In Vitro and In Vivo Ligand Binding to the $5HT_3$ Serotonin Receptor Characterised by Time-Resolved Fluorescence Spectroscopy

Pascal Vallotton, Ruud Hovius, Horst Pick, and Horst Vogel*[a]

The binding of the fluorescein-labelled antagonist GR-flu ([1,2,3,9-tetrahydro-3-[(5-methyl-1H-imidazol-4-yl)methyl]-9-(3-amino-(N-fluoresceinthiocarbamoyl)propyl)-4H-carbazol-4-one]) to a purified, detergent-solubilised ligand-gated ion channel, the type-3 serotonin (5-hydroxytryptamine, 5HT) receptor ($5HT_3R$), was characterised by frequency-domain time-resolved fluorescence spectroscopy (TRFS). Detailed understanding of how ligands interact with the homopentameric receptor was obtained. While a 1:1 stoichiometry was observed for the GR-flu – receptor complex, the agonist quipazine bound cooperatively to the receptor, suggesting multiple binding sites for this ligand. The GR-flu-binding site of the receptor was proven to provide an acidic environment as shown by determining the fraction of bound GR-flu in the protonated state. Fluorescence anisotropy relaxation experiments indicated a hindered but still high mobility for the receptor-bound GR-flu. Hence, the binding site is expected to present a wide opening to the ligand. Finally, we succeeded in measuring the binding of GR-flu to $5HT_3$ receptors in live cells. These results show that the purified and the native receptor behave identically and demonstrate that time-resolved fluorescence measurements are suited to selectively investigate biomolecular interactions in live cells.

KEYWORDS:

drug research · fluorescence spectroscopy · ion channels · molecular recognition · receptors

Introduction

Ligand-gated ion channels play a central role in fast signal transduction in the nervous system. Despite intensive research in this field, the detailed molecular mechanism underlying this process has not been elucidated yet. The best studied ligand-gated ion channel is the nicotinic acetylcholine receptor,[1–5] which can be purified relatively easily in quantities sufficient for further studies. It belongs to a superfamily that includes ionotropic receptors for serotonin (5-hydroxytryptamine, 5HT), γ-amino-*n*-butyric acid (GABA) and glycine. This report concerns another ligand-gated ion channel: the serotonin type-3 receptor ($5HT_3R$),[6] which can be found in tiny amounts in the enthorinal cortex, the hypocampus, the nucleus tractus solaritus and the area postrema.[7] It has also been detected in the peripheral system in the gut, skin and blood vessel walls. We recently overexpressed $5HT_3R$ in several mammalian cell lines and succeeded in purifying the functional receptor in detergent-solubilised form in sufficient quantities for biophysical studies.[8] The great pharmaceutical interest in $5HT_3R$ stems from its involvement in emesis caused by anticancer chemotherapy, in colonal dysfunction and possibly in schizophrenia and drug abuse.[9] $5HT_3R$ assembles as a functional homopentameric receptor.[8, 10] A four-transmembrane domain topology is predicted for each subunit, based on sequence analysis.[11] The secondary structure of purified $5HT_3R$ was shown to be rich in α helices (50%) and β strands (24%).[8]

Here, we concentrate on the characterisation of the receptor's ligand-binding site by time-resolved fluorescence spectroscopy (TRFS). First, we exploit the dependence of the fluorescence lifetime of GR-flu on its microenvironment (Figure 1) to monitor and quantify ligand binding to the receptor. By using this technique, the local pH value of the binding site could be determined as well as the stoichiometry for the binding of GR-flu

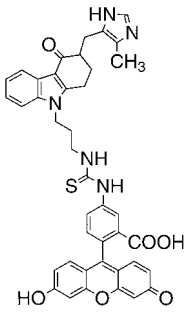

Figure 1. *Structure of the fluorescent $5HT_3$ antagonist GR-flu. The xanthene hydroxy group is deprotonated under basic conditions.*

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to the receptor. This reaction was also investigated by time-resolved fluorescence anisotropy experiments. We could determine the orientational order parameter of the fluorescein moiety of the receptor-bound ligand[12–14] as well as the time scale for the segmental movements of this fluorophore, thereby providing insight into the conformation and dynamics of the complex.

