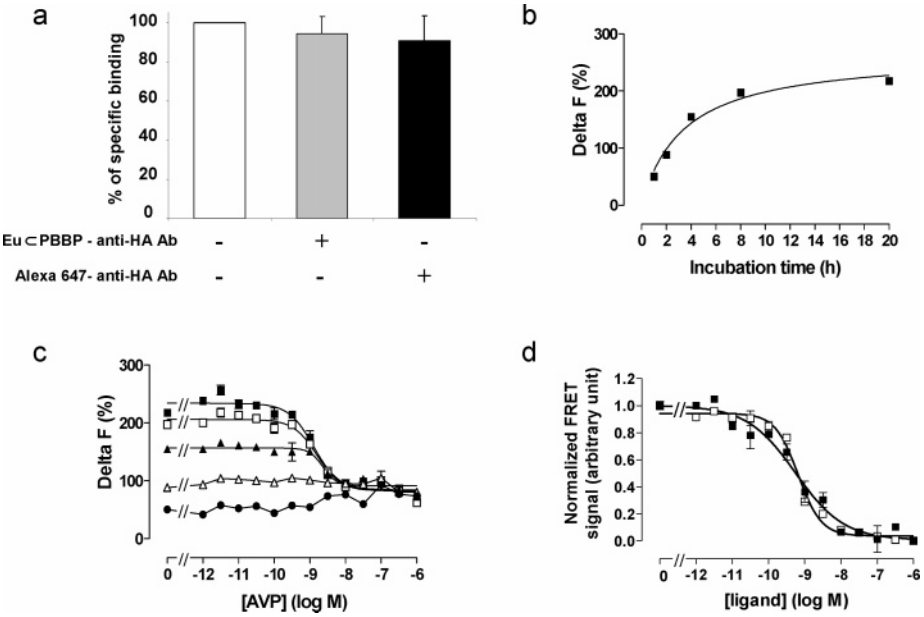

Figure 6. HTRF binding assays performed on Cos-7 cells expressing human vasopressin HA-tagged V_{1a} receptor in a 96-well plate. (a) Effect of tagged antibody on ligand binding: cells were incubated in the presence of [^{3}H]AVP (1−3 nM) and, when specified, in the presence of europium cryptate-PBBP-labeled antibody (500 pM) or Alexa 647-labeled antibody (3 nM). Nonspecific was determined by addition of an excess of vasopressin (1 μM). (b) Kinetic curve of time-resolved FRET experiments performed on cells which were incubated in the presence of the [Lys^8(Eu⊂PBBP)]-PVA (2 nM) and the Alexa 647-labeled antibody (3 nM) for 1 h, 2 h, 4, 8, and 20 h at 4 °C. (c) Competition experiments with AVP. Cells were incubated in the presence of the [Lys^8(Eu⊂PBBP)]PVA (2 nM) and Alexa 647-labeled antibody (3 nM) and increasing concentration of AVP (1 pM to 1 μM) for 1 h (●), 2 h (Δ), 4 h (▲), 8 h (□), and 20 h (■) at 4 °C. After a 20-h incubation, the inhibition constant (K_i) is 0.56 nM for AVP. (d) Competition experiments with V_{1a} antagonists. Cells were incubated with the [Lys^8(Eu⊂PBBP)]PVA (2 nM) and the Alexa 647-labeled antibody (3 nM) increasing concentration of HO-LVA ([□]) or SR49059 (▲) (1 pM to 1 μM) for 20 h at 4 °C. The K_i are 0.24 nM for HO-LVA and 0.65 nM for SR49059. The time-resolved FRET signal (DeltaF %) measured at 665 nm after excitation was calculated as described in the Experimental Section. Each curve is representative of at least three independent experiments performed in triplicate samples. Values are means ± SEM of triplicates. The solid line corresponds to the best fit obtained with the Graphpad Prism Software package when using the "sigmoidal with variable slope factor fitting" subroutine.

