Articles

Mapping the Antagonist Binding Site of the Serotonin Type 3 Receptor by Fluorescence Resonance Energy Transfer†

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ABSTRACT: We have measured fluorescence resonance energy transfer (FRET) between a fluorescent antagonist, bound to the purified detergent-solubilized serotonin type 3 receptor, and a lipophilic acceptor probe partitioned into the micelle surrounding the detergent-solubilized receptor. The experimentally observed FRET efficiency was evaluated on the basis of the characteristic dimensions of the receptor−micelle complex and the average number of acceptor molecules in such micelles. The binding site was determined to be 5.4 ± 0.9 nm above the center of the detergent micelle. The experiments were performed below the critical micellar concentration of the detergent ($C_{12}E_9$) used to solubilize the receptor, under which conditions it was demonstrated that the ligand binding activity was fully preserved. This reduces considerably the fluorescence background arising from probes not associated with the receptor, allowing a precise determination of the transfer efficiency.

In contrast to water-soluble proteins, most integral membrane proteins still resist high-resolution X-ray structure determination because it is difficult to obtain sufficient amounts of purified protein and to crystallize them. Today, only a few membrane proteins have been resolved at atomic detail. Any alternative noncrystallographic method for obtaining structural information about membrane proteins is therefore welcome. Fluorescence techniques have in this context experienced a renaissance for probing the structure and function of membrane proteins (*1*). In particular, fluorescence resonance energy transfer (FRET)[1] measurements provide a "molecular ruler" for determining molecular distances between so-called donor and acceptor chromophores (*2*, *26*). Among the various possibilities to introduce suitable donor−acceptor pairs into the system of interest, the most promising are the site-specific chemical labeling of amino acids in proteins (*3*), the genetic fusion of the green fluorescent protein (GFP) and analogues (*4*), the insertion of fluorescent derivatives of amino acids into proteins by suppressor tRNA technology (*5*), or the partitioning of fluorescent lipids into micelles or membranes (*6*).

This paper focuses on a ligand-gated ion channel: the serotonin type 3 receptor ($5HT_3R$)[2] which is involved in emesis caused by anti-cancer chemotherapy, in colonal dysfunction, and possibly in schizophrenia and drug abuse (*8*). The $5HT_3R$ assembles as a functional homopentameric receptor with a four-transmembrane domain topology predicted for each subunit based on sequence analysis, electron microscopy and biochemical experiments (*9*−*11*). We recently overexpressed the $5HT_3R$ in several mammalian cell lines and succeeded in purifying the functional receptor in detergent-solubilized form in sufficient quantities for biophysical studies (*11*).

Here, we determined by FRET measurements the height of the $5HT_3R$ ligand binding site above the center of the detergent micelle surrounding the purified receptor. Together with the known overall dimensions of the receptor (*10*), this allows positioning of the ligand binding site within the receptor.

To achieve this, FRET was measured between a receptor-bound fluorescent ligand acting as donor and amphipathic fluorescent acceptor molecules integrated in the receptor micelles. The antagonist GR-flu was chosen as the fluores-

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[1] Abbreviations: $5HT_3R$, serotonin type 3A receptor; $C_{12}E_9$, nonaethyleneglycol monododecyl ether; CMC, critical micellar concentration; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; GR-flu, 1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-amino-(*N*-fluorescein-thiocarbamoyl)-propyl)-4*H*-carbazol-4-one; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.

[2] Two subtypes of $5HT_3$ receptors are known, designated $5HT_{3A}$ and $5HT_{3B}$ (*7*). Here, we work exclusively with the $5HT_{3A}$ receptor, referred to as $5HT_3R$ in the text.