In the final part of this work, time-resolved fluorescence measurements were performed on $5HT_3R$ in live cells. Differences in the properties of native and purified receptors in most instances strongly limit the relevance of detailed biophysical studies on purified material. In the present case, however, the results obtained in vivo and in vitro agreed well. The purified receptor is therefore representative of the native receptor. This was further confirmed by confocal microscopy, which was used to deliver an estimate of the dissociation constant of the receptor – ligand complex in whole cells. These results shed new light on an important receptor – ligand system and, together with previous work,[15–17] they illustrate the possibilities offered by time-resolved fluorescence spectroscopy for the selective investigation of ligand – receptor interactions under minimal sample consumption and even in a complex environment such as live cells.

Experimental Section[18]

Preparation of purified $5HT_3R$: The protein was overexpressed in HEK 293 cells and purified as described elsewhere.[8] The receptor preparations were stored at $-80\,^{\circ}C$ in buffer A (10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 0.4 mM nonaethylene glycol monododecyl ether ($C_{12}E_9$), pH 7.4) until use.

Fluorescence measurements: Steady-state fluorescence measurements were performed with a SPEX Fluorolog II apparatus (Instruments S.A., Stanmore, UK) as described elsewhere.[19] Time-resolved data (total intensity and anisotropy decays) were acquired with an ISS K2 multifrequency cross-correlation phase and modulation fluorimeter (ISS Inc., Champaign, USA). Fluorescence confocal imaging was performed using an LSM 510 microscope (Zeiss, Jena, Germany).

Processing of fluorescence data: The fluorescence intensity decay $I(t)$ could be represented according to Equation (1),

$$I(t) = I_0 \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i) \quad (1)$$

where I_0 is the intensity at time $t = 0$ of excitation and α_i is the preexponential factor of the fluorescent species of lifetime τ_i. The anisotropy decay for a single fluorescent species can be described by Equation (2),

$$r(t) = r_0 \sum_{j=1}^{m} \beta_i \exp(-t/\phi_i) \quad (2)$$

where r_0, the limiting anisotropy, is the value of the anisotropy at time $t = 0$ of excitation and β_i is the fractional amplitudes with associated rotational correlation time ϕ_i. The specification of a photophysical model for the sample uniquely defines the expression to be used for fitting of its anisotropy decay. These equations, and their corresponding expressions in the frequency domain, were generated automatically by the software used in this work (Globals Unlimited and Miniglobal packages, Laboratory for Fluorescence Dynamics, University of Illinois, Urbana-Champaign, USA).[20]

Results

Characterisation of GR-flu by TRFS

First, the sensitivity of GR-flu to the pH value was characterised in buffer solutions. When freshly prepared in 10 mM HEPES at pH 7.4, GR-flu displayed a single fluorescence lifetime of 3.7 ± 0.1 ns. The presence of 0.4 mM of the detergent $C_{12}E_9$ did not change the fluorescence lifetime. Upon lowering the pH, an additional lifetime species of 0.9 ± 0.3 ns appeared suggesting that in the investigated pH range, GR-flu is in equilibrium between a deprotonated and a protonated form. To obtain the lifetime values given above for these two forms as well as the pK_a for the chemical equilibrium, we performed a series of independent experiments at pH values of 4.6, 5.0, 5.4, 5.6, 6.0, 6.7 and 7.8, and analysed all the corresponding phase and modulation data simultaneously using the global analysis approach. The lifetime values for the protonated and deprotonated species were linked individually across all experiments during the global χ^2 minimisation because only the proportion between the protonated and deprotonated forms of GR-flu, but not their individual lifetimes are expected to vary with pH. A value of $\chi^2 = 6.38$ was obtained for the global fit, which could not be improved by introducing a third lifetime species. Generally, one would expect values around one for χ^2 if a model is suited to describe a data set. We suspect that the standard errors on the phase and modulation which we have used (0.2° and 0.04, respectively, as advised by the manufacturer of the instrument) underestimate the real errors when the fluorescence signals are low as in our case, creating this apparent discrepancy.