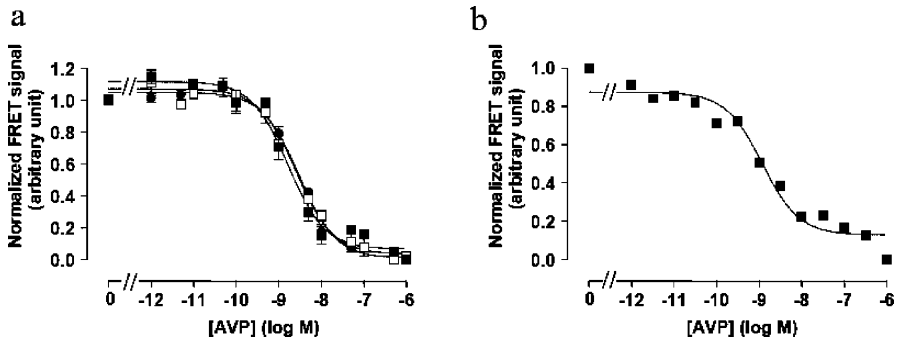

Figure 7. Fluorescent binding assays performed on Cos-7 cells expressing human HA-tagged vasopressin V_{1a} receptor. (a) Competition experiments with AVP. Cells (■, ●) or membrane preparations (□) were incubated in a 96- (■, □) or 384- (●) well plates in the presence of the [Lys^8-(Eu⊂PBBP)]PVA (2 nM) and the Alexa 647-labeled antibody (3 nM) and an increasing concentration of AVP (1 pM to 1 μM) for 20 h at 4 °C. The time-resolved FRET signal (DeltaF %) measured at 665 nm after excitation at 337 nm was calculated as described in the Experimental Section. The inhibition constants (K_i) are 0.56 nM, 1.13 nM, and 1.3 nM for AVP on cells in a 96-well plate, membranes in a 96-well plate, and cells in a 384-well plate, respectively. (b) Competition experiments with AVP. Cells were incubated in the presence of the [Lys^8(Alexa 647)]PVA (3 nM) and the europium cryptate-PBBP-labeled antibody (500 pM) and increasing concentration of AVP (1 pM to 1 μM) for 20 h at 4 °C. The DeltaF (%) measured at 665 nm after excitation at 337 nm was calculated as described in the Experimental Section. The K_i is 0.4 nM for AVP on cells in a 96-well plate. Each curve is representative of at least three independent experiments. Values are means ± SEM of triplicates. The solid line corresponds to the best fit obtained with the Graphpad Prism Soft-ware package when using the "sigmoidal with variable slope factor fitting" subroutine.

mentioned that previous studies have already reported that according to the nature of the radioligand used to perform binding experiments, variations in ligand K_i values can be observed.[15,34]

Both polarization and HTRF binding assays have been performed with the fluorescent peptides in various configurations. Indeed, they have been done in 96- and 384-well plate formats, and HTRF assays can be performed either on a membrane preparation or on intact cells transiently transfected. Since the polarization method is a single-label method, it is more sensitive to nonspecific binding. By contrast, the HTRF method is a dual-label method but nonspecific binding only weakly affects the signal/noise ratio. Moreover, compared to other FRET assays, the HTRF assays exhibit two attractive properties: first it presents an interesting signal/noise ratio due to the time-resolved measurements, and second it allows to consider only receptors targeted to the membrane and not the receptors stocked into the subcellular compartments.

