Ligand Binding to the Serotonin $5HT_3$ Receptor Studied with a Novel Fluorescent Ligand†

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ABSTRACT: The thermodynamics and kinetics of ligand binding to the purified serotonin $5HT_3$ receptor and the local environment of the bound ligand were studied by fluorescence spectroscopy using a novel fluorescein-labeled ligand GR-flu [1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-amino-(*N*-fluorescien-thiocarbamoyl)-propyl)-4*H*-carbazol-4-one]. Electrophysiological investigations demonstrated GR-flu to be an antagonist, and radioligand competition assays delivered a dissociation constant of 0.32 nM. Changes in the fluorescence intensity and anisotropy upon specific binding to the receptor yielded dissociation constants of ∼0.2 nM. Fluorescence measurements showed that selective $5HT_3$ receptor ligands competed for GR-flu binding with a rank order of potency identical to that established with the radioligand [^{3}H]-GR65630. The kinetics of GR-flu binding to the $5HT_3$ receptor revealed a bimolecular association process with an on-rate constant of 1.17×10^6 s^{-1} M^{-1} and a biphasic dissociation reaction with off-rate constants of 275×10^{-6} and 43×10^{-6} s^{-1}. The temperature dependence of the dissociation constant yielded an enthalpic term of −26 kJ mol^{-1} and an entropic term of 94 J K^{-1} mol^{-1} for the binding of GR-flu to the receptor, indicating that both quantities contribute equally to the reaction. An activation enthalpy $\Delta H^{\#}_{on}$ and entropy $\Delta S^{\#}_{on}$ of binding of 50 kJ mol^{-1} and 43 J mol^{-1} K^{-1} were obtained, indicating that the entropy facilitates the initial steps of GR-flu binding to the $5HT_3$ receptor. The fluorescence anisotropy of receptor-bound GR-flu and the environmental sensitivity of the fluorescent probe suggest that the binding site has a wide entrance and that it is 0.8 pH unit more acidic than the bulk solution.

The serotonin $5HT_3$[1] receptor is the only ligand-gated ion channel that has been found among the 14 known 5HT receptors (*1*). It is involved in rapid signal transduction in the central nervous system and the peripheral nervous system. Strong interest for this receptor has been provoked by the ability of $5HT_3$ receptor antagonists to treat emesis caused by anticancer chemotherapy (*2*). Moreover, antagonists for this receptor show promise for the treatment of colonal dysfunction (*3*).

Its gene structure (*4*) and amino acid sequence (*5*) indicate that the $5HT_3$ receptor resembles the channel-forming receptor proteins of the nicotinic acetylcholine (nACh) receptor family comprising the glycine receptor and the γ-aminobutyric acid type A ($GABA_A$) receptor. The receptors of this family are composed of five homologous subunits, each containing four transmembrane domains as inferred from hydropathy analysis (*6*). A common feature of ligand-gated ion channels is that agonist occupancy on the receptor leads to receptor activation followed by the opening of a transmembrane channel allowing ions to flow across the otherwise

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[1] Abbreviations: $5HT_3$ receptor, serotonin $5HT_3$ receptor; 5HT, 5-hydroxytryptamine, or serotonin; GR-flu, GR186741X, or 1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-amino-(*N*-fluorescien-thiocarbamoyl)-propyl)-4*H*-carbazol-4-one; GR-H, GR119566X, or 1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-aminopropyl)-4*H*-carbazol-4-one; [^{3}H]-GR65630, 3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-[^{3}H]-methyl-1*H*-indol-3-yl)-propanone; $C_{12}E_9$, nonaethyleneglycol monododecyl ether; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; nACh receptor, nicotinic acetylcholine receptor; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; EGTA, ethylene glycol *O*,*O*′,bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; K_d, dissociation constant of the ligand; K_i, dissociation constant obtained by inhibition of competitors; EC_{50}, half-maximal effective concentration; IC_{50}, concentration of a competing ligand that displaced 50% of the specific binding; k_{on}, association rate constant; k_{off}, dissociation rate constant; mCPBG, 1-(*m*-chlorophenyl)biguanide; PBG, phenylbiguanide; p*K*, −log(dissociation constant in M).

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impermeable membrane. Prolonged presence of agonists, however, results in a time-dependent, reversible closure of the ion channel; a process called desensitization (*1*).

Affinity cross-linking and site-directed mutagenesis studies have identified residues implicated in the ligand binding site and the channel-lining region of the nACh receptor (reviewed in refs *7* and *8*). From a comparison of the primary sequences of the different members of the nACh receptor family, it has been observed that conserved regions are involved in ligand binding (*9*).

Fluorescent receptor ligands have proven to be valuable tools for the investigation of the interactions of different receptors with their ligands in complement to mutagenesis studies. They have been used to study, for example, the mechanism of ligand binding (*10*−*12*), the movement and internalization of receptors in living cells (*13*), the distances between ligands and fluorescently labeled amino acids (*14*, *15*), the physical nature of the binding pocket (*16*), and the development of receptor-based biosensors (*17*, *18*).

Here we report the use of spectrofluorometric methods to study the interaction between the serotonin $5HT_3$ receptor and the fluorescent ligand GR-flu [1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-amino-(*N*-fluorescien-thiocarbamoyl)-propyl)-4*H*-carbazol-4-one]. Fluorescein was used to modify the high-affnity $5HT_3$ receptor ligand GR-H [1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-aminopropyl)-4*H*-carbazol-4-one] as its fluorescence is highly sensitive to local pH and polarity (*19*).

We first showed that GR-flu is a high-affinity antagonist for the $5HT_3$ receptor displaying a pharmacological profile virtually identical to that obtained with a radiolabeled ligand. The thermodynamic quantities involved in the binding of GR-flu to the $5HT_3$ receptor were determined from the temperature dependence of equilibrium binding and the kinetics of ligand binding measured by fluorescence spectroscopy. Comparison of the fluorescence spectra and their pH dependence of GR-flu free in bulk solution and bound to the receptor revealed an acidic character of the binding site of the $5HT_3$ receptor. Fluorescence anisotropy measurements indicated that the fluorescein moiety of receptor-bound GR-flu has a high mobility, suggesting that the binding site has a wide opening.

EXPERIMENTAL PROCEDURES

Materials

The radioligand 3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-[^{3}H]-methyl-1*H*-indol-3-yl)propanone ([^{3}H]-GR65630; 61 Ci/mmol) was from NEN-DuPont (Boston, MA). The antagonists granisetron, ondansetron, and 1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-aminopropyl)-4*H*-carbazol-4-one (GR-H) were obtained from Glaxo Wellcome (Geneva, Switzerland), as was the fluorescent ligand 1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-amino-(*N*-fluorescien-thiocarbamoyl)-propyl)-4*H*-carbazol-4-one (GR-flu). The agonists phenylbiguanide (PBG) and serotonin (5HT) were obtained from Aldrich (Buchs, Switzerland) and Sigma (Buchs, Switzerland), respectively, and quipazine and 1-(*m*-chlorophenyl)biguanide (mCPBG) from Tocris-Cookson (Langford, UK). Monododecyl nonaethylene glycol ($C_{12}E_9$) was from Fluka (Buchs, Switzerland). All other products used were of the highest quality available and purchased from regular sources.

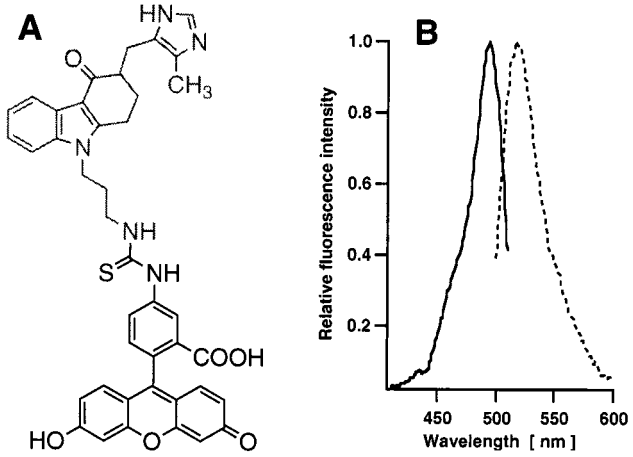

FIGURE 1: Chemical structure and fluorescence excitation/emission spectra of GR-flu: (A) The fluorescein moiety is attached via a thiourethane bound to the pharmacophore GR-H (gray). (B) GR-flu in 0.4 mM $C_{12}E_9$/10 mM HEPES, pH 7.4, exhibits an excitation spectrum with a maximum at 495 nm (emission at 520 nm, solid line) and an emission spectrum with a maximum at 520 nm (excitation at 495 nm, dashed line).

Methods

Preparation of Solubilized $5HT_3$ Receptor Purified from Mammalian Cells. A $5HT_3$ receptor, carrying a C-terminal hexa-histidine tag, was expressed in baby hamster kidney cells using Semliki Forest virus vectors (*20*). For purification, a suspension of cell membranes was solubilized in $C_{12}E_9$ and the receptor was purified in one step by immobilized metal-ion chromatography (*21*). The receptor was exchanged into 10 mM HEPES and 0.4 mM $C_{12}E_9$, pH 7.4, by gel filtration (Sephadex G-25 column, NAP-10, Pharmacia, Uppsala, Sweden). The preparation was stored at −80 °C until further use.

Preparation of the Fluorescent Ligand GR-flu. To characterize the $5HT_3$ receptor by fluorescence spectroscopy, GR-flu (Figure 1) was prepared by reacting GR-H with fluorescein-5-isothiocyanate (Molecular Probes, Eugene, OR) in the presence of triethylamine (dimethylformamide, 3 h at room temperature) and purifying by HPLC. Its fluorescence properties in aqueous solutions are similar to those of fluorescein: excitation maximum at 495 nm and emission maximum at 520 nm.

Electrophysiological Measurements. Human embryonic kidney 293 cells were transiently transfected using lipofectin (*22*) with 1 μg mL^{-1} of the mammalian reporter vector pCMVβ (Clontech, Palo Alto, CA) where the β-galactosidase gene was replaced by a 1.49 kb *Sma*I/*Not*I fragment containing the cDNA encoding the mouse $5HT_3$ receptor with or without C-terminal hexahistidine tag. Cotransfection with cytosolic green fluorescent protein (0.5 μg mL^{-1}; Clontech) was used to identify cells expressing the $5HT_3$ receptor. Whole-cell currents were recorded 18−48 h after transfection using an EPC9 patch-clamp amplifier (HEKA, Lambrecht, Germany), at an acquiring rate of 1 kHz; data were analyzed using the software Pulse and Pulsefit 8.02 (HEKA). Patch pipets (4−7 MΩ) contained (mM) 140 NaCl, 10 EGTA, and 10 HEPES. External solution was (mM) 147 NaCl, 10 HEPES, 12 glucose, 2 KCl, 2 $CaCl_2$, and 1 $MgCl_2$. Both

solutions were maintained at 300−315 mOsmol L^{-1} and pH 7.3, respectively. All experiments were performed at room temperature and at a holding potential of −60 mV. The agonist 5HT was applied by a U-tube delivery system. GR-flu and GR-H were added to both bath superfusate and U-tube solution. Antagonist concentration−inhibition curves were obtained from individual cells using a concentration of 30 μM 5HT and progressively increasing the antagonist concentration.

