Fluorescent Agonists and Antagonists for Vasopressin/Oxytocin G Protein-Coupled Receptors: Usefulness in Ligand Screening Assays and Receptor Studies

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Abstract : Different series of fluorescent agonists and antagonists have been developed and characterized for arginine-vasopressin and oxytocin G protein-coupled receptors. Both cyclic and linear peptide analogs of the neurohypophysial hormones are useful tools for investigating receptor localization and trafficking, analysing receptor structural organization, and developing new receptor-selective high-throughput ligand screening assays.

Key Words: Arginine-vasopressin, oxytocin, G protein-coupled receptor, structure-activity relationships, fluorescent peptide, fluorescence resonance energy transfer, fluorescence polarization, high-throughput screening assays.

INTRODUCTION

G protein-coupled receptors (GPCRs) constitute the largest family of integral membrane proteins, participate in the regulation of most physiological functions and are the targets of approximately 50% of currently marketed drugs [1, 2]. Investigating receptor localization and trafficking, receptor molecular structural analysis and developing new receptor-selective high-throughput ligand screening assays, are presently crucial tasks. Because of the increased sensitivity of the fluorescence readers and of the brightness of the fluorophores, most of these studies can be performed using fluorescence-based technologies. Fluorescent ligands selective for a given receptor family or a given receptor subtype are consequently very useful labeling and pharmacological tools [3].

Arginine-vasopressin (AVP) and oxytocin (OT) receptors are typical members of the class A GPCRs (rhodopsin-like) and are considered as prototypes of GPCRs for which small peptides and hormones are endogenous ligands [4, 5]. The two neurohypophyseal hormones are disulfide bridge-containing cyclic nonapeptides (CYFQNCPRG-NH_2 and CY-IQNCPLG-NH_2, respectively) which regulate well known peripheral physiological functions [6, 7]. For instance, AVP participates in the regulation of blood pressure acting on the vessel V1a receptor subtype, controls the kidney water reabsorption through binding and activating V2 receptor subtype. AVP also plays a crucial role in the adaptative response to stress, anxiety or emotional situations by regulating corticotropin secretion from the anterior pituitary when interacting with its third receptor, the V1b subtype. OT is a major hormone in most reproductive functions, such as labor/delivery by inducing the uterine contractions, and lactation by triggering milk ejection. All the OT activities are mediated by a unique OT receptor (OTR). The AVP/OT receptors have been cloned in many different mammal species [8-11]. More recently, the central effects of AVP/OT hormones have been described. Indeed, the two peptides are neuromediators released in many different areas of the central nervous system (CNS), and modulate many aspects of mammalian behavior [12]. They act mainly on social behavior and precisely control social recognition, pair bonding, maternal care, social education, sexual behavior, trust. These actions were confirmed through the development of knockout mice for OT, OTR, V1a or V1b, and raise the possibility that these receptors may have important implications for developmental disorders characterized by deficits in social behavior like autism [13].

Since the original chemical synthesis of the cyclic nonapeptides AVP and OT [14, 15], these two hormones have been the focus of intensive structure-activity, conformation-activity and analog design studies for decades. Hundreds of peptide or non peptide agonists and antagonists of the AVP/OT receptors, several being of potential clinical value, have been synthesized and characterized, and presently are very useful pharmacological tools [16]. Paradoxically, only a few fluorescent analogs of AVP and OT have been developed. Nevertheless, as indicated above, such ligands have many potential applications. This review is mainly focused on the recent progress in the design of selective high-affinity fluorescently-labeled peptide agonists and antagonists for AVP/OT receptors and their usefulness in studying receptor cellular distribution and receptor molecular structural organization at the cell membrane, or in developing new nonradioactive high-throughput drug screening procedures. For an understanding reading, we chose to follow a chronological description of the different generations of fluorescent analogs of AVP/OT hormones, clearly describing the improvement either in ligand design or fluorophore properties. In addition, agonist ligands are illustrated in Fig. (**1**) and Fig.

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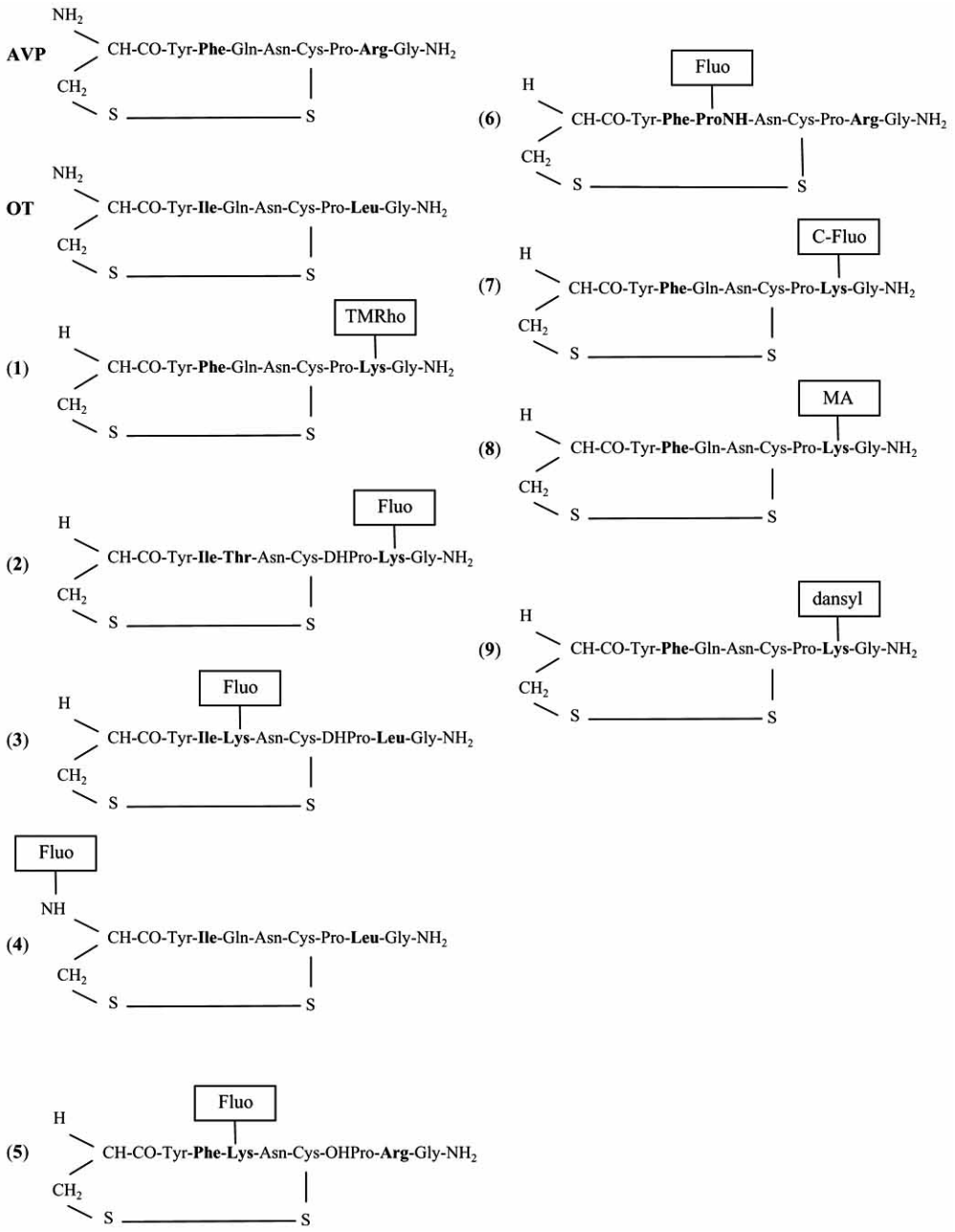

