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Site-directed mutagenesis of the putative human muscarinic M_2 receptor binding site

Freddy Heitz [a,1], James A. Holzwarth [a], Jean-Pierre Gies [b], Rebecca M. Pruss [a,2], Suzanne Trumpp-Kallmeyer [c], Marcel F. Hibert [b], Chantal Guenet [a,*,2]

[a] *Marion Merrell Research Institute, rue d'Ankara, 67080, Strasbourg Cedex, France*
[b] *Faculté de Pharmacie, Université Louis Pasteur, 74, route du Rhin, BP24, 67401 Illkirch Cedex, France*
[c] *Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI, 48105, USA*

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Abstract

Experimental probing of the model of the muscarinic M_2 receptor binding site proposed by Hibert et al. [Hibert, M.F., Trumpp-Kallmeyer, S., Bruinsvels, A., Hoflak, K., 1991. Three-dimensional models of neurotransmitter G-binding protein-coupled receptors. Mol. Pharmacol. 40, 8–15.] was achieved by mutating each amino-acid proposed to interact with muscarinic ligands. Pharmacological analysis of the different mutant receptors transiently expressed in human embryonic kidney (HEK/293) cells was performed with a variety of agonists and antagonists. D103A, Y403A and N404A mutations prevented binding of [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate with a reduction in affinity greater than 100-fold, indicating essential contributions of these residues to the binding site for the radioligands. W400A and W155A mutations had very large effects on the binding of [^{3}H] *N*-methylscopolamine (150-fold, 960-fold) but modest effects on the binding of [^{3}H] quinuclidinyl benzilate (4-fold, 17-fold). In addition, binding of oxotremorine-M, oxotremorine, arecoline and pilocarpine to W155A resulted in a greater than 100-fold decrease in affinity. Threonine mutations (T187A and T190A) alter binding of most agonists but not of antagonists. W99 makes little contribution ($<$ 10-fold) to the binding site of the M_2 receptor. D103, W155, W400, Y403 and N404 are likely to be part of the binding site for *N*-methylscopolamine and also to contribute to the binding site for quinuclidinyl benzilate. Some of the predicted residues do not seem to be part of the M_2 receptor binding site but W155 is important for proper ligand binding on the muscarinic M_2 receptor, as predicted by the proposed model.

Keywords: Muscarinic M_2 receptor; Human; Site-directed mutagenesis; G-protein coupled receptor

1. Introduction

Muscarinic cholinergic receptors belong to a large family of plasma membrane receptors which mediate signal transduction via coupling to heterotrimeric G-proteins. For the muscarinic acetylcholine receptors, five different subtypes (M_1–M_5) have been cloned (Caufield, 1993). They share a large number of amino acids particularly within the predicted transmembrane domains. Muscarinic receptors are separated into two groups according to their biological function: M_1, M_3 and M_5 stimulate phospholipase C activity whereas M_2 and M_4 preferentially inhibit adenyl cyclase activity. To identify residues responsible for ligand binding, mutagenesis, structural and modelling studies have been performed. Mutagenesis studies on the β-adrenoceptor (Dixon et al., 1987a,b) and structural studies on rhodopsin and bacteriorhodopsin (Findlay and Pappin, 1986; Henderson et al., 1990), have suggested that ligand binding occurs within the hydrophobic core of these receptors. Additional site directed mutagenesis of the β-adrenoceptor (Strader et al., 1988) and muscarinic M_1 receptor (Fraser et al., 1989) and affinity labelling studies on muscarinic M_1 receptor (Curtis et al., 1989) have shown that ligand binding appears to be mediated by ion–ion interaction between the amine moiety of the ligand and an aspartic acid present in the third transmembrane α helix. This residue being conserved in a large number of bio-

* Corresponding author. Tel.: +33-388-608780; fax: +33-388-459070
[1] Also corresponding author. The Babraham Institute, Laboratory of Cognitive and Developmental Neuroscience Babraham, Cambridge, CB2 4AT, UK.
[2] Present address: Synthélabo Biomoléculaire, rue d'Ankara, 67080, Strasbourg Cedex, France.

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genic amine binding receptors, it is likely that specificity for ligand binding is not defined only by this aspartate residue. Side chains of amino-acids located inside the hydrophobic transmembrane domain must also contribute to ligand binding.

