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A high-affinity monoclonal antibody with functional activity against the 5-hydroxytryptaminergic (5-HT_4) receptor

Rehab Kamel [a], Pierre Eftekhari [b], Simone Garcia [c], Magali Berthouze [d], Isabelle Berque-Bestel [e], Jean-Christophe Peter [a], Frank Lezoualc'h [d], Johan Hoebeke [a,*]

[a] *UPR 9021 du C.N.R.S., Immunologie et Chimie Thérapeutiques, I.B.M.C., 15, Rue René Descartes, F-67084 Strasbourg, France*
[b] *FORENAP Therapeutic Discovery, Institute of Pharmacology, School of Medecine, 11, Rue Humann, F-67000 Strasbourg, France*
[c] *Fnd. Oswaldo Cruz, Brotas, Salvador CERP40295-001, Brazil*
[d] *Inserm U-446, Faculté de Pharmacie, 92296 Châtenay-Malabry, France*
[e] *UMR-8076, CNRS, Faculté de Pharmacie, 92296 Châtenay-Malabry, France*

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Abstract

Splenocytes from a BALB/c mouse immunised with a synthetic peptide corresponding to the second extracellular loop of the 5-HT_4 receptor were fused with SP2/O myeloma cells to produce a monoclonal antibody. The monoclonal antibody was of the IgG2b isotype. The antibody recognised the human 5-$HT_{4(g)}$ (h5-$HT_{4(g)}$) receptor by immunoblots and by immunofluorescence on chinese hamster ovary (CHO) cells expressing this 5-HT_4 receptor isoform. Epitope mapping of the antibody suggested the recognition of a conformational epitope, encompassing the N- and C-terminal fragments of the second extracellular loop. Kinetic experiments using surface plasmon resonance showed that the antibody had a picomolar affinity for its cognate peptide. Inhibition experiments using the same methodology confirmed the specificity of the interaction. The antibody at a concentration of 500 pM competitively inhibited inverse agonist GR113808 binding and showed an inverse agonist effect on the basal activity of CHO cells expressing the 5-$HT_{4(g)}$ receptor. The antibody decreased the effect of 5-HT at 500 and 50 pM concentrations but it increased 5-HT-induced cAMP levels at 5 pM. The dual effect of the monoclonal antibody could be ascribed to mono- or bivalent recognition of the receptor. The antibody described here is the first example of a high-affinity modulator of the 5-HT_4 receptor.

Keywords: 5-HT_4 receptor; Antagonist; cAMP; Epitope mapping; Monoclonal antibody; Surface plasmon resonance

1. Introduction

G protein-coupled receptors (GPCR) belong to a superfamily of membrane proteins with seven α-helices spanning the membrane lipid bilayer. They are widely expressed throughout the body and play a fundamental role in physiology and pathophysiology. They possibly represent the most important target class of proteins for drug discovery. Over 30% of clinically marketed drugs are active at this receptor family [1,2].

The 5-HT_4 receptor is a member of this family of receptors that is coupled to Gs proteins and through stimulation of adenylyl cyclase activity increases intracellular cAMP [3,4]. The 5-HT_4 receptors are localised in the central nervous system, heart, gastrointestinal tract, adrenal cortex and bladder [5,6]. They are involved in a variety of pathological disorders like cognitive dysfunction, irritable bowel syndrome, gastroparesis, urinary incontinence and cardiac arrhythmias [7–9]. It has been shown in vitro that 5-HT_4 receptor agonists stimulate the release of the non-amyloidogenic soluble amyloid precursor protein, which has neuroprotective and memory-enhancing properties, suggesting that these receptors may represent a novel target for the treatment of Alzheimer's disease [10]. A recent study demonstrated that activation of 5-HT_4 receptors in the medullary respiratory center represents a method

Abbreviations: CHO cells, Chinese hamster ovary cells; GPCR, G protein-coupled receptors; RU, resonance units; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SPR, surface plasmon resonance

* Corresponding author. Tel.: +33 3 88 41 70 24; fax: +33 3 88 61 06 80.
E-mail address: J.Hoebeke@ibmc.u-strasbg.fr (J. Hoebeke).

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for treatment of respiratory depression induced by opioids without loss of analgesia [11]. Consequently, these receptors constitute a valuable target for the design of new drugs.

The second extracellular loop of GPCR represents a preferential target for functional autoantibodies [12]. This was demonstrated in pathological cases, where autoantibodies were identified against several receptors [13–17]. Cross-reactivity was found between the peptide derived from the second extracellular loop of the 5-HT_4 receptor and the SSA/Ro52 ribonucleoprotein, which is an antigenic target strongly associated with the autoimmune response in mothers whose children develop neonatal lupus [18]. The affinity-purified polyclonal antibodies raised in rabbits against the second extracellular loop of the 5-HT_4 receptor had no intrinsic activity but were able to depress 5-HT-induced L-type calcium channel activation in human atrial cells [19]. These results suggested that the 5-HT_4 receptor could be a molecular target for autoantibodies in mothers at risk of giving birth to children with neonatal atrio-ventricular block. It has also been demonstrated that 5-HT_4 receptor is the antigenic target of physiopathological autoantibodies in pups of mice immunised with 5-HT_4 receptor peptides [20].

In the present work, in order to unravel the mechanism by which anti-5-HT_4 receptor autoantibodies exert their potential deleterious function, we produced monoclonal antibodies (mAb) against a peptide corresponding to the second extracellular loop of the 5-HT_4 receptor. Indeed, monoclonal antibodies are homogeneous and very specific reagents for their target antigen, which may be considered as new interesting tools for studying receptor function and developing target-specific ligands [21]. The physico-chemical parameters of the antibody interaction with the target were defined, the fine specificity of the recognised epitope was established and its pharmacological activity on the 5-HT_4 receptor was assessed.