Fig. (1). fluorescent agonist analogs of AVP/OT.

(**2**), whereas cyclic and linear antagonist analogs are depicted in Fig. (**3**) and Fig. (**4**), respectively.

1. FIRST GENERATIONS OF FLUORESCENT PROBES FOR AVP AND OT RECEPTORS

1.1. Agonist Ligands

A desaminated analog of the porcine hormone lysine-vasopressin (LVP), namely d[Lys^8]VP (dLVP), was used as a chemical matrix to develop the first fluorescent probe of the AVP/OT family more than twenty years ago [17]. Indeed, a rhodamyl derivative of dLVP (see Fig. (**1**)), d[Lys^8(tetramethylrhodamyl)]VP (**1**), for which the coupling of the fluorophore can only be limited to the ε-amine group of lysine at position 8 (the NH_2 terminal has been replaced by a hydrogen atom at position 1), allowed to detect the V2 receptor in the principal cells of rabbit collecting ducts [18]. This study directly demonstrated that the principal cells constitute the primary target cell for vasopressin (VP) in the kidney. This analogue of VP, whose binding properties in terms of affinity, kinetics and internalization, resemble those of the natural hormone in all respects, was then used to directly measure the lateral mobility of the renal-type V2 receptor in the basal plasma membrane of the LLC-PK1 porcine epithelial cells by microphotolysis (photobleaching) [19]. The V2 receptor was shown to be highly mobile at physiological temperature. This result reported the first measurement of the lateral mobility in membranes of a polypeptide hormone receptor that is coupled to adenylyl cyclase. As shown in Fig. (**1**), analogs of OT having a fluoresceinyl

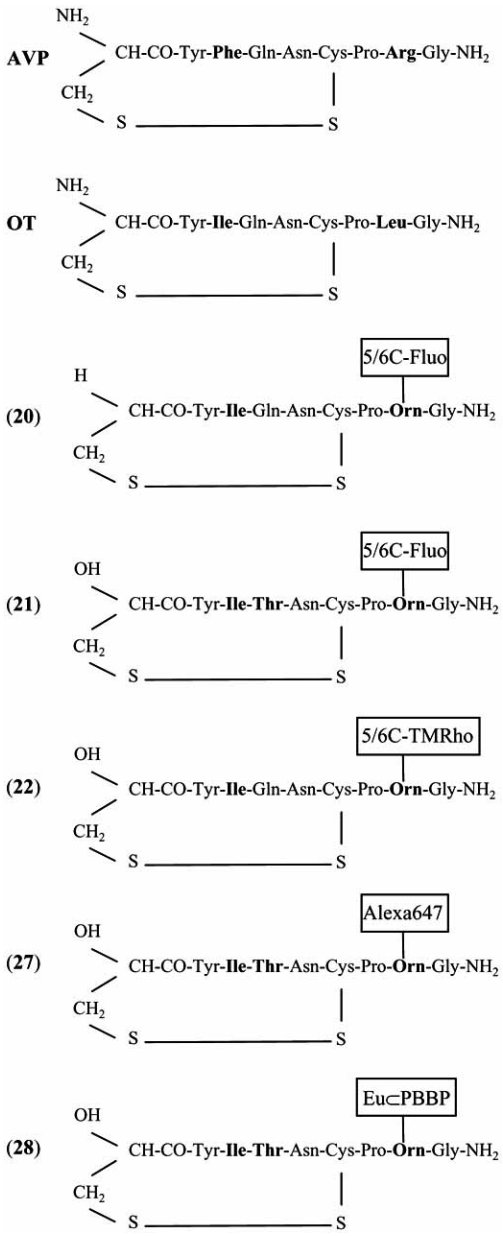

Fig. (2). fluorescent agonist analogs of AVP/OT.

