Characterization of the Ligand-binding Site of the Serotonin 5-HT₃ Receptor

THE ROLE OF GLUTAMATE RESIDUES 97, 224, AND 235

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Ligand-gated ion channels of the Cys loop family are receptors for small amine-containing neurotransmitters. Charged amino acids are strongly conserved in the ligand-binding domain of these receptor proteins. To investigate the role of particular residues in ligand binding of the serotonin 5-HT₃ receptor (5-HT₃R), glutamate amino acid residues at three different positions, Glu⁹⁷, Glu²²⁴, and Glu²³⁵, in the extracellular N-terminal domain were substituted with aspartate and glutamine using site-directed mutagenesis. Wild type and mutant receptor proteins were expressed in HEK293 cells and analyzed by electrophysiology, radioligand binding, fluorescence measurements, and immunoccopy. A structural model of the ligand-binding domain of the 5-HT₃R based on the acetylcholine binding protein revealed the position of the mutated amino acids. Our results demonstrate that mutations of Glu⁹⁷, distant from the ligand-binding site, had little effect on the receptor, whereas mutations Glu²²⁴ and Glu²³⁵, close to the predicted binding site, are indeed important for ligand binding. Mutations E224Q, E224D, and E235Q decreased Kᵣ values 5–20-fold, whereas E235D was functionally expressed at a low level and had a more than 100-fold increased ECₛₒₐ value. Comparison of the fluorescence properties of a fluorescein-labeled antagonist upon binding to wild type 5-HT₃R and E235Q, allowed us to localize Glu²³⁵ within a distance of 1 nm around the ligand-binding site, as proposed by our model.

The question of how ligand binding to certain receptor proteins eventually gates ion channels is of central importance in cellular signaling but still unresolved at a molecular level. In order to enhance our understanding of the molecular mechanisms, we used a combined biomolecular and biophysical approach to study a serotonin-gated ion channel.

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The 5-hydroxytryptamine (5-HT³; serotonin) type 3 receptor (5-HT₃ receptor) is the only ligand-gated ion channel found among the serotonin receptors. Its gene structure (1) and amino acid sequence are similar to those of other members of the Cys loop receptor family including the nicotinic acetylcholine (nAChR), ionotropic γ-aminobutyric acid, and glycine receptors. They are composed of five homologous or identical subunits, each comprising four predicted transmembrane regions and a large extracellular N-terminal domain containing the ligand-binding site. Among them, the nAChR is most closely related to the 5-HT₃ receptor. Both receptors form ion channels that are permeable to cations and share about 20–30% amino acid sequence identity.

So far, three different 5-HT₃ receptor subunits, A, B, and C, have been cloned as well as a short splicing variant of the A subunit. Expression of only A subunits results in functional ion channels of similar properties as for 5-HT₃ receptors in native tissues, suggesting that this receptor is active as a homopentamer (2). Expression of solely the B (3) or C (4) subunits did not result in functional receptors; however, their coexpression with the A subunit yields in functional receptor proteins with slightly different channel properties.

Site-directed mutagenesis and biochemical studies, combined with amino acid sequence alignments, have identified amino acid residues and sequence regions (the so-called loops A–F; see Fig. 1) in the N-terminal extracellular domain implicated in the ligand-binding site of the nicotinic acetylcholine and 5-HT₃ receptors (for reviews, see Refs. 5–7 and references therein).

For the nAChR, these experiments together with the recently resolved three-dimensional structure of the acetylcholine-binding protein (AChBP) (8) indicate that the binding site is located at the interface between two adjacent subunits and that the binding loops A–F are forming the binding pocket. According to this model, loops A–C would form the binding site on one subunit, whereas loops D–F of the adjacent subunit would contribute to a lesser extent to ligand binding. In the case of the 5-HT₃ receptor, only few details are known about the ligand-binding site. Published data indicate that certain residues, especially in loops C and D (see Fig. 1A), are important for ligand binding.

[¶] The abbreviations used are: 5-HT, serotonin; 5-HT₃, serotonin 5-HT₃ receptor; AChBP, acetylcholine-binding protein; C₂₀E₉, monododecyl nonaethylene-glycol; GR65630, 3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-propanone; GR-Ru, 1,2,3,9-tetrahydro-3-(5-methyl-1H-imidazol-4-yl)methyl-9-(3-amino-(6′-fluorescein-thiocarbamoyl)-propyl)-4H-carbazol-4-one; mCPBG, m-chlorophenylbiguanide; nAChR, nicotinic acetylcholine receptor; WT, wild type.