Using the Henderson – Hasselbalch equation [Eq. (3)]

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{base}]}{[\mathrm{acid}]} \quad (3)$$

a pK_a of 6.0 ± 0.2 was determined. The changes in the phase and modulation data could be reversed when the pH was adjusted to its original value excluding the possibility that they might have been caused by hydrolysis of the probe. We have also investigated the influence of the polarity of the medium on the fluorescence of GR-flu by measuring the phase and modulation data using either methanol, ethanol, butanol or octanol, but observed no significant changes in the lifetime as compared to an aqueous solution at a pH value equal to 7.4.

Ligand binding measured by TRFS

In the absence of receptor, GR-flu showed a single fluorescence lifetime of $\tau_1 = 3.7 \pm 0.1$ ns at pH 7.4. Hence, under these conditions GR-flu is deprotonated. In the presence of receptor, an additional lifetime component was obtained, similar to the one measured for the protonated form of GR-flu in aqueous solution without receptor. The contribution of this short lifetime species increased with the amount of receptor added. This indicates that the receptor-bound probe is in equilibrium between a deprotonated and a protonated form. Therefore, the time-resolved fluorescence decay can be described by Equation (4).

$$I(t) = I_0[\alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) + \alpha_3 \exp(-t/\tau_3)] \quad (4)$$

Subscript "1" corresponds to the free deprotonated ligand, subscript "2" designates the bound deprotonated ligand and subscript "3" the bound protonated ligand. Actually, the fluorescence decay was fitted with a biexponential function as the quality of the fits did not improve by introducing a third component. This is reasonable as τ_1 and τ_2 both represent the lifetime of the deprotonated free and bound forms of GR-flu, respectively, which are obviously identical.

A representative example of the experimental data is shown in Figure 2. The phase and modulation data of all independent experiments performed at different receptor/ligand molar ratios

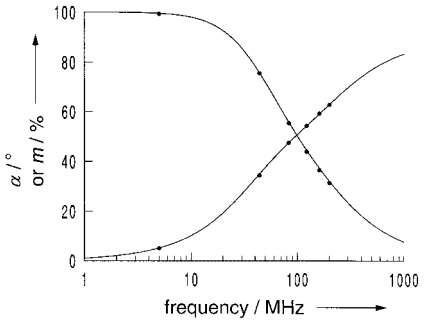

Figure 2. *Frequency response of phase (α) and modulation (m) data for GR-flu bound to purified $5HT_3R$ at a receptor/ligand molar ratio of 6:1. The fit delivered lifetime values of $\tau_1 = \tau_2 = 3.5 \pm 0.1$ ns for the deprotonated species and $\tau_3 = 0.90 \pm 0.2$ ns for the protonated species. The corresponding preexponential factors were $\alpha_1 + \alpha_2 = 0.43 \pm 0.02$ and $\alpha_3 = 0.57 \pm 0.02$. Buffer A was used, the concentration of GR-flu was 3 nM, the excitation wavelength was 488 nm, emission was collected through an interference filter (full width at half-maximum = 45 nm at 530 nm).*

were analysed globally[20] to recover the lifetimes and preexponential factors of the deprotonated and protonated ligands. The recovered lifetimes for the deprotonated and protonated species were equal to 3.6 ± 0.1 ns and 0.8 ± 0.3 ns, respectively ($\chi^2 = 11.9$), which is in excellent agreement with the results of experiments performed at various pH values in the absence of receptor. Figure 3 shows the preexponential factor α_3, corresponding to the protonated, bound ligand, as a function of the receptor/ligand molar ratio. The dissociation constant obtained by fitting these results (see Supporting Information) was equal to $K_d = 0.6 \pm 0.2$ nM and the concentration ratio (c_2/c_3) of the bound deprotonated to the bound protonated ligand was equal to 0.38.