group attached to the ε-amine group of a lysine at position 4, d[Lys^4(fluoresceinyl), dihydro-Pro^7]OT (**2**), or 8, d[Thr^4, dihydroPro^7, Lys^8(fluoresceinyl)]OT (**3**), or at position 1 in the case of OT itself, [Cys1(fluoresceinyl)]OT (**4**), were also developed [20]. Those with fluorescein at position 8 retained good uterine and milk ejection activities (only a 4-fold decrease in activity compared to OT) and represented potentially useful molecular tools for studying receptor localization, whereas those having the fluorophore at positon 1 or 4 gave very low or moderate activities, respectively. Fluorescent analogs of AVP derivatized with a fluoresceinyl (illustrated in Fig. (**1**)) in either position 4 (d[Lys^4(fluoresceinyl), OHPro^7]AVP) **(5)** or 7 (d[Pro^7(NH-fluoresceinyl)]AVP) (**6**), with or without a spacer arm inserted between the lysine in position 4, were demonstrated to keep good antidiuretic activity (a 15-fold decrease if compared to AVP) and were proposed to be valuable probes in studying receptor visualization [21]. The synthesis and biological activity of novel fluorescent VP analogs was published in 1990 [22]. All these analogs were prepared by coupling again the appropriate fluorochrome to the ε-amine group of the lysine residue in dLVP (see Fig. (**1**)). The d[Lys^8(carboxyfluorescein)]VP (**7**) and the d[Lys^8(methylanthranilamide)]VP (**8**) were demonstrated to retain high affinity for the V2 receptor present in canine renal plasma membranes (32 and 8.8 nM whereas that of AVP was around 1 nM) and whole LLC-PK1 cells (390 and 38 nM compared to 9 nM for AVP), and able to stimulate adenylyl cyclase activity as efficiently as AVP. On the other hand, the dansyl derivative of dLVP (d[Lys^8(dimethylaminonaphtalene-1-sulfonyl)]VP (**9**), was unable to bind the receptor and activate cyclic adenosine monophosphate (cAMP) production. The first three ligands were defined as useful for the study of renal AVP receptors by fluorescence microscopy, particularly the fluorescein and rhodamine-labeled agonists. Whereas fluorescein exhibits low photostability, which restricts its use for imaging events occurring over long time periods, the rhodamine fluorophore is ideal in that it possesses a high molar extinction coefficient and does not photobleach readily. Taking into account these studies, designing of a fluorescent analog of AVP or OT retaining all agonistic properties of the natural hormones, was obviously much more successful when introducing a fluorophore at the side chain of the residue 8 of the peptide.

1.2. Cyclic and Linear Antagonist Peptides

The primary fluorescent antagonists of AVP/OT receptors were described in 1992 but were also nonselective. First, following the discovery that requirement of cyclic conformation of peptide antagonists in binding to AVP receptor is not necessary [23], the classical fluorophore fluorescein was added to the N-terminus of a linear antagonist having a high affinity for both the V1a and V2 receptor subtypes [24]. The resulting peptide N-α-fluoresceinyl-thiocarbamoyl-D-Tyr(Et)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2 (**10**), shown in Fig. (**4**), was unable to stimulate a phospholipase C activity from WRK1 cells measured by the accumulation of inositol phosphates (IP), and consequently exhibited pure antagonistic properties. However, this ligand was not useful at all for labeling cells and visualizing AVP receptors because it displayed a very weak affinity compared to that of its parent peptide (a 1000-fold decrease). Following the description of the well-established V1a selective antagonist, d$(CH_2)_5$[Tyr(Me)^2]AVP [25], and of some of its V1a/OTR nonselective analogues, such as d$(CH_2)_5$[D-Tyr(Et)^2, Val^4, Lys^8]VP [26], the fluorescent cyclic peptide d$(CH_2)_5$[D-Tyr(Et)^2, Val^4, Lys^8(carboxytetramethylrhodamine)]VP (**11**) was synthesized (see Fig. (**3**)) and characterized [22]. It was shown to bind nonselectively the V2 receptor from LLC-PK1 and the V1a receptor from A-10 cells (79 and 32 nM respectively). This ligand failed to increase cAMP accumulation in LLC-PK1 cells or intracellular calcium concentration in A-10 cells. In contrast to the prototype fluorescent agonist d[Lys^8(tetramethylrhodamyl)]VP, this antagonist did not undergo receptor endocytosis in either cell type. The authors concluded that AVP receptor occupancy in the absence of receptor activation is insufficient to elicit receptor-mediated internalization. Later, interestingly, it was shown that linear pep-