However, both methods have a few drawbacks. First, although we were able to perform experiments in 384-well plate format, the sensitivities of the assays remain a little bit lower than those of radioactivity binding, particularly when radioiodinated ligands are used. The amplitudes of the specific signal are not very important. This signal can probably be improved in the case of the HTRF assay by decreasing the distance between donor and acceptor fluorophores. This requires the substitution of a smaller fluorescent molecule on the antibody.[35,36] Second, although the estimated K_i values by HTRF assays are in the same range as those determined by radioligand binding, we observed a variability in the data. However, both technologies are promising approaches since they can offer attractive advantages to perform ligand-GPCR HTS. Indeed more than for the precise determination of the K_i values of new compounds, these assays are particularly appropriate to efficiently screen and to identify new hits in a chemical bank. First, polarization and HTRF are homogeneous technologies which allow kinetic measurements. Moreover the homogeneous format is especially easier and faster to automate because of the absence of washing steps. No separation of the unbound and bound ligand fractions is necessary to read the fluorescence signal and to evaluate the pharmacological properties of the drugs tested. Second, the long signal acquisition times make the radioactive assays difficult to use in automated screening platforms while the polarization or HTRF readings take only a few minutes per plate. Third, these techniques should be carried out on small quantities (nanomolar range) of reagents used in small volumes, reducing costs. Finally, these assays are nonradioactive, therefore safer to perform.

The efficiency of these methods is linked to the ability to obtain fluorescent ligands. One can imagine that the addition of bulky fluorescent groups would have prevented the achievement of specific high-affinity fluorescent ligands, especially for the bioamine receptors. However, numerous fluorescent ligands have been synthesized for a wide variety of GPCR (for a review, see ref 37); fluorescent agonists and antagonists for the adrenergic, serotonin, and muscarinic receptors have already been described. These methods can therefore be extended to other GPCR models to perform efficient drug HTS.

Experimental Section

Fluorescent Analogue Synthesis. Synthesis of the Peptide. The peptide 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2 ([Lys^8] PVA) was synthesized as previously described.[19] The fluorescent labeling was monitored on an analytical HPLC, and purifications of the fluorescent peptides from the nonfluorescent peptides were performed by semipreparative HPLC.

Analytical HPLC. The monitoring was performed on a 4 × 250-mm C18 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).[19] 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 C18 columns:[19] 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. 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).

Peptide Labeling with Europium Pyridine-bis-bipyridine Cryptate Fluorophore. 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(Eu⊂PBBP)-NH_2·CF_3CO_2H. The lyophilized peptide 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2·2CF_3CO_2H[19] (120 nmol) was coupled with Eu⊂PBBP−NH_2 cryptate (US Patent 7,087,384)[38] using succinimidyl 3-(2-pyridyldithio)proprionate/succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate protocol.[39] The coupling was monitored by RP-HPLC, the free peptide peak (ϵ_{275} = 3 200 M^{-1} cm^{-1}) decreased, the more lipophilic fluorescent conjugate appeared (t_R = 17.5 mn, $\epsilon_{325}/\epsilon_{275}$ = 1.44), and the labeling agent in excess gave a peak ($\epsilon_{325}/\epsilon_{275}$ = 1.88) with shorter retention. After acidification (150 μL of 1% aqueous CF_3CO_2H), the labeled peptide was isolated as the trifluoroacetate salt by semipreparative RP-HPLC and lyophilization, yield 35 nmol (29%) based on UV absorbance (ϵ_{325} = 18 000 M^{-1} cm^{-1} for the cryptate moiety). The structure was checked by MALDI-TOF mass spectroscopy and found ([Lys^8-(Eu⊂PBBP)]PVA − H)$^+$ = 2331.03 calculated for $C_{107}H_{125}EuN_{24}O_{25}S$ (2330.8).

Peptide Labeling with Alexa Fluorophores. 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(Alexa 488)-NH_2·CF_3CO_2H. The lyophilized peptide 4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2·2CF_3CO_2H[19] (1.8 mg, 1.3 μmol, in excess) was dissolved in anhydrous Me_2SO (100 μL, spectroscopic grade), and we added Alexa 488 tetrafluorophenyl ester of the 5-carboxy pure isomer (1 mg sample, Molecular Probes) and *i*-Pr_2EtN (3 μL), mixed (Vortex and ultrasounds), and allowed to react for 1 h, with RP-HPLC monitoring. The peptide (eluted by 34% MeCN, $\epsilon_{214}/\epsilon_{254}$ = 26) disappeared as a new fluorescent, more hydrophilic compound (adduct) appears (eluted by 33% MeCN, $\epsilon_{214}/\epsilon_{254}$ = 1.7). After acidification (5 μL CF_3CO_2H), it was isolated as trifluoroacetate salt by semipreparative RP-HPLC and was lyophilized. The structure was checked by MALDI-TOF mass spectroscopy and calculated for $C_{75}H_{88}N_{16}O_{22}S_2$: (*m*/*z*) 1628.55. Found: $[M + H]^+$ = 1629.55.