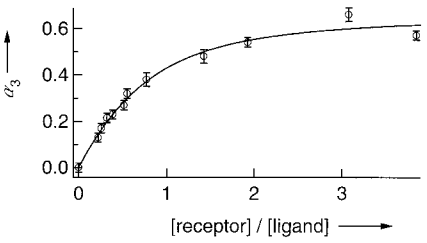

Figure 3. *Saturation binding of GR-flu to purified $5HT_3R$. The graph represents the preexponential factor α_3 corresponding to the bound protonated form of the ligand as a function of the receptor/ligand molar ratio. The fit (see Supporting Information) yielded $K_d = 0.6 \pm 0.2$ nM. Conditions were identical to those described in Figure 2.*

Applying the Henderson – Hasselbalch equation [Eq. (3)] to the protonation equilibrium of the bound ligand and using the measured value of $pK_a = 6.0$ for GR-flu, the local pH value of the binding site is estimated to be 5.6. Analysing each set of phase and modulation data individually delivered similar results although the scattering of the data points was higher. There was no systematic change of the individual lifetimes as a function of the receptor/ligand molar ratio. Such a tendency would have been expected if several ligands would bind simultaneously to the receptor leading to fluorescence resonance energy homotransfer. Hence, the experimental results strongly indicate that only one GR-flu molecule binds per receptor molecule.

Competition between GR-flu and quipazine for binding to $5HT_3R$ analysed by TRFS

The specificity of the binding of GR-flu to $5HT_3R$ was investigated in the presence of increasing concentrations of the competitive, nonfluorescent agonist quipazine. Both probes were mixed prior to incubation with the receptor and the phase and modulation data for the independent experiments were evaluated by global analysis as before. The two recovered lifetimes were equal to 3.6 ± 0.1 ns and 0.7 ± 0.2 ns (global $\chi^2 = 3.7$) in good agreement with the lifetimes determined before for the deprotonated and protonated forms of the ligand. The preexponential factor [Eq. (4)] corresponding to the bound protonated ligand is reported in Figure 4 as a function of the concentration of

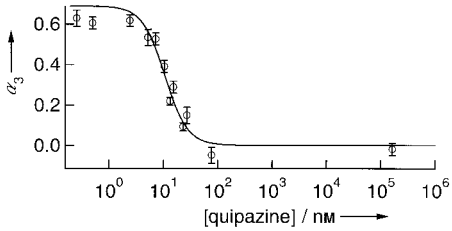

Figure 4. *Competition of GR-flu with the agonist quipazine for binding to purified $5HT_3R$ monitored by the preexponential factor α_3 of the protonated bound ligand. The fit yielded a K_d value of 2.1 ± 0.4 nM and a Hill coefficient of 2.6 ± 0.7 for quipazine. Conditions were identical to those described in Figure 2.*

competitor. Fitting of the data (see Supporting Information) yielded an IC_{50} value of 12.8 ± 2 nM and a Hill coefficient of 2.6 ± 0.7. To reduce the number of free parameters during the analysis, the concentration ratio $c_3/(c_2 + c_3)$ of the bound deprotonated probe to that of the total bound probe was taken to be equal to the one obtained by fitting the saturation binding curve. This choice reduces the visual quality of the fit in Figure 4, but increases the robustness of the parameter recovery. A dissociation constant of $K_d = 2.1 \pm 0.4$ nM as calculated with the Cheng – Prusoff equation[19] was obtained for the quipazine – receptor complex (see Supporting Information). When analysing the spectra individually, no systematic trend in the lifetime values as a function of the concentration of the competitor was observed.