5HT and antagonist concentration−response curves for each cell were fitted by a least-squares method to

$$I = I_{max}/\{1 + (EC_{50}/[5HT])^n\} \quad (1)$$

and

$$I = I_o/\{1 + (IC_{50}/[\text{antagonist}])^{-n}\} \quad (2)$$

respectively, where I is the peak current in the presence of a certain ligand concentration, I_{max} is the maximal peak current inducible by 5HT, I_0 is the peak current in the absence of antagonist, EC_{50} and IC_{50} are the half-maximal effective and inhibitory concentrations, respectively, and n is the Hill coefficient. Curve fitting was performed with an iterative Levenberg−Marquardt algorithm minimizing chi-square using the software package Igor (WaveMetrics Inc., Lake Oswego, OR). The graphs were drawn by averaging results from all experiments and fitting a single concentration−response curve to the combined data.

Binding Assay Using Radioligands. The amount of ligand binding sites was determined by incubation of samples containing ~0.2 pmol of $5HT_3$ receptor for 30 min at room temperature in 10 mM HEPES/0.4 mM $C_{12}E_9$, pH 7.4, with six concentrations of [^{3}H]-GR65630, ranging from 0.1 to 40 nM in a final volume of 1 mL. The incubation was terminated by rapid filtration through Whatman GF/B filters [presoaked for 15 min in 0.5% (w/v) polyethylenimine] followed by two washes with 3 mL of ice-cold 10 mM HEPES, pH 7.4. Filters were transferred into scintillation vials, and 4 mL of Ultima Gold (Packard, Meridan, CT) was added. The radioactivity was measured in a Tri-Carb 2200CA liquid scintillation counter (Packard) and corrected for quenching and counting efficiency. Nonspecific binding was determined in the presence of 1 μM quipazine. All experiments were performed in triplicate.

The binding affinity of the receptor to different pharmacologically active compounds was determined by competition binding assays. Samples containing 0.2 pmol of $5HT_3$ receptor, ~0.8 nM [^{3}H]-GR65630 in 10 mM HEPES/0.4 mM $C_{12}E_9$, pH 7.4, and increasing concentrations of the competitors in a final volume of 1 mL were incubated for 30 min at room temperature. The dissociation constant K_d of [^{3}H]-GR65630, the total concentration of $5HT_3$ receptor (expressed as concentration of binding sites), and Hill coefficients n were evaluated by fitting experimental data with the following binding isotherm:

$$[\text{GR-65630}]_{bound} = [5HT_3\text{-R}]_{total}/\{1 + (K_d/[\text{GR-65630}]_{free})^n\} \quad (3)$$

Binding inhibition curves were fitted to

$$B = B_0/\{1 + (IC_{50}/[\text{competitor}])^{-n}\} \quad (4)$$

where B and B_0 are the bound [^{3}H]-GR65630 in the presence and absence of unlabeled competitor, respectively, and IC_{50} is the concentration of competing ligand that displaced 50% of the specifically bound [^{3}H]-GR65630. The dissociation constant of inhibition K_i of competitors was estimated from the Cheng−Prusoff equation (*23*):

$$K_i = IC_{50}/\{1 + ([L]/K_d)\} \quad (5)$$

where [L] and K_d are the concentration and the dissociation constant of [^{3}H]-GR65630, respectively.

Binding Assay by Fluorescence Measurements. Fluorescence measurements were performed on a SPEX Fluorolog II (Instruments S.A., Stanmore, U.K.) using 1.0 and 1.35 nm band-passes for excitation and emission, respectively. Quartz cuvettes of 10 × 4 × 25 mm^3 or 3 × 3 × 5 mm^3 (Hellma, Müllheim, Germany) were placed in a temperature-controlled holder; the larger cuvette was continuously stirred with a magnetic bar. All spectra shown were obtained after blank subtraction and smoothing using the Sawitzky−Golay procedure (five points, second order, software Igor).

Routinely, the binding of GR-flu to the purified $5HT_3$ receptor was followed in real time by measuring continuously the concomitant fluorescence intensity change of the ligand at 20 °C. A solution of GR-flu in 10 mM HEPES/0.4 mM $C_{12}E_9$, pH 7.4, was prepared, and the fluorescence was measured at 520 nm upon excitation of the chromophore at 495 nm. $5HT_3$ receptor was added to obtain final concentrations of GR-flu of 2 or 4 nM and of receptor ranging from 0.3 to 8 nM. Upon establishment of a final stable fluorescence intensity, excitation spectra were acquired (emission at 520 nm). Possible fluorescence changes arising from nonspecific GR-flu interactions were determined by preincubating the $5HT_3$ receptor with 1 μM of the nonfluorescent ligand quipazine before its addition to the fluorescent ligand. All experiments were performed at least twice.

The dissociation constant K_d and the Hill coefficient n for the interaction of GR-flu with the $5HT_3$ receptor were determined by fitting the binding isotherm to the experimental fluorescence intensity data F_i at the particular receptor concentrations $[5HT_3\text{-R}]_i$ to

$$F_i = F_0 + \Delta F_{max}/\{1 + (K_d/[5HT_3\text{-R}]_i)^n\} \quad (6)$$

where F_0 is the fluorescence of GR-flu in the absence of receptor and ΔF_{max} the decrease in fluorescence in the presence of a large excess of receptor.

k_{on}, the rate constant of binding of GR-flu to the receptor, was obtained from the dependence of the initial rate of the fluorescence change observed upon the addition of various concentrations of $5HT_3$ receptor (0.3−8 nM) to a GR-flu solution (final concentrations 2 or 4 nM). Therefore, the time course of the fluorescence intensity signal F_t was converted to the concentration of receptor-bound GR-flu according to the relation

$$[\text{GR-flu}]_{bound,t} = [\text{GR-flu}]_{total}(F_0 - F_t)/\Delta F_{max} \quad (7)$$

where $[\text{GR-flu}]_{bound,t}$ is the concentration of GR-flu bound to the receptor at time t, $[\text{GR-flu}]_{total}$ the total GR-flu concentration, and F_0 is the fluorescence signal observed in

the absence of receptor. Alternatively, the time courses of the binding of GR-flu to the different receptor concentrations were fitted to an integrated rate equation which assumes a 1:1 interaction binding of ligand to receptor that takes the changes in free receptor and ligand concentrations during the progression of the binding reaction into account (*24*). The increase of receptor-bound GR-flu with time was fitted by the integrated form of

$$d[\text{GR-flu}]_{\text{bound},t}/dt = k_{\text{on}}[\text{R}]_0[\text{GR-flu}]_{\text{free},t} - k_{\text{off}}[\text{GR-flu}]_{\text{bound},t} \quad (8)$$

giving

$$[\text{GR-flu}]_{\text{bound},t} = [\text{GR-flu}]_{\text{total}} - \frac{b[de^{ft} - 1] + f[de^{ft} + 1]}{2c[1 - de^{ft}]} \quad (9)$$

where $a = k_{\text{off}}[\text{GR-flu}]_{\text{total}}$, $b = k_{\text{on}}([\text{GR-flu}]_{\text{total}} - [\text{R}]_{\text{total}}) - k_{\text{off}}$, $c = -k_{\text{on}}$, $d = (-2k_{\text{on}}[\text{GR-flu}]_{\text{total}} + b - f)/(-2k_{\text{on}}[\text{GR-flu}]_{\text{total}} + b + f)$, and $f = (b^2 - 4ac)^{0.5}$ and where $[\text{R}]_{\text{total}}$ and $[\text{GR-flu}]_{\text{total}}$ represent the total concentrations of $5HT_3$ receptor and GR-flu, which are known and entered and held fixed during fitting using the global fitting routine of the software Igor.

k_{off}, the rate constant of dissociation of GR-flu from the $5HT_3$ receptor, was determined by dissociating bound GR-flu from the receptor by the addition of 100 μM of a competing nonfluorescent ligand to 2.8 nM $5HT_3$ receptor pre-equilibrated with 2 nM GR-flu. The increase with time of the fluorescence intensity of the GR-flu was fitted by a sum of exponential functions.

The changes in enthalpy $\Delta H°$ and entropy $\Delta S°$ accompanying the binding of GR-flu to the $5HT_3$ receptor were obtained from the evaluation of K_d measured at a range of temperatures T according to the van't Hoff equation

$$\ln(K_d) = (\Delta H°/RT) - (\Delta S°/R) \quad (10)$$

where R is the gas constant. The activation enthalpy $\Delta H^{\#}_{\text{on}}$ and activation entropy $\Delta S^{\#}_{\text{on}}$ of binding were evaluated using the Eyring equation

$$\ln(k_{\text{on}}/T) = (\Delta S^{\#}_{\text{on}}/R) - (\Delta H^{\#}_{\text{on}}/RT) + \ln(k/h) \quad (11)$$

with the Boltzmann constant k and the Planck constant h.

The affinity of various pharmaceutically interesting, nonfluorescent ligands for the $5HT_3$ receptor was determined by a competition assay: 2 nM GR-flu and 3.6 nM $5HT_3$ receptor were incubated with a wide range of competitor concentrations for 30 min at room temperature, after which time an excitation spectra was recorded as above. Changes in fluorescence intensity versus competitor concentration were fitted by a binding isotherm, yielding particular values of the Hill coefficient and IC_{50}, from which the dissociation constant K_i of the corresponding competitor was estimated as described above.

Fluorescence of GR-flu in Different Solvents. Dependence of the excitation spectra of GR-flu on the polarity of the solvent was studied by using mixtures of increasing amounts of dioxane in 10 mM HEPES, pH 7.4. The influence of pH on the excitation spectra of GR-flu was investigated in 10 mM buffers of acetate (pH 3.8−5.4), phosphate (pH 6.2−7.0), or Tris (pH 7.4−9.0), with or without 0.4 mM $C_{12}E_9$. Individual pH values were adjusted with either NaOH or HCl to an accuracy of 0.05 pH unit.

Fluorescence Anisotropy of GR-flu Bound to the $5HT_3$ Receptor. Glan Taylor polarizers (Halbo Optics, Chelmsford, U.K.) were placed in the excitation and emission light paths directly adjacent to the cuvette holder to enable polarized excitation and emission detection. Excitation was at 490 nm, and fluorescence was measured at 520 nm either with a 1.8 nm band-pass or with omission of the emission monochromator using an OG530 high pass filter (Schott, Mainz, Germany) to remove scattered excitation light. The anisotropy r of GR-flu was calculated from the vertically I_{vv} and horizontally I_{vh} polarized fluorescence intensity measured upon vertical excitation

$$r = \frac{I_{\text{vv}} - GI_{\text{vh}}}{I_{\text{vv}} + 2GI_{\text{vh}}} \quad (12)$$

where G is the ratio of the vertically I_{hv} and horizontally I_{hh} polarized fluorescence intensity measured upon horizontal excitation to correct for the different detection efficiencies of vertically and horizontally polarized fluorescence. For both setups values of 1.00 ± 0.02 were obtained for G.

GR-flu (2 nM) was equilibrated with $5HT_3$ receptor (0−20 nM) at 25 °C for 1 h before determination of the anisotropy of GR-flu. Nonspecific effects due to the presence of the receptor on the anisotropy of GR-flu were measured in the presence of 1 μM quipazine.

The anisotropy of receptor-bound GR-flu r_{bound} was obtained by fitting the dependency of the observed anisotropy r_{exp} on the concentration of the $5HT_3$ receptor to

$$r_{\text{exp}} = \frac{r_{\text{bound}}[5HT_3\text{-R}]_{\text{free}}F_{\text{rel}} + r_{\text{free}}K_d}{[5HT_3\text{-R}]_{\text{free}} + K_d} \quad (13)$$

where r_{free} is the anisotropy of GR-flu in the absence of receptor and F_{rel} the ratio of fluorescence intensity of receptor-bound to free GR-flu. The segmental motion of receptor-bound GR-flu was estimated from

$$r_{\text{bound}} = r_{\text{calc}}[0.5(3\cos^2\beta - 1)] \quad (14)$$

where r_{calc} is the anisotropy calculated for a fluorescein moiety immobilized to a $5HT_3$ receptor of 280 kDa (*21*) and β the mean angle over which the fluorophore is free to rotate (*25*). The fluorescence lifetime and the limiting anisotropy of GR-flu were determined to be 3.6 ns and 0.21, respectively (data not shown).