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cent donor because it was shown recently to bind in a 1:1 stoichiometry to the homopentameric $5HT_3R$ (*12*, *13*, *22*), thus simplifying the FRET data interpretation. This stoichiometry suggests that GR-flu is either a competitive antagonist, binding with very strong negative cooperativity to one of the five presumed agonist binding sites of the homopentameric receptor, or an allosteric inhibitor, binding on the symmetry axis of the receptor. To date, no experimental data are available for either of these propositions. The acceptor probe 2-dodecylresorufin was chosen as it offers optimal spectroscopic properties for measuring distances around 5 nm and was found to leave the biological activity of the receptor such as ligand binding intact upon micelle partitioning. Moreover, it shows a much lower fluorescence in aqueous phase than in micelles.[3] This is important as the main experimental difficulty inherent to this approach was to keep the fluorescence background arising from non-receptor-associated acceptor probe molecules as low as possible in order to allow for precise measurement of the fluorescence of the receptor-bound donor. For the same reason, we also worked below the critical micellar concentration (CMC) of $C_{12}E_9$, thus avoiding the partitioning of the acceptor probe into receptor-free detergent micelles. We found that the ligand binding activity of the receptor was fully preserved when working under such conditions. To test whether the receptor is still monomeric at low detergent concentration, fluorescence correlation spectroscopy (FCS) measurements were conducted.

Based on the dimensions of the $5HT_3R$ (*10*) and of the nicotinic acetylcholine receptor (*27*, *28*) obtained from low- and medium-resolution electron microscopic images, and from assumptions about the spatial distribution of the acceptor molecules in the receptor micelles, the position of the GR-flu binding site on the receptor was estimated from experimental FRET results. The determination of the binding site position represents an important step toward the complete 3-D structure of the $5HT_3R$. We are confident that the method presented here could be also applied to other membrane receptors.

MATERIALS AND METHODS

Materials. The $5HT_3R$ was heterologously overexpressed in mammalian cells and purified in detergent-solubilized form as described elsewhere (*11*). It was stored at −80 °C until use. 2-Dodecylresorufin (Molecular Probes, Eugene, OR) was dissolved in ethanol and stored at −15 °C until use. The $5HT_3R$ antagonist GR-flu [1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-amino-(*N*-fluorescein-thiocarbamoyl)-propyl)-4*H*-carbazol-4-one] was obtained from the former Geneva Biomedical Research Institute (Glaxo Wellcome, Geneva, CH). The compound was shown to be pure by high-pressure liquid chromatography and thin-layer chromatography. It was dissolved in dimethyl sulfoxide, aliquoted, and stored at a concentration of 1 μM at −80 °C. The ^{3}H-labeled radioligand [^{3}H]-GR65630 was from NEN-Du Pont (Boston, MA).

[3] These measurements (results not shown) were performed by absorbance and fluorescence spectroscopy. In both cases, a considerable increase of both the absorbance and the fluorescence intensity of dodecylresorufin was observed when upon increasing the concentration of $C_{12}E_9$, the detergent's CMC was reached.

Absorbance measurements were recorded using a Beckman DU7500i spectrophotometer (Beckman Coulter International S. A., Nyon, CH). Steady-state fluorescence spectra were acquired using a SPEX Fluorolog II (Instruments S. A., Stanmore, U.K.). They were smoothed using a Sawitzki-Golay filter of order 5 (*14*). Fifty microliter volume fluorescence cuvettes (Hellma, Müllheim, GE) were used.

Methods. The stock solution of the purified receptor contained 0.4 mM $C_{12}E_9$ (i.e., corresponding to 5 times the CMC of $C_{12}E_9$), and the concentration of binding sites was equal to 77 nM as determined by saturation radioligand binding assay as described elsewhere (*11*). Fifty-five microliters of a solution of 3 nM GR-flu in 10 mM HEPES, pH 7.4, was freshly prepared, mixed with 4 μL of the receptor solution, and incubated for 30 min at room temperature to reach binding equilibrium. A fluorescence spectrum was then acquired (λ_{ex} = 460 nm). One microliter of a particular concentration of the acceptor 2-dodecylresorufin in ethanol was mixed with the sample. Another spectrum was taken after 5 min, the time necessary to reach partitioning equilibrium. This procedure was repeated with different acceptor concentrations. Addition of corresponding volumes of pure ethanol did not affect the fluorescence spectrum of either receptor-bound GR-flu or free GR-flu. No change of the fluorescence signal of GR-flu free in solution was observed upon addition of 2-dodecylresorufin concentrations used in the FRET experiments, excluding collisional quenching from possible artifacts.