2. Materials and methods

2.1. Materials

Polyethelene glycol solution 1450, Tween 20, 3,3′,5,5′-tetramethyl benzidine (TMB), Dulbecco's Modified Eagle's Medium (DMEM), 5-hydroxytryptamine (5-HT), 3-isobutyl-1-methylxanthine (IBMX), 4′,6-diamidino-2-phenylindole (DAPI) and paraformaldehyde were from Sigma–Aldrich. Iscove's Modified Dulbecco's Medium (IMDM) was from BioWhittaker, France. Bovine foetal serum, goat serum, sodium pyruvate, penicillin, streptomycin, gentamycin, hypoxanthine, aminopterine, thymidine (HAT) supplements were purchased from Invitrogen, France. Activated thiol sepharose 4B was from Pharmacia Diagnostics AB, Uppsala, Sweden. β-Mercaptoethanol was from Carl Roth GmbH, Karisruhe, Germany. BSA and antiprotease cocktail tablets were from Roche Diagnostics, Mannheim, Germany. Affinity-purified goat anti-mouse IgG, Fc fragment-specific antibodies conjugated to horse-radish peroxidase (HRP) were from Jackson Immunoresearch Laboratories, San Diego, CA. HEPES-buffered saline (HBS) was from BIACORE, Uppsala, Sweden. SPOTs cellulose membrane and Fmoc-amino acid-active esters were from Sigma Genosys, Cambridge, UK. Skimmed milk was from Bio-Rad Laboratories, Hercules, CA. Alexa Fluor 594 goat anti-mouse IgG (H + L) was from Molecular Probes, OR, USA. DAKO fluorescent mounting medium was from Dako Corporation, Carpinteria, USA. HEPES and Hanks balanced saline solution (HBSS) were from Bio Media, Boussens, France. ML10375 (2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate) was kindly provided by Dr. Michel Langlois, Faculty of Pharmacy at Châtenay-Malabry, France. The mouse monoclonal IgG2b anti-H2B histone was kindly provided by Dr. Marc Monestier, Temple University School of Medicine, Philadelphia, USA. GR113808 (1-[(2-methylsulphonyl) amino] ethyl-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate) was a gift from Glaxo Research Group (Ware, Hertfordshire, UK) [3H] GR113808 was purchased from Amersham Biosciences (UK).

2.2. Peptides

Two peptides, corresponding to the sequences derived from the human 5-HT_4 receptor (G21V and C15Q) [18] as well as a control peptide H19C corresponding to the second extracellular loop of the human β_2 adrenergic receptor [22], were synthesised in an automatic peptide synthesiser as previously described [23]. The peptides were purified by HPLC and checked by mass spectrometry. Sequences are shown in Table 1.

2.3. Production of monoclonal antibodies

The spleen of a BALB/c mouse immunised with G21V peptide (as previously described [18]) was harvested for fusion. Mouse splenocytes and myeloma cells SP2/O were fused according to the standard procedures. Briefly, a ratio of one to two splenocyte/myeloma cells were gradually mixed with 2 ml polyethylene glycol solution (PEG 1450) [24]. The mixture was diluted within a total time of 7 min with a 10-fold volume of Iscove's Modified Dulbecco's Medium supplemented with 10% bovine foetal serum, 1% sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol. The cells were kept for

Table 1
Sequences of different peptides derived from human 5-HT_4 receptor and β_2-adrenergic receptor

G21V (165–185)	GIIDLIEKRKFNQNSNSTYCV
C15Q (164–177)	(C) IGIIDLIEKRKFNQ
H19C (172–190)	HWYRATHQEAINCYANETC

10 min at 37 °C prior to centrifugation at 265 × *g* for 7 min. The pellet was suspended in 25 ml of complete IMDM medium supplemented with 2% hypoxanthine, aminopterine, thymidine. Cells were distributed in seven pre-coated 96-well culture plates with peritoneal macrophages of BALB/c mice and cultured for 8 days in a humidified incubator at 37 °C under an atmosphere of 5% CO_2. Secreting clones were screened by direct enzyme linked immunoassay and sub-cloned at least twice, by the limiting dilution method [25].

2.4. Purification of produced antibodies

The supernatant of cell cultures in a MINIPerm bioreactor (Vivascience, Sartorius group, Göttingen, Germany) was affinity-purified on C15Q peptide coupled to activated thiol-Sepharose 4B column according to the standard procedure. The supernatant was passed on the column at 4 °C. The bound antibodies were eluted with 0.2 M glycine pH 2.7, collected in tubes containing 1M Tris buffer pH 8 and immediately dialyzed against PBS (phosphate 10 mM, NaCl 150 mM, KCl 27 mM, pH 7.4) overnight at 4 °C and stored at −20 °C until use.

2.5. Mouse monoclonal antibody ($C_{10}A_{11}$) isotyping

Isotyping of the monoclonal antibody was performed on hybridoma supernatants using mouse monoclonal antibody typing kit (ICN Immunobiologicals, France).

2.6. Enzyme-linked immunosorbent assay

Five micrograms per millilitre of peptides were coated in 100 mM carbonate buffer at pH 9.6, for 1 h on microtiter plates (Falcon, Oxnard, CA) at 37 °C. The wells were then saturated with PBS supplemented with 0.1% Tween 20 and 1% BSA for 1 h at 37 °C. Fifty microliters of hybridoma supernatants or purified monoclonal antibody at different dilutions in PBS–Tween–BSA were added to the saturated plate for 1 h at 37 °C. After washing the wells three times with PBS–Tween, affinity-purified goat anti-mouse IgG, Fc fragment-specific antibodies (diluted 1/5000 in saturation buffer) conjugated to horse-radish peroxidase were allowed to react for 1 h at 37 °C. The wells were then washed three times with PBS–Tween followed by PBS before revealing the bound antibodies by addition of 3,3′,5,5′-tetramethyl benzidine in the presence of H_2O_2. Reaction was stopped with 1N HCl and absorbance was read at 450 nm in a microplate reader.