Time-resolved fluorescence anisotropy measurements

First, the anisotropy decay of GR-flu in 10 mM HEPES at pH 7.4 was measured. The best fit ($\chi^2 = 4.6$) was obtained by using a monoexponential anisotropy decay of rotational correlation time of the free ligand $\Phi^f = 0.31 \pm 0.04$ ns and a limiting anisotropy $r_0 = 0.34 \pm 0.04$. The lifetime of the deprotonated form of GR-flu, 3.7 ± 0.1 ns, was used as a fixed parameter in the present and in all the anisotropy decay analyses that follow in order to facilitate the parameter recovery. The same holds for the protonated form of GR-flu having a lifetime of 0.8 ± 0.2 ns. Unexpectedly, considering the asymmetric shape of the probe, the fit of the anisotropy decay was not improved when we used two rotational correlation times. The presence of 0.4 mM of $C_{12}E_9$ had no influence on the observed anisotropy decay of GR-flu indicating that the probe did not partition significantly into the detergent micelles. The results of this analysis were used to fix the parameters describing the free GR-flu species in the following receptor titration experiments. For these experiments, eight sets of phase and modulation data were measured at different receptor/ligand molar ratios and fitted globally, assuming the three species (as defined above) to be present. In contrast to the fluorescence lifetime experiments, these forms can now all be resolved because the bound and free deprotonated species undergo different rotational motions. The bound probe was assumed to undergo restricted motions, resulting in an anisotropy decay described by Equation (5).[21]

$$r(t) = (r_0 - r_\infty)\exp(-t/\phi^b) + r_\infty \quad (5)$$

Considering that the protonation state which distinguishes the second and third species has most probably no influence on their rotational motions, the parameters describing the anisotropy decay for these two receptor-bound species were assumed to be identical. Hence, each of them was characterised by the same rotational correlation times Φ^b and the same limiting anisotropy.[22] Moreover, we have assumed that the limiting anisotropies r_0 for the three species were identical, expecting that neither the interaction of the probe with the receptor, nor its protonation state would affect this parameter. Indeed, relaxing this constraint had little influence on the recovered parameters. A global χ^2 value of 4.3 was obtained by fitting the data as described. It has been shown[12] that independent of the exact shape of the anisotropy decay curve, the ratio r_0/r_∞ is equal to the square of the orientational order parameter S of the fluorophore. S is defined by Equation (6) as

$$S = \langle(3\cos^2\theta - 1)/2\rangle \quad (6)$$

where θ is the angle between an instantaneous orientation of the fluorophore and the average orientation. The angle brackets denote the average of the orientational distribution of the fluorophore within its lifetime. Therefore, S is a measure of the orientational order of the fluorophore in this time regime.[12–14, 23] S^2 was recovered to be 0.21, indicating considerable segmental motions of the fluorophore in the excited state. The time scale of these movements is equal to the rotational correlation time Φ^b of the bound probe for which the fit recovered a value of 0.76 ± 0.2 ns. This value is shorter than the average lifetime displayed by the bound probe, meaning that the probe has sufficient time to explore its configurational space during the anisotropy decay, in agreement with the important segmental movements observed.

The sum of the fluorescence intensity decay preexponential factors $\alpha_2 + \alpha_3$ corresponding to the bound species for each experiment performed at a different receptor/ligand molar ratio is shown in Figure 5. By proceeding as described in the Supporting Information, a value of $K_d = 0.8 \pm 0.2$ nM was obtained by fitting these data. In the presence of an excess (10 μM) of the competitor quipazine, the preexponential factor for the free ligand, α_1, was recovered to be 1.0 indicating the absence of nonspecific binding.

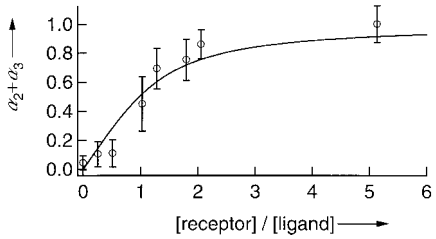

Figure 5. *Anisotropy decay binding curve obtained by plotting the fraction $\alpha_2 + \alpha_3$ of the fluorescence decay that can be attributed to the bound ligand as a function of the receptor/ligand molar ratio. The fit delivered a K_d value of 0.8 ± 0.2 nM. Conditions were identical to those described in Figure 2.*

Measurements on whole cells

In order to verify the relevance of the data obtained on the detergent-solubilised receptor and to demonstrate the possibility to perform ligand-binding studies by TRFS in vivo, measurements were also performed on living cells.