A few years ago, several three-dimensional models of muscarinic receptors were constructed (Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992; Nordvall and Hacksell, 1993). Using bacteriorhodopsin as a template, Hibert et al. (1991) and Trumpp-Kallmeyer et al. (1992), defined a general model for the putative interactions between cationic neurotransmitters and their receptor binding site. This model was revised after the description of the two-dimensional structure of bovine rhodopsin at 9 Å resolution (Shertler et al., 1993) and mutagenesis results on the rat muscarinic M_3 receptor (Wess et al., 1991). In the particular case of muscarinic acetylcholine receptors, the refined model predicts that ligand binding would take place in an intramembrane cleft at 10–15 Å from the cell surface. Numerous amino acids, mainly located on transmembrane helices III, IV, V and VI, as well as on the second extracellular domain of the receptor would be implicated in the binding of acetylcholine. Binding would be driven by ion–ion interaction between the positively charged amino head group of muscarinic ligands and the negatively charged aspartate (D103) on helix 3. This interaction would be stabilised by a hydrophobic cleft formed by three aromatic residues, W99, W400 and Y403. Additional hydrophobic interactions between the ligands and residue W155 and hydrogen bonding between the ligands and the residues N404, T187, T190 or Q179 were postulated.

Mutations of amino acids that are highly conserved among all muscarinic receptor subtypes have shown that two threonine (T231 and T234) and four tyrosine residues (Y148, Y506, Y529, Y533, mutated to phenylalanine) of the rat muscarinic M_3 receptor are important for high affinity binding of acetylcholine and carbachol, but not for antagonists (Wess et al., 1991). The key role of three conserved proline (P201, P242 and P540) residues in muscarinic M_3 receptor expression, ligand binding and receptor activation was shown, whereas three tryptophan residues (W192, W503 and W530) were found to be less important (Wess et al., 1993). However, a systematic study of the putative binding site of a muscarinic receptor has never been undertaken.

On the muscarinic M_2 receptor binding site, few mutagenesis studies have been described. In this study, we report results obtained by site-directed mutagenesis of the residues implicated in ligand binding to the human muscarinic M_2 receptor. Residues were selectively mutated to alanine. Aspartate D103 and asparagine N404 were also mutated to glutamate and glutamine, respectively. The different mutated cDNAs were transiently transfected into Human Embryonic Kidney (HEK/293) cells. Insertion of a Flag sequence at the N-terminal end of the receptors provided a means to assay for accurate expression of the mutated receptors, particularly when binding properties were lost. We investigated the effects of the mutations on ligand binding with a variety of muscarinic agonists and antagonists and on receptor expression by immunofluorescence. Our results show that mutations of the putative muscarinic M_2 receptor agonist binding site differentially affect agonist and antagonist binding efficiencies and that structurally related ligands may have different binding modes. In addition, differences are observed between the muscarinic M_2 receptor studied in the present work and highly homologous muscarinic receptor subtypes, as reported earlier (Hibert et al., 1995; Matsui et al., 1995).

2. Materials and methods

2.1. Materials

The pcDNAI/Amp vector was purchased from Invitrogen and the pBS-KSII plasmid from Stratagene. [^{3}H] *N*-methylscopolamine (79.5 or 84 Ci/mmol) and [^{3}H] quinuclidinyl benzilate (45.4 Ci/mmol) were from Du Pont, New England Nuclear. Arecoline, atropine, carbachol, carbamyl-β-methylcholine chloride (bethanechol), oxotremorine and pilocarpine were from Sigma, McN-A343 (McNeal-A343), pirenzepine, *N*-methylscopolamine and oxotremorine-M were from Research Biochemicals BIBN 99, (5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl)ethylamino]propyl]-1-piperidinyl]ace-tyl]-6*H*-pyrido [2,3-*b*][1,4]benzodiazepin-6-one) was synthesised in houseby Dr. Gilbert Marciniak. pfHHSiD (*para*-fluorohexahy-drosiladifenidol) was a gift from Prof. Günther Lambrecht, University of Frankfurt. All tissue culture reagents were from Gibco-BRL. Oligonucleotides were synthesised on an Applied Biosystems DNA/RNA Synthesiser Model 392. DNA sequence analysis was performed using an Applied Biosystems Model 373A DNA sequencer with the Taq Dyedeoxy Terminator Cycle reagents (Applied Biosystems). Amplification steps using Polymerase Chain Reaction were carried out with a Perkin Elmer DNA thermal cycler 480 using the GeneAmp kit reagents (Perkin Elmer Cetus). The anti-Flag M_2 monoclonal antibody was purchased from Kodak Biotechnologies.