2.7. Surface plasmon resonance

Measurements were performed using BIACORE 3000 system. G21V and H19C peptides were immobilised on the sensor chip CM5 (BIACORE, Uppsala, Sweden) by the thiol groups of their cysteines as previously described [26]. $C_{10}A_{11}$ was diluted in HEPES-buffered saline, the sensor surface chip was regenerated after each experiment using 50 mM HCl. Active concentration of monoclonal antibody was measured under conditions of partial mass transport limitation using different flow rates [27]. Kinetic parameters were measured using increasing concentrations of antibody, which were injected on immobilised peptides at a flow rate of 30 μl/min for 5 min followed by dissociation for 10 min at the same flow rate. BIAeval 3.1 software (Biacore) was used for kinetic analysis using a monovalent binding with mass transfer model. This model, taking into account the diffusion rate of the antibody towards the reactive surface, yielded the best fit to the experimental results. In view of the low dissociation rate, the k_{off} was calculated from the linear fit $k_{obs} = k_{on}[Ab] + k_{off}$ in which k_{obs} is the observed association rate at different concentrations of antibody [Ab], k_{on} and k_{off} being, respectively, the association and dissociation rate constants.

2.8. Inhibition assay using surface plasmon resonance

H19C and C15Q peptides were immobilised on the sensor chip as described above. $C_{10}A_{11}$ (250 pM) alone or mixed with different concentrations (0.156–100 nM) of G21V and C15Q was injected at a flow rate of 20 μl/min for 2 min followed by dissociation for the same time at the same flow rate. Inhibition percentages were calculated from the initial association slopes of sensorgrams representing binding of immobilised peptide with monoclonal antibody alone or mixed with peptides. IC_{50}'s were calculated by the logistic three parameters equation as used by Sigma-Plot 2000 software. The inhibitor constant (K_i) of peptides in solution were calculated from the IC_{50}s according to [28]:

$$K_i = \frac{\sqrt{2}[IC_{50} - m_{Ab}]}{2 + \sqrt{2}}$$

2.9. Epitope mapping of monoclonal antibody and alanine replacement scanning

A set of 11 undecapeptides, overlapping by 10 residues, covering the sequence 165–185 of the h5-HT_4 receptor was synthesised using spot method on cellulose membrane [29] as modified by Molina et al. [30]. To assess the reactivity of the set of immobilised peptides, the membrane was blocked overnight in TBS (Tris-buffered saline: Tris 10 mM; NaCl 140 mM; pH 7.4) supplemented with 0.1% Tween 20 and 5% skimmed milk. It was incubated with 25 pM of $C_{10}A_{11}$ in blocking buffer for 3 h at room temperature followed by an affinity-purified goat anti-mouse IgG, Fc fragment-specific antibodies conjugated to HRP, diluted 1/6000 in blocking buffer for 2 h. Positive peptides were identified using the ECL plus kit (Amersham Biosciences, UK).

By the same method, a set of 21 decapeptides corresponding to the regions defined in previous experiment was prepared by substituting alanine for every residue. Recognition of the modified peptides was performed as described above.

2.10. Membrane preparation

CHO cells expressing the h5-$HT_{4(g)}$ receptor [31] or non-transfected CHO cells were disrupted in lysis buffer (Tris 300 mM, NaCl 150 mM, antiprotease cocktail tablets, pH 8) for 1 h at 4 °C followed by centrifugation at 739 × *g* for 10 min at the same temperature. The pellets were suspended in PBS supplemented with antiprotease cocktail tablets and homogenised. The suspensions were centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatants ultracentrifuged at 58,000 × *g* for 40 min at 4 °C. The pellets were resuspended in PBS supplemented with antiprotease cocktail tablets. The protein amount was assessed using BCA Protein Assay Reagent Kit (PIERCE, Rockford, USA).

2.11. Immunoblotting

Membrane proteins (200 μg/gel) from transfected (expressing the h5-$HT_{4(g)}$ receptor) and non-transfected CHO cells, were submitted to SDS-PAGE [32] and subjected to electrotransfer to nitrocellulose membrane according to the standard procedures [33]. The membrane was blocked for 2 h at room temperature in TBS (pH 7.4) supplemented with 0.1% Tween 20 and 5% skimmed milk. It was incubated with 1.8 pM of purified $C_{10}A_{11}$ in blocking buffer for 1 h at room temperature followed by an affinity-purified goat anti-mouse IgG, Fc fragment-specific antibodies conjugated to HRP, diluted 1/10,000 in blocking buffer for 1 h. Antibody detection was performed using the ECL plus kit.

2.12. Immunocytochemistry

CHO cells expressing the 5-$HT_{4(g)}$ receptor and untransfected cells were deposited on SuperFrost Plus slides (Menzel-Glazer, Germany) and fixed for 2 min in paraformaldehyde 4%. They were washed with PBS, PBS–Tween 20 (0.1%) and saturated with PBS supplemented with 0.1% Tween 20 and 5% bovine foetal serum for 1 h at room temperature. They were incubated with 6 pM $C_{10}A_{11}$, for 2 h at room temperature followed by 1 μg/ml 4′,6-diamidino-2-phenylindole and Alexa Fluor 594 goat anti-mouse IgG (H + L) diluted 1/200 for 1 h at the same temperature. The slides were mounted using DAKO fluorescent mounting medium.

2.13. Radioligand-binding assay

Radioligand-binding studies were performed in 250 μl of HEPES buffer (50 mM, pH 7.4), 20 μl of the studied antibody, 20 μl of [^{3}H]GR113808 at a concentration of 0.2 nM (K_d) and 50 μl of membrane preparations from CHO cells transfected with 5-$HT_{4(g)}$ receptor (80 μg of protein). Non-specific binding was determined with 10 μM GR113808. Competition assays were performed in the presence of four concentrations of control monoclonal antibody or $C_{10}A_{11}$ (from 10^{-11} to 4.5×10^{-10} M) and a concentration of [^{3}H] GR113808 corresponding to the K_d value. Saturation experiments were performed using [^{3}H] GR113808 at five concentrations ranging from 0.01 to 3.75 nM in presence of 450 pM of control monoclonal antibody or $C_{10}A_{11}$.