4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-(Alexa 546)-NH_2·CF_3CO_2H. The same peptide (1.66 mg, 1.2 μmol, in excess) was added to the succinimidyl ester of Alexa 546 (1 mg, 0.9 μmol, Molecular Probes), freshly dissolved in anhydrous Me_2SO (100 μL) and *i*-Pr_2EtN (5 μL), and mixed quickly (Vortex, ultrasounds). A new, more lipophilic compound appeared (46% MeCN, $\epsilon_{214}/\epsilon_{254}$ = 1.4). The mixture was acidified (6 μL CF_3CO_2H), and the fluorescent peptide was isolated by semipreparative RP-HPLC and was lyophilized. The structure was checked by MALDI-TOF mass spectroscopy and calculated for $C_{94}H_{118}Cl_3N_{17}O_{23}S_3$: (*m*/*z*) 2053.68. Found: $[M + H]^+$ = 2054.68.

4-HOPh$(CH_2)_2$CO-DTyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(Alexa 647)-NH_2·CF_3CO_2H. The lyophilized peptide (1.4 mg, 1.04 μmol, in excess) was added to Alexa 647 succinimidyl ester (1 mg, 0.8 μmol, MW ≈ 1250 env) dissolved in Me_2SO (100 μL, UV grade, anhydrous) and *i*-Pr_2EtN (4 μL) and mixed (Vortex, ultrasound). After 1 h, analytical RP-HPLC showed residual peptide (eluted by 34% MeCN, $\epsilon_{214}/\epsilon_{254}$ = 30) and a new, more hydrophilic compound (30% MeCN, $\epsilon_{214}/\epsilon_{254}$ = 4.3). The compound was isolated by semipreparative RP-HPLC, monitored at 214 and 647 nm, and was lyophilized and characterized ($[M + H]^+$ = 1954.11 found), This value cannot be compared to the theoretical mass since the structure of Alexa 647 succinimidyl ester was not revealed by Molecular Probes.

Cell Culture. The CHO cell lines, which stably express the human vasopressin V_{1a}, V_{1b}, V_2 or oxytocin receptors, and Cos-7 cell lines were maintained in culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units/mL penicillin and streptomycin in an atmosphere of 95% air and 5% CO_2 at 37 °C. The expression levels of CHO expressing either the V_{1a} or V_{1b} receptors are in the range of 0.7 and 1 pmol/mg protein depending on the confluence of the cell and the number of

passage. Regarding the V_2 and oxytocin receptors, the expression levels were in the range of 2 to 8 pmol/mg protein and 0.3−0.5 pmol/mg, respectively.

The Cos-7 cells were transiently transfected by electroporation as previously described[40] with 1 μg of vector coding for HA-tagged human V_{1a} receptor and empty vector to a final amount of 10 μg. Under this condition, the expression level is in the range of 0.5 to 2 pmol/mg protein.

Membrane Preparations. Culture dishes of Cos-7 or CHO cells expressing the human vasopressin V_{1a}, V_{1b}, V_2 or oxytocin receptors were washed twice in PBS without calcium and magnesium, and cold lysis buffer (15 mM Tris:HCl, 2 mM $MgCl_2$, 0.3 mM EDTA, pH 7.4) was added. Cells were scraped with a rubber, homogenized, and centrifuged at 100*g* for 5 min at 4 °C. Supernatants were recovered and centrifuged at 44 000*g* for 30 min at 4 °C. Pellets were resuspended in a suspension medium (50 mM Tris:HCl, 5 mM $MgCl_2$, pH 7.4) centrifuged at 44 000*g* for 30 min at 4 °C. Pellets were resuspended in an appropriate volume of the same buffer. For each membrane preparation, protein content was evaluated, and membranes were then aliquoted and frozen in liquid nitrogen.