2.2. Construction and mutagenesis of muscarinic receptors

Human muscarinic M_2 receptor cDNA was amplified by polymerase chain reaction from plasmid pSV4M_2, as previously described (Heitz et al., 1995), using FH1 5′ AAA GCT TTA TTT GCT ACT GGC TAC TG 3′ and FH2 5′ CCT GAA TTC CGT TTT GTA ATC AGAC 3′ as forward and reverse primers. Amplification conditions were 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 25 cycles. The amplified fragment was restricted with *Hin*dIII and *Eco*RI, subcloned in pBS-KS and fully sequenced.

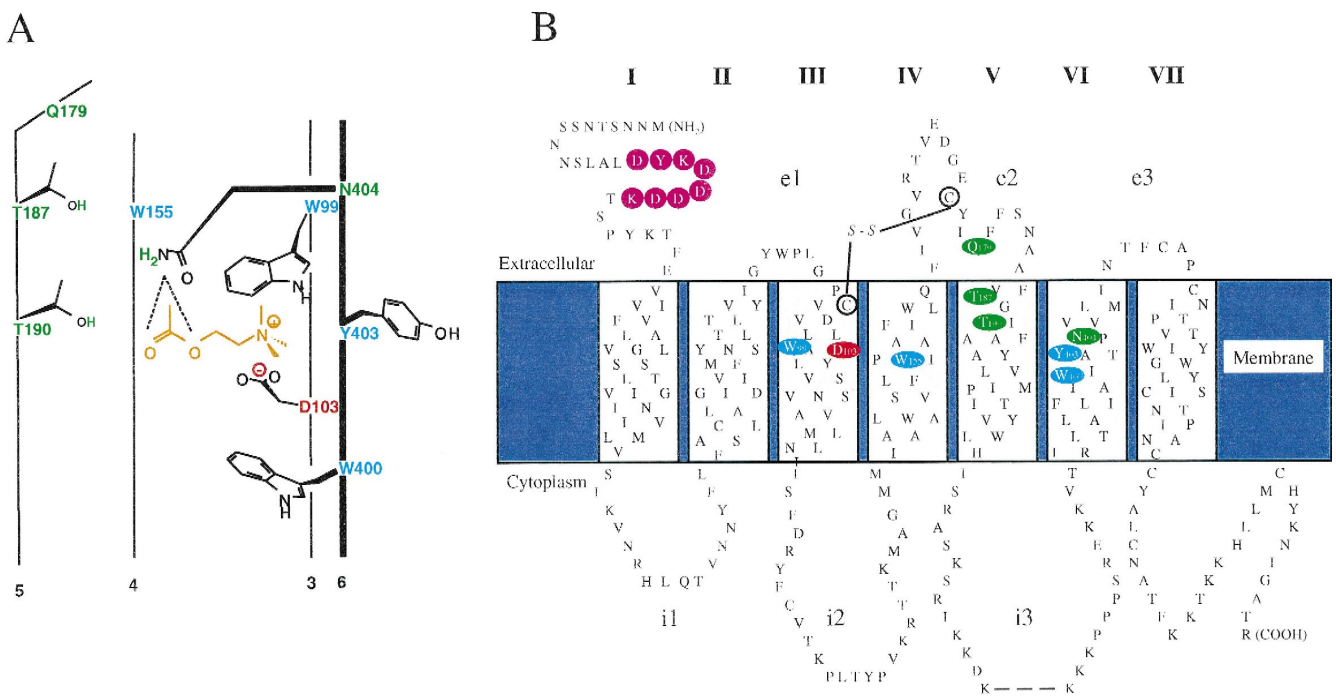

Fig. 1. Model of the human muscarinic M_2 receptor. (A) Representation of the predicted ligand binding site. Helix axes are represented as vertical lines and numbered 3 to 6. Acetylcholine is shown in orange. Residues that have been mutated in this study are highlighted. In red, D103 residue predicted to provide the counter-ion. The aromatic residues (W99, W155, W400, Y403) are shown in blue. Residues supposed to interact by hydrogen bonding (Q179, T187, T190, N404) are shown in green. (B) Representation of the putative seven transmembrane topology of the muscarinic M_2 receptor. The Flag sequence inserted near the N-terminus is highlighted in pink. The mutated residues are shown with the same color code as in (A).