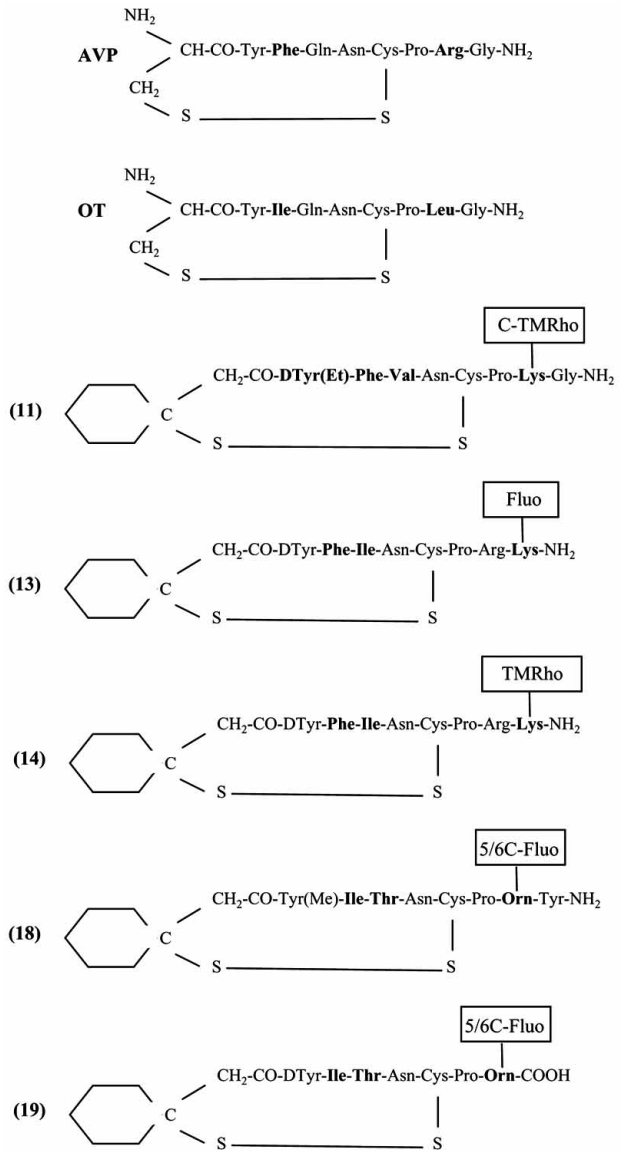

Fig. (3). fluorescent cyclic antagonist analogs of AVP/OT.

tide antagonists can accommodate a lysine(NH_2) residue at position 9 and retain very high affinity and selectivity for the V1a receptor subtype *versus* V2 [27]. Aminomethyl coumarin (AMCou) was coupled onto the ε amino group of this lysine[9] residue through its N-hydroxysuccinimide ester to give the [phenylacetyl[1], D-Tyr(Me)[2], Arg[6], Arg[8], Lys[9](Nε-7-amino-4-methylcoumarin-3-acetamide)NH_2]VP (**12**), illustrated in Fig. (**4**) [28]. The AMCou fluorophore was chosen as it exhibits bright blue fluorescence at neutral pH, is maximally excited at near ultraviolet wavelengths and is relatively resistant to photobleaching. All these factors are important to distinguish AMCou fluorescence from background cell autofluorescence. The AMCou-labeled ligand behaved as the first selective fluorescent antagonist to the V1a receptor (affinity was 0.44 nM), was unable to produce IP accumulation, and demonstrated to be useful for labelling WRK-1 cells expressing the V1a receptor. Finally, two new fluorescent cyclic antagonists having the fluorophore attached to a lysine[9] residue were synthesized and characterized, confirming that position 9 in these peptides can accept derivatization with more or less bulky chemical groups, as published for linear peptide antagonists [29]. The lateral mobility of the adenylyl cyclase-coupled V2 AVP receptor from LLC-PK1 cells was studied using fluorescence photobleaching with these two fluorescent peptides (depicted in Fig. (**3**)), $d(CH_2)_5$ [D-Tyr[2], Ile[4], Lys[9](N^6-fluoresceinylaminothiocarbonyl)]AVP (**13**) and $d(CH_2)_5$[D-Tyr[2], Ile[4], Lys[9](N^6- tetramethylrhodamylaminothiocarbonyl)]AVP (**14**), and compared to that measured with the agonist d[Lys[8](tetramethylrhodamyl)]VP (**1**). The results reported that antagonistic properties of the V2 fluorescent ligands were not correlated to a decreased receptor lateral mobility. Altogether, these studies with fluorescent antagonists demonstrated that derivatization of linear and cyclic peptides both at position 8 and 9 with different fluorophores allows characterization of high-affinity ligands for V1a and V2 receptors.

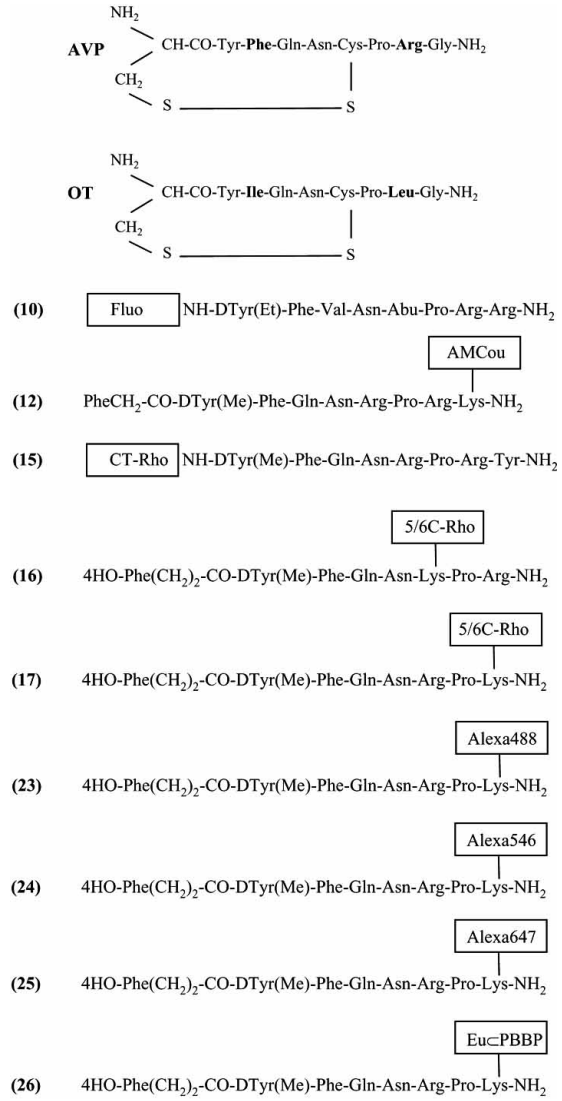

Fig. (4). fluorescent linear antagonist analogs of AVP/OT.

The synthesis and characterization of first generations of fluorescent agonists and antagonists for AVP/OT receptors led to the discovery of very promising pharmacological tools but their use was limited to fluorescence microscopy techniques applied to the investigation of the cellular expression

and localization of receptors. Indeed, the sensitivity of the fluorophores attached to these ligands was far from being equivalent to that of usual radioisotopes (^{3}H, ^{125}I), and ligand binding assays could not generally be developed.