Radioligand Binding Assays. Competition experiments were performed on membranes of CHO cells expressing human vasopressin V_{1a}, V_{1b}, V_2 or oxytocin receptors, as previously described.[32] Briefly, membranes were incubated for 1 h at 30 °C with [^{3}H]-AVP (1−2 nM) and with increasing concentrations of nonradioactive ligands ranging from 1 pM to 1 μM. Nonspecific binding was determined with an excess of AVP (1 μM). Bound tritiated vasopressin ([^{3}H]AVP) fractions were separated from the free tritiated vasopressin by filtration. We used Whatman GF-C filters preincubated in bovine serum albumin (10 mg/mL). Filtration was performed on a Brandel apparatus. Radioactivity on the filters was counted on a beta-counter Tri-carb 2100TR (Perkin-Elmer Life and Analytical Sciences).

Inositol Phosphate Assays. The accumulation of inositol phosphates was determined by IP-One ELISA test kindly provided by CIS bio international or by measurement of [^{3}H]inositol phosphate production. The immunoassay IP-One ELISA is based on the competition between free IP1 (*myo*-inositol 1-phosphate) and IP1-horse radish peroxidase (IP1-HRP) conjugate (for more information on the principle, see http://www.htrf.com/products/gpcr/iponeelisa/). Briefly, CHO cells expressing the human vasopressin V_{1a} receptor were seeded at a density of 8×10^5 cells/well in 96-well culture plates for 24 h (37 °C + 5% CO_2) in Dulbecco's modified Eagle's medium. Cells were preincubated with various concentrations of fluorescent ligands (1 pM to 1 μM) for 15 min and then stimulated for 30 min with 2 nM of AVP and lysed for 30 min. After transfer of the supernatant into the ELISA-coated plates, plates were incubated for 3 h at room temperature under shaking with IP1-HRP conjugate and anti-IP1 Mab. After a washing step, the addition of HRP substrate (3,3′,5,5′-tetramethylbenzidine) for 30 min at room temperature in a dark environment revealed enzymatic activity. After addition of the "stop solution", the optical density was read at 450 nm with correction for optical imperfections in the plate between 610 and 650 nm on a Dynatech MR5000 apparatus.

The inositol phosphate accumulation experiments on CHO-expressing oxytocin receptors were performed as previously described by Goudet and collaborators.[41] Briefly, experiments were performed in 96-well plates (80 000 cells/well) after overnight labeling with ^{3}H-*myo*-inositol (0.5μCi/well). Cells were preincubated with various concentrations of fluorescent ligands (1 pM to 1 μM) for 15 min and then stimulated for 30 min with 50 nM OT in a medium containing 10 mM LiCl. The reaction was stopped by replacing the medium with 0.1 M formic acid. Supernatants were recovered, and inositol phosphates were purified on 96-well plates by ion exchange chromatography. Radioactivity was measured on a Wallac 1450 Microbeta microplate liquid scintillation counter (Perkin-Elmer life Analytical Sciences). Data were expressed as the amount of total inositol phosphates produced over the amount of radioactivity remaining in the membranes plus the produced inositol phosphates.

Polarization Binding Assays. Unless otherwise stated, assays were performed in black 96-well plates (Costar) in a total volume of 200 μL. Membranes (10−15 μg/assay) of CHO cells expressing human vasopressin V_{1a} receptor were incubated for 2 h at room temperature with [Lys^8(Alexa 488)]PVA (0.5 nM) or [Lys^8(Alexa 546)]PVA (2 nM) or [Lys^8(Alexa 647)]PVA (3 nM) and with increasing concentrations (1 pM to 1 μM) of nonfluorescent competing ligands. Competition experiments in a 384-well plate (Costar) were performed in a final volume of 50 μL with membranes at a concentration of 3−5 μg protein /assay. The polarization signal was measured on an Analyst apparatus (Molecular Devices). Appropriate excitation and emission filters were selected depending on the fluorescent ligand used: the excitation and emission wavelengths were respectively 485 and 530 nm for Alexa 488, 535 and 580 nm for Alexa 546, and 620 and 670 nm for Alexa 647.

