Fluorescent ligands to investigate GPCR binding properties and oligomerization

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Abstract

Fluorescent ligands for GPCRs (G-protein-coupled receptors) have been synthesized for a long time but their use was usually restricted to receptor localization in the cell by fluorescent imaging microscopy. During the last two decades, the emergence of new fluorescence-based strategies and the concomitant development of fluorescent measurement apparatus have dramatically widened the use of fluorescent ligands. Among the various strategies, TR (time-resolved)-FRET (fluorescence resonance energy transfer) approaches exhibit an interesting potential to study GPCR interactions with various partners. We have derived various sets of ligands that target different GPCRs with fluorophores, which are compatible with TR-FRET strategies. Fluorescent ligands labelled either with a fluorescent donor (such as europium or terbium cryptate) or with a fluorescent acceptor (such as fluorescein, dy647 or Alexa Fluor® 647), for example, kept high affinities for their cognate receptors. These ligands turn out to be interesting tools to develop FRET-based binding assays. We also used these fluorescent ligands to analyse GPCR oligomerization by measuring FRET between ligands bound to receptor dimers. In contrast with FRET strategies, on the basis of receptor labelling, the ligand-based approach we developed is fully compatible with the study of wild-type receptors and therefore with receptors expressed in native tissues. Therefore, by using fluorescent analogues of oxytocin, we demonstrated the existence of oxytocin receptor dimers in the mammary gland of lactating rats.

Introduction

Fluorescent ligands have long been considered as potentially interesting tools to study GPCRs (G-protein-coupled receptors). Many fluorescent analogues of GPCR ligands have been synthesized and have been particularly well illustrated for the vasopressin and oxytocin ligand family [1]. However, their use was restricted to show the expression of their cognate receptor at the cell surface or to study receptor internalization by conventional fluorescent microscopy (e.g. [2–5]). During the last two decades, the concomitant discovery of bright fluorophores with the emergence of concepts such as GPCR oligomerization, has driven the development of new fluorescent-based strategies to investigate GPCR interactions with various partners.

RET (resonance energy transfer)-based approaches have attracted attention as they are particularly well adapted to investigating molecular interactions. These approaches are based on an energy transfer between a donor and an acceptor. The transfer can operate only if: (i) donor and acceptor exhibit energy compatibility, i.e. the emission spectrum of the donor overlaps the absorption spectrum of the acceptor; (ii) the two molecules are well oriented: dipole moments should not be perpendicular; and (iii) the distance between donor and acceptor is short, although it depends on the pair of donor/acceptor, it should usually not exceed 10 nm.

Although the principle of RET was described in the 1940s by Förster [6], RET-based approaches have only been widely used during the last two decades. Variants of energy transfer strategies have been developed (for review see [7–9]): FRET, BRET (bioluminescence RET; for which the donor is a bioluminescent protein e.g. luciferase) and TR (time-resolved)-FRET, which uses lanthanides as donors. These approaches do not display the same signal/noise ratio; this ratio is highly dependent on the ability to stimulate the donor efficiently without exciting the acceptor and to discriminate the fluorescence of the acceptor from that of the donor. Therefore the two absorption spectra or the two emission spectra should overlap as little as possible. The autofluorescence of the medium or of the biological preparation is another parameter that deeply impacts the signal/noise ratio.

TR-FRET

Two lanthanides can be used in TR-FRET strategies: europium or terbium (Figure 1a). These two elements form complexes with chelates or cryptates to enable the labelling

Key words: europium, fluorescent ligand, G-protein-coupled receptor, lanthanide, terbium, time-resolved FRET.

Abbreviations used: Cy5, indodicarbocyanine; FRET, fluorescence resonance energy transfer; GLP-1, glucagon-like peptide 1; GPCR, G-protein-coupled receptor; RET, resonance energy transfer; TR, time-resolved; YFP, yellow fluorescent protein.