2. NOVEL GENERATIONS OF FLUORESCENT ANALOGS FOR AVP AND OT RECEPTORS

Since 1992, the different AVP/OT receptor genes or cDNAs were all cloned from different mammals, lower vertebrate and invertebrate species [8-11]. Molecular cloning of this receptor family has confirmed that AVP/OT receptors are typical GRPCs consisting of seven hydrophobic transmembrane α-helices with an extracellular N-terminus and a cytoplasmic C-terminus. The knowledge of their nucleotide sequence and consequently of their primary structure (the amino acid residue sequence) constituted a starting point to receptor structure-function relationships analysis. We and others have undertaken many studies that were dedicated to the identification of the hormone binding sites at a molecular level [5, 30-35]. Extensive receptor mutational analysis combined with receptor three-dimensional molecular modelling or by direct receptor covalent photolabeling have led to very valuable information concerning peptide agonist and peptide and nonpeptide antagonist binding domains of the AVP/OT receptor family [36-39]. Indeed, the AVP/OT receptor binding pocket is buried into a 15-20 Å deep central cavity defined by the transmembrane helices and surrounded by the extracellular loops [40]. The hydrophobic part of the ligands dives deeply into the binding cavity for interacting with hydrophobic residue clusters, whereas the more hydrophilic part of the peptides bind to the transmembrane edge. Only the side-chain of residue 8 of the peptides is pointing towards the extracellular loops of the receptors and is potentially less constrained than all other parts of the ligands [32, 33, 41]. Thus it is not surprising *a posteriori* that derivatization of AVP/OT analogues with bulky fluorophores like fluorescein or rhodamine at this particular position (amino acid residue 8) led to the successful development of numerous fluorescent ligands retaining high affinity, selectivity, and functional activity for these receptors. This residue is finally not crucial for binding, although it has been demonstrated to be involved in receptor subtype binding selectivity [32, 33].

2.1. Linear Peptide Antagonists with Fluoresceinyl and Tetramethylrhodamyl Fluorochromes

While analysing the structure/function relationships of AVP/OT receptors and particularly identifying the ligand binding sites of these receptors, we also decided to develop new fluorescent ligands, and we first added fluorophores to linear VP antagonists which possess very high affinities for the V1a receptor subtype and are much more stable than disulfide-bridged molecules [42]. The hydrophilic compounds LVA, $PhCH_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2, and HO-LVA, 4-$HOPhCH_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2, were chosen as the parent peptides in order to counterbalance the hydrophobicity introduced by tetramethylrhodamyl and fluoresceinyl groups. The coupling of tetramethylrhodamyl at position 1 to give 1-tetramethylrhodamyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**15**) was first realized (see Fig. (**4**)), but the ligand displayed very weak affinity (K_i > 500 nM). This result was equivalent to the one obtained a few years earlier for the first linear peptide antagonist to be labelled with a fluorophore at position 1, N-α-fluoresceinyl-thiocarbamoyl-D-Tyr(OEt)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2 (**10**). It confirmed that position 1 is not suitable for introducing bulky hydrophobic groups such as fluoresceinyl and tetramethylrhodamyl fluorophores. More interestingly, the substitution of Lys for Arg at position 6 (4-$HOPh(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Lys(5/6C-tetramethyrhodamyl)-Pro-Arg-NH_2 (**16**) or at position 8 (4-$HOPh(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5/6C-tetramethyrhodamyl)-NH_2 (**17**) allowed the coupling of fluorophores through an amide link instead of the thiocarbamoyl link, being more stable and giving higher quantum yields (the peptides **16** and **17** are illustrated in Fig. (**4**)). The closer to the C-terminus the fluorophore is, the higher the affinities of the fluorescent derivatives for the human AVP V1a receptor are, confirming that position 8 is ideal for derivatization (these ligands displayed a 0.1-1 nM affinity range for the V1a subtype). The position of the link between the fluorophore and the peptide (5C- or 6C- for both carboxyfluoresceinyl and carboxytetramethylrhodamyl) only slightly affected the affinity of the ligands. These different fluorescent ligands retained full antagonistic activity, were very selective for V1a versus V2 or V1b receptor subtypes. Interestingly, they also displayed a high affinity for the OTR subtype. For instance, the K_i for 4-$HOPh(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5C-carboxytetramethylrhodamyl)-NH_2 ([Lys^8(tetramethylrhodamyl)]PVA) to V1a and OTR were 0.07 and 1.2 nM, respectively, making this ligand useful for two different receptors [42]. The 4-$HOPh(CH_2)_2CO$-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5C-carboxyfluoresceinyl)-NH_2 ([Lys^8(fluoresceinyl)]PVA) displayed equivalent properties. With such an affinity, these fluorescent peptides are the highest affinity fluorescent V1a receptor ligands reported to date. Because of this property, these ligands are well adapted to fluorescent techniques. They were demonstrated to be efficient for labelling AVP receptors in epifluorescence and could easily be used for flow cytofluorometric studies due to a low concentration needed to label cells because of a very high affinity. Indeed, the ([Lys^8(fluoresceinyl)]PVA analog made possible the visualization of cell surface AVP V1a receptors in rat hepatocytes [43, 44]. A high affinity is also crucial for cellular and subcellular analysis and colocalization with other markers in fluorescence microscopy. Fluorescence recovery after photobleaching could also be used to study the diffusion of fluorescent probes in a tissue or in one cell. Fluorescence correlation spectroscopy which allows the detection of interactions between one receptor molecule and one ligand molecule is also a very interesting application. Structural information for ligand-receptor interactions could also be obtained by fluorescence quenching, polarization, or FRET (fluorescence resonance energy-transfer) experiments using these compounds.