For expression, the human muscarinic M_2 receptor cDNA was inserted in pcDNAI/Amp at the *Hin*dIII and *Xba*I restriction sites generating M_2pcDNA. Mutagenesis was achieved by polymerase chain reaction using oligonucleotide primers with base mismatches (Higuchi et al., 1988) and M_2pcDNA as template. For epitope tagging of the wild-type and mutant receptors, the eight amino acid Flag sequence (DYKDDDDK) (Hopp et al., 1988) was inserted after the leucine 14 residue at the N-terminal part of the receptor. All constructs and mutants were verified by sequencing.

2.3. Nomenclature used

The expressed wild type human muscarinic M_2 receptor is referred as wt. The wild type receptor harbouring the Flag epitope is designated as Flwt. The different mutant receptors are named according to the following example: FlD103A for the mutant receptor displaying a Flag sequence (Fl) in which aspartate 103 was replaced by an alanine (D103A).

2.4. Cell culture and transfection

HEK/293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 100 μl/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified 5% CO_2 incubator. HEK/293 cells were transfected by the calcium phosphate

Table 1
Binding affinities of muscarinic M_2 receptor mutants for [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzylate
Dissociation constants (K_D) for the radioligands were determined in direct binding assays. ND means that specific binding was not detectable. B_{max} values indicate maximum number of binding sites/mg of membrane proteins. Data are presented as mean ± SEM for three to five independent experiments each performed in triplicate. Concentrations of radioligands up to 10 nM final were used.

Receptor	[^{3}H] *N*-methylscopolamine		[^{3}H] quinuclidinyl benzylate	
	K_D (pM)	B_{max} (fmol/mg)	K_D (pM)	B_{max} (fmol/mg)
wt	260 ± 30	750 ± 40	180 ± 20	900 ± 90
Flwt	240 ± 30	760 ± 60	200 ± 20	750 ± 190
FlD103A	ND		ND	
FlD103E	840 ± 60	380 ± 10	200 ± 10	490 ± 10
FlN404A	ND		ND	
FlN404Q	ND		2640 ± 200	430 ± 80
FlT187A	730 ± 100	1060 ± 120	540 ± 30	1460 ± 160
FlT190A	900 ± 160	1270 ± 50	170 ± 20	1570 ± 50
FlT187A + T190A	840 ± 50	470 ± 90	190 ± 40	620 ± 140
FlQ179A	180 ± 40	630 ± 60	100 ± 20	720 ± 100
FlW155A	ND		3000 ± 300	260 ± 50
FlW99A	270 ± 20	510 ± 50	410 ± 50	960 ± 140
FlW400A	ND		800 ± 170	480 ± 20
FlY403A	ND		ND	

precipitation method (Sambrook et al., 1989). Briefly, 1 day before transfection, 10^4 cells/cm^2 were seeded in 140 mm dishes. Two hours before transfection fresh medium was added and chloroquine (100 μM) was added just before transfection. For transfections 12 μg of plasmid was used per plate. Four hours after transfection, fresh