RESULTS

In the following we present a detailed characterization of the interaction of the fluorescent ligand GR-flu with a histidine-tagged serotonin $5HT_3$ receptor. The receptor was expressed with the aid of Semliki Forest virus vectors in suspension cultures of baby hamster kidney cells, resulting in high yields of recombinant receptor protein (*20*, *26*), and purified as earlier described (*21*). In the first part, the new fluorescent ligand GR-flu was demonstrated to be a competitive antagonist for the $5HT_3$ receptor using electrophysiology and radioligand binding assays. Fluorescence spectroscopy was then used to determine the thermodynamics and kinetics

of binding GR-flu to the $5HT_3$ receptor, as well as to probe the molecular environment of the ligand binding site of the receptor. Finally, the pharmacology of the receptor was determined by a fluorescence assay.

Electrophysiological Measurements. The electrophysiological behavior of the GR-flu and the parent pharmacophore GR-H was investigated in mammalian cells transfected with plasmids encoding the $5HT_3$ receptor. Both the wild type receptor and the C-terminally His-tagged receptor were studied.

First, the concentration dependence of 5HT activation was determined, and for both receptors an EC_{50} of 5 ± 2 μM and a Hill coefficient close to unity were obtained (data not shown). Neither GR-flu nor GR-H showed agonist activity at concentrations up to 30 nM. Subsequently, the effect of GR-flu on 5HT-evoked currents was investigated. GR-flu inhibited 5HT-evoked currents in a dose-dependent fashion with an IC_{50} of 0.46 nM ($K_i = 0.066$ nM) and a Hill coefficient of 1.49 (Figure 2 and Table 1). The nonlabeled ligand GR-H displayed similar behavior, with a slightly lower IC_{50} of 0.085 nM ($K_i = 0.011$ nM) and a Hill coefficient of 1.09. Data shown in Figure 2 are from cells expressing the wild type $5HT_3$ receptor; similar results were obtained from cells expressing the His-tagged $5HT_3$ receptor (data not shown).

Radioligand Binding Assays. The affinity of the GR-flu for the purified His-tagged $5HT_3$ receptor, solubilized in detergent, was determined by a competition assay using the radioligand [^{3}H]-GR65630, which strongly interacts with the receptor showing a K_d of 0.24 nM and a Hill coefficient of unity (*21*). When the concentration of GR-flu increased, the binding of [^{3}H]-GR65630 to the $5HT_3$ receptor decreased in a monophasic manner (Figure 3) characterized by an IC_{50} of 1.19 nM ($K_i = 0.27$ nM) and a Hill coefficient of 1.4. The parent GR-H inhibited radioligand binding with an IC_{50} of 0.27 nM ($K_i = 0.06$ nM) and a Hill coefficient of 1.1. Table 1 summarizes the results of several experiments.

GR-flu Binding to the $5HT_3$ Receptor by Fluorescence Measurements. Electrophysiological measurements and radioligand displacement experiments established that the new fluorescent ligand GR-flu is a high-affinity antagonist for the $5HT_3$ receptor (Table 1). Therefore, fluorescence spectroscopy was applied to monitor its binding to the receptor.

In buffer solution, GR-flu exhibited excitation and emission spectra similar to those of fluorescein (Figure 1). Upon addition of $5HT_3$ receptor to GR-flu, the fluorescence intensity decreased, reaching a limiting value after some time, depending on the concentration of the interacting species (Figure 4A). The fluorescence intensity changed in a dose-dependent fashion, and at sufficiently high receptor concentrations the final signal decrease approached a saturation value (curves f and g). To prove that the observed decrease in fluorescence intensity was effectively due to binding of GR-flu to the $5HT_3$ receptor, the receptor was preincubated with a large excess (1 μM) of the nonfluorescent ligand quipazine ($K_i = 1.4$ nM versus GR-flu; Table 3): subsequent addition to GR-flu caused no change in fluorescence (curve h). This indicates that the fluorescence intensity decrease observed after the addition of $5HT_3$ receptor to GR-flu (Figure 1 curves a−g) is a consequence of the specific binding of GR-flu to the $5HT_3$ receptor.

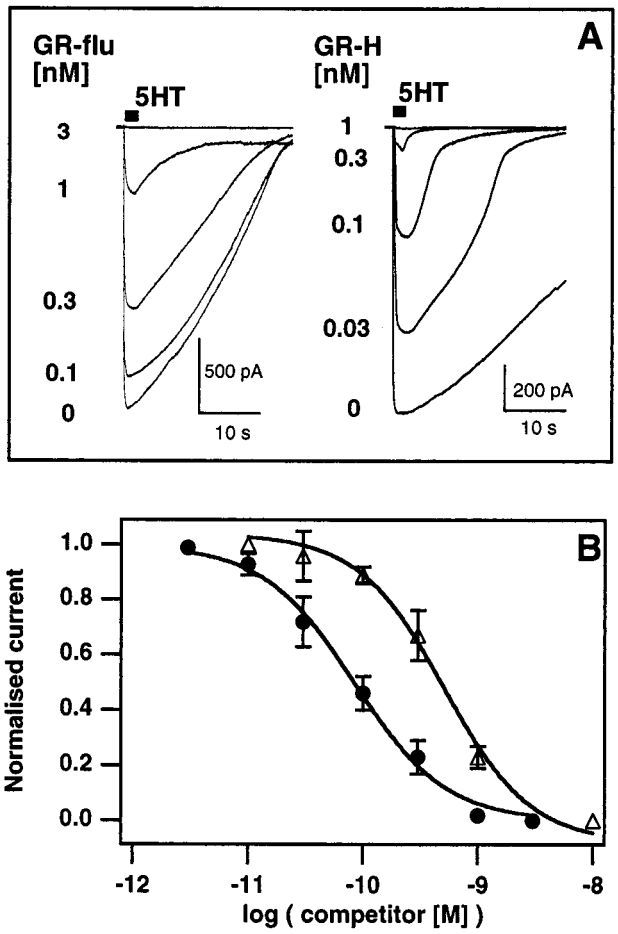

FIGURE 2: Antagonistic effects of GR-flu and GR-H versus 5HT-induced ion currents. Human embryonic kidney 293 cells transfected with $5HT_3$ receptor encoding plasmids were stimulated with 30 μM 5HT ($EC_{50} = 5$ μM): (A) superimposed recordings of currents evoked in individual cells by applying 30 μM 5HT for 2 s (indicated as a bar on top of the recordings) in the absence and presence of increasing ligand concentrations of the fluorescent ligand GR-flu (left) and GR-H (right). (B) Peak currents observed in the presence of either GR-flu (△) or the nonmodified ligand GR-H (●) were normalized to the peak current in the absence of antagonist. The inhibition curves were fitted by the Hill equation, yielding Hill coefficients of 1.49 ± 0.08 and 1.09 ± 0.08 and IC_{50} values of 0.46 ± 0.04 and 0.085 ± 0.006 nM resulting in dissociation constants of 71 ± 4 and 11 ± 1 pM for GR-flu and GR-H, respectively. Each point represents the mean value ± SD of three to five experiments.

Evaluation of the equilibrium fluorescence intensities yielded a K_d of 0.24 nM and a Hill coefficient of 0.8 (Figure 4B); similar values have been obtained from the radioligand competition assays (Table 1).

The initial rate of GR-flu binding to the $5HT_3$ receptor, determined from the time course of the decrease of the GR-flu's fluorescence upon receptor addition, was linearly dependent on the receptor concentration, yielding an on-rate constant of $(1.17 \pm 0.08) \times 10^6$ s^{-1} M^{-1} (Figure 4C). This observation indicates that the binding of GR-flu to the $5HT_3$ receptor is first order with respect to the receptor concentration. Repeating the experiment with a 2-fold higher concentration of GR-flu, that is, 4 nM, resulted in 2-fold higher initial rates of GR-flu binding and an on-rate constant of $(1.1 \pm 0.1) \times 10^6$ s^{-1} M^{-1}. This indicates that the binding of GR-flu to the $5HT_3$ receptor is also first order with respect

Table 1: Affinity of GR-flu and GR-H to the $5HT_3$ Receptor[a]

	GR-flu		GR-H	
	pK[b]	Hill coefficient	pK[b]	Hill coefficient
electrophysiology	10.18 ± 0.03	1.49 ± 0.08	10.92 ± 0.04	1.09 ± 0.08
radioligand assay	9.49 ± 0.20	1.37 ± 0.07	9.92 ± 0.42	1.11 ± 0.07
fluorescence				
equilibrium binding	9.62 ± 0.23	0.80 ± 0.30		
kinetics	9.58 ± 0.10	1[c]		
anisotropy	9.66 ± 0.08	1[c]		

[a] Radioligand competition assays were performed by incubating purified $5HT_3$ receptor with 0.8 nM [^{3}H]-GR65630 (K_d = 0.24 nM) and increasing concentration of competitor (GR-flu or GR-H); the results were fitted to binding isotherms. Electrophysiological measurements were performed on human embryonic kidney 293 cells transfected with $5HT_3$ receptor encoding plasmids. Inhibition curves were obtained by stimulating the cells with 30 μM 5HT (EC_{50} = 5 μM) at a particular competitor concentration. The observed peak currents were fitted by a binding isotherm yielding the concentration of half-inhibition IC_{50}, from which the dissociation constant of each ligand was calculated (see Methods). Fluorescent assays were performed by mixing 2 nM GR-flu with increasing amounts of purified, detergent-solubilized $5HT_3$ receptor (0.3−20 nM). Final equilibrium fluorescence intensity changes and anisotropy values were fitted by binding isotherms. Evaluation of the kinetics of binding and dissociation yielded rate constants of binding and dissociation from which the dissociation constant is calculated. For details see text. Data are mean values ± SD for two to five experiments. [b] pK is −log(dissociation constant in M). [c] A Hill coefficient of 1 was assumed in the evaluation.

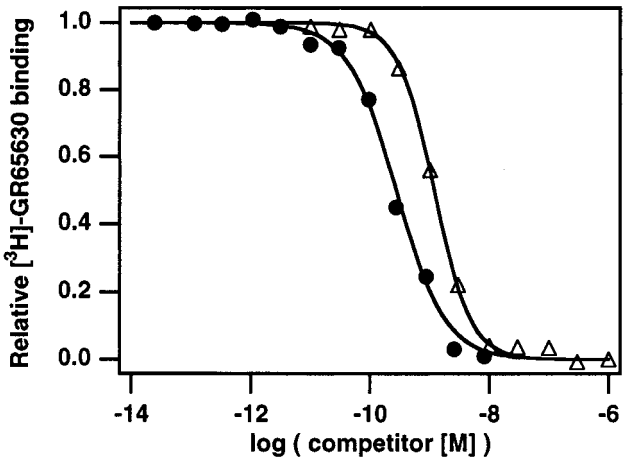

FIGURE 3: Inhibition of radioligand binding to purified $5HT_3$ receptor by GR-flu and GR-H. Radioligand displacement by competing ligands was assessed by incubating the purified 0.2 nM $5HT_3$ receptor and 0.8 nM [^{3}H]-GR65630 (K_d = 0.24 nM) with GR-flu (△) or the parent compound GR-H (●) at the indicated concentrations. The specific binding of the radioligand was inhibited progressively by increasing competitor concentrations. The data obtained for GR-flu and GR-H were fitted to binding isotherms and yielded Hill coefficients of 1.42 ± 0.08 and 1.06 ± 0.10 and half-inhibition concentrations IC_{50} values of 1.19 ± 0.05 and 0.27 ± 0.03 nM, resulting in dissociation constants of 0.27 ± 0.02 and 0.06 ± 0.01 nM, respectively. Data of a representative experiment are shown.

to the GR-flu concentration. Moreover, an integrated rate equation, describing a binding reaction with a 1:1 ligand/receptor stoichiometry, which takes into account depletion of reactants during binding (*24*), was used to fit the time course of fluorescence decrease. As can be seen in Figure 4A, the fits describe the experimental traces well. The on-rates thus obtained were identical to the on-rates determined from the initial rates of binding in Figure 4C.