To correct for the contribution of 2-dodecylresorufin to the total fluorescence emission at the wavelength of maximal emission of GR-flu (516 nm) where the FRET efficiency is calculated, the experiments were repeated under identical conditions but without adding GR-flu. The experimental fluorescence intensities were also corrected by subtracting the contribution of free GR-flu, which cannot undergo FRET with 2-dodecylresorufin. For the experimental conditions applied, it can be calculated, using the determined dissociation constant for the receptor−ligand interaction (Table 1), that only 8−12% of the total amount of GR-flu was not receptor-bound, accounting for 26−37% of the fluorescence intensity.

FCS measurements were performed as described by Wohland et al. (*12*). Briefly, all measurements were done in 10 mM HEPES, pH 7.4, containing either 0.8 or 5 times the CMC of the detergent $C_{12}E_9$. The laser was focused into a droplet of 50 μL of 1.5 nM GR-flu solution deposited on a coverslip. The receptor was added from a stock solution of 4.8 nM in steps of 3, 4, 8, 7, and 30 μL. For every titration step, the fluorescence intensity was measured 10 times over a 30 s interval, and each trace was then autocorrelated.

MODEL

Molecular distances from the experimentally measured FRET efficiencies are evaluated on the basis of the model of the receptor−ligand complex integrating the following facts and hypotheses (Figure 1): (i) According to low-resolution electron microscopy images, the $5HT_3R$ has a cylindrical shape with a radius r_1 of 4 nm (*10*). This might be an oversimplification, as medium-resolution electron microscopy images demonstrated that the outer surface of the homologous nicotinic acetylcholine receptor is highly

irregular (*27*, *28*). (ii) The detergent $C_{12}E_9$ used to solubilize the receptor forms a circular belt with an outer radius of 2.6 nm that replaces the original lipid bilayer around the waist of the receptor (*15*−*17*). (iii) The 2-dodecylresorufin molecules partition into this micellar belt such that the chromophore moiety is distributed on the surface of a torus (see eq 3) described by an outer radius $r_2 = 1.5$ nm, the average length of the aliphatic dodecyl chains in the liquid state (*15*). The thickness of the hydrophobic part of the membrane around the nicotinic acetylcholine receptor has been reported to be approximately 30 Å (*27*), corresponding to twice the radius r_2 of the hydrophobic micelle core used in this model. (iv) The ligand binding site is assumed to be lying on the main symmetry axis of the receptor, which is the simplest model in accord with the experimental finding that only one GR-flu molecule is bound per receptor homopentamer (*12*). However, we cannot exclude that the binding site for GR-flu might be located elsewhere in the receptor protein (see Discussion).

The height h of the binding site above the hydrophobic waist of the receptor is determined by FRET measurements. To evaluate the FRET efficiency between a pointlike donor and acceptor molecules distributed over a two-dimensional surface, averaging over every allowed configuration and over the statistical distribution of the number of acceptors per micelle is required. We essentially adapted the calculations of Jones et al. (*18*) to our model and refer the reader to this publication and to Wolber et al. (*19*) for further details.