Samples were incubated at 25 °C for 30 min and the reaction was terminated by filtration through Whatman GF/B Filter paper using the Brandel 48R cell harvester. Filters were presoaked in a 0.1% solution of polyethylenimine, washed with ice-cold buffer (50 mM Tris–HCl, pH 7.4) and placed overnight in 4 ml of ready-safe scintillation cocktail. Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. K_d and B_{max} values for [^{3}H] GR113808 in the presence of 450 pM $C_{10}A_{11}$ or control antibodies were calculated by non-linear regression using Sigma-Plot 2000 software. The data are the results of one determination in triplicate.

2.14. Cyclic AMP assay

Transfected and non-transfected CHO cells were distributed in six-well plates, treated overnight at 37 °C with serum free Dulbecco's Modified Eagle's Medium supplemented with 1% geneticin (for transfected cells), 100 U/ml penicillin, 100 μg/ml streptomycin. The cells were incubated with 1 ml Hanks balanced saline solution (HBSS) buffered with 10 mM HEPES containing 100 μM 3-isobutyl-1-methylxanthine for 30 min at 37 °C and treated with different concentrations of $C_{10}A_{11}$, of mouse monoclonal IgG2b anti-H2B histone or with 5 μM ML10375 [34] for 30 min. Treatment with 500 nM 5-HT was for 15 min at 37 °C. Supernatants were then aspirated and 1 ml of boiling water added to each well. cAMP assay was performed following acetylation procedure using cAMP competitive enzyme immunoassay (Amersham Pharmacia Biotech, Orsay, France) and the protein amount was assessed using BCA Protein Assay Reagent Kit (PIERCE, Rockford, USA).

3. Results

3.1. Physico-chemical parameters of the peptide antibody interaction

Kinetic parameters were measured at 25 °C; different concentrations of the antibody (0.625–10 nM) were injected on the immobilised peptides (G21V and H19C) at a flow rate of 30 μl/min for 5 min, followed by a dissociation phase for 10 min (Fig. 1). The values obtained

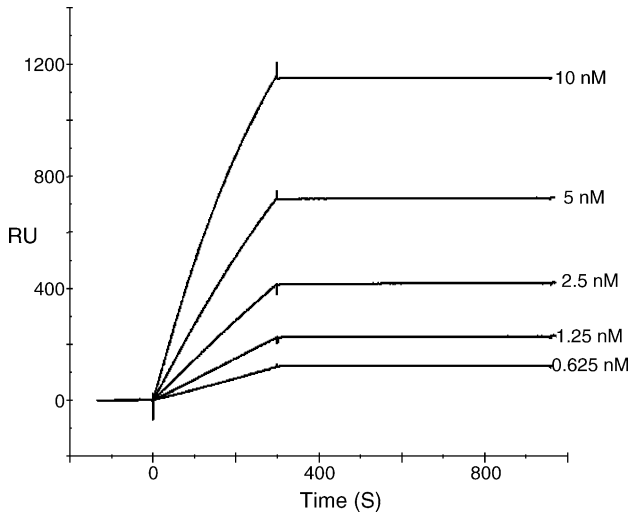

Fig. 1. Assessment of antibody–peptide interaction using surface plasmon resonance. The sensorgrams represent the interaction of increasing concentrations (0.625–10 nM) of monoclonal antibody $C_{10}A_{11}$ with immobilised G21V peptide. An association phase of 5 min was followed by a dissociation phase of 10 min. The response was measured in resonance units (RU) vs. time.

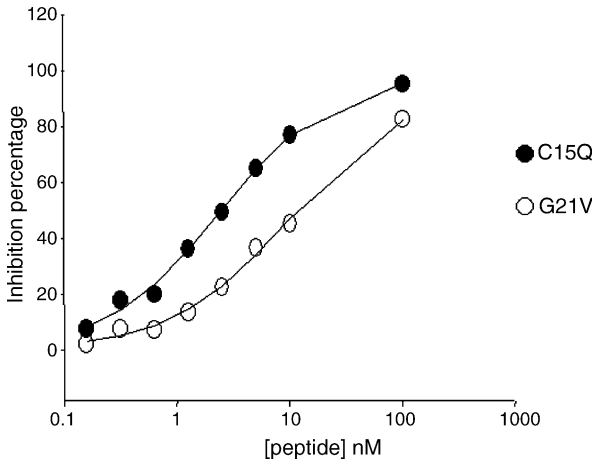

Fig. 2. Inhibition percentages curves for different concentrations of G21V and C15Q peptides are represented. Inhibition percentages were calculated relative to the monoclonal antibody response using surface plasmon resonance without being mixed with peptides. The values of pIC_{50} for G21V and C15Q were 8.03 ± 0.06 and 8.64 ± 0.04, respectively. The respective calculated values for K_i were of 3.8 and 0.9 nM for G21V and C15Q.

for the association rate constant (k_{on}) and dissociation rate constant (k_{off}) were $4.29 \pm 0.07 \times 10^5\ M^{-1}\ s^{-1}$ and $1.4 \pm 0.3 \times 10^{-4}\ s^{-1}$, respectively, yielding a dissociation constant, $K_d = 330$ pM. The quasi-irreversible complex as expressed by the absence of dissociation on the sensorgrams is due to a combination of bivalent binding (avidity) and reassociation of the released antibodies.

3.2. Peptide-specificity of monoclonal antibody

In order to demonstrate the specificity of $C_{10}A_{11}$ against C15Q peptide, an inhibition assay was performed using surface plasmon resonance phenomenon. Different concentrations of C15Q and G21V peptides were mixed with 250 pM of antibody. A concentration-dependent inhibition of immobilised C15Q–antibody interaction was observed for both peptides (Fig. 2). The calculated K_i was of 3.8 and 0.9 nM for G21V and C15Q, respectively.

3.3. The monoclonal antibody $C_{10}A_{11}$ recognises the 5-HT_4 receptor

It has been demonstrated that polyclonal rabbit anti-G21V antibodies recognise by immunoblotting the 5-HT_4 receptor in CHO cell membranes [18]. Here, we showed that the $C_{10}A_{11}$ recognises a protein band of 60 kDa corresponding to the expected molecular mass for glycosylated 5-HT_4 receptor (Fig. 3). A band of 120 kDa, probably, corresponding to the receptor dimer was also detected. No immunoreactivity was observed for either non-transfected CHO cells or transfected CHO cells treated only with secondary antibody (Fig. 3).