2.2. Cyclic Peptide Agonists and Antagonists with Classical Fluoresceinyl and Tetramethylrhodamyl Fluorochromes

We next reported the design and synthesis of new fluorescent OT antagonists and agonists and their pharmacological properties for the AVP/OT receptors. Again, fluoresceinyl

and tetramethylrhodamyl groups were coupled to parent peptides through an amide bond to the side chain amino group at position 8 [45]. Two antagonists, d(CH_2)$_5$[Tyr^2(Me), Thr^4, Orn^8(5/6carboxyfluoresceinyl), Tyr^9-NH_2]VT (**18**) and des-Gly-NH_2^9, d(CH_2)$_5$[D-Tyr^2, Thr^4, Orn^8(5/6carboxyfluoresceinyl)]VT (**19**) were obtained (shown in Fig. (**3**)), the first one only having a good affinity for the OTR (K_i was around 6 nM). More interestingly, it was selective for this receptor. These properties rendered the ligand very useful for specifically labelling cell surface OTR in tissues, freshly dissociated cells or in primary cell cultures. All other ligands of the series were fluorescent agonists deriving from OT agonists lacking the α-NH_2 group at position 1 and for which the leucine residue at position 8 was replaced with an ornithine or a lysine for attachment of the fluorophore [45]. Nine different fluorescent agonists exhibited very good affinities for the OTR with K_i values in the 0.5 nM range. One is very selective to the OTR, d[Orn^8(5/6C-Fluoresceinyl)]VT (**20**) while two other peptides, [HO^1][Thr^4, Orn^8(5/6C-fluoresceinyl)]VT (**21**) and [HO^1][Orn^8(5/6C-tetramethylrhodamyl]VT (**22**), can be used to label both OTR and the V1a subtype and are selective *versus* V1b and V2 (the three peptides are shown in Fig. (**2**)). Fluorescence microscopy labelling of CHO and HEK cells stably or transiently expressing the human OTR, respectively, was realized in order to study the distribution of the receptor and its internalization process. Incubation of the two cell lines with d[Orn^8(5/6C-Fluoresceinyl]VT for 1 h at 37°C revealed a specific punctate labeling. Because tetramethylrhodamine is less sensitive to photobleaching than fluorescein, the peptide [HO^1][Orn^8(5/6C-tetramethylrhodamyl]VT was next used to label the different cells. An equivalent labeling was observed for 1 h incubation. Because a punctate labeling observable at 37°C and not at 0°C suggested an endocytosis of the ligand-receptor complex, a confocal analysis was undertaken for various periods of time. Small fluorescent endosome-like particles were seen widespread into the cytoplasm when cells were incubated at 37°C but not at 0°C. These endosome-like particles were not concentrated in the perinuclear region, even for longer incubation periods (3 h), in agreement with a recycling pathway. Interestingly, the punctate labelling observed was equivalent to what has already been described for the V1a receptor, but suggested that the internalization process of the OTR is different from that of the V2 receptor [46]. Indeed, it has been demonstrated that the V2 receptor is addressed to a perinuclear region of LLC-PK1 cells labelled with the classical fluorescent agonist d[Lys^8(tetramethylrhodamyl)]VP [47]. [HO^1][Orn^8(5/6C-tetramethylrhodamyl]VT would be a very useful ligand to study desensitization and internalization of the ligand-OTR complex in OTR-expressing myocytes of human myometrum during parturition. These fluorescent ligands could also be used to perform fluorescence recovery after photobleaching to study the diffusion of molecules in a tissue or in a single cell or to obtain structural information for ligand-receptor interactions by fluorescence quenching, polarization or FRET.

2.3. Linear Peptide Antagonists with Novel Alexa and Europium Cryptate Fluorochromes

As indicated in the introduction, GPCRs represent the single largest molecular target of therapeutic drugs currently on the market, but are also the most common target in high-throughput screening (HTS) assays for identifying potential new drug candidates. Most of these HTS procedures are still presently based on radioligand binding competition assays. Fluorescence approaches, based on the development of fluorescent ligands, offer a nonradioactive alternative to radioligand binding assays. Fluorescence polarization and FRET (such as homogenous-time resolved fluorescence (HTRF)) techniques are even more useful since they can be achieved without the separation of the bound and free ligand fractions, leading to the development of homogenous microplate format assays with cell membrane preparation or whole cell samples. The development of these techniques has stemmed first from the elaboration of numerous sensitive microplate readers, and second from the synthesis of new fluorescent probes. Among the new fluorophores, Alexa series exhibit greater photostability and enhanced fluorescence emission than those of the classical fluoroprobes such as fluorescein and rhodamine. These properties increase the sensitivity of the assays, facilitating the development of fluorescence-based techniques. Other fluoroprobes, such as lanthanide complexes, have also been developed because of their long fluorescence lifetime which allows specific signal to be separated from non-specific signal. This makes these fluorophores convenient for homogenous time-resolved fluorescence (HTRF) approaches. Altogether, these different parameters make possible fluorescence-based HTS assays.

The previous series of fluorescein- or rhodamine-conjugated peptides developed for AVP/OT receptors were generally not useful for HTS assays, because of a low quantum yield and a low photostability of their fluorophores. It is interesting to note that a HTS fluorescence polarization assay was developed using a fluoresceinyl analog of VP and membrane preparations of cells transfected with the human V1a receptor [48]. Although the competition binding assay was successful, this agonist fluoresceinyl-VP is not useful for other fluorescence applications. A novel series of fluorescent ligands labelled with europium pyridine-bis-bipyridine cryptate (Eu⊂PBBP) or with Alexa 488, 546 or 647 were consequently designed for AVP/OT receptors, synthesized and characterized to develop fluorescence polarization or HTRF binding assays [49]. As described previously, we chose to anchor the new fluorophores onto the linear peptide antagonist 4-HOPh(CH_2)$_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2. This peptide has been demonstrated to display a very high affinity for AVP V1a receptor and for the OTR and the presence of the lysine at position 8 permits addition of bulky fluorescent groups without loosing the high affinity, in agreement with all previous receptor modelling and mutagenesis studies. In addition, the new fluorophores were linked to the side-chain of this lysine using a short spacer, avoiding excessive rotation of the fluorophore which would have prevented the measurement of any modification in polarization intensity. The different Alexa 488, 546 and 647 as well as the Eu⊂PBBP were covalently linked to the parent peptide to obtain the corresponding fluorescent ligands (illustrated in Fig. (**4**)). [Lys^8(Alexa488)]PVA (**23**), [Lys^8(Alexa546)]PVA (**24**), [Lys^8(Alexa647)]PVA (**25**) and [Lys^8(Eu⊂PBBP)]PVA (**26**), all exhibited a very high affinity for both human V1a and OTR subtypes and were highly selective to these receptors *versus* the V1b and V2 subtypes (

a nM range affinity compared to a µM range affinity). This result confirmed that very large fluorophores can be coupled to the residue 8 of these linear peptide antagonists without modifying their affinity or loosing specificity. Moreover, these fluorescent ligands are all antagonists for both human V1a and OTR subtypes, like the 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2.