Briefly, the number of acceptor molecules per micelle is assumed to follow a Poisson distribution of average L. The transfer efficiency is then given by

$$E = 1 - \int_0^\infty e^{-[u+L(1-M(u))]}\,du \qquad (1)$$

where the configuration integral $M(u)$ is

$$M(u) = \int_R e^{-u(R_0/R)^6} W(R)\,dR \qquad (2)$$

R_0 is the Förster distance for the donor−receptor couple, and $W(R)$ is the distribution of the distance R between the donor and the acceptor. The configuration integral has to be evaluated in the case of our geometry, where the surface containing the acceptor is the outer half of a torus (Figure 1):

$$\begin{aligned} x &= r_1\cos(\alpha) + r_2\cos(\beta)\cos(\alpha) \\ y &= r_1\sin(\alpha) + r_2\cos(\beta)\sin(\alpha) \\ z &= r_2\sin(\beta) \end{aligned} \qquad (3)$$

with $\alpha \in [0, 2\pi]$ and $\beta \in [-\pi/2, \pi/2]$.

Expressing R as a function of β, the configuration integral can be rewritten

$$M(u) = \int_{-\pi/2}^{\pi/2} e^{-u(R_0/(r_1^2+r_2^2+2r_1r_2\cos(\beta)+h^2-2hr_2\sin(\beta))^{1/2})^6} \times \frac{2\pi(r_1 + r_2\cos(\beta))r_2}{S_0}\,d\beta \qquad (4)$$

where S_0 is the area of the surface in which the acceptor can move freely.

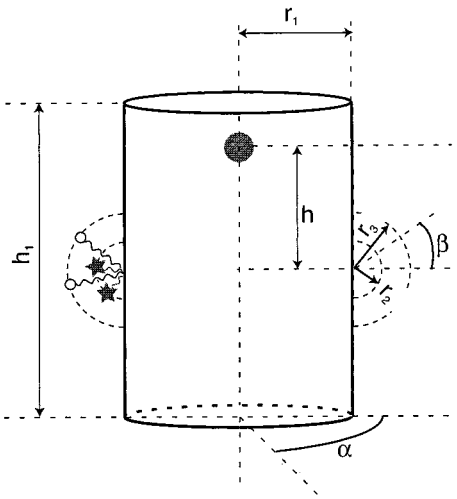

FIGURE 1: Model of the detergent-solubilized $5HT_3R$. The receptor is depicted as a cylinder (radius $r_1 = 4$ nm, height $h_1 = 11$ nm) surrounded by a torus (radius $r_3 = 2.6$ nm) of $C_{12}E_9$ detergent molecules. The fluorescence acceptor 2-dodecylresorufin (★) partitions into this torus at maximal depth with its dodecyl chain, shaping an inner torus with radius equal to the length of a dodecyl aliphatic chain ($r_2 = 1.5$ nm). The bound fluorescent donor GR-flu (●) is located on the main symmetry axis at an unknown height h above the center of the torus. The angles α and β are used to define the detergent torus (see eq 3).

The FRET efficiency was measured at various concentrations of acceptor in independent experiments, thus yielding information on how the acceptor probe partitions into receptor micelles. The data could be well fitted by the following expression:

$$L = N_{aggr}\frac{[a]}{[c] + [a]} \qquad (5)$$

giving the average number L of acceptor molecules partitioning into a receptor micelle. Here N_{aggr} is the number of detergent molecules surrounding the receptor, and [a] and [c] are the total molar concentrations of 2-dodecylresorufin and of detergent in the sample. Hence, in the concentration range studied, the acceptor probe behaves like a $C_{12}E_9$ molecule. The recovered height h of the binding site was the only free fitting parameter. The integrals were evaluated numerically using the *Mathematica* software (Wolfram Research, Inc., Champaign, IL).

RESULTS AND DISCUSSION

Determination of the Förster Distance for the FRET Couple 2-Dodecylresorufin−GR-flu. To determine the height h of the binding site with respect to the middle plane of the detergent torus (Figure 1), the Förster distance R_0 for the couple GR-flu−2-dodecylresorufin is required (eq 2). R_0 depends on the spectral properties and on the relative orientation of the two fluorophores (*20*):

$$R_0^6 = (8.8 \times 10^{-25})(\kappa^2 n^{-4}\phi_d J)\ (\text{cm}) \qquad (6)$$

where the spectral overlap integral J is

$$J = \int_0^\infty \lambda^4 F_D(\lambda)\epsilon_A(\lambda)\,d\lambda \qquad (7)$$