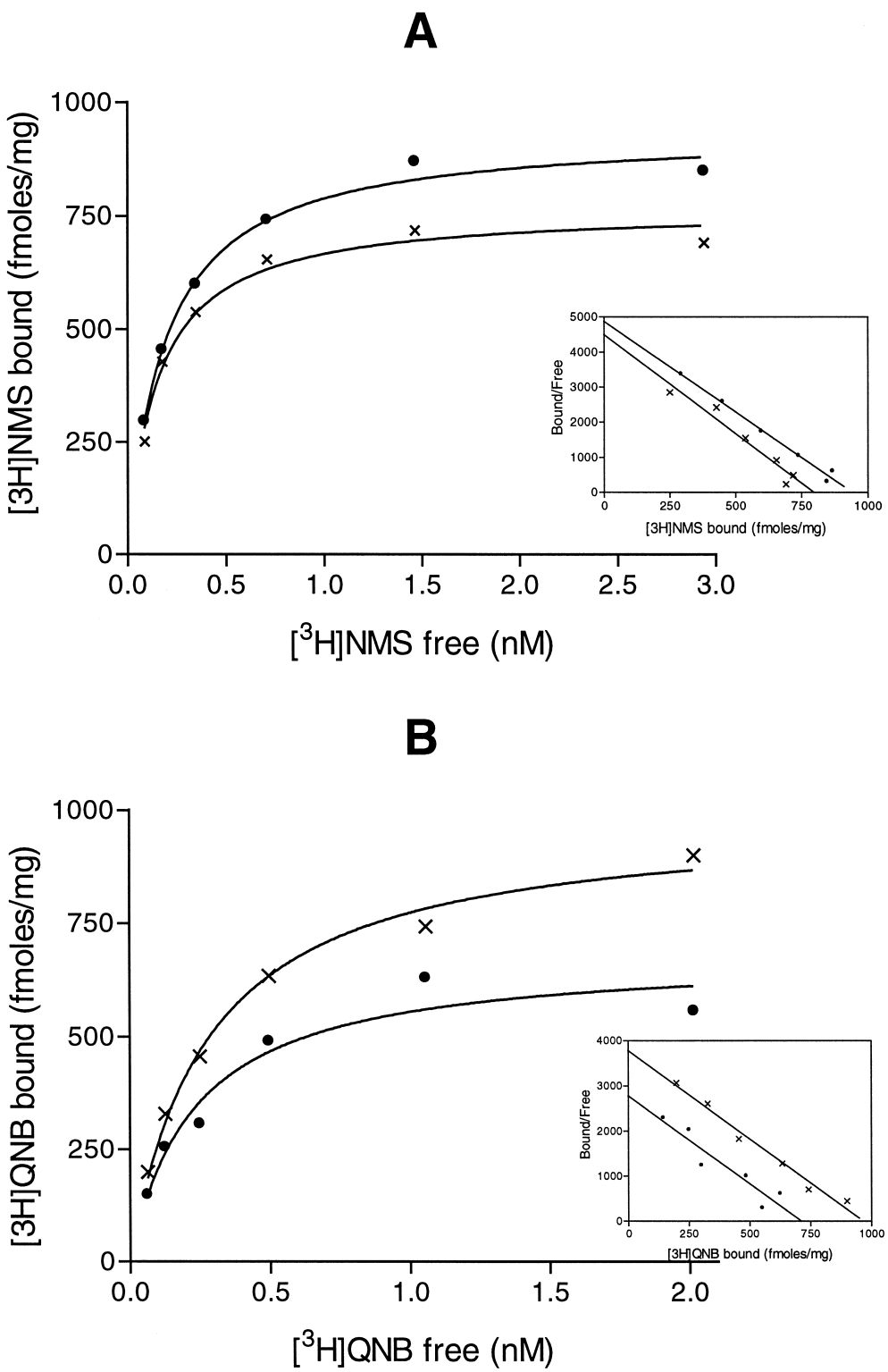

Fig. 2. Saturation of [^{3}H] *N*-methylscopolamine (A) and [^{3}H] quinuclidinyl benzylate (B) binding. Saturation binding was carried out as described under experimental procedures on membranes expressing the wt receptor (x) or the Flwt construct (•). Insets, Scatchard transformation of specific [^{3}H] *N*-methylscopolamine (A) and [^{3}H] quinuclidinyl benzylate (B) saturation data. The data shown are the average of triplicate determinations at each point and are representative of at least five separate experiments.

medium was added and cells were cultured at 37°C until harvesting.

2.5. Binding assays

Forty-eight to seventy-two hours after transfection, cells were harvested and resuspended in 25 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, 100 μg/ml Bacitracin. Membrane homogenates were prepared as follows: the cells were broken with a Polytron for 20 s and centrifuged at $40\,000 \times g$ for 10 min. After one washing, the pellet was resuspended in the same buffer. Protein concentrations were determined by the Bradford method (Bradford, 1976) using a Bio-Rad kit. [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate saturation isotherms were carried out in the presence of 15 or 50 μg of membrane protein in 0.25 or 0.5 ml total volume for 1 h at room temperature. Six different concentrations of radioligands ranging from 60 pM to 2 nM were used. In the case of mutants with drastically reduced affinities, 3 h incubation time and concentrations of radioligands up to 10 nM final were used. Non specific binding was determined in the presence of 5 μM atropine. The reactions were stopped by rapid filtration through GF/B filters.

In competition binding, membrane homogenates were incubated for 60 min with increasing concentrations of cold competing ligands and 300 pM [^{3}H] *N*-methylscopolamine for the wt, Flwt, FlW99A and FlQ179A receptors, or 300 pM [^{3}H] quinuclidinyl benzilate for the wt, Flwt, FlD103E, FlT187A, FlT190A receptors, for the double mutant FlT187A + T190A and FlW400A or 3000 pM [^{3}H] quinuclidinyl benzilate for the FlW155A and FlN404Q mutants.

The competition data were analysed according to a four-parameter logistic equation to determine IC