The fluorescent properties of these new AVP/OT analogues make them ideal for undertaking HTS assays using polarization or FRET experiments. The affinity of several unlabeled well-known ligands was determined using homogenous assays on membrane preparations expressing the V1a receptor into 96- and 384-well plate formats. The [Lys^8(Alexa488)]PVA (**23**) was particularly demonstrated to be well-suited for developing fluorescence polarization screening assays. Indeed, the specific/non-specific signal ratio is good, whereas that for [Lys^8(Alexa546)]PVA (**24**) and [Lys^8(Alexa647)]PVA (**25**) is too low. Affinities for AVP, HO-LVA [50], and the nonpeptide antagonist SR49059 [51], determined using fluorescence polarization assays with Lys^8(Alexa488)]PVA were similar to those defined using [^{3}H]AVP in a radioligand binding assay.

Eu⊂PBBP exhibits particular fluorescent properties such as a long fluorescent lifetime and is then suitable as a donor for FRET experiments. Combined with the use of an Alexa 647 fluorescent probe as an acceptor, it is convenient to perform HTRF experiments. Thus, an HTRF competition binding assay based on a FRET signal between the two fluorophores, the Eu⊂PBBP carried by the ligand and Alexa 647 coupled to an antibody specific for an epitope tag located at the N-terminus of the receptor (HA tag and anti-HA antibody 12CA5), was developed. The proximity of the two fluorophores when the ligand binds 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. The FRET signal was measured for different periods of incubation at 4°C for avoiding receptor internalization; 8 h was necessary for reaching a plateau. As for fluorescence polarization assays, affinities for different unlabeled ligands were found to be equivalent to those determined using [^{3}H]AVP in a radioligand binding method. Competition HRTF experiments were also conducted using membrane preparations and similar results were obtained.

Both polarization and HTRF assays are promising approaches and offer attractive advantages for performing ligand-GPCRs HTS procedures. Compared to other binding assays, these methods are nonradioactive, safe to perform, very sensitive and homogenous, easier and faster to automate. The different peptide antagonists [Lys^8(Eu⊂PBBP)]PVA, [Lys^8(Alexa488)]PVA, [Lys^8(Alexa546)]PVA, [Lys^8(Alexa647)]PVA are the first fluorescent AVP/OT analogs adapted to different HTS procedures for screening ligand libraries and discovering novel potential therapeutic compounds. These methods can therefore be applied to other GPCRs.

3. CONCLUDING REMARKS

The availability of the Alexa/lanthanide-labeled peptide ligands for AVP/OT receptors opens new perspectives in receptor studies. First, structural organization of these membrane receptors at the surface of target cells is still an open question. For instance, dimerization/oligomerization of GPCRs has been demonstrated in heterologous cell expression systems using co-immunoprecipitation, bioluminescence resonance energy transfer or FRET techniques with highly-expressed tagged-receptors or luminescent fusion proteins. However, no evidence of direct receptor-receptor interactions has yet been provided in native tissues. We have recently used a FRET approach to address this question with pairs of labeled ligands, one with the Alexa 647 the other with the Eu⊂PBBP [52]. Indeed, OTR dimers/oligomers in the mammary glands of lactating rats were unambiguously identified using a pair of labelled antagonists, [Lys^8(Eu⊂PBBP)]PVA and [Lys^8(Alexa647)]PVA, but also a pair of fluorescent agonists, [HO^1][Thr^4, Orn^8(Eu⊂PBBP)]VT (**27**) and [HO^1][Thr^4, Orn^8(Alexa647)]VT (**28**). These two agonists are seen in Fig. (**2**). Interestingly, due to a high affinity for the V1a receptor, these pairs of ligands should be useful to the demonstration of V1a dimers/oligomers in native tissues. This would be crucial as well to apply this strategy to the V2 and V1b subtypes in order to demonstrate whether these receptors behave as dimers in the kidney and the pituitary/CNS, respectively. Such a FRET strategy represents an interesting alternative to other energy transfer strategies. The specific recognition of ligands by receptors as well as their small size compared to antibodies or luminescent proteins make ligands ideal tools for the study of GPCR dimers/oligomers in native tissues without altering the physiological context. The use of lanthanide-labeled ligands deserves a specific comment, particularly in the context of native tissues/cells which express very low levels of endogenous receptors. It is obvious that the sensitivity of the fluorophore is crucial in terms of receptor expression levels. With most fluorophore-labeled ligands, the signal-to-noise ratio is worse than the one obtained with the corresponding radioligands (tritiated or iodinated), whatever the methodology used. This is probably due to the hydrophobic nature of the AVP/OT ligands but also that of the fluorophores themselves covalently-bound to these ligands, in addition to the low sensitivity of the fluorescent molecules. In comparison, lanthanides are advantageous since their detection by time-resolved fluorescence results in a specific signal with a low background, and thus the signal-to-noise ratio is somewhat equivalent to that measured with radioligands. Consequently, detection of very low levels of receptor expression in native tissues/cells should be much easier and reproducible with these new generations of fluorescent probes.