$F_D(\lambda)$ is the normalized fluorescence emission of the donor

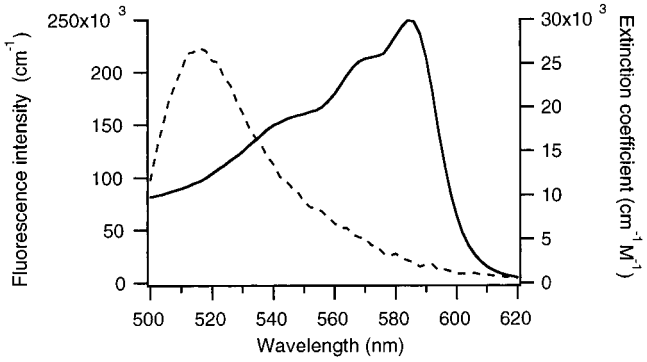

FIGURE 2: Calculation of the Förster distance. The normalized emission spectrum of receptor-bound GR-flu (dashed) overlaps with the absorption spectrum of 2-dodecylresorufin in $C_{12}E_9$ micelles (continuous line), resulting in a Förster distance $R_0 = 5.1 \pm 0.4$ nm.

at the wavelength λ (cm), $\epsilon_A(\lambda)$ is the extinction coefficient of the acceptor (M^{-1} cm^{-1}), ϕ_d is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium between the donor and acceptor, and κ^2 is a geometric factor that depends on the mutual orientation of the donor and acceptor chromophores.

The absorption spectrum of 2-dodecylresorufin has to be measured under identical conditions as used for FRET experiments because the spectroscopic properties of 2-dodecylresorufin are known to be sensitive to the environment (*21*). Unfortunately, it was not possible to obtain suitable absorption spectra under these conditions because of the relatively high concentrations of receptor required for such measurements. Therefore, the absorption spectrum of 2-dodecylresorufin in a buffer (10 mM HEPES, pH 7.4) containing 5 CMC (0.4 mM) of $C_{12}E_9$ was measured, and was shown to be identical to the fluorescence excitation spectrum of the acceptor in the presence of the receptor. This indicates that the environment probed by the acceptor in detergent micelles is similar to that in detergent−receptor micelles, justifying the use of the above measured absorption spectrum to evaluate the Förster distance. The emission spectrum of GR-flu in the presence of excess $5HT_3R$ was also measured, and the overlap integral (Figure 2) was finally calculated to be $J = 1.26 \times 10^{-13}$ M^{-1} cm^3. A value of $\kappa^2 = 2/3$ was used for the orientation factor, first, because the fluorescein moiety of receptor-bound GR-flu (donor) was shown by fluorescence anisotropy measurements to be highly mobile (*13, 22*) and, second, because the acceptor dipole moment is randomly oriented within the detergent micelle surrounding the receptor (Figure 1). This reduction in the uncertainty of κ^2 is an important advantage of using a randomly distributed acceptor probe. The refractive index of the medium between the donor and acceptor is not known precisely, and was assumed here to be that of water ($n = 1.33$). Taking instead a value generally taken for proteins of $n = 1.45$, R_0 would decrease by approximately 5%. The quantum yield of the donor, i.e., receptor-bound GR-flu, was estimated from its 2.5-fold lower fluorescence intensity as compared to the free ligand. This diminished fluorescence intensity is due to a decrease of both extinction coefficient and quantum yield. There is convincing evidence that the bound ligand senses a local pH of 6.2 (*13, 22*). Transfer from pH 7.4 (the bulk) to pH 6.2 (the binding site) results in an approximate 2-fold decrease of the absorption coefficient of fluorescein (*21*). Additionally, at pH 6.2 the quantum yield of GR-flu is reduced by a factor of 0.81 as compared to its quantum yield at pH 8. Assuming the quantum yield of GR-flu at pH 8 to be identical to that of fluorescein, for which an absolute quantum yield of 0.93 was determined elsewhere (*23*), a value of 0.75 is obtained for the quantum yield of receptor-bound GR-flu. Finally, a distance of $R_0 = 5.1 \pm 0.4$ nm was calculated for the Förster distance.