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In this paper, we investigate the role of glutamate residues in ligand binding to the mouse subunit A of 5-HT₃ₐ receptor, hereafter denominated as 5-HT₃ₐR, at three different positions, Glu⁹⁷, Glu²²⁴, and Glu²³⁵, by replacing them with glutamine and aspartate using site-directed mutagenesis. Glu⁹⁷, present in loop D, is conserved in both 5-HT₃ₐR subunits A and B but neither in the 5-HT₃₈ subunit nor in nAChR subunits. Glu²²⁴ and Glu²³⁵, located in loop C, are present in all 5-HT₃₈ subunits A but absent in the B and C subunits. An acidic residue at a position corresponding to Glu²³⁵ is conserved in all nAChR subunits. Amino acid residues in both loops have been shown to influence ligand binding (21). Here, wild type (WT) and mutant 5-HT₃₈ proteins were transiently expressed in HEK293 cells and were characterized functionally by whole cell patch clamp and radioligand binding experiments; the presence of receptor proteins in the plasma membrane was investigated by immunocytochemistry and receptor binding of the GR-flu. The question whether the Glu residues investigated here create the local acidic environment sensed by receptor-bound GR-flu was studied by fluorescence spectroscopy. A structural model of the N-terminal domain of 5-HT₃₈ based on the structure of AChBP was conceived in order to interpret our results.

We present experimental evidence that, in contrast to Glu⁹⁷, Glu²²⁴ and Glu²³⁵ are deeply involved in ligand binding of the 5-HT₃₈, because a decrease in both ligand affinity and efficacy of 1–3 orders of magnitude was observed when these residues were mutated. Moreover, for the first time, direct biophysical measurements are presented, restricting the location of Glu²³⁵ within 1 nm of the ligand-binding site of the 5-HT₃₈. The structural model corroborated these results.

MATERIALS AND METHODS

The radioligands 3-(5-methyl-1H-imidazol-4-yl)-1-(1H-methyl-1H-indol-3-yl)-propanone ([H]GR65630; 61 Ci/mmol) and n-methyl-Biguanydine ([H]mCPBG; 20 Ci/mmol) were from PerkinElmer Life Sciences. Granisetron and the fluorescent antagonist GR-flu were obtained from GL Biochem (Shanghai, China) and Sigma. Monododecyl nonaethylene-glycol (C₁₂E₉ was from Panreac Quimica, S-A, Barcelona, Spain. Other chemicals were of the highest quality available and purchased from regular sources.

Site-directed Mutagenesis of 5-HT₃₈ cDNA—Full-length cDNA encoding the short splicing variant of the murine 5-HT₃₈ subunit (1) was cloned as a 1.5-kb SmallIdNot DNA fragment in the eukaryotic expression vector pCMVβ (Clontech, Palo Alto, CA) by replacing the β-galactosidase reporter gene. Site-directed mutagenesis was performed using the QuikChange™ kit (Stratagene). Glutamates 97, 224, and 235 were mutated to glutamine and aspartate using mutagenic oligonucleotides that introduced as well a silent mutation to create a new restriction site to facilitate mutant sampling (Table 1 of Supplementary Material). Mutations were confirmed by restriction pattern analysis and verified through sequence analysis.

Transient Transfection of HEK293 Cells—Human embryonic kidney cells (HEK293 cells) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2.2% fetal calf serum in a humidified 5% CO₂ atmosphere at 37 °C. Adherently growing cells were subcultivated once or twice a week at ratios between 1:10 and 1:100.

To investigate the function of mutant receptors in cells by electrophysiology, immunolocalization, or binding the fluorescent ligand GR-flu, HEK293 cells (60–80% confluent), growing in either six-well plates or 35-mm cell culture dishes, were transfected using Effectene™ lipofection according to the protocol of the manufacturer (Qiagen, Hilden, Germany) with 0.2 or 1.0 µg of plasmids containing the coding region of WT or the mutant 5-HT₃₈. After 4 h, the transfection solution was replaced by fresh medium. Experiments were performed 24–46 h after transfection.