Second, excepted the first generations of fluorescent agonist and antagonist peptides useful for fluorescence microscopy techniques applied to the investigation of receptor cellular expression and localization, no specific peptide ligand labelled with Alexa or lanthanide series has been synthesized and characterized for the V1b and V2 receptors so far. Unfortunately, the search for a peptide agonist/antagonist selective for the human V2 receptor with respect to the V1a, V1b and OTR is still a challenging goal [16]. V2 antagonists with high affinity but which are nonselective have been developed [53]. For instance, the affinity of d$(CH_2)_5$[D-$Tyr(Et)^2$,Pro^3, Val^4]AVP for the human V2 receptor is 0.5 nM [16], rendering this ligand a good candidate for replacing the arginine[8]

with a lysine and derivatization of the lysine[8] with Alexa or lanthanide fluorophores. This ligand would probably be not selective but would be useful for HTS fluorescence polarization and HTRF assays using membranes or cells expressing the V2 receptor. On the other hand, several agonists being selective to the human and rat V1b receptors might be promising tools. Indeed, one can imagine that the V1b-selective d[Cha[4],Lys[8]]VP and d[Leu[4],Lys[8]]VP [54], could be directly derivatized with Alexa and Eu⊂PBBP fluorophores on the lysine residue 8, behaving as efficient tools for fluorescence polarization and HTRF HTS assays as well as for the direct demonstration of V1b dimers/oligomers in the CNS or the pituitary.

Third, future generations of fluorescent ligands for AVP/OT receptors will certainly be developed from nonpeptide compounds. Several selective nonpeptide antagonists have been developed and characterized by pharmaceutical companies for each AVP/OT receptor [51, 55-58]. Three nonpeptide V2 antagonists are currently in clinical trial: Tolvaptan, Lixivaptan and Satavaptan [59]. More recently, at least three companies have reported nonpeptide agonists for the V2 receptor and for the OTR [60, 61]. To date, there are no reports on nonpeptide V1a or V1b agonists. None of these nonpeptide agonists and antagonists has been covalently-labeled with a fluorophore. Thus today, there are no available fluorescent nonpeptide ligands for AVP/OT receptors. Taking into account available three-dimensional models of nonpeptide ligands docked into AVP/OT receptors should help in the design of new fluorescent compounds. Based on these models, it is commonly accepted that the nonpeptides mostly interact with receptor residues located deeply in the binding pocket, particularly with aromatic and hydrophobic clusters. Because their size is much smaller than that of AVP/OT peptide analogs, derivatizing this class of ligands for obtaining useful fluorescent probes would probably necessitate adding a spacer arm long enough to allow the fluorophore being at the receptor surface, in a position equivalent to that of the corresponding peptide residue 8. Characterizing a fluorescent nonpeptide ligand retaining a high affinity but also selectivity for a given receptor subtype of the AVP/OT family is even more challenging.

The ability to measure and quantify the binding of ligands to GPCRs has been and remains a key element of drug discovery. Conventional methods based on radioactively-labeled ligands still contribute a major aspect of this activity. It is clear, however, that fluorescent molecules offer a safer, more powerful and more versatile alternative to radioligands in molecular pharmacology and drug discovery. There is indeed a need to develop both more robust fluorescence-based techniques and highly receptor-specific fluorophore-tagged ligands. Apart from AVP/OT receptors, many fluorescent ligands targeted at other peptidic GPCR families have been developed [for review, 62-64]. Different labels, such as fluorescein, rhodamine, Bodipy, Alexa488, Alexa594, Alexa633, Texas red, Cy3, Cy5, Oregon green, were covalently-attached to peptidic and small molecule nonpeptidic ligands for peptide GPCRs. These ligands were useful for studying different aspects of opioid, neurotensin, somatostatin, cholecystokinin, melanocortin, neuropeptide Y, chemokine receptors. Even lanthanide-labeled ligands have been reported and have been used successfully in evaluating receptor-ligand interactions, for instance for neurokinin, neurotensin or chemokine receptors [for review, 65]. Moreover, lanthanide labels have many advantages over radiolabels or other fluorolabels because they are highly sensitive, easy to automate, synthesized with relative ease, have a much longer lifetime and can be used in multilabel experiments. The lanthanide-labeled ligands are particularly well adapted to receptor-ligand screening assays but they also should revolutionize the study of GPCRs in their native cellular environment. With such powerful tools, coupled with the rapidly advancing complementary imaging technologies such as fluorescence correlation spectroscopy, one is therefore becoming able to investigate therapeutically important GPCRs and their molecular relationships with bioactive ligands at the single cell level and even at the single molecule level. This will open the way to study compartmentalization and structural organization of GPCRs in specialized microdomains of the cell plasma membrane.

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Abbreviations

AVP	=	Arginine-vasopressin
OT	=	Oxytocin
LVP	=	Lysine-vasopressin
VP	=	Vasopressin
VT	=	Vasotocin
OTR	=	Oxytocin receptor
GPCRs	=	G protein-coupled receptors
CHO	=	Chinese hamster ovary cells
CNS	=	Central nervous system
HEK	=	Human embryonic kidney cells
Abu	=	Aminobutyric acid
Cha	=	Cyclohexylalanine
MA	=	Methylanthranilamide
dansyl	=	Dimethylaminonaphthalene-1-sulfonyl
AMcou	=	Aminomethylcoumarin
TMRho	=	Tetramethylrhodamine
CTMRho	=	Carboxytetramethylrhodamine
fluo	=	Fluorescein
Bodipy	=	Bordifluoropyrromethene
Cy	=	Cyanine
PVA	=	Phenylpropionyl linear vasopressin antagonist

HO-LVA = Hydroxyphenylacetyl linear vasopressin antagonist

FRET = Fluorescence resonance energy transfer

HTRF = Homogeneous time-resolved fluorescence

HTS = High-throughput screening

Eu⊂PBBP = Europium pyridine-bis-bipyridine cryptate

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