Confocal microscopy

Laser confocal microscopy was performed in 10 mM HEPES at pH 7.4 on whole cells stably overexpressing $5HT_3R$. Addition of low concentrations of GR-flu resulted in labelling of extended regions in the plasma membrane (Figure 6A). In the presence of 10 μM of the nonfluorescent competitive agonist quipazine, this staining was almost absent, indicating that nonspecific binding was very low (Figure 6B). A binding curve was constructed by calculating the normalised average pixel intensity per cell resulting from bound GR-flu as a function of GR-flu concentration (Figure 6C). Based on these data, the dissociation constant was estimated as $K_d = 0.6 \pm 0.2$ nM.

Time-resolved fluorescence spectroscopy

Under the experimental conditions chosen, the excess of receptor-binding sites as compared to the ligand was fivefold so that most of the ligand is expected to be bound. By using an optimal combination of excitation wavelength and interference filter, it was possible to select the GR-flu fluorescence emission and exclude the Raman scattering peak. Indeed, the contribution

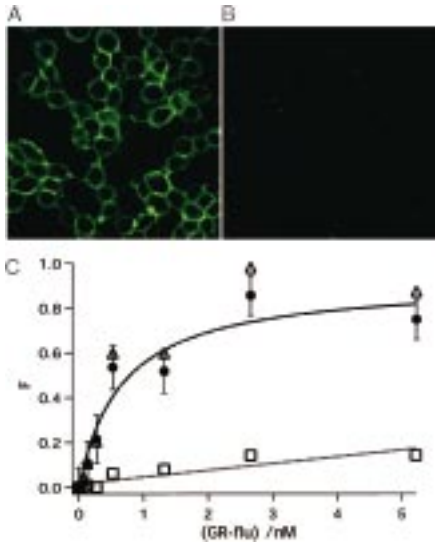

Figure 6. *Binding of GR-flu to $5HT_3R$ in live cells. A) HEK 293 cells stably overexpressing $5HT_3R$ were incubated in buffer A in the presence of 0.5 nM of GR-flu for 15 min at 20 °C. B) Same conditions as in Figure A, but 10 μM quipazine were present. C) Average fluorescence signal F per cell as function of the concentration of GR-flu in the surrounding medium. The signal of the specifically bound GR-flu ligands (●) was obtained by subtracting the background fluorescence signal (□) as obseved in figure B) from the total fluorescence signal (△) as observed in figure A). Fitting the data with a binding isotherm yielded a K_d value of 0.6 ± 0.2 nM.*

of GR-flu to the total fluorescence signal was higher than 96% as determined by measuring cells before and after incubation with GR-flu. To analyse the data, we fitted the spectrum using two lifetime components whose values were fixed to those obtained previously for the deprotonated and protonated GR-flu (3.7 ns and 0.9 ns, respectively). The preexponential factors were recovered to be equal to $(\alpha_1 + \alpha_2) = 0.41 \pm 0.02$ and $\alpha_3 = 0.59 \pm 0.02$, respectively, in good agreement with the values obtained for the purified receptor when the latter was in excess over the ligand (see Figure 3). In the presence of 10 μM quipazine, binding of GR-flu to the receptor was suppressed as shown by a monoexponential decay of fluorescence lifetime equal to 3.5 ± 0.2 ns. The morphology of the cells as examined by widefield optical microscopy and laser confocal imaging after they had been measured by time-resolved fluorescence spectroscopy gave no indication that they had suffered from this treatment. Moreover, examining the same cells by confocal imaging confirmed that nonspecific binding was less than 5% under the conditions chosen.

Anisotropy decay

Binding of GR-flu to whole cells was also investigated by using time-resolved anisotropy measurements. The samples were prepared exactly as for the whole-cell lifetime experiments. Unfortunately, we did not succeed in performing a complete time-resolved anisotropy analysis on live cells. However, the difference in the GR-flu anisotropy decay phase and modulation data between cells incubated in the absence or in the presence of a 10 μM concentration of quipazine was striking, as was the similarity between the anisotropy spectrum performed on stable cells and the corresponding spectrum for the purified receptor (Figure 7).