To investigate mutant receptors in either cell membranes or solubilized in detergent micelles (radioisogrd or GR-flu binding), HEK293 cells were seeded (1 × 10⁶ cells/ml) into 150-cm² flasks. 16–20 h after splitting, cells were transfected with calcium phosphate-precipitated DNA as described (22). For each transfection, 75 µg of WT or mutant 5-HT₃₈ cDNAs diluted in 1.5 ml of CaCl₂ (250 mM) was added to an

FIG. 1. Amino acid sequence of the N-terminal extracellular domain of the mouse 5-HT₃₈ (SwissProt accession number P23979) and alignment of loops C and D with related receptor subunits. Amino acid residues of the N-terminal extracellular domain of the mouse 5-HT₃₈ comprising the signal sequence (residues 1–23, indicated by dots); the first transmembrane domain would start at residue 246. Underlined are the binding loops A–D; indicated by plus signs are residues for which experimental data suggest importance for ligand binding (12, 16, 21, 28–30). The glutamate residues investigated by mutation in this paper are indicated as asterisks, and sequences corresponding to the oligonucleotides used for mutagenesis are shaded. In B, amino acid sequences of loops D and C of different nAChRs and 5-HT₃₈s are aligned. The studied glutamates (asterisks) and the corresponding conserved anionic residues in the other receptor subunits are indicated (gray shading).
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FIG. 2. Electrophysiological response of single cells expressing 5-HT₃R WT, E235Q, or E235D to 5-HT measured in whole cell configuration. Currents versus time traces are representative of measurements on at least three different cells.

5-HT at a concentration inducing 90% of Iₘ₉ of the respective mutant in the absence of granisetron.

Binding Assay Using Radioligands—The affinity of 5-HT₃R mutant proteins for radioligands and the total amount of ligand-binding sites were determined as described (23) with the modification that samples containing ~0.2 pmol of 5-HT₃R were incubated for 60 min at room temperature in 10 mM HEPES, pH 7.4, with increasing concentrations of [³H]GR65630 (up to 20 nM) or [³H]mCPBG (up to 120 nM) in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of 1 μM quipazine (PK = 9.0 ± 0.3 (9)). All experiments were performed in triplicate.

The affinities of the receptor for granisetron were determined by competition binding assays. Samples comprised ~0.8 μM [³H]GR65630 or 20 nM [³H]mCPBG in 10 mM HEPES, pH 7.4, and 5-HT₃R-containing membranes to produce 100–2000 cpm of specifically bound radioactive ligand; they were incubated at increasing concentrations of the competitor in a final volume of 0.2 ml for 60 min at room temperature.

The dissociation constant Kᵦ of the radioligand, the total concentration of 5-HT₃R (expressed as concentration of binding sites), and Hill coefficients (n) were evaluated by fitting experimental data as follows.

\[ [L]_{\text{bound}} = \frac{[5-HT_3R]_{\text{total}}}{[1 + (K_i/[L])]} \]

(Eq. 3)

Binding inhibition curves were fitted to the following equation.

\[ [B] = \frac{[L]_0}{[1 + (IC_{50}/[\text{competitor}])]} \]

(Eq. 4)

[B] and [B₀] are the concentrations of bound radioligand in the presence and absence of unlabeled competitor, respectively, and IC₅₀ is the concentration of competing ligand that displaced 50% of the specifically bound radioligand. The dissociation constant of inhibition Kᵦ of competitors was estimated from the Cheng-Prusoff equation (23).

\[ K_i = IC_{50}/[1 + ([L]/K_i)] \]

(Eq. 5)

where [L] and Kᵦ are the concentration and dissociation constant of radioligand, respectively.

Binding of GR-flu to Living Cells—HEK293 cells grown on glass coverslips in six-well plates were transfected as described above. 40 h after transfection, the coverslips were transferred into a sample holder and covered with 400 μl of phosphate-buffered saline. Then, during image recording using an LSM 510 confocal microscope (Zeiss, Oberkochen, Germany) equipped with appropriate excitation and emission filters for fluorescein and using identical settings for all samples when not stated otherwise, GR-flu was added to reach either 1.5 or 15 nM final concentration. Nonspecific binding of GR-flu to cells, measured in the presence of 1 μM quipazine, was negligible.