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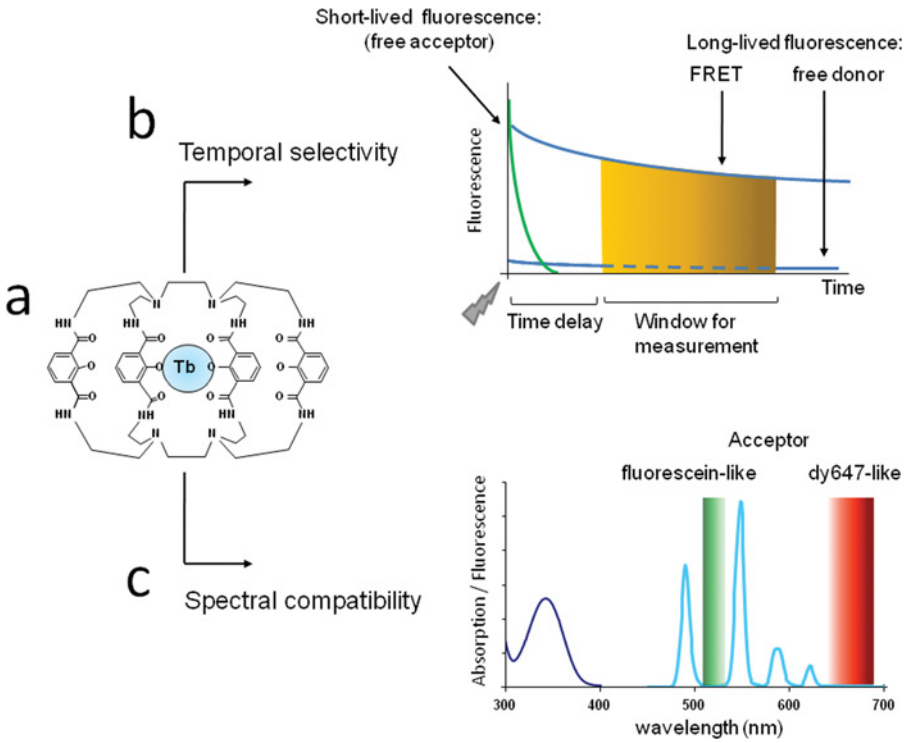
Figure 1 | **Fluorescent properties of a lanthanide cryptate**

(**a**) Structure of terbium cryptate, Lumi4-Tb. (**b**) Principle of TR-FRET: lanthanide cryptates display long-lasting luminescence with a time decay faster than 1 ms. Fluorescence measurement during a time window (orange area) is delayed after the flash allowing the short-lived fluorescence to decrease to 0. (**c**) Absorbance (dark blue) and emission (light blue) spectra of Lumi4-Tb. Emission of fluorescein-like and dy647- or Cy5-like fluorophore is symbolized by the green and red areas respectively, illustrating the compatibility of these fluorophores with Lumi4-Tb to perform TR-FRET.



of receptors of interest. These cages play different roles: first, they are essential to label proteins of interest with lanthanides. By incorporating reactive groups in their structure, it allows for protein labelling. Secondly, these chromophores behave as antennae that induce a dramatic increase in the absorption of the complex, compared with lanthanides alone. Finally, cryptates, in contrast with chelates, prevent lanthanide quenching by water [10]. Cryptate–lanthanide complexes also exhibit a better stability than chelate–lantahanide complexes.

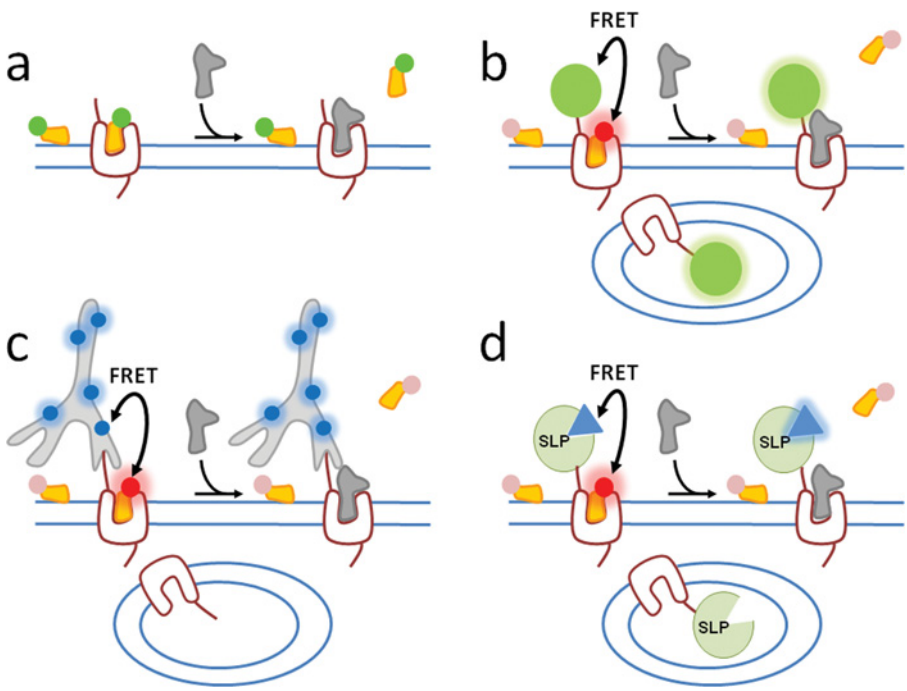
Lanthanides display attractive luminescent properties. Their luminescence half-lives are longer than 1 ms; this is approximately 100 000-fold greater than the half-lives of conventional fluorophores (usually less than 20 ns). The separation of lanthanide luminescence from short-lived fluorescence is therefore quite easy when introducing a time delay between a pulsed excitation and the measurement of the luminescence emission [11,12] (Figure 1b). Moreover, lanthanide cryptates are excited at approximately 300–350 nm and display a very large Stoke shift. For example, the europium pyridine-bis-bipyridine cryptate has four emission peaks at 595, 615, 680 and 705 nm; it is therefore compatible with deep red dy647- or Cy5 (indodicarbocyanine)-like fluorophores to carry out TR-FRET experiments. Lumi4-Tb, a terbium cryptate [13] also displays four emission peaks at approximately 490, 550, 585 and 620 nm (Figure 1c). Lumi4-Tb is compatible with fluorescein-like and dy647- or Cy5-like fluorophores, allowing for multiplexing if necessary. Notably, the use of RET with lanthanides is not, strictly speaking, equivalent to FRET because lanthanide luminescence is not fluorescence; however, as the transfer follows the same rule it has been associated with FRET [10].