Table 1: Ligand Binding to $5HT_3R$ Solubilized by Different $C_{12}E_9$ Concentrations[a]

$C_{12}E_9$[b]	K_d (nM)	binding sites (pM)
5 CMC	0.43 ± 0.06	42 ± 8
0.5 CMC	0.42 ± 0.04	38 ± 6
0.2CMC	0.39 ± 0.04	37 ± 5

[a] Saturation binding of [^{3}H]-GR65630 to identical amounts of detergent-solubilized $5HT_3R$ at different $C_{12}E_9$ concentrations. The experimental data could be fitted well with Langmuir isotherms yielding dissociation constants K_d and concentrations of ligand binding sites. Data represent mean ± deviation of 2 experiments. [b] The CMC of $C_{12}E_9$ is 80 μM.

Table 2: Diffusion of $5HT_3R$ Solubilized by Different $C_{12}E_9$ Concentrations[a]

	autocorrelation time (μs)		
$C_{12}E_9$[b]	free GR-flu	GR-flu in micelles	$5HT_3R$-bound GR-flu
5.0 CMC	62.3 ± 2.7	485.8 ± 43.5	652.5 ± 347
0.8 CMC	57.8 ± 1.5		640 ± 162

[a] FCS measurements of GR-flu (1.4 nM) in the presence of increasing concentrations of detergent-solubilized $5HT_3R$ at $C_{12}E_9$ concentrations of 5 and 0.8 CMC. At a detergent concentration of 5 CMC, a 3-particle fit was needed to describe the experimental autocorrelation function, yielding the diffusion time of GR-flu free in solution, GR-flu partitioned in receptor-free detergent micelles, and GR-flu bound to the receptor. At a detergent concentration of 0.8 CMC, a 2-particle fit was sufficient, yielding diffusion times of GR-flu either free in solution or bound to the receptor, indicating the absence of receptor-free $C_{12}E_9$ micelles. The receptor does not aggregate below the CMC of $C_{12}E_9$ as similar autocorrelation times were measured for receptor-bound GR-flu at $C_{12}E_9$ concentrations above (5 times) and below (0.8 times) its CMC. [b] The CMC of $C_{12}E_9$ is 80 μM.

FRET measurements have been performed at a $C_{12}E_9$ concentration equivalent to 0.33 CMC of the pure detergent to abolish fluorescence from acceptor molecules in receptor-free detergent micelles. Under such conditions, the receptor is still functional and present in a nonaggregated form as shown by the following experiments. (i) Radioligand binding experiments performed at 5, 0.5, and 0.2 times the CMC of $C_{12}E_9$ yielded virtually identical values for both the dissociation constant and the total amount of ligand binding sites (Table 1). These data indicate that the ligand binding properties of the $5HT_3R$ are preserved below the CMC, and, moreover, that the receptor remained stable in solution. (ii) In addition, fluorescence correlation spectroscopy experiments were performed to check for receptor aggregation at detergent concentrations below the CMC. The experimental autocorrelation function at a $C_{12}E_9$ concentration of 5 CMC was well fitted by a 3-particle model, as GR-flu is present free in solution, partitions in detergent micelles, and binds to the $5HT_3R$ (Table 2) (*12*). Below the CMC of $C_{12}E_9$, a 2-particle model was sufficient to fit the experimental autocorrelation function, yielding diffusion times similar to those obtained for GR-flu free in solution and bound to the receptor above the CMC. These data demonstrate that at