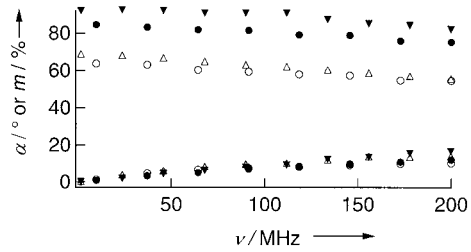

Figure 7. *Anisotropy phase (α) and modulation (m) data for GR-flu under various conditions: bound to $5HT_3R$-expressing cells suspended in buffer (○), added to a suspension of $5HT_3R$-expressing cells in the presence of 10 μM quipazine (●), bound to purified, detergent-solubilised $5HT_3R$ (△), added to purified, detergent-solubilised $5HT_3R$ in the presence of 10 μM quipazine (▼). The ratio of the modulated intensities (upper curves) and the phase difference between the vertical and horizontal polarisations (lower curves) is plotted.*

Discussion

Stoichiometry and cooperativity

The fitting of the time-resolved saturation binding curve for GR-flu provided no indication of cooperativity effects, suggesting that either a single ligand molecule binds per receptor or that several ligand molecules bind per receptor to identical and independent binding sites. Our measurements favour the former conclusion because several GR-flu molecules bound on the receptor would undergo fluorescence energy homotransfer and the efficiency of the process would depend on the number of ligand molecules bound. Comparing the Förster distance for the couple fluorescein – fluorescein (4.4 nm)[24] with the diameter of the receptor (8 nm) estimated by electron microscopy,[10] we expect such an effect to be easily measurable. We observed no change in either the short or the long lifetime component in any of the experiments. Thus, the binding of one GR-flu molecule seems to exclude the binding of other GR-flu molecules. This issue has also been investigated by fluorescence correlation spectroscopy,[25] and those results confirm our conclusions.

In contrast to the antagonist GR-flu, the agonist quipazine displayed a Hill coefficient equal to 2.6 in competition with GR-flu, a hallmark for positive cooperativity. A Hill coefficient of 2.5 was found for quipazine by analysis of the recovery of ionic conductance of desensitised receptors.[26] Electrophysiology measurements performed on HEK 293 cells overexpressing $5HT_3R$ delivered a Hill coefficient of 2.2 for the natural agonist serotonin.[35] This indicates that several agonist molecules are able to bind to one receptor. In this respect, the behaviour of the antagonist GR-flu is surprising and suggests that the probe binds close to the fivefold symmetry axis of the receptor, sterically hindering the binding of other GR-flu molecules. Several smaller ligands like quipazine possibly bind to the same site without sterical hindrance. Strong inhibitory allosteric mechanisms, however, cannot be completely excluded as the cause of the 1:1 stoichiometry observed for GR-flu.

The K_d values that we have measured for the GR-flu – receptor complex by frequency-domain TRFS ($K_d = 0.6 \pm 0.2$ nM), time-resolved fluorescence anisotropy ($K_d = 0.8 \pm 0.2$ nM) and confocal microscopy ($K_d = 0.6 \pm 0.2$ nM) are consistent. They also agree

with a value of $K_d = 0.8 \pm 0.2$ nM that we have determined by steady-state fluorescence anisotropy (data not shown). The dissociation constant for quipazine of $K_d = 2.1 \pm 0.4$ nM recovered from the competition experiment with GR-flu compares favourably with values spreading around $K_d = 2$ nM reported by other authors for the wild-type receptor.[27–30]

Fluorescence anisotropy decay experiments

For evaluating our time-resolved anisotropy data, various parameters had to be fitted (two fluorescence intensity pre-exponential factors, $\alpha_1 + \alpha_2 + \alpha_3 = 1$; one rotational correlation time, Φ^b; and the limiting anisotropy of the bound probe, r_∞) reflecting the fact that the technique addresses aspects of the biological system at a very detailed level. The number of free fitting parameters was limited to only these four by measuring the other relevant parameters such as those describing the decay of the free probe or the lifetimes in independent experiments. The recovery of the unknown parameters was also made possible by using the global analysis approach, which allowed to simultaneously analyse all the experiments performed at different receptor/ligand molar ratios. This technique demanded that reasonable hypotheses be made for the photophysical model but increased the confidence in the recovered parameters.