Use of TR-FRET-based assay for drug screening

Various attempts have been carried out to substitute fluorescent assays for radioactive-based ones (for a review see [14]). Fluorescent derivatives are less hazardous and constraints for their use are less restrictive. Fluorescent ligands have been synthetized and binding assays were first based on the measurement of fluorescence intensity of the fraction of ligands bound to the receptors. These assays require the separation of the ligand-bound fraction from the ligand-free fraction and are therefore not homogeneous assays. Moreover, the addition of a fluorophore to the ligand can increase the non-specific binding leading to a decrease of the sensitive of the assay (Figure 2a).

Figure 2 | Principle of various fluorescent ligand-based binding assays

(**a**) Binding properties of competitors are estimated by measuring the fluorescence bound to the cells or membrane preparation after separation of the bound- and free-ligand fractions. (**b**) FRET-based assays have been developed between fluorescent ligands and various mutants of YFPs fused to the N-terminus of GPCRs. It is noteworthy that receptors trapped inside the cells are fluorescent and their signal may have an impact on sensitivity of the binding assay. (**c**) TR-FRET assay between fluorescent ligands and receptors labelled with fluorescent antibodies against epitopes fused to the N-terminus of the receptors. Owing to their size, antibodies can generate an important steric hindrance. (**d**) TR-FRET assay between fluorescent ligands and receptors labelled with the Tag®-lite strategy. An SLP (self-labelling protein) is fused to the N-terminus of receptors and cells expressing such chimaeric receptors are incubated in the presence of the fluorescent substrate. The SLP catalyses the transfer of the fluorescent moiety of the substrate on to itself, leading to the labelling of the chimaeric receptor. In the last two methods, endogenous receptors are not labelled and do not contribute to the fluorescence signal.



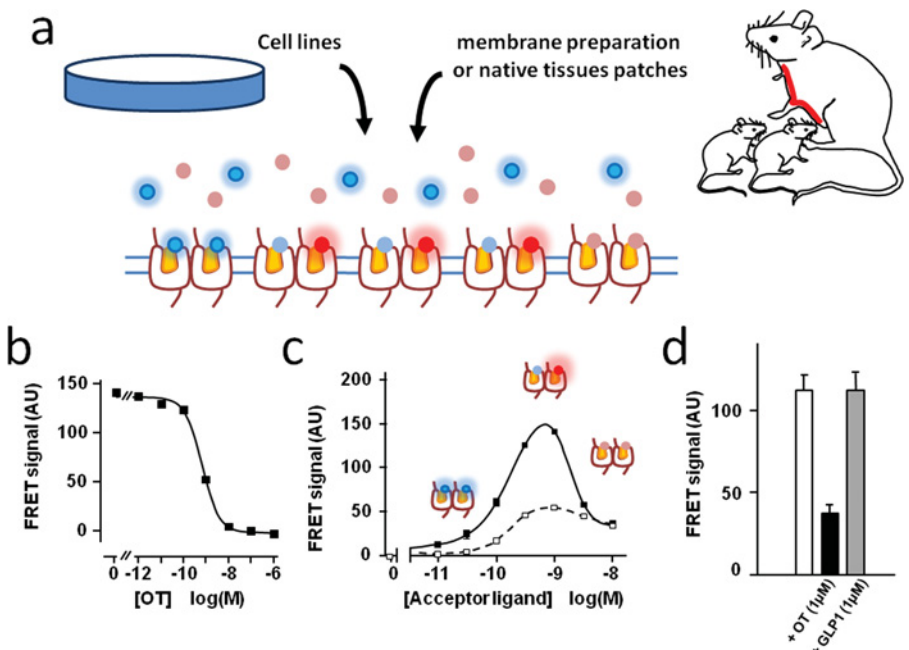
Fluorescence polarization-based assays have also been developed and, although they are interesting because the assays can be performed in homogeneous conditions, they display various sensitivities owing to the nature of ligands, and notably, their hydrophobicity [15]. Moreover, the measurement window is often very narrow, which explains why this approach, although is inexpensive and fast [16], has not been extensively used [15,17–19].