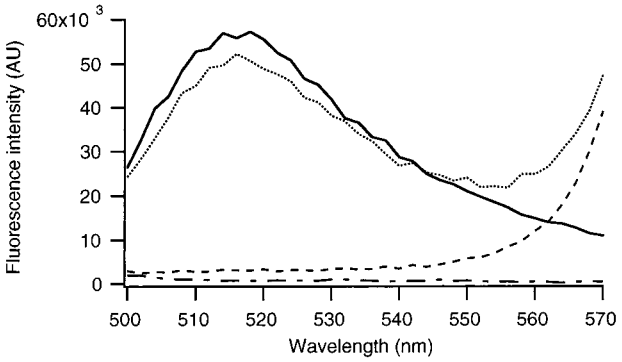

FIGURE 3: Typical FRET experiment is shown. The fluorescence emission spectrum of GR-flu (3 nM) bound by 5 nM $5HT_3R$. is measured before (continuous line) and after (dotted line) adding a 166 nM sample of the acceptor 2-dodecylresorufin ($\lambda_{ex} = 460$ nm). The contribution of 2-dodecylresorufin (dashed line) and the contribution of free GR-flu are subtracted for the FRET calculation. The blank measurement (dotted−dashed line) is done in the presence of receptor. Spectra are shown only to 570 nm for clarity.

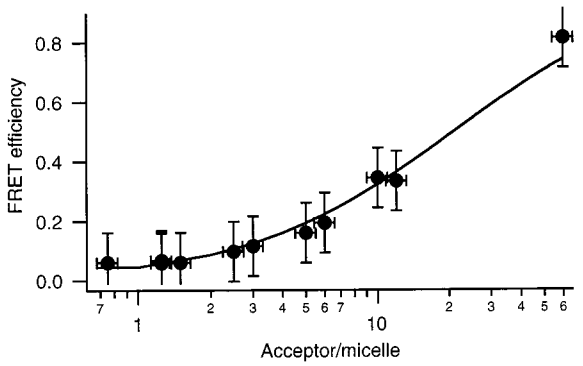

FIGURE 4: FRET efficiency E measured as a function of the average number L of acceptor 2-dodecylresorufin molecules in the detergent-receptor micelles. A value equal to 5.4 ± 0.9 nm was calculated for the height of the binding site above the waist of the receptor by fitting the data with eq 1.

$C_{12}E_9$ concentrations below the CMC the receptor is monomeric, and that no receptor-free micelles are present.

Position of the Binding Site. For estimation at which height h above the center of the micelle the ligand binding site of the $5HT_3R$ is located, N_{aggr}, the number of detergent molecules per receptor micelle, is required. A value of $N_{aggr} = 600 \pm 200$ was obtained by the following approaches. (i) N_{aggr} was estimated from the volumetric ratio between a free micelle and the detergent−receptor micelle presented in Figure 1. According to Tanford et al. (*15*), the volume of a $C_{12}E_9$ micelle is 63 nm^3, comprising 120 detergent molecules, close to the value of 124 measured by FCS (*12*). The volume of the detergent torus surrounding the receptor can be calculated from our geometrical model to be 267 nm^3, corresponding to 523 detergent molecules per detergent−receptor micelle. (ii) Under the experimental conditions used, the molar ratio between $C_{12}E_9$ (2.64 μM) and $5HT_3R$ (5.1 nM) was 515. (iii) The molecular mass of the detergent-solubilized ligand−receptor complex has been estimated from FCS measurements using four different fluorescent ligands; at a $C_{12}E_9$ concentration equal to 5 CMC, an average mass of 630 ± 100 kDa was obtained (*12*). On the basis of the amino acid sequence, a molecular mass of 270 kDa is calculated for the homopentameric $5HT_3R$, leaving 360 ± 100 kDa for the detergent belt, corresponding to 620 ± 170 detergent molecules.