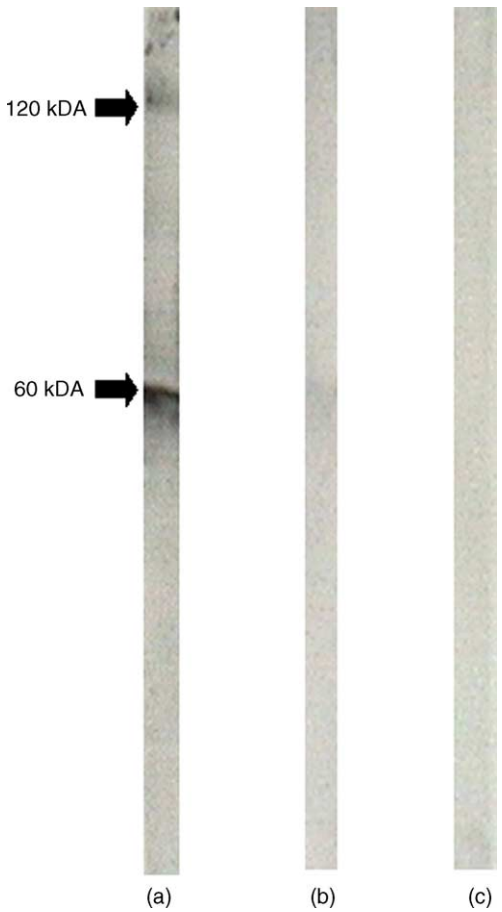

Fig. 3. Immunoblots on membrane preparation from untransfected and 5-$HT_{4(g)}$ receptor transfected CHO cells. *Lane a:* bands of 60 and 120 kDa corresponding to the 5-HT_4 receptor monomer and dimer, respectively, were revealed with $C_{10}A_{11}$. *Lane b:* preparation from untransfected CHO cells showing the absence of recognition of the receptor. *Lane c:* membrane proteins from transfected CHO cells incubated with secondary antibody conjugated to HRP. This immunoblot is representative of three independent experiments.

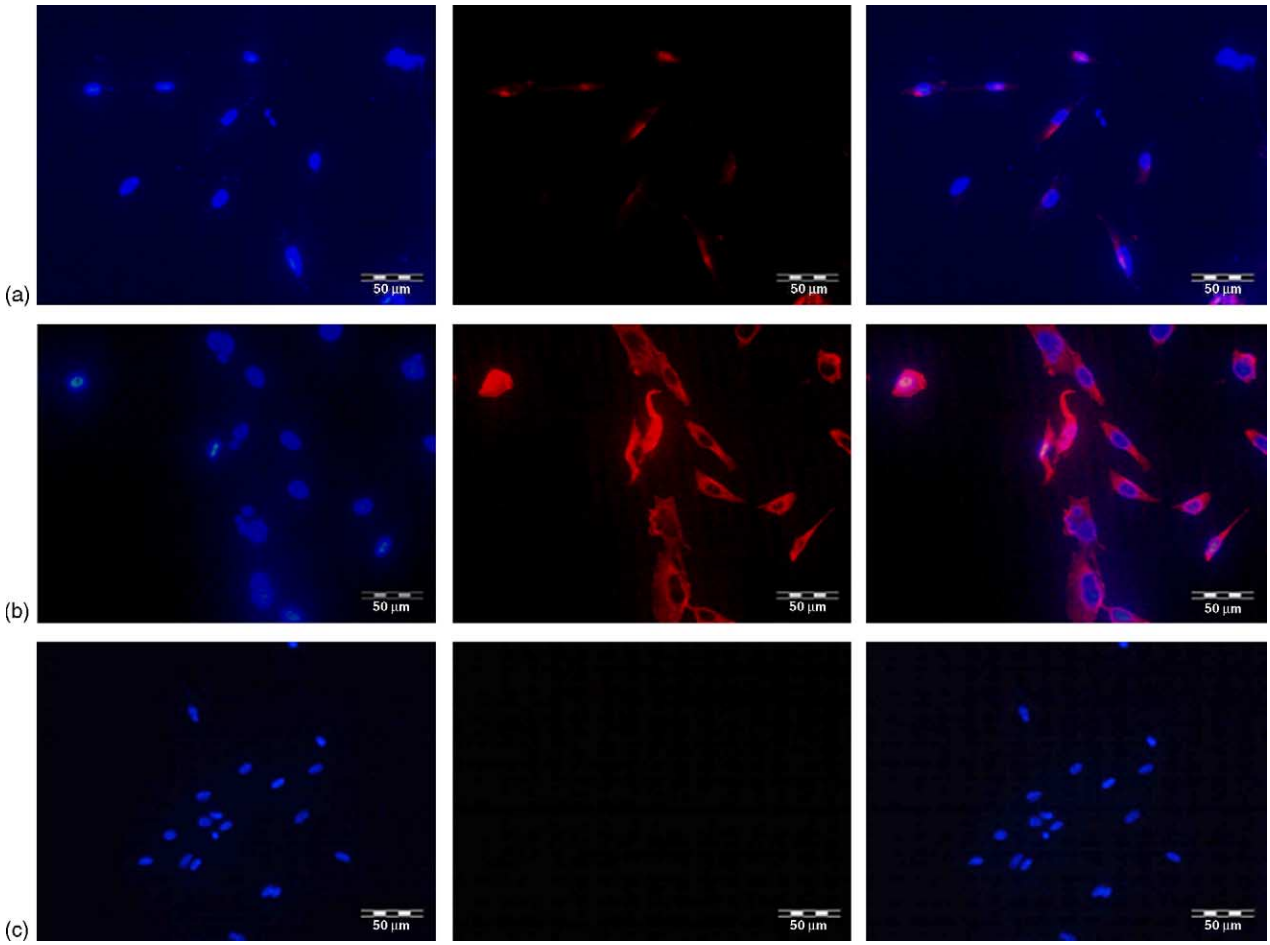

Fig. 4. Immunocytochemical characterisation of $C_{10}A_{11}$. *Middle panels:* immunolabeling of (a) non-transfected and (b) 5-$HT_{4(g)}$ receptor transfected CHO cells. A membrane and cytoplasmic-specific labelling is observed for transfected cells compared with non-transfected which show a slight unspecific nuclear labelling. (c) 5-$HT_{4(g)}$ receptor transfected CHO cells only treated with Alexa Fluor 594 goat anti-mouse IgG (H + L). *Left panels:* the DAPI nuclear coloration of the corresponding cells. *Right panels:* the merge of left and middle panels. The figure is representative of three separate experiments.