Binding of GR-flu to Detergent-solubilized 5-HT₃R—HEK293 cells harvested from one T150 flask were resuspended in 1 ml of 10 mM HEPES, 0.5 mM EDTA, pH 7.4, pelleted by centrifugation (10 min at 3200 × g at 4 °C), resuspended in 1 ml of total volume of the same buffer, and finally homogenized for 10 s with an Ultra-Turrax T25 (IKA, Staufen, Germany). Membranes obtained by this procedure were pel-
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### RESULTS

**Electrophysiology**—The functional properties of WT and mutant receptor proteins were investigated by whole cell patch clamp measurements. Typical electrical responses upon application of 5-HT with peak currents around 1–4 nA at saturating 5-HT concentrations (Fig. 2) indicated that WT and mutant receptor proteins were functionally expressed at similar levels. As an exception, in the case of E235D only small currents were observed at millimolar concentrations of 5-HT. From dose-response curves (Fig. 3), the EC$_{50}$ (Table I) and the Hill coefficient $n$ for 5-HT were evaluated. Whereas both mutant proteins of Glu$^{97}$ showed no difference compared with WT, the EC$_{50}$ values for E224Q, E224D, and E235Q were increased by a factor of 4, 10, and 20, respectively. In the case of E235D, no EC$_{50}$ could be determined, since only small currents of some 100 pA could be recorded repetitively at 20 mM 5-HT, close to the solubility limit. Neither with 5-HT nor with the agonist mCPBG, which has a higher affinity to the WT receptor than 5-HT, could saturating ligand binding conditions be reached. Control experiments verified that nontransfected HEK293 cells did not respond electrically upon application of 5-HT or mCPBG concentrations up to 20 mM. This suggests that the EC$_{50}$ of E235D is shifted more than 1000-fold to higher concentrations. The Hill coefficient $n$ was similar for all mutant (except E235D) and WT receptors and ranged from 1.5 to 1.9. In contrast, the kinetics of channel activation and desensitization of E235Q were considerably slower than for Glu$^{97}$ and WT (Fig. 2).

Antagonist-agonist competition experiments yielded similar results (Fig. 4 and Table I). After incubation for 2–5 min with increasing concentrations of the antagonist granisetron, the response to 5-HT was recorded. The 5-HT concentration applied evoked 90% of $I_{\text{max}}$ of the respective mutant in absence of granisetron. For mutant proteins of Glu$^{97}$, granisetron inhibited 5-HT-induced currents equally well as for the WT; for E224Q, E224D, and E235Q, the IC$_{50}$ of granisetron was shifted to higher concentrations by a factor of 3, 9, and >100, respectively. The Hill coefficient varied between 1.0 and 1.6. For E235D, this experiment could not have been carried out in a

### Table 1

| 5-HT$_3$R Protein | Electrophysiology: 5-HT-induced currents | Radioligand binding |
|-------------------|-----------------------------------------|---------------------|
|                   | EC$_{50}$ | Granisetion inhibition K$_{IC50}$ | Kd | Granisetion inhibition K$_{d}$ | KIC50 | mCPBG inhibition K$_{IC50}$ |
| WT                | 2.1 ± 0.4 | 0.4 ± 0.1 | 12.7 ± 1.9 | 2.0 ± 0.6 | 1.4 ± 0.3 | 1.4 ± 0.4 | 11 ± 0.4 |
| E97Q              | 2.0 ± 0.4 | 0.19 ± 0.1 | 29 ± 12$^a$ | 4.3 ± 2.1 | 2.3 ± 0.8 | 0.8 ± 0.3$^a$ | 5.3 ± 1.8$^a$ |
| E97D              | 2.1 ± 0.9 | 0.25 ± 0.1 | 12.8 ± 3.5 | 1.4 ± 0.4 | 3.5 ± 0.3$^a$ | 1.8 ± 0.4 | 12 ± 2 |
| E224Q             | 7.5 ± 3.3 | 0.47 ± 0.3 | 13.0 ± 4.5 | 2.4 ± 0.4 | 4.5 ± 0.7$^a$ | 6 ± 2$^a$ | 9 ± 4 |
| E224D             | 21 ± 5$^a$ | 1.2 ± 0.4$^a$ | 37 ± 5$^a$ | 7.0 ± 1.5$^a$ | 12 ± 3$^a$ | 11 ± 4$^a$ | 41 ± 11$^a$ |
| E235Q             | 44 ± 12$^a$ | 22 ± 5$^a$ | >60$^a$ | >22$^a$ | >20$^a$ | 12 ± 5$^a$ | 150 ± 20$^a$ |
| E235D             | >2-10$^a$ | NR$^a$ | NR | NR | NR | NR |

$^a$ Significantly different from WT ($p < 0.05$).

$^b$ NR, not relevant.

$^c$ NB, no specific binding could be detected.