The polarization signal was defined as follows : $P = (I_{||} - I_{\perp})/(I_{||} + I_{\perp})$ where P is the polarization fluorescence intensity, $I_{||}$, the parallel component of the emitted light, and $I_{\perp}$, the perpendicular component of the emitted light. For convenience, results are expressed in mP (1000 mP = 1 P).

Fluorescent Binding Assays. HTRF experiments were performed as described previously.[17] These experiments involve the transfer of energy from europium pyridine-bis-bipyridine cryptate (Eu⊂PBBP) as donor fluorophore to Alexa 647 as acceptor fluorophore. We used the ligand labeled with Eu⊂PBBP and the monoclonal anti-HA antibody (12CA5) labeled with Alexa 647 kindly provided by CIS bio international research group. After transfection, 100 000 Cos-7 cells expressing human vasopressin HA-tagged V_{1a} receptor per well were dispatched into a black 96-well assay plate (Costar) or used to prepare membranes as described above.

Competition experiments were performed in 100 μL of the following medium: Tris/Krebs buffer (20 mM Tris-HCl, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$, pH 7.4) supplemented with 0.1% bovine serum albumin. Cells or membranes (25 μg/assay) were incubated with 2 nM [Lys^8(Eu⊂PBBP]PVA, 3 nM Alexa 647-labeled anti-HA antibody (or 0.5 nM Eu⊂PBBP-labeled anti-HA antibody, 3 nM [Lys^8(Alexa 647)]PVA), and increasing concentrations (1 pM to 1 μM) of unlabeled ligand (AVP, HO-LVA, or SR49059). As a negative control, Cos-7 cells or membranes were incubated only with the donor fluorophore-labeled antibody. Competition experiments in a 384-well plate were performed with 50 000 cells per well in a final volume of 50 μL.

After an incubation of 20 h at 4 °C, preparations were excited at 337 nm (excitation of Eu⊂PBBP) and fluorescence emissions were measured on a RubyStar fluorometer (BMG Labtechnologies) at 620 nm and at 665 nm, wavelengths which correspond to the total europium cryptate emission and to the FRET signal, respectively. A 400 μs reading was measured after a 50 μs delay to remove the short-lived fluorescence background from the signal. The specific signal was calculated using the following equation : DeltaF $= (R - R_{neg})/(R_{neg})$ where R is the ratio (fluorescence 665 nm/fluorescence 620 nm) calculated for each assay and R_{neg} is the same ratio for the negative control.

Data Analysis. All binding data were analyzed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The K_i values for unlabeled analogues were calculated from binding competition experiments according to the Cheng and Prusoff equation:[42] $K_i = IC_{50} \times (K_d/K_d + [L])$, where IC_{50} is the concentration of unlabeled analogue leading to half-maximal inhibition of specific binding, K_d is its affinity for the receptor studied, and [L] is the concentration of the radioligand present in the assay. It should be mentioned that for the polarization and HTRF experiments, the K_d values of the fluorescent tracers are assumed to be equal to the K_i values determined by competition binding experiments with radioactive tracer since polarization or HTRF assays did not allow the precise determination of the tracer K_d values. In all polarization and HTRF

experiments, the tracer is in excess over receptors species. Concentrations of analogue leading to half-maximal inhibition (IC_{50}) of IP accumulation were calculated from functional studies using GraphPad PRISM. The apparent inactivation constants (K_{inact}) 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] = 2 nM, and K_{act} is the concentration of AVP inducing half-maximal stimulation of total inositol phosphate accumulation ($K_{act} = 0.32$ nM). Results are expressed as the mean ± SEM of at least three independent experiments.

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