FRET-based binding assays between receptors fused to fluorescent proteins such as GFP (green fluorescent protein) or YFP (yellow fluorescent protein) and fluorescent ligands have opened new perspectives [20,21] (Figure 2b). Indeed FRET depends on the proximity of the fluorescent ligand to the fluorescently tagged receptor. The distance between the two fluorophores is compatible with the observation of a FRET signal when a ligand binds specifically to a receptor. By contrast, the non-specific ligand binding does not generate any FRET since the distance between the two fluorophores is generally too large. Therefore FRET-based assays should in theory increase the signal/noise ratio, but in practice they suffer of few drawbacks. First, the spectra of donor and acceptor are usually not fully compatible owing to the existence of overlaps between the absorption spectra and/or between the emission spectra. Secondly, autofluorescence due to biological preparation or medium is often not negligible at the emission wavelength of the fluorophores used.

We have developed TR-FRET-based binding assays [15,22] that present some advantages. As mentioned above, the TR mode allows for discrimination of the specific FRET signal from non-specific fluorescence and thus increases the signal/noise ratio. Two strategies have been developed to label receptors. The first one consists in a non-covalent labelling of receptors with lanthanide-conjugated antibodies against epitopes fused to the N-terminus of the receptors [23] (Figure 2c). Although it is convenient to label receptors, this method is dependent on the accessibility of the epitope to the antibody, and equilibrium depends on the binding of the antibody and of the fluorescent ligand to the receptors [15]. The second method is using the basis of receptor covalent labelling, which can be performed by different

Figure 3 | **Analysis of GPCR oligomerization with fluorescent ligands**

(**a**) The technique has first been validated on receptors expressed in cell lines and then on oxytocin receptors expressed in the mammary gland of lactating rats. Cells or membranes are incubated in the presence of fluorescent ligands derived with donor or acceptor fluorophores. Proximity between fluorescent ligands bound to receptor oligomers leads to a FRET signal. (**b**) Competition between fluorescent ligands and unlabelled ligands leads to a competition sigmoidal curve. (**c**) Variation of the acceptor/donor fluorescent ligand ratio leads to a bell-shaped curve. For low and high ratios, binding sites preferentially bind two donor ligands or two acceptor ligands respectively, and only a marginal FRET signal can be observed. By contrast, FRET can be measured when receptor oligomers bind two different fluorescent ligands. (**d**) TR-FRET experiments have been performed on patches of tissue incubated in the presence of donor and acceptor fluorescent ligands. The signal is significantly reduced in the presence of an excess of oxytocin (1 μM) but not affected by an excess of the unrelated peptide GLP-1 (1 μM). OT, oxytocin; AU, arbitrary unit.



strategies (Figure 2d). GPCRs can be labelled by fusing their N-terminus to self-labelling proteins (also known as suicide enzymes) such as SNAP- or CLIP-tag and by adding fluorescent substrates BG [24–28] or BC [29] respectively.

Fluorescent analogues derived with lanthanide cryptates have successfully been synthesized and used to label GPCRs [30–32]. An advantage of these techniques is that 100% of the receptors can be labelled in an irreversible manner [32].

Such assays have been validated on a large scale and the K_i (inhibition constants) determined for various competitors are in accordance with those ascertained by radioactive binding assays [22].