In order to verify the capacity of the monoclonal antibody to recognise the native receptor, immunocytochemistry using untransfected and transfected CHO cells with 5-$HT_{4(g)}$ receptor was performed. Specific labelling was shown to occur with the transfected cells at membrane and cytosolic level compared with untransfected cells. No labelling was found after treatment of transfected cells with the Alexa Fluor 594 goat anti-mouse IgG (H + L) only (Fig. 4).

3.4. Epitope mapping of $C_{10}A_{11}$

To characterise the epitope recognised by the antibody, epitope mapping by the spot method was performed using overlapping undecapeptides encompassing the G21V sequence. The monoclonal antibody was shown to react with two regions of the peptide corresponding to the N-terminal sequence IDLIEKRKF and the C-terminal sequence FNQNSNSTYC, respectively (Fig. 5a). In an attempt to determine the critical residues of the recognised epitope, alanine replacement for each residue was successively done. Densities of obtained spots were measured using MacBas software. The critical residues detected were Arg 173, Phe 175 and Cys 184 (a respective decrease in signal of 75, 80 and 81% of the value obtained with the control peptide (Fig. 5b). A significant loss of signal was observed when Lys 174 (40%) and Tyr 183 (51%) were replaced by alanine suggesting that these residues also contribute to antibody recognition.

3.5. Effect of the monoclonal antibody on [^{3}H] GR113808 binding

To assess the effect of the monoclonal antibody on 5-HT_4 receptor ligand binding, analysis of 0.2 nM [^{3}H] GR113808 binding in presence of increasing concentrations of $C_{10}A_{11}$ or control monoclonal antibody 6H8 (which is directed against the second extracellular loop of the β_2-adrenergic receptor) was done. A concentration-dependent decrease in radioligand-binding could be observed, with nearly 50% inhibition obtained at 450 pM $C_{10}A_{11}$. The control antibody had no effect on [^{3}H] GR113808 binding (Fig. 6a). Analysis of the radioligand-binding curve at 450 pM antibody showed a com-

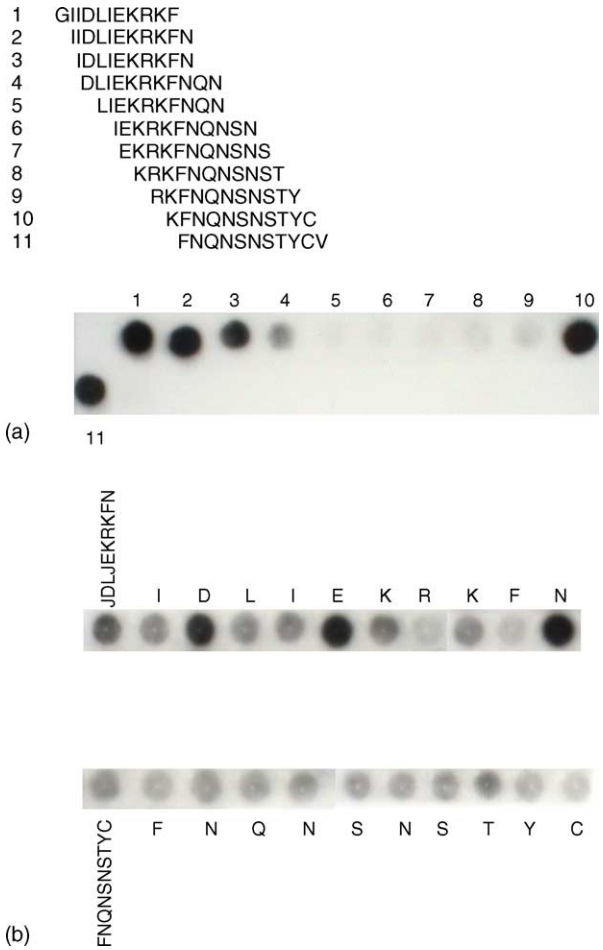

Fig. 5. Epitope analysis of $C_{10}A_{11}$. (a) Epitope localisation using overlapping undecapeptides. The sequences IDLIEKRKF (amino acids 167–175) and FNQNSNSTYC (amino acids 175–184) were shown to be immunoreactive. (b) Identification of key residues required for monoclonal antibody binding by alanine scan. Each residue of the immunoreactive peptides was successively replaced by alanine. Critical residues were found to be Arg 173, Phe 175 and Cys 184. Results shown are representative of two separate experiments.

petitive inhibition with increasing K_d values (0.43 nM versus 0.11 nM) and B_{max} values of, 0.04 and 0.028 nM, respectively (not significantly different) in the presence and absence of $C_{10}A_{11}$ (Fig. 6b).