The binding of GR-flu to the $5HT_3$ receptor can thus be described by a bimolecular reaction, where a single ligand molecule binds to one receptor.

Dissociation of GR-flu from the $5HT_3$ Receptor. (a) Fluorescence Measurements. To gain more insight into the mechanism of the ligand binding reaction, we studied the dissociation of the GR-flu/receptor complex induced by the addition of excess concentrations of a competitive, nonfluorescent ligand. This is illustrated in Figure 5A for the agonist mCPBG: the dissociation occurred over a very long time period (up to 20 h). Moreover, the fluorescence intensity increase appeared to be biphasic and was well fitted by a biexponential function, yielding fast and a slow dissociation rate constants of 255 × 10^{-6} s^{-1} and 43 × 10^{-6} s^{-1}, respectively (Table 2). This was similar for all competitive ligands used, irrespective of whether they were agonists or antagonists. The relative contribution of the two phases varied between 30 and 70% without a clear pattern. The fast dissociation rate constant agrees relatively well to the product of the K_d determined from equilibrium measurements (Figure 4B) and the on-rate constant k_{on} (Figure 4C): $K_d k_{on}$ = 280 × 10^{-6} s^{-1}.

(b) Electrophysiology Measurements. In a second approach the dissociation of GR-flu from the $5HT_3$ receptor was investigated by dilution. $5HT_3$ receptor expressing cells were preincubated with a saturating concentration of GR-flu to completely block 5HT activation of currents. To remove the GR-flu, the cells were washed with a continuous stream of buffer without this ligand while challenging the cells each 2 min with a short pulse of 5HT. After a lag phase of ∼5 min, the 5HT-evoked current response recovered in the first instance relatively rapidly to ∼55% of the initial value after ∼1500 s; further recovery was very slow (Figure 5B).

These observations made by fluorescence spectroscopy and electrophysiology suggest that the mechanism of ligand−receptor interaction is more complex than R + L ⇔ RL, possibly involving either a slow conformational change after ligand binding or the binding of additional ligand molecules.

Thermodynamics and Kinetics of GR-flu Binding to the $5HT_3$ Receptor. The thermodynamics and kinetics of binding reaction of GR-flu to the receptor deliver important information on the nature of the corresponding ligand−receptor interaction.

First, the binding of GR-flu to the $5HT_3$ receptor was determined at different temperatures between 5 and 35 °C. The affinity of the fluorescent ligand for the receptor decreased with increasing temperature. Figure 6A shows an evaluation of the data according to the van't Hoff relation, yielding a change in the enthalpy of ΔH° = −26 ± 9 kJ mol^{-1} and in the entropy of ΔS° = 94 ± 32 J mol^{-1} K^{-1};

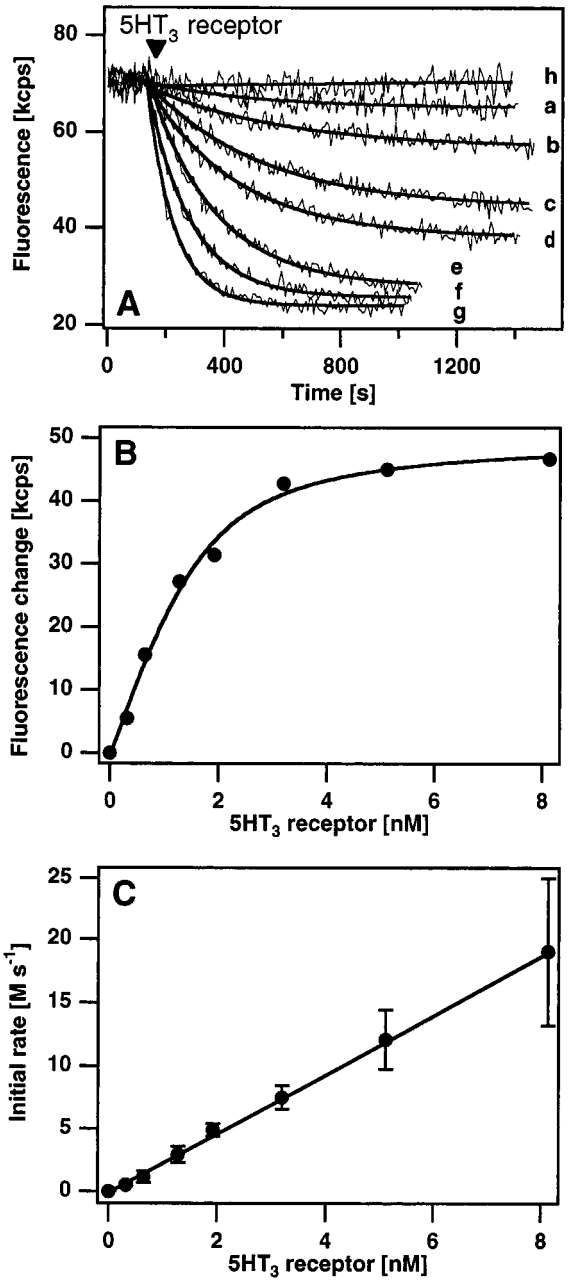

FIGURE 4: Binding of GR-flu to the $5HT_3$ receptor observed by fluorescence spectroscopy: (A) Increasing concentrations of purified $5HT_3$ receptor were added to a solution of 2 nM GR-flu, yielding the following final nanomolar receptor concentrations: (a) 0.32, (b) 0.64, (c) 1.28, (d) 1.92, (e) 3.20, (f) 5.12, (g) 8.13. The binding of GR-flu to the $5HT_3$ receptor was observed in real-time by measuring the concomitant fluorescence decrease at 520 nm upon excitation at 490 nm. To prove that the fluorescence decrease resulted from the specific binding of GR-flu to the receptor, the $5HT_3$ receptor was preincubated with 1 μM of the nonfluorescent ligand quipazine ($K_i = 1.4$ nM) before addition to GR-flu; no change in fluorescence intensity was observed (h). The time course of fluorescence decrease due to the binding of GR-flu to the $5HT_3$ receptor was fitted to the integrated rate equation (see Methods), which takes into account the concentration change of reactants with time. (B) The affinity of GR-flu for the receptor was determined by plotting the fluorescence intensity decrease at equilibrium versus the receptor concentration and fitting with the Hill equation. A dissociation constant of 0.24 ± 0.08 nM and a Hill coefficient of 0.8 ± 0.3 were obtained. Data are representative of two independent experiments. (C) The association rate constant of GR-flu binding to the $5HT_3$ receptor was estimated to be $k_{on} = 1.17 \pm 0.08 \times 10^6$ s^{-1} M^{-1} from the linear dependence of the initial rate of fluorescence change on the receptor concentration.

Table 2: Displacement of the GR-flu Bound to the $5HT_3$ Receptor by Nonfluorescent Ligands[a]

	rate constant of GR-flu dissociation from the $5HT_3$ receptor	
displacing ligand	$k_{off,1}$ ($s^{-1} \times 10^{-6}$)	$k_{off,2}$ ($s^{-1} \times 10^{-6}$)
antagonist		
granisetron	261 ± 14	42.8 ± 0.6
ondansetron	257 ± 15	42.3 ± 1.8
agonist		
quipazine	284 ± 11	42.2 ± 1.4
mCPBG	255 ± 12	43.2 ± 1.7
5HT	308 ± 11	46.7 ± 0.6
PBG	274 ± 17	40.0 ± 1.2

[a] The GR-flu bound to the $5HT_3$ receptor was displaced by high concentrations (0.1 mM) of pharmaceutically active ligands at 20 °C. The apparent biphasic change of the fluorescence intensity was described by a sum of two exponentials, where $k_{off,1}$ and $k_{off,2}$ are the corresponding apparent dissociation rate constants of the faster and slower phases, respectively. Average values ± SD are indicated.

Table 3: Pharmacology Profile of Various Ligands for the $5HT_3$ Serotonin Receptor[a]

	GR-flu		[^{3}H]-GR 65630	
competitor	pK_i	Hill coefficient	pK_i	Hill coefficient
antagonist				
GR-H	9.80 ± 0.13	1.46 ± 0.13	9.92 ± 0.42	1.11 ± 0.07
granisetron	8.94 ± 0.06	1.11 ± 0.16	9.42 ± 0.03	0.93 ± 0.09
ondansetron	8.35 ± 0.12	1.04 ± 0.04	8.76 ± 0.04	1.08 ± 0.11
agonist				
quipazine	8.86 ± 0.04	1.08 ± 0.09	9.33 ± 0.07	1.00 ± 0.09
mCPBG	7.73 ± 0.03	0.82 ± 0.04	8.29 ± 0.02	0.80 ± 0.02
5HT	7.25 ± 0.06	1.11 ± 0.14	7.33 ± 0.05	0.84 ± 0.07
PBG	6.05 ± 0.03	0.83 ± 0.05	6.93 ± 0.05	0.80 ± 0.04

[a] Competition assays were performed with two different labeled ligands: the fluorescent ligand GR-flu and the radioactive ligand [^{3}H]-GR65630. The competition curves were fitted by a binding isotherm, yielding a Hill coefficient close to unity. The dissociation constant K_i of each ligand was calculated as described under Methods. Data are mean values ± SD for two to three experiments.

this indicates that the enthalpy and entropy contribute almost equally to the change in free energy of binding of $\Delta G° = -54 \pm 13$ kJ mol^{-1} at 293 K.

Finally, evaluation of the temperature dependence of k_{on} according to the Eyring relation, which assumes a transition state in the reaction coordinate between the free and bound states, yielded an activation enthalpy of binding of $\Delta H^{\#}_{on} = 50 \pm 1$ kJ mol^{-1} and activation entropy of binding of $\Delta S^{\#}_{on} = 43 \pm 1$ J mol^{-1} K^{-1} (Figure 6A), allowing the calculation of the corresponding activation free energy of $\Delta G^{\#}_{on} = 37 \pm 1$ kJ mol^{-1} at 293 K. The corresponding thermodynamic quantities of dissociation of the GR-flu/receptor complex were calculated by employing the relation $\Delta X^{\#}_{off} = \Delta X^{\#}_{on} - \Delta X°$, where X stands for a thermodynamic parameter: $\Delta H^{\#}_{off} = 76 \pm 9$ kJ mol^{-1}, $\Delta S^{\#}_{off} = -51 \pm 32$ J mol^{-1} K^{-1}, and $\Delta G^{\#}_{off} = 91 \pm 9$ kJ mol^{-1} at 293 K.

Pharmacology of the $5HT_3$ Receptor Determined by Fluorescence Measurements. A major bottleneck in the discovery of new drugs is the availability of fast screening methods. Fluorescence assays offer an alternative to the current methods as they are extremely sensitive, noninvasive, and nonradioactive and they offer on-line (kinetics) information of binding. GR-flu would be ideally suited for such assays, because upon binding to the $5HT_3$ receptor its

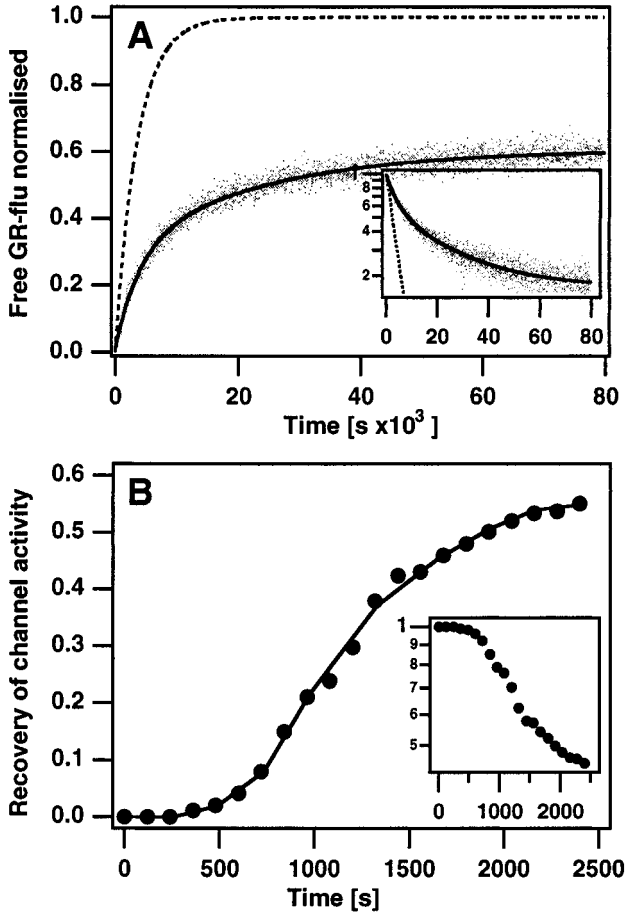

FIGURE 5: Dissociation of GR-flu from the $5HT_3$ receptor: (A) GR-flu (2 nM) prebound to the $5HT_3$ receptor (0.3 nM) was displaced from the receptor by the addition of 0.1 mM of the nonfluorescent agonist mCPBG. The fluorescence signal increased biphasically in time (dots) and could be fitted well with a biexponential function (solid line), yielding two dissociation rate constants of $k_{off} = 255$ and 43×10^{-6} s^{-1}. Similar biphasic dissociation was observed with the other ligands tested for the displacement (Table 2). For comparison, the time course of the expected monophasic dissociation using the dissociation rate constant of 280×10^{-6} s^{-1} obtained from $k_{off} = K_d k_{on}$ (Figure 4) is shown (dashed line). (B) Whole cell currents were recorded continuously of human embryonic kidney 293 cells expressing the $5HT_3$ receptor. After a preincubation with 3 nM GR-flu, the cells were washed with buffer without GR-flu and 2 s pulses of 30 μM 5HT were applied at 2 min intervals. The dissociation of GR-flu from the $5HT_3$ receptor is evident from the slow recovery in time of the 5HT-evoked peak currents. In the inserts, the change in response with time is evaluated by plotting (1 − response) versus time in a semilogarithmic graph. The nonlinearity clearly indicates a complex dissociation process.

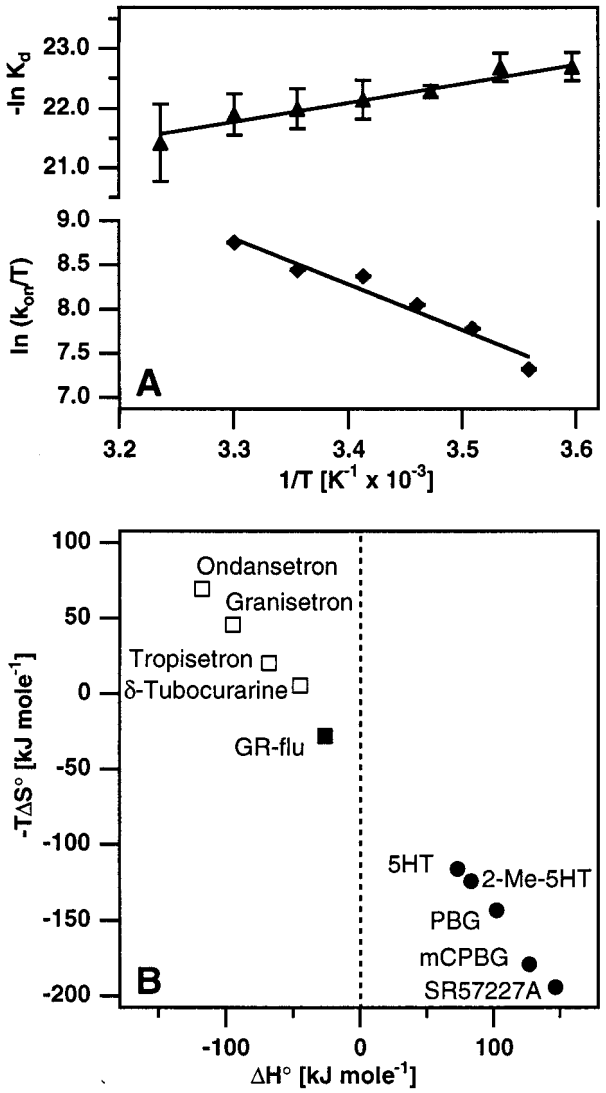

FIGURE 6: Energetics of GR-flu binding to the $5HT_3$ receptor: (A) The dependence of K_d (▲) and k_{on} (◆) on the temperature was determined by following the fluorescence signal upon the addition of $5HT_3$ receptor (0.32−8.13 nM) to a solution of GR-flu (2 or 4 nM) at the indicated temperatures. The K_d of the GR-flu increased with increasing temperature, and from the linear van't Hoff plot (correlation coefficient of 0.88) the changes in enthalpy and entropy of receptor binding were estimated to be $\Delta H° = -26 \pm 9$ kJ mol^{-1} and $\Delta S° = 94 \pm 32$ J mol^{-1} K^{-1}. From the linear Eyring plot (correlation coefficient of −0.98) an activation enthalpy of binding of $\Delta H^{\#}_{on} = 50 \pm 1$ kJ mol^{-1} and an activation entropy of binding of $\Delta S^{\#}_{on} = 43 \pm 1$ J mol^{-1} K^{-1} were found. (B) The thermodynamic quantities of the binding of GR-flu (■) to the $5HT_3$ receptor are compared to those reported for several antagonists (□) and agonists (●) as determined from radioligand competition experiments (*47*). The entropic term $-T\Delta S°$ is plotted against the enthalpy $\Delta H°$ for the binding of the various ligands to the $5HT_3$ receptor at $T = 310$ K. A clear discrimination between the agonists and antagonists is observed: Agonists bind in an endothermic fashion, whereas antagonists react exothermically.

fluorescence properties change considerably. However, one first has to establish that the pharmacological profile obtained by fluorescence measurements corresponds to that obtained by classical radioligand binding assays.

Therefore, competition binding experiments were performed to determine the efficacy of $5HT_3$ receptor antagonists and agonists to displace both GR-flu and [^{3}H]-GR65630, for comparison, from the $5HT_3$ receptor. Figure 7A shows that increasing competitor concentrations progressively inhibited the binding of GR-flu to the receptor, as reflected by the reduced fluorescence intensity decrease. At the highest concentrations tested, all competing ligands inhibited completely the binding of GR-flu. The competition binding curves were all monophasic and could be fitted well by binding isotherms with Hill coefficients close to unity, suggesting a single class of binding sites. The rank order of potencies for the various ligands obtained by employing the fluorescence assay with GR-flu is closely matched by the one determined using the established radioligand [^{3}H]-GR65630 (Figure 7B and Table 3). Slightly higher pK_i values were found with the radioactive assay, but the

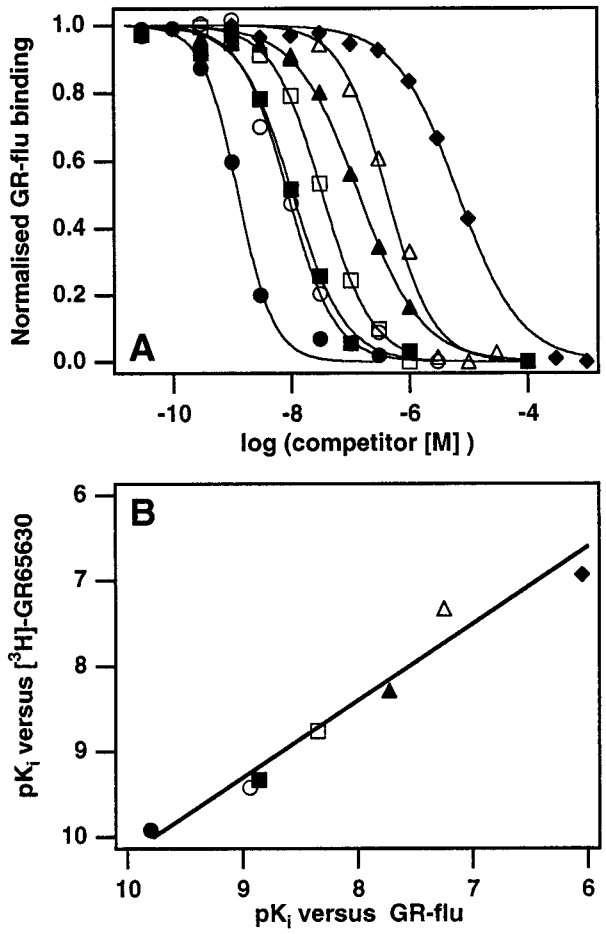

FIGURE 7: Pharmacological profile of the $5HT_3$ receptor. The affinity of unlabeled pharmaceutically interesting compounds was determined by competition assays incubating $5HT_3$ receptor with either GR-flu or [^{3}H]-GR65630 and increasing concentrations of unlabeled receptor ligands: (A) For the fluorescence assay, 3.6 nM $5HT_3$ receptor and 2 nM GR-flu were incubated in the presence of the indicated concentrations of the antagonists GR-H (●), granisetron (○), ondansetron (□), and the agonists quipazine (■), mCPBG (▲), 5HT (△), and PBG (◆). Specific binding of GR-flu, measured from its fluorescence decrease upon receptor binding, was normalized to that in the absence of competitor. The data were fitted to $1/\{1 + (IC_{50}/[\text{competitor}])^{-n}\}$ (solid lines), where IC_{50} is the concentration of half-inhibition and n the Hill coefficient. pK_i values were calculated as described under Methods (see also Table 3). (B) Comparison of the pK_i values obtained from the fluorescent and radioactive competition assays. Data points can be fitted to a straight line (solid line) with a correlation coefficient of 0.98 and a slope of 0.9. Symbols are as in (A). Data are means of two independent experiments; the range falls within the symbols.

sequence remained unaltered, as is illustrated by their linear correlation in Figure 7B.

Molecular Properties of the Ligand Binding Site of the $5HT_3$ Receptor. A significant decrease of the fluorescence of GR-flu was observed upon binding to the receptor (Figure 4A). This effect is due to specific binding to the receptor because pre- (Figure 4A, curve h) or co-incubation (Figure 7A) of the receptor with 1 μM of the competing, nonfluorescent ligand quipazine prevented this spectral change. As the fluorescence properties of fluorescein are reported to be sensitive to the molecular environment of the chromophore (*19*), especially to pH and hydrophobicity, the changes in the GR-flu fluorescence upon binding report on the molecular properties of the ligand binding site of the $5HT_3$ receptor. We investigated this effect more closely by comparing the excitation spectra of GR-flu in different solutions with those of GR-flu bound to the receptor.

Figure 8A shows excitation spectra of free and receptor-bound GR-flu in an aqueous buffer containing detergent. Next to the obvious differences of fluorescence intensity, more subtle changes in the shape of the spectra are visible. The ratio between fluorescence intensities upon excitation at 495 and 440 [I(495/440)], known to be a sensitive indicator of the local pH, changed from 9.5 for the free ligand to 7.9 upon binding to the receptor; the wavelength of excitation, where maximum fluorescence intensity occurs, remained unchanged (curves 1 and 2 in Figure 8A). To define whether these spectral changes upon binding to the $5HT_3$ receptor originated from local polarity and/or pH effects, we characterized the behavior of GR-flu in more detail.

First, the polarity of the buffer was changed by the addition of different percentages of dioxane. With increasing concentrations of dioxane, the fluorescence intensity decreased drastically and the excitation maximum shifted 8 nm to higher wavelengths (Figure 8B). As the receptor-bound GR-flu showed the same maximum in the excitation spectrum as the free ligand, it seems unlikely that the ligand binding site has a strong hydrophobic character.

Subsequently, the effect of the pH on the excitation spectrum of GR-flu was investigated. When the pH decreased from 9 to 6, the fluorescence intensity at 495 nm decreased >10-fold, without a shift of the maximum excitation wavelength (Figure 8C). With further decrease in the pH, the intensity diminished more, until the original shoulder around 440 nm eventually became the major peak. Figure 8D depicts a sigmoidal dependence of I(495/440), from which an apparent pK_a of 6.2 was estimated for the fluorescein moiety. This value was independent of the absence of the detergent $C_{12}E_9$.

Comparing the value of the I(495/440) = 7.9 of receptor-bound GR-flu with the pH dependence of this ratio for the free GR-flu, one can estimate that the receptor-bound GR-flu senses a pH of about 6.75, which is 0.65 pH unit below the pH of the bulk buffer phase. Also, the decrease in fluorescence intensity observed upon receptor binding indicated that GR-flu sensed a local pH of ~6.6 at the receptor.

To verify that GR-flu perceived a local low pH in the binding pocket, we determined the pH dependence of I(495/440) for the receptor-bound GR-flu. As the binding of ligands to the $5HT_3$ receptor itself is very pH-dependent with a sharp optimum around pH 7.4 (Figure 9), the very slow dissociation of GR-flu from the receptor was exploited. That is, GR-flu was first allowed to bind to the $5HT_3$ receptor at pH 7.4, and an excitation spectrum was measured (Figure 8A, curve 2). The pH of the medium was then rapidly changed by the addition of either HCl or NaOH, and an excitation spectrum was recorded again (Figure 8A, curve 3). Finally, the reversibility of the pH-induced changes was investigated by adding an equivalent amount of NaOH or HCl to return to pH 7.4 and acquiring again an excitation spectrum (Figure 8A, curve 4). Curves 2 and 4 were virtually indistinguishable, indicating that the rapid changes of pH had not caused a dissociation of the GR-flu from the receptor. From the variation with the pH of the bulk buffer of I(495/440) for the receptor-bound GR-flu, a pK_a of 7.0 was determined, indicating that the bound GR-flu sensed a pH of 0.8 unit below that of the buffer. Thus, in a buffer of pH

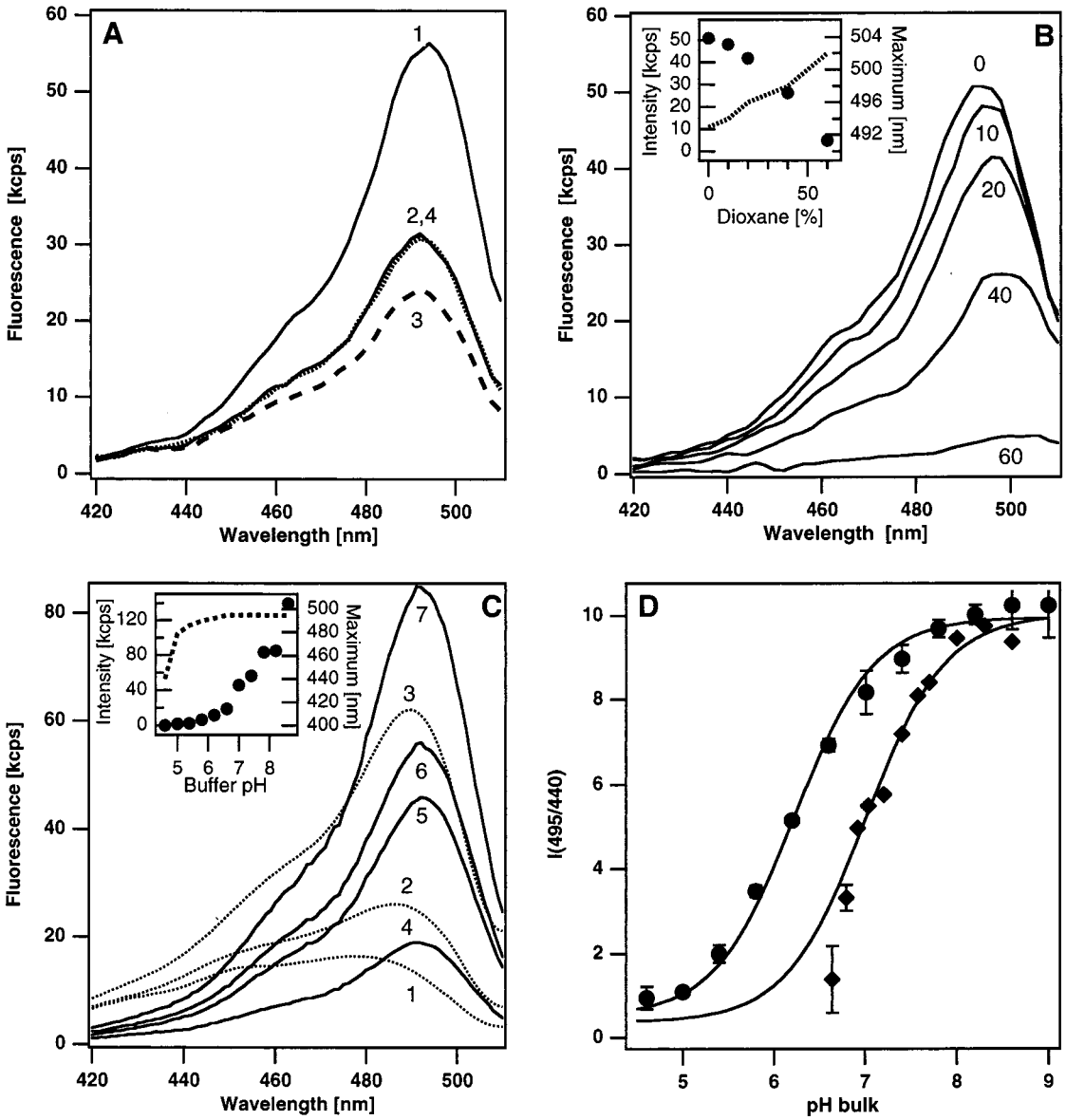

FIGURE 8: Molecular environment of GR-flu bound to the $5HT_3$ receptor: (A) Excitation spectra of 2 nM GR-flu free in solution (line 1) and bound by 4.3 nM $5HT_3$ receptor (line 2) were both recorded in 0.4 mM $C_{12}E_9$/10 mM HEPES, pH 7.4. Binding to the receptor decreased the fluorescence intensity to ~45% of the initial value while the peak maximum remained unchanged. pH titrations of receptor-bound GR-flu were performed to determine the local pH sensed by the bound ligand [see (D)]. For example, addition of 2 mM HCl diminished the fluorescence intensity of the bound GR-flu (dashed line 3). This effect was completely reversed by addition of 2 mM NaOH, as the spectrum (dashed line 4) recovered its initial intensity (solid line 2). (B) The influence of the polarity of the medium on the fluorescence of GR-flu was investigated by addition of dioxane [indicated in %(v/v)]. When the polarity of the solvent decreased, the fluorescence intensity of the excitation spectra of 2 nM GR-flu decreased and a concomitant red shift up to 10 nm of the excitation spectra was observed. The inset summarizes the changes of the excitation wavelength (dashed line, right-hand scale in nm) and the fluorescence intensity (●, left-hand scale, relative fluorescence intensity) induced by increasing concentrations of dioxane. (C) The influence of the pH on the fluorescence of free GR-flu was investigated by recording excitation spectra of 2 nM GR-flu in buffers of different pH values containing 0.4 mM $C_{12}E_9$. The numbered curves correspond to particular pH values: 1 (pH 5.0), 2 (pH 5.4), 3 (pH 5.8), 4 (pH 6.6), 5 (pH 7.0), 6 (pH 7.4), 7 (pH 8.2). Note that the intensity of the dashed spectra is multiplied by a factor of 10 to be visible on the same scale. The fluorescence intensity of the excitation spectra (emission at 495 nm) decreased considerably with decreasing pH. Between pH 9 and pH 6 the wavelength of the fluorescence maximum remained constant, but lowering the pH resulted in a further blue shift of this maximum and the shoulder around 440 nm became more pronounced: the shape of the fluorescence spectra is pH sensitive. The inset summarizes the effect of the pH on the excitation wavelength (dashed line, right-hand scale in nm) and the fluorescence intensity (●, left-hand scale, relative fluorescence intensity). Identical results were obtained in the absence of detergent. (D) Local pH was sensed by GR-flu bound to the $5HT_3$ receptor. The ratio of the fluorescence intensities of the fluorescein moiety upon excitation at 495 and 440 nm is a measure of the local pH sensed by the fluorophore. This ratio was determined and plotted as function of pH for receptor-bound [◆, for methods see (A)] and free GR-flu [●, for methods see (C)]. A sigmoid dependence on the pH was observed in both cases, characterized by pK_a values of 6.22 ± 0.04 for free and 7.01 ± 0.05 for receptor-bound GR-flu. Data are means ± standard deviation of three independent experiments.

7.4 the fluorescein moiety of receptor-bound GR-flu senses a pH of 6.6.

To rule out that the decrease in fluorescence intensity of the GR-flu upon binding to the $5HT_3$ receptor might have been due to quenching of its fluorescence, the fluorescence lifetimes of free and receptor-bound GR-flu were measured. In both cases a lifetime of ~3.6 ns was found (data not shown), excluding quenching as a possible mechanism of

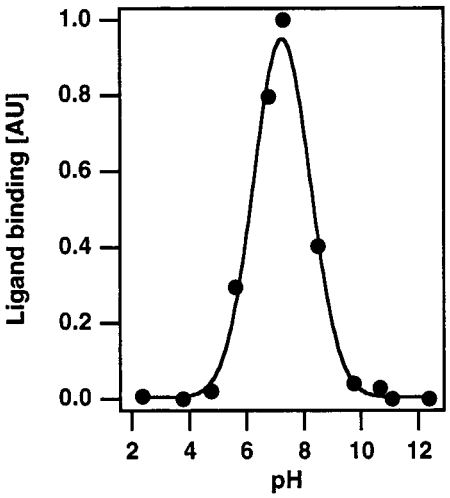

FIGURE 9: pH dependence of [^{3}H]-GR65630 binding to the 5HT$_3$ receptor. Radioligand binding assays were performed by incubating homogenized baby hamster kidney cells expressing the 5HT$_3$ receptor with 0.3 nM of [^{3}H]-GR65630 at the indicated pH. Maximal ligand binding was observed around pH 7.4. The specific binding observed at the different pH values was normalized to that at pH 7.4.

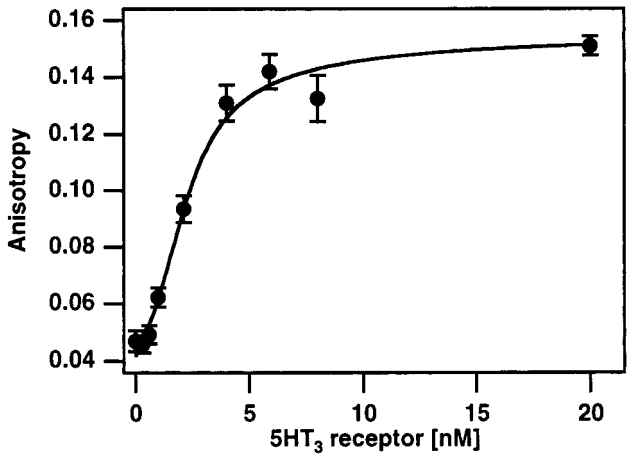

FIGURE 10: Anisotropy of GR-flu bound to the 5HT$_3$ receptor. The anisotropy of 2 nM GR-flu was deterimed in the presence of the indicated concentrations of 5HT$_3$ receptor. With increasing receptor concentration the anisotropy of GR-flu augmented to a limiting value. The data were fitted to a bimolecular binding reaction (see Methods for details), yielding a dissociation constant of 0.22 ± 0.04 nM and an anisotropy of 0.156 ± 0.003 and 0.044 ± 0.002 for GR-flu bound to the receptor and GR-flu free in detergent containing solution, respectively. Each point represents the mean value ± SD of two to four experiments.

the fluorescence intensity decrease. This corresponds to the notion that GR-flu is bound in a local acidic environment, as the lifetime of fluorescein is relatively constant in the pH range concerned, whereas the extinction coefficient decreases rapidly with increasing acidity of the medium (*27*).

Taken together, all of these observations suggest that GR-flu is bound at the 5HT$_3$ receptor in a relatively acidic environment.

To further characterize the microenvironment of the receptor-bound GR-flu, we measured its fluorescence anisotropy to estimate its mobility. Therefore, GR-flu was incubated with increasing 5HT$_3$ receptor concentrations, and for each sample the fluorescence anisotropy was determined (Figure 10). The anisotropy increased with receptor concentration and saturated at receptor concentrations >10 nM. In the presence of 1 μM of the nonfluorescent receptor ligand quipazine no increase in anisotropy was observed. This indicates that the observed increase in anisotropy in the presence of receptor is due to the specific binding of the fluorescent ligand to the receptor. Evaluation of the binding isotherm yielded anisotropies of 0.044 and 0.156 for the free and receptor-bound GR-flu, respectively, and a dissociation constant of 0.22 nM (Table 1).

The anisotropy of 0.156 of receptor-bound GR-flu corresponds to the rotational motion of a molecule with a mass of 25.8 ± 2.0 kDa, which is considerably smaller than that of the 5HT$_3$ receptor complex (280 kDa) (*21*). An anisotropy of 0.2035 is expected for a fluorescein moiety completely immobilized to the receptor. Therefore, the fluorescein moiety of receptor-bound GR-flu features a high segmental mobility on the nanosecond time scale. A mean angle of 23.2 ± 0.8° was calculated over which the fluorophore is free to rotate.

DISCUSSION

Fluorescent receptor ligands have proven to be useful tools for the study of ligand−receptor interactions and to be a valuable supplement to classical methods such as radioligand binding and site-directed mutagenesis. Such ligands provide an attractive alternative to radioligands in receptor studies, circumventing several drawbacks associated with radioactivity such as high costs, potential health hazards, and waste disposal problems. Moreover, fluorescence techniques are noninvasive, deliver insight into the local environment of the fluorophore, and offer the possibility to obtain on-line information on the interactions studied. Thermodynamic and kinetic parameters can thus be accessed directly. Here, the interaction of a novel fluorescent 5HT$_3$ receptor ligand to its receptor was investigated in detail.

GR-flu Is a Genuine 5HT$_3$ Receptor Ligand. The high affinity of GR-flu for the 5HT$_3$ receptor and its antagonism of 5HT-evoked receptor activation were demonstrated by electrophysiological measurements, radioligand displacement studies (Figures 2 and 3), and fluorescent binding assays (Figures 4 and 10). Attachment of the fluorophore fluorescein to GR-H influenced receptor binding only little, as is evident from the only slightly diminished affinity of GR-flu (Table 1). The lower pK_i values observed in the radioligand binding assays compared to the electrophysiological experiments are most probably due to differences in the environment of the receptor, detergent versus intact membranes, as this was also observed for the binding of [^{3}H]-GR65630 to the 5HT$_3$ receptor in membranes and to the detergent-solubilized purified receptor (*21*). It was furthermore shown that both rank order and the absolute values of the affinities of several unlabeled compounds determined by the fluorescent competition assay presented are in complete agreement with those obtained in radioligand competition experiments using purified 5HT$_3$ receptor [Figure 7, (*21*)], 5HT$_3$ receptor expressing neuroblastoma cell lines (*28*), or native brain tissue (*29*). Recently, GR-flu has been applied for the development of a novel bioanalytical assay, combining the immobilization of the 5HT$_3$ receptor to a chemically modified sensor surface and ultrasensitive fluorescence detection, with

which the affinity of unlabeled receptor ligands was determined successfully (*17*).

We therefore conclude that GR-flu is a $5HT_3$ receptor ligand displaying high-affinity antagonism and suitable for studying the interaction between the $5HT_3$ receptor and its ligands. This is facilitated by the significant decrease of the fluorescence intensity (Figure 4A) and increase of the anisotropy (Figure 10) upon the specific binding to the $5HT_3$ receptor. These features can be exploited for two purposes: First, binding of GR-flu to the receptor can be measured with omission of separation of free and receptor-bound ligand by employing steady-state fluorescence intensity measurements. Second, due to the well-characterized dependence of the fluorescence properties of fluorescein on the local molecular environment of the fluorophore, information can be obtained on the nature of the ligand binding site on the receptor.

Kinetics of GR-flu Binding to the 5HT$_3$ Receptor. The values of the rate constants of binding (10^6 M^{-1} s^{-1}) and dissociation (43×10^{-6} and 275×10^{-6} s^{-1}) determined in this paper are relatively low as compared to published data. Reported rate constants of binding for antagonists range from 4×10^{-6} to 150×10^6 M^{-1} s^{-1} (*30, 31*). What is consistent, however, is that binding could be described by a single exponential in these papers, corresponding to our case. The off-rate constants reported in the literature range from 3.5×10^{-3} to 8×10^{-3} s^{-1} (*31, 32*) for monophasic dissociation and from 2×10^{-3} to 70×10^{-3} s^{-1} and from 60×10^{-6} to 500×10^{-6} s^{-1} for the fast and slow off-rates for the biphasic dissociation (*30*). In the case of biphasic dissociation, as in our study, the fast k_{off} corresponded well to the product of the determined K_d and k_{on} (*30*).

These lower rate constants found in our study are not an artifact due to the use of a detergent-solubilized, purified, expressed His-tagged receptor protein, because the binding of [^{3}H]-GR65630 to the same receptor preparation is characterized by rate constants of binding of 83×10^6 M^{-1} s^{-1} and of dissociation of 23×10^{-3} M^{-1} s^{-1} (data not shown), values comparable to the literature values mentioned above. Therefore, the slower kinetics of GR-flu might result from the chemical modification of our ligand. The ~2-fold increase in mass upon labeling should have no measurable effect on the diffusion rates of the GR-flu, as diffusion rates scale with the inverse cube of the molecular mass. It is more probable that the slower reaction rates are caused by steric factors or by the negative charges of the fluorescein moiety, because, in general, $5HT_3$ receptor ligands are positively charged (*33*).

Stoichiometry of GR-flu Binding to the 5HT$_3$ Receptor. The presence of five binding sites per $5HT_3$ receptor might be expected from its putative homopentameric nature (*5, 21, 34*), but the actual number of agonists or antagonists that do bind is not known.

A 1:1 stoichiometry for ligand binding to the $5HT_3$ receptor, however, is suggested by several observations presented in this paper: (i) the linear dependence of the initial rate of binding on the concentrations of both GR-flu and $5HT_3$ receptor (Figure 4B); (ii) Hill coefficients of close to unity observed for the concentration dependence of 5HT-activation of ion currents and for the binding of GR-flu to the receptor (Figure 4B); and (iii) Hill coefficients of close to unity for the inhibition by receptor ligands of both the activation of currents by 5HT (Figure 2) and the binding of radioligand (Figure 3) and GR-flu (Figure 7) to the $5HT_3$ receptor. Furthermore, this 1:1 stoichiometry is corroborated by the specific radoligand binding activity of 5 nmol/mg of protein reported for the purified receptor used in this paper (*21*). One has to be careful interpreting Hill coefficients as they indicate only a lower limit of the number of binding sites; a value of 1 might correspond to the presence of more that one independent binding site with an identical affinity.

From our experiments, however, the interaction of GR-flu with the $5HT_3$ receptor cannot be described by the simple bimolecular equilibrium $R + L \leftrightarrow RL$, because (i) the observed EC_{50} (5 μM) and IC_{50} (50 nM) for 5HT are very different, which has recently been observed as well for the $5HT_3$ receptor in NG108-15 neuroblastoma cells (*35*) and was interpreted by the presence of a low-affinity active state and a high-affinity desensitized state; and (ii) the dissociation of GR-flu from the receptor showed a rather complex behavior and seemed to be composed of two processes with distinct time constants (Figure 5 and Table 2). The fast dissociation phase is characterized by a time constant that equals the product of the determined K_d and k_{on} (Figure 4) and might correspond to the dissociation of the active or nondesensitized receptor−ligand complex RL. The slower off-rate might originate from a subsequently formed, desensitized state. Biphasic ligand dissociation of radioligands from the $5HT_3$ receptor has also been observed for granisetron (*30*), but also monoexponential dissociation was described for the same ligand (*32*).

No consensus has been reached in the literature on the number of ligands binding to the $5HT_3$ receptor (*5, 29, 35*−*38*): The data suggest that for channel activation the binding of at least one to three agonists is required, that desensitization is induced by the binding of four to five agonists, and that at least one antagonist has to bind to inhibit channel opening. The presence of several binding sites on the $5HT_3$ receptor or several receptor conformations is suggested by the observation that preincubation with one ligand altered the affinity of a second ligand (*39*). In the present study, no differences were observed in the ability of agonists or antagonists to dissociate the GR-flu/receptor complex (Table 2).

Recent experiments with the homopentameric α_7 acetylcholine receptor, which shares a 30% sequence homology with the $5HT_3$ receptor, indicated that five molecules of the antagonist methyllycaconitine bind per receptor (*40*). The model used in the paper that described the recovery of the α_7 acetylcholine receptor channel from antagonist blockage was applied also here to the recovery of the $5HT_3$ receptor channel activity from GR-flu inhibition (Figure 5B). The values obtained are difficult to understand, as the best fit was obtained for a stoichiometry of eight GR-flu molecules per receptor homopentamer.

Therefore, although GR-flu binding to the $5HT_3$ receptor seems to be well described by a 1:1 ligand/receptor stoichiometry, our data do not allow us to conclude the exact number of sites. Consequently, the apparent rates measured in these experiments are likely to be complex functions of several competing processes where several distinct conformational states of the $5HT_3$ receptor have been assigned to particular desensitized states (*41*).

Energetics of GR-flu Binding to the 5HT$_3$ Receptor. Analysis of the thermodynamic and kinetic parameters of

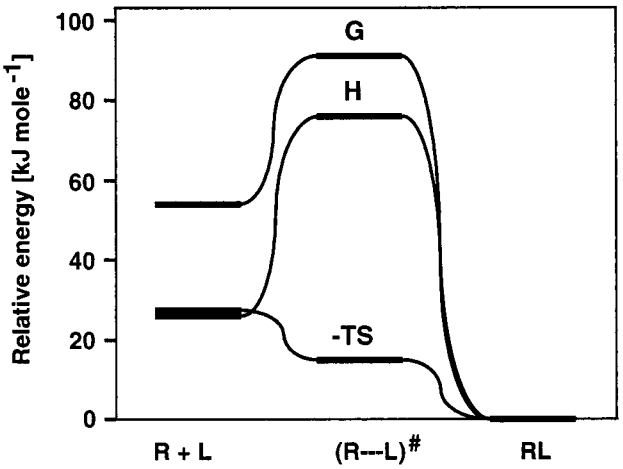

FIGURE 11: Thermodynamic profile of the binding of GR-flu to the $5HT_3$ receptor. The relative energy levels of the thermodynamic parameters are plotted against the proposed reaction coordinate. The binding scheme, where binding of the ligand L to the receptor R passes via a transition state R- -L$^{\#}$, is depicted along the reaction coordinate. ΔG is calculated for a temperature of 293 K.

the interaction of a ligand with its receptor can shed light on the mechanism of ligand binding and on receptor activation processes. Here we have determined not only the changes of the thermodynamic quantities upon ligand binding but also the activation thermodynamics of binding and dissociation. The results are summarized graphically in Figure 11. Here we have applied the Eyring theory, which assumes an activated intermediate between the free and bound state: it was found that for GR-flu to bind to the $5HT_3$ receptor an enthalpic barrier has to be surmounted, whereas the changes in entropy are favorable for both the formation of the transition complex RL$^{\#}$ and the final bound state RL. On the other hand, dissociation of the GR-flu/receptor complex has to overcome both enthalpic and entropic barriers.

How can this be interpreted? GR-flu is slightly hydrophobic, because it does not dissolve in water at high concentrations ($>1\ \mu$M). Therefore, one can expect that the binding of this hydrophobic molecule to the receptor would liberate water molecules that were constrained in its hydration shell, enhancing entropy. Moreover, water molecules might interact with the empty binding site, and these could be expulsed from the binding site upon the arrival of the ligand, thus increasing the entropy as well. Another source of entropy enhancement might be the induction of a loosened structure of the receptor upon ligand binding. However, a large structural change is not expected, as the change in specific heat upon ligand binding is close to zero, as is interpreted from the linear van't Hoff plot (Figure 6A). Moreover, we have recently shown that the secondary structure, as determined by circular dichroism, did not change upon agonist or antagonist binding (*21*).

The enthalpic barrier might be caused by the disruption of hydrogen bonds or electrostatic interactions between water molecules and the ligand or between water molecules and the binding site on the receptor. The final drop in enthalpy from the transition state to the final GR-flu/receptor complex might be caused by the formation of more favorable H-bonds and van der Waals forces between the receptor and the ligand. Alternatively, the free water molecules, liberated from their restrained position in the hydration shell of the ligand or binding site, might form in bulk more or stronger H-bonds. It is virtually impossible to estimate the number of H-bonds implicated as the enthalpy of a hydrogen bond in proteins varies between 8 and 40 kJ mol^{-1}. Only a small number of hydrogen bonds seem to be involved, judging from the measured binding enthalpy of $\Delta H^\circ = -26$ kJ mol^{-1}.

The determined activation enthalpies fall in the range of what has been reported for the nACh receptor (*42*, *43*) and $GABA_A$ receptor (*44*, *45*), where values of 23−125 and 40−80 kJ mol^{-1} have been obtained for the activation energy of binding and dissociation, respectively.

Recently, two groups employed radioligand competition experiments to determine the thermodynamic parameters of unlabeled $5HT_3$ receptor ligands (*46*, *47*). Both studies reported that binding of agonists is endothermic ($\Delta H^\circ > 0$), whereas binding of antagonists is exothermic (see also Figure 7B). This is in complete agreement with our finding that GR-flu (i) antagonizes 5HT-evoked currents and (ii) binds exothermically to the $5HT_3$ receptor. However, some points of divergence exist. Antagonist binding was found to lead to either an increase [(*46*) and this study] or a decrease (*47*) in entropy, and both linear [(*46*) and this study] and curved (*46*) van't Hoff plots were obtained. Moreover, very different values for ΔH° (19 versus 83 kJ mol^{-1}) and ΔS° (179 versus 401 J mol^{-1} K^{-1}) were found for 5HT [(*46*) versus (*47*)]. There are no apparent explanations from the experimental description for these discrepancies. In this study, the binding of GR-flu was determined directly, not in a competition assay, and might therefore be more precise.

Interestingly, it seems to be a general phenomenon that one can distinguish agonists from antagonists by their thermodynamic properties of binding to their receptors [(*46*) and references cited therein]. This distinction between agonists and antagonists has been also observed for other receptor ligand-gated ion channels and seems to be a general thermodynamic property. Therefore, thermodynamic studies might be a good complement to functional experiments of receptor activation, like electrophysiological measurements, to demarcate the character of new compounds toward the studied receptor.

Local Microenvironment of Receptor-Bound GR-flu. Serotonin, the natural ligand for the $5HT_3$ receptor, and all known synthetic agonists and antagonists contain a basic nitrogen and an aromatic system (*33*). Therefore, it is expected that the ligand binding site contains complementary, compatible groups. The experiments presented here (Figure 8) indicate, from the observed fluorescence intensity decrease and the pK_a of the bound ligand, that receptor-bound GR-flu finds itself in an acidic microenvironment, suggesting close proximity of carboxylate-containing amino acids, that is, aspartate and or glutamate.

The observed decrease of the fluorescence intensity of GR-flu upon receptor binding is not due to fluorescence quenching, because the fluorescence lifetime of GR-flu was unaffected by binding. This experiment also excludes the possibility of resonance energy transfer between two or more GR-flu molecules bound to the same receptor, as this would result in a reduction of the fluorescence lifetime. Also, the dissociation constants obtained from $5HT_3$ receptor concentration dependence of the decrease of the fluorescence

intensity and of the increase of the fluorescence anisotropy of GR-flu were identical (Table 1).

Involvement of charged groups in binding is also suggested from the strong pH dependence of the specific binding of the [^{3}H]-GR65630, as depicted in Figure 9. The sharp pH dependence might involve (de-)protonation of acidic amino acids in the ligand binding site and amines of the GR-65630 or a structural rearrangement, even denaturation, of the receptor. The binding of [^{3}H]-zacopride to the $5HT_3$ receptor, however, increased sigmoidally with pH (*48*). This difference might originate from the different structures of the radioligand used and might be due to the presence of an indol moiety in GR65630 instead of a primary amine in zacopride.

Little is known about the involvement of specific amino acids in ligand binding by the $5HT_3$ receptor. Recently, Glu^{106} has been shown to be involved in the binding of ligands to the $5HT_3$ receptor, as mutations of this residue strongly affected ligand binding and channel activation (*49*). This residue is conserved in all known primary sequences of the $5HT_3$ receptor (mouse (*5*), rat (*50*), human (*51*), and guinea pig (*52*)] and is located in a region of the primary sequence that corresponds to the so-called loop A, shown to be involved in ligand binding in the nACh receptor (*8, 53, 54*). Moreover, other acidic residues might be implicated in ligand binding. In the nACh receptor, δAsp^{180} and δGlu^{189} [mouse muscle (*55*)] and αAsp^{200} [*Torpedo* (*56*)] have been shown to play a role in ligand binding: these residues are conserved in all four $5HT_3$ receptor sequences known. The exact role of acidic residues in the binding of ligand to the $5HT_3$ receptor is not clear, because chemical modification of these residues resulted only in a slight inhibition of radioligand binding (*57*). The same authors demonstrated that radioligand binding was strongly impaired by chemical modification of Trp (−70%) and to a lesser extent (−39 to −27%) upon modification of His, Tyr, and Arg. Also, Trp^{66} seems to be important for the binding of the atypical ligand curare to the $5HT_3$ receptor: upon mutation to Phe the affinity decreased 10-fold (*58*). Therefore, both hydrophilic and hydrophobic residues seem to be implicated in ligand binding.

Also, in the case of the nACh receptor the exact nature of the residues involved in ligand binding of acetylcholine is not quite clear. Next to the role of the negatively charge residues (see above), aromatic residues have been implied (for reviews see refs *7*, *8*, and *59*). It has been proposed that aromatic residues in the binding site might accommodate the positive charge of the quaternary ammonium group of the acetylcholine through interactions with the aromatic π-electrons [(*60*) and references discussed].

Fluorescently labeled ligands have been used to investigate the nature of the ligand binding site of several ligand-gated ion channels. The benzodiazepine binding site of the $GABA_A$ receptor seems to be rather hydrophilic as the fluorescence intensity of an NBD-labeled benzodiazepine decreased upon binding (*61*). To the nACh receptor, fluorescent agonists seem to bind in a hydrophilic pocket (*42, 62*), whereas fluorescent antagonists are bound in a somewhat hydrophobic site (*63*). From these later findings one might interpret that agonist binding to the nACh receptor is more enthalpically driven, whereas antagonist binding is entropically favored, again a thermodynamic distinction between agonists and antagonists.

The rather low fluorescence anisotropy of receptor-bound GR-flu indicates that the fluorescein moiety has a relatively high degree of motional liberty. This suggests that the ligand binding site of the $5HT_3$ receptor presents itself to ligands either as a broad opening with a diameter of at least 12 Å, considering the mean angle of segmental motion of 23° and the dimensions of the fluorescein moiety, or as a shallow site on the surface of the receptor protein. This suggests that water molecules might have easy access to the fluorescein moiety of the bound GR-flu and corroborates the proposed hydrophilic environment of the bound GR-flu (Figure 8).

In conclusion, we demonstrated that the novel fluorescein-labeled ligand GR-flu was a high-affinity antagonist for the $5HT_3$ receptor. The spectral changes of GR-flu upon receptor binding enabled us to determine the affinity for the $5HT_3$ receptor of unlabeled compounds, offering possible applications in pharmaceutical drug screening. Analysis of GR-flu binding to the $5HT_3$ receptor and of its temperature dependence suggests that most probably one GR-flu binds per receptor, followed by conformational change of the ligand/receptor complex. Combination of the thermodynamic data with the demonstrated mobility of the bound fluorophore and the acidic character of the ligand binding site indicates that electrostatic and hydrogen-bonding interactions involving acidic amino acids play an important role in the binding of GR-flu to the $5HT_3$ receptor.

The novel fluorescent ligand opens a variety of possibilities to probe the function of the $5HT_3$ receptor. Presently, we investigate the accessibility of the bound ligand and by fluorescence correlation spectroscopy the clustering of the receptor on living cells.

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