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**Granisetron competition for 5-HT binding measured with whole cell patch clamp.** After incubation with increasing concentrations of granisetron, the maximal electrical response upon the addition of 5-HT (concentration corresponding to 90% $I_{\text{max}}$ of the respective mutant) is recorded for 5-HT$_3$R WT ($\times$) and mutants E97Q (□), E97D (○), E224Q (■), E224D (△), and E235Q (▲). E235D could not be measured, since no comparable agonist concentration could be reached. Currents are normalized to the electrical response evoked by the respective 5-HT concentration before the incubation with the antagonist. Representative curves are shown.

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**Localization of 5-HT$_3$Rs in Cells by Immunofluorescence**—Microscopy glass cover slides with attached cells transiently expressing either WT or mutant 5-HT$_3$Rs were washed three times with PBS and incubated in ice-cold 4% paraformaldehyde in PBS for fixation of cells. To immunolabel the N-terminal extracellular domain, pAb120 antiseraum, containing antibodies directed against the first 13 residues of the mature protein; i.e., until 6 residues before the start of the first transmembrane domain) was aligned to the sequence of the AChBP (residues 1–198 of the recently solved crystal structure (Protein Data Bank entry 1BBB.pdb)). Optimal alignment resulted in three gaps of 2, 1, and 3 residues, respectively, between residues Asp$^{193}$, Glu$^{195}$, Ala$^{197}$, Thr$^{198}$, and Asp$^{195}$, Pro$^{197}$ in the sequence of the AChBP. The alignment was submitted to SWISS-MODEL for the generation of a homology model of the N-terminal domain of the 5-HT$_3$R based on the template of the structure of AChBP using Swiss-PdbViewer 3.7 as interface (25). The structural model thus obtained was inspected using either Swiss-PdbViewer or Rasmol version 2.7.

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**Structural Model of Extracellular N-terminal Domain of 5-HT$_3$R**—The N-terminal domain of the murine 5-HT$_3$R (SwissProt entry P23997, residues 14–217 of the mature protein; i.e., until 6 residues before the start of the first transmembrane domain) was aligned to the sequence of the AChBP (residues 1–198 of the recently solved crystal structure (Protein Data Bank entry 1BBB.pdb)). Optimal alignment resulted in three gaps of 2, 1, and 3 residues, respectively, between residues Asp$^{193}$, Glu$^{195}$, Ala$^{197}$, Thr$^{198}$, and Asp$^{195}$, Pro$^{197}$ in the sequence of the AChBP. The alignment was submitted to SWISS-MODEL for the generation of a homology model of the N-terminal domain of the 5-HT$_3$R based on the template of the structure of AChBP using Swiss-PdbViewer 3.7 as interface (25). The structural model thus obtained was inspected using either Swiss-PdbViewer or Rasmol version 2.7.
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**Localization of the 5-HT₃R WT and mutant receptor proteins transiently expressed in HEK293 cells.** To probe the presence of functional receptor proteins on the plasma membrane, life cells were incubated with 15 nM GR-flu for 30 min, after which fluorescence images were acquired by confocal fluorescence microscopy (left column) using equivalent settings, except for the image of the GR-flu incubation of E235D, where the sensitivity of the microscope was increased to its limits, as can be seen from the enhanced background. The cellular distribution of WT and mutant 5-HT₃R proteins was investigated using fluorescein-labeled streptavidin and a biotinylated secondary antibody. All images are 50 × 50 μm².

**Binding Experiments Using Radioactive Ligands.**—To investigate whether the effects due to the different mutations observed by electrophysiology are caused by changes in ligand affinity or channel gating, the ligand-binding properties of the WT and mutant 5-HT₃R were investigated using the radiolabeled agonist mCPBG and antagonist GR65630. Both the dissociation constants $K_d$ of the binding of these ligands to the receptor proteins and the inhibition of radioligand binding by granisetron or mCPBG were investigated. The results are summarized in Table I. The expression level of the different mutant proteins as compared with WT inferred from the saturation binding of $[^{3}H]$GR65630 ranged from 0.3-fold (E97Q and E224Q) to 1.5-fold (E97D), except for E235D, for which less than 0.03-fold was observed which is in the range of the experimental detection limit.