Concerning the photophysical model itself, we assumed that the protonated and deprotonated forms of the bound ligand are distinct species (undergoing identical rotational motions). This model is only sound if the time scale of protonation is much greater than the fluorescence lifetime of the ligand. Indeed, the typical time scale for protonation in the pH range and ionic concentration explored here is three orders of magnitude greater than the fluorescence lifetime of GR-flu.[31]

Although the overall mobility of GR-flu was restricted, we found considerable rotational freedom for the chromophore of the bound probe in the nanosecond time regime. As has been shown by comparing various ligands specific for $5HT_3R$, the pharmacophore seems to be bound by three major functional groups in the receptor binding site. A close look at the structure of GR-flu shows that it shares the structural features of a typical $5HT_3R$ ligand indicated by Cappelli et al.[32] Therefore, it is likely that the GR moiety is immobilised in its binding site and that the rotational motions of the fluorescein moiety are due mostly to the internal flexibility of the GR-flu molecule. The structure of the molecule (Figure 1), notably at the level of the propyl linker, indicates that such internal movements are possible. Moreover, this high internal flexibility could explain why the fit of the anisotropy decay for free GR-flu could not be improved by introducing a second rotational correlation time. Indeed, the time scale of the internal motions, given by the rotational correlation time, was much shorter than the lifetime of the probe so that, on this time scale, the probe appears spherical.

Previous experiments performed by two of us[33] to characterise 5-carboxyfluorescein have established that the lifetimes corresponding to the deprotonated and protonated species described in the present contribution had lifetime values equal to 4.0 and 3.0 ns, respectively. Identical values, although for fluorescein, were obtained by Klonis and Sawyer.[34] Hence, the properties of the dye seem not to depend significantly on the functional groups attached to its benzene ring. In fact, it is common practice to assume that a dye and its conjugates have similar spectrophysical properties. In the light of our present data, however, the situation is different for GR-flu. The lifetime of the protonated GR-flu species was determined to be equal to 0.8 ns as compared to 3 ns for fluorescein. To explain this difference, we propose that the fluorescein moiety is quenched by the GR moiety by a mechanism that precisely relies on the high internal flexibility of the molecule. The aromatic amine portions of the pharmacophore are potential quenchers[21] for this putative mechanism.

An essential advantage of anisotropy decay experiments over other fluorescence assays is the absolute character of the results. For example, if for any reason, only a fraction of the ligand was able to bind, the assay would recover naturally this fraction. For comparison and in the same situation, we would only attribute a reduced anisotropy to the bound species if only steady-state anisotropy measurements were performed. This potential problem is particularly acute in the case of GR-flu because of the limited stability of this ligand in an aqueous environment. As shown by our results, the asymptotic value for the bound fraction is close to 100% (Figure 5).

Conclusion

The fluorescence experiments delivered a detailed picture of the complex formed between GR-flu and $5HT_3R$. The receptor offers an acidic binding site providing a large opening to the fluorescent ligand. As a result, an equilibrium between the protonated and the deprotonated form of the ligand is observed and the latter moves relatively freely. This interaction was also studied in whole cells. The similarity of the results obtained on whole cells with those obtained on purified receptors indicate 1) that the purified receptor is fully functional and 2) that TRFS can be used to study receptor – ligand interactions in their native environment. Considering that all these experiments were performed at very low (nanomolar) concentrations of receptor, we are confident that the assays as developed here will be useful, for example, in the rapidly expanding field of high-throughput screening of ligand – receptor interactions. Experiments based on site-directed mutagenesis are in progress to determine whether and which amino acids are responsible for the acidity sensed by the bound probe, thus permitting to map the binding site.

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