3.6. Functional characterisation of $C_{10}A_{11}$

Functional effect of $C_{10}A_{11}$ was assessed by measuring cAMP production in CHO cells transfected with the h5-$HT_{4(g)}$ receptor. 5-HT increased cAMP levels in a specific manner since this increase was inhibited with the selective 5-HT_4 receptor antagonist ML10375. We found that $C_{10}A_{11}$ significantly decreased 5-HT-induced cAMP production when used at a concentration of 500 and 50 pM. At a concentration of 5 pM, the mAb had no intrinsic agonist activity, but significantly enhanced the effect of 5-HT. Decreasing the concentration of $C_{10}A_{11}$ to 0.5 pM leads to restoration of 5-HT activity (Fig. 7a). ML10375 (5 μM) and the mAb alone at 500 pM significantly decreased basal

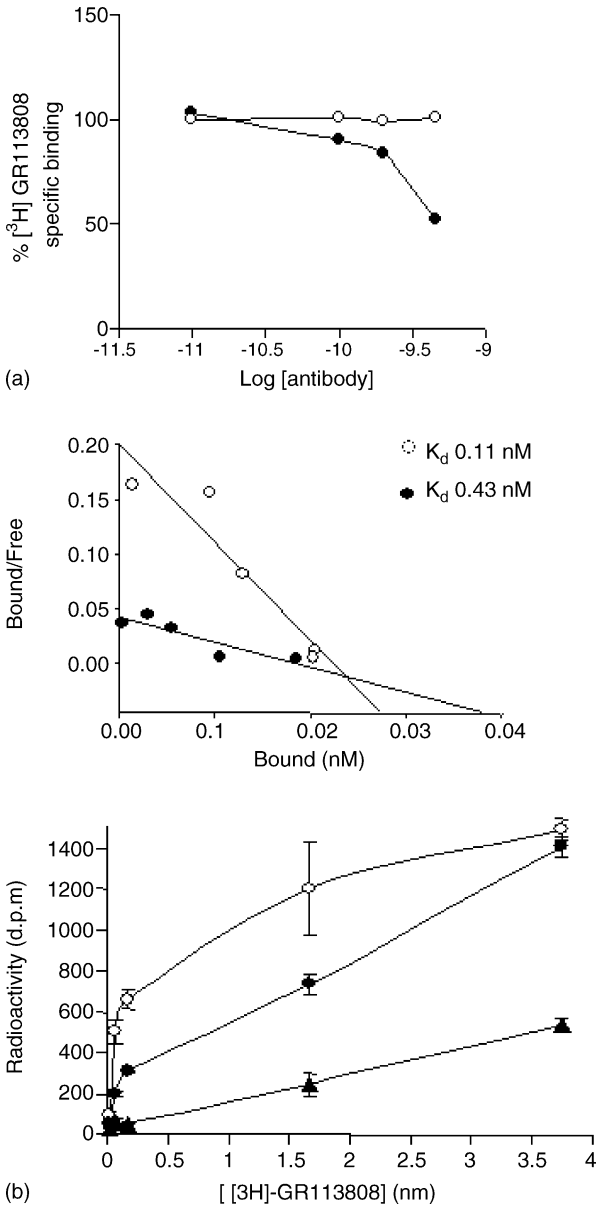

Fig. 6. Binding analysis of $C_{10}A_{11}$. (a) Analysis of 0.2 nM [^{3}H] GR113808 binding in the presence of increasing concentrations of $C_{10}A_{11}$ or control monoclonal antibody. The effect of various concentrations of $C_{10}A_{11}$ or the control monoclonal antibody on radioligand-binding was determined on membranes from CHO cells stably expressing the h5-$HT_{4(g)}$ receptor isoform. The radioactivity corresponding to the total binding and non-specific binding was 2224 ± 18 and 55 d.p.m, respectively. (b) Effect of $C_{10}A_{11}$ on [^{3}H] GR113808 binding to the 5-$HT_{4(g)}$ receptor stably expressed in CHO cells. Membranes harvested from stably transfected CHO cells were incubated with different concentrations of [^{3}H] GR113808 in the presence of 0.45 nM control monoclonal antibody (○) or $C_{10}A_{11}$ (●). Non-specific binding (▲) was determined with 10 μM GR113808.

cAMP levels (Fig. 7b). The inverse agonist activity of ML10375 on CHO cells transfected with the same receptor has previously been reported [31].

The mouse monoclonal IgG2b anti-H2B histone had no effect on 5-HT-induced cAMP production when used at the same concentrations of $C_{10}A_{11}$ (data not shown). Neither 5-HT nor $C_{10}A_{11}$ at 500 pM had any effect on cAMP production in non-transfected cells (data not shown)).

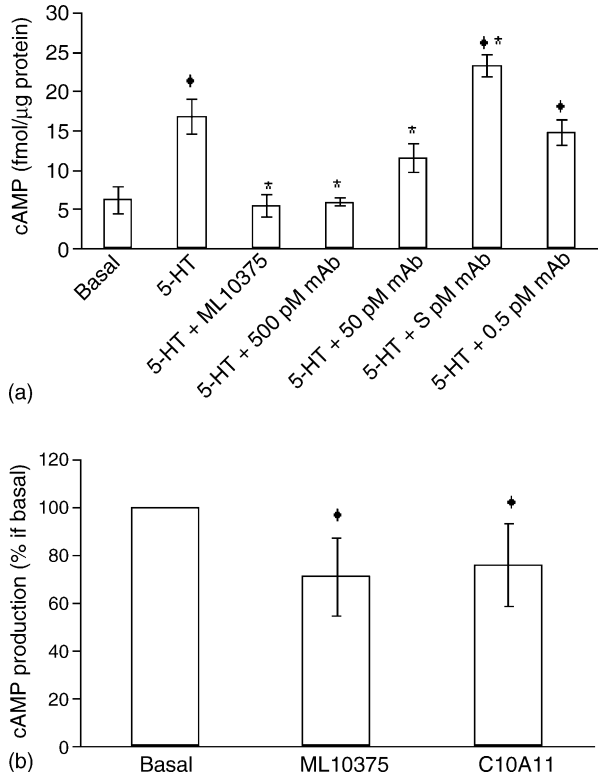

Fig. 7. Effect of $C_{10}A_{11}$ on intracellular cAMP production in transfected CHO cells with 5-$HT_{4(g)}$ receptor. (a) CHO cells were treated with 500 nM 5-HT. The effect of 5 μM ML10375 as well as different concentrations of $C_{10}A_{11}$ on 5-HT-induced cAMP production was also tested. cAMP production is expressed as fmol/μg proteins. (b) The effect of 5 μM ML10375 and 500 pM $C_{10}A_{11}$ on basal cAMP production is shown. The basal cAMP level (6.2 fmol/μg proteins) is obtained with untreated transfected cells. Values presented are means ± S.D. of two representative independent experiments performed in triplicate. Significantly different, $P < 0.05$ vs. control (♦) and 5-HT (*) values, respectively, by Student *t*-test.