To determine precisely the height h of GR-flu bound to the $5HT_3R$ with respect to the detergent micelle, the ligand−receptor complex was titrated with various concentrations of the FRET acceptor 2-dodecylresorufin in separate experiments (Figure 3). The FRET efficiency E was calculated from the fluorescence intensities of the donor in the presence (F_{da}) and in the absence (F_d) of acceptor using the relationship:

$$E = 1 - \frac{F_{da}}{F_d} \qquad (8)$$

Fluorescence intensities were measured at 516 nm, the emission maximum for GR-flu. FRET also resulted in an increase of the fluorescence of the acceptor, but this effect was not exploited to calculate transfer efficiencies. A value of $h = 5.4 \pm 0.9$ nm was finally obtained by fitting the dependence of FRET on L, the average number of acceptor molecules per receptor micelle (Figure 4).

This result has to be discussed in light of the assumptions made (i) on the localization of the binding site on the central axis of the receptor and (ii) on the shape of the receptor, i.e., the radii of the receptor (r_1) and micelle (r_2) used in the model. These possibilities were evaluated by adapting the configuration integral (eq 4) accordingly. Assuming that the GR-flu binding site is not located on the central axis reduces the height h. For example, a displacement of the binding site of 4 nm away from the central axis, i.e., the ligand would be present on the outer surface of the receptor, would yield a height h of 4.9 nm. This value is not significantly different from the height $h = 5.4 \pm 0.9$ nm obtained in the case where the binding site is located on the central symmetry axis of the receptor. Also, variations of ±10% in r_1 (±4 Å) and r_2 (±1.5 Å) lead to changes of about 1.5 and 3 Å, respectively, in the height h of the ligand binding site above the center of the detergent micelle. The apparent insensitivity of the height h to the geometry of the model is caused by the distance between the FRET donor and acceptor, of which the average is 7.3 or 7.8 nm when the binding site is located on or 4 nm away from the central axis. Accurate determination of distances in that range demands a FRET donor−acceptor pair having a Förster distance R_0 of approximately 75 Å, which is not available (*26*).

CONCLUSION

The experimental FRET results were evaluated according to the model presented in Figure 1 based on two major assumptions. First, the GR-flu binding site was positioned on the major symmetry axis of the $5HT_3R$. This is the simplest model to account for the findings by recent FCS (*12*) and time-resolved fluorescence spectroscopy (*13*) experiments that only one GR-flu molecule binds per homopentameric $5HT_3R$. This symmetrical model is also compatible with the absence of allosteric effects in the binding isotherms (Table 1). However, other locations of the ligand binding site cannot be excluded on the basis of the data presented. Second, the FRET acceptor 2-dodecylresorufin behaves like $C_{12}E_9$ with respect to micelle formation. This is strongly suggested by the structures of the 2-dodecylresorufin and $C_{12}E_9$ molecules in relation to the hydrophobic forces that pack the molecules together in a micelle.

Moreover, the substantial spectroscopic changes displayed by 2-dodecylresorufin upon insertion into receptor micelles indicate a dramatic change of the fluorophore's microenvironment. The fact that the experimental FRET data were described well by the model supports the assumptions made.

The stability of $5HT_3R$ at detergent concentrations below the CMC indicates that the hydrophohic transmembrane surface of the receptor is shielded from the water by the detergent. From the arguments presented before, at a $C_{12}E_9$ concentration of 0.33 CMC sufficient detergent is present to form a closely packed detergent torus around the receptor.

The height of the binding site above the center of the detergent torus was determined to be $h = 5.4 \pm 0.9$ nm. In turn, this value can be exploited to estimate the position of the binding site relative to a biological membrane if one assumes that a detergent micelle and a biological membrane are localized identically around the receptor. Comparing the relative magnitudes of the height *h* with a typical membrane thickness of 4 nm, the binding site protrudes clearly into the extracellular space by about 3.4 nm. It is interesting to compare our results with those obtained for a related ionotropic receptor, the nicotinic acetylcholine receptor from *Torpedo*. Arias et al. state that the binding site of acetylcholine is lying 3−3.5 nm above the membrane surface (*24*, *25*). This agrees well with our findings for the $5HT_3R$, and suggests that structurally the nicotinic acetylcholine receptor and $5HT_3R$ might be similar.

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