Fluorescent ligands used to investigate GPCR oligomerization in native tissues

The concept of GPCR oligomerization emerged two decades ago ([32a] and also reviewed in [9,33,34]). GPCRs have been shown to have the propensity to interact together to form homo-oligomers, when the receptors are identical, or hetero-oligomers, when receptors are different. At first, complexes were described as strict dimers but larger complexes have since been described, for example tetramers for $GABA_B$ [30,32] and dopamine [35] receptors, or larger oligomers for rhodopsin [36,37]. To demonstrate the existence of GPCR dimers or larger complexes, FRET-based approaches between labelled receptors are relevant because, as mentioned above, signals can only be measured if receptors interact together.

Most analyses have been performed on chimaeric receptors fused to luminescent or fluorescent proteins or to self-labelling proteins on the C- or N-terminus, expressed in cell lines by transfection. Although these experiments give a lot of information regarding the propensity of GPCRs to oligomerize and their potential roles, the proof of their existence in native tissues remains elusive. This is probably the reason why the concept of GPCR oligomerization remains a matter of debate.

Performing experiments on receptors in a native context imposes various constraints, notably that the sequence of receptors and their level of expression should not be modified. To demonstrate the existence of oligomers in native tissues, we resort to an indirect labelling of receptor with fluorescent ligands derived either with a donor such as europium or

terbium cryptate or with an acceptor such as fluorescein or dy647. The strategy was first validated on different receptors expressed in cell line vasopressin V_{1a} and V_2 receptors, oxytocin and dopamine D_2 receptors with various sets of fluorescent ligands [38] (Figure 3a). Previous structure–activity relationship analyses have shown that some peptidic analogues of vasopressin and oxytocin tolereate the presence of fluorophores on residue 8 without dramatically losing affinity for their receptors [2,3]. Surprisingly, smaller non-peptidic ligands for the dopamine D_2 receptor such as NAPS [*N*-(*p*-aminophenethyl)spiperone] or PPHT [(±)-4′-amino-2-(*N*-phenethyl-*N*-propyl)-amino-5-hydroxytetralin] have also been successfully derived with fluorophores such as Lumi4-terbium or D_1 while keeping high affinity [38]. The first experiments carried out in homogeneous conditions on COS-7 cells transiently expressing vasopressin, oxytocin or dopamine D_2 or on membrane preparation incubated with donor- and acceptor-fluorescent ligands led to a specific FRET signal. The specificity of the signal was attested by (i) its dramatic decrease after adding an excess of unlabelled ligand; (ii) the observation of a sigmoidal curve when performing a competition with unlabelled ligand; and (iii) the bell-shaped curve of the FRET signal when performing competition between the fluorescent ligands. Noteworthily, the FRET signal obtained with two fluorescent agonists is marginal compared with the signal obtained with two fluorescent antagonists. This is in accordance with the negative co-operative binding for agonists previously observed [39] and the absence of any co-operative binding or a positive one for antagonists. Thus it reveals an asymmetry in the structure of the dimer when agonists bind on the receptors. Moreover, since fluorescent ligands are non-permeant, it proves that dimers are present on the cell surface.

Similar experiments have been performed on mammary gland membrane preparation of lactating rat [38] (Figure 3a). During lactation, the mammary gland expresses a high level of oxytocin receptors and therefore constitutes an excellent model to validate our strategy on a native model. As previously observed with transfected cells, binding of donor- and acceptor-derived ligands leads to a significant FRET that is abolished in the presence of an excess of unlabelled ligand (Figure 3b). Similarly, we observed a difference in the FRET amplitude depending on the nature of the ligands. Indeed, fluorescent antagonists lead to a greater signal than fluorescent agonists (Figure 3c). As for receptors expressed in cell lines, we have suspected different co-operative binding processes for agonists and antagonists. To validate the hypothesis, we performed radioligand saturation and dissociation kinetics, and observed that agonists display negative co-operative binding whereas antagonists exhibit no co-operative binding or a positive one.

These data indicate the existence of receptor oligomers in membrane preparation of the mammary gland. We performed similar experiments on patches of mammary gland tissue (Figure 3d) and observed a significant FRET that is abolished in the presence of excess oxytocin but not affected by the presence of an unrelated ligand such as GLP-1 (glucagon-like peptide 1). This indicates the presence of oxytocin receptor oligomers on the cell surface of the mammary gland.

In conclusion, the interest in using fluorescent ligands has been renewed during the last two decades owing to the concomitant development of bright fluorophores, the development of faster and more sensitive fluorescence detection systems and the emergence of new concepts highlighting the importance of molecular interactions. Fluorescent ligands represent promising tools to investigate the binding properties of GPCR and are perfectly well adapted to be used in high-throughput screening. Receptor interaction, and more specifically, the use of fluorescent ligands to detect GPCR oligomers is interesting because they can be used on native tissue and in a physiological context.

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