4. Discussion

Although antibodies are widely used as markers for tagging membrane proteins and receptors, their use as pharmacological agents is not widespread. One of the reasons of this neglected property of antibodies is the more difficult characterisation of their physico-chemical properties and interaction mechanisms compared to more classical drugs, binding to a pharmacophore pocket. The possibility, however, to produce monoclonal antibodies, which are perfectly characterised molecules and antibody fragments derived from such monoclonals together with new sophisticated technologies to study molecular interactions has made such molecules more affordable for pharmacological evaluation. In particular, the determination of the second extracellular loop of G protein-coupled receptors as an epitopic target for functional anti-receptor antibodies has allowed the production and characterisation of monoclonal antibodies directed against the β_1-adrenergic receptors [35], β_2 adrenergic receptors [22] and the M_2 muscarinic receptor [36] and has stressed the importance of receptor dimerisation for their agonist-like activity [36,37].

The existence of autoantibodies against the 5-HT_4 receptor with blocking activity against 5-HT in anti-Ro52/SSA antibody positive mothers (a risk marker for congenital heart block) [18] and the induction of congenital heart block in neonates of female mice immunised with a peptide derived from the second extracellular loop of the 5-HT_4 receptor [20] has prompted us to produce and characterise monoclonal antibodies directed against this domain of the 5-HT_4 receptor. Among the 27 obtained clones, only three were shown to be positive for the immunogenic peptide and only one passed through two sub-cloning protocols to assure its monoclonality. This antibody, of the IgG2b isotype, was shown to be monoclonal by cloning of the variable fragment of its heavy chain and by N-terminal amino acid sequencing of its light chain (data not shown). Although the producing hybridoma was a poor antibody secretor, it was possible to purify the secreted monoclonal antibody in sufficient amounts for its characterisation.

Determination of the physico-chemical parameters of the antigen–antibody interaction showed an extremely high affinity (K_d = 330 pM), mainly due to the formation of a nearly irreversible complex with an estimated half-life of approximately 2 h ($t_{1/2} = \ln 2/k_{off}$). The structural basis of this recognition is conformational in nature. Indeed, epitope analysis showed that the N- and C-terminal part of the second extracellular loop are involved in antibody recognition with essential amino acids distributed over both fragments. The low percentage of a conformer, approaching the two fragments, in the free peptides explains the lower affinity (in the nM range) of the antibody for the free peptides compared to the peptides immobilised on the surface plasmon resonance chip by their cysteine moieties. Since these fragments must be neighbours in the receptor in view of the distance between the fourth and the fifth transmembrane regions, the existence of such an epitope seems straightforward. The antibody not only recognised the 5-HT_4 receptor in Western blots, in which the receptor is normally partially denatured, but it also recognised the receptor in situ on cells transfected with the 5-$HT_{4(g)}$ receptor as determined by immunofluorescence. The distribution of the fluorescence on membrane patches but also on cytosolic patches is compatible with receptor recognition at the plasma membrane level and in the cytoplasmic vesicles, trafficking the receptor from the Golgi apparatus towards the plasma membrane [38].

The antibody exerted a functional effect on the 5-HT_4 receptor. It competitively inhibited the binding of the inverse agonist GR113808 on the receptor. This is in contrast with other anti-GPCR antibodies showing a non-competitive [13] or uncompetitive [14] antagonist binding inhibition. We, therefore, must consider that the second extracellular loop forms an essential part of the inverse agonist-combining site. This observation is in line with the recent finding that the second extracellular loop of the C5a receptor is a negative regulator of receptor activity

[39]. Binding results were confirmed by functional results since the antibody, in a similar way as ML10375 [31,40] decreased constitutive receptor activity and could thus be considered as an inverse agonist. The inverse agonist effect showed itself at concentrations corresponding to the affinity constant, as defined by the monovalent binding model. Since monovalent antibody fragments, directed against the second extracellular loop of GPCR receptors, also show inverse agonist activity [41,42], the hypothesis can be made that, with an excess of antibody-combining sites, the antibody monovalently binds to the receptor.

The effect of the antibody on 5-HT-induced 5-HT_4 receptor activation was more complex. Indeed, at excess of antibody-combining sites (50 and 500 pM), the antibody antagonises the activation. However, at a lower concentration (5 pM), the antibody exerted an additional agonistic effect over the 5-HT effect. This apparently paradoxical result can be explained by the equivalence of the antibody with its antigen. In excess of antibody, the chance that both its binding sites are involved in receptor recognition is statistically very low since monovalent binding will be favoured. At concentrations equimolar to the receptor concentration (in the pM range), the chance of bivalent recognition of two receptors by one antibody increases since excess of binding sites is limited. As shown for other anti-G protein-coupled receptors, bivalency is necessary for agonist-like activity, while monovalent antibody recognition has an antagonist effect. The biphasic curve due to the polyvalency of antibodies, which is already a well know observation in immunological reactions [43] is here made use of in a special way; at antibody excess (500 and 50 pM), the antibody behaves as an antagonist favouring monovalent binding to receptors, at equivalency (5 pM) favouring stabilisation of receptor-active dimers, induced by the agonist 5-HT and under equivalency (0.5 pM) loosing both effects. The antibody indeed has no intrinsic ‘‘agonist-like’’ activity at 5 pM, suggesting that its effect on 5-HT activation at this concentration is a facilitating and not an additive effect. These results are in line with the fact that GR113808 has an extended binding site compared to 5-HT [44], extension which could partially cover the second extracellular loop, recognised by the antibody.

The antibody presented here is thus an exquisite tool to sense differences in the binding pockets between agonist and antagonist and the different conformations stabilised by different ligands, again stressing the conformational flexibility of GPCRs [21,45].

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