The agonist $[^{3}H]$mCPBG bound with comparable affinity to the mutant receptor protein E97D and E224Q as to WT, whereas for the mutants E97Q and E224D, 2.5–3-fold lower affinities were observed. For E235Q, the dissociation constant was increased more than 5 times, whereas for E235D no significant binding could be observed. The Hill coefficient was close to unity in all cases. Inhibition by the antagonist granisetron of the binding of agonist $[^{3}H]$mCPBG to the various 5-HT₃R mutant proteins showed a similar trend.

The affinity of the antagonist $[^{3}H]$GR65630 for both E97 mutant proteins and E224Q was slightly less (2–3-fold) as compared with WT, whereas for E224D and E235Q a 10-fold or more reduced affinity was observed. As for the agonist $[^{3}H]$mCPBG, no binding of $[^{3}H]$GR65630 to E235D could be detected. Antagonist inhibition of $[^{3}H]$GR65630 binding was about 2-fold stronger (E97Q), similar (E97D), or about 5-fold (E224Q) and 10-fold (E224D and E235Q) weaker then for WT. A similar trend was observed for agonist inhibition of $[^{3}H]$GR65630 binding.

In general, large effects on radioligand binding were observed for those mutants, featuring decreased potencies of ligand in electrophysiology.

**Localization of 5-HT₃R in Cells.**—To investigate whether the absence of significant whole cell currents upon 5-HT addition and of radioligand binding to E235D is either due to the mutation in an otherwise intact protein or caused by defect biogenesis of the protein, we applied both binding of GR-flu and immunolocalization, using a monoclonal antibody recognizing the extracellular domain of the receptor (24).

A distinct fluorescence signal due to specific binding of GR-flu was observed for all receptor proteins except E235D (Fig. 5, left column). Staining with the receptor-specific antibody (Fig. 5, middle column) demonstrated the presence of WT and all 5-HT₃R mutants on the surface of the intact HEK293 cells but
was less evident for E97Q and E235D. In permeabilized cells, intracellular localized receptor was detected, especially in the case of E235D. These data suggest that mutation E235D impairs folding, assembly, and/or trafficking of the 5-HT₃R to the cell surface.

**Fluorescence Measurements**—Binding of the competitive antagonist GR-flu to WT 5-HT₃R results in a ~70% decrease of its fluorescence intensity, due to a local acidic pH of the binding site; concomitantly, the fluorescence anisotropy increases (9, 11). To assess whether one of the Glu residues under investigation is responsible for this fluorescence intensity decrease, fluorescent ligand binding studies were performed on detergent-solubilized membranes of cells expressing the different receptor proteins. Binding of GR-flu to the receptor can be shown unequivocally by an increase of the fluorescence anisotropy, whereas the local environment sensed by the receptor-bound GR-flu is indicated by its fluorescence intensity. Fig. 6 summarizes the results obtained for both the fluorescence intensity and anisotropy of receptor-bound GR-flu. The Glu⁹⁷ and Glu²²⁴ mutant proteins yielded high fluorescence anisotropies and low fluorescence intensities, comparable with WT, albeit Glu²³⁵ mutant proteins did not bind to GR-flu, suggesting that Glu²²⁴ is located not too distant from the ligand binding site, either allowing a direct interaction of this residue with the chromophore or provoking a protein structural change that affects the fluorescent properties of the chromophore.

**DISCUSSION**

Negatively charged amino acids have been implicated in ligand binding to ligand-gated ion channels of the Cys loop family. Here, we investigated the role of 3 glutamate residues located in the binding loops C and D, which are conserved in the nAChR, a corresponding Asp in the mouse muscle –subunit (δAsp²⁰²) was mutated to Asn without any effect on agonist affinity or efficacy (18). An explanation of these different results might be that Glu²³⁵ in the 5HT₃R is located in loop C, whereas the mouse muscle –subunit contributes with loops D–F to the ligand binding site (i.e. the sequence corresponding to loop C in the δ-subunit is not involved in ligand binding). To our knowledge, no equivalent mutation has been reported for the α-subunit of the nAChR.

**Residue Glu²²⁴**—Mutation of Glu²³⁵ to Asp had a dramatic effect on both the functional properties of the receptor and its biogenesis. Exceedingly high ligand concentrations were needed to observe a low but significant channel conductivity, indicating that the efficacy of 5-HT was reduced by at least 3 orders of magnitude. The presence of functional receptor protein in the plasma membrane of cells could not be detected by
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Glutamate residues Glu224 and Glu235 are involved in formation of hydrogen bonds implicated in ligand binding or channel gating. Finally, the results show that the structural model is reliable and forms an important base to direct further biochemical and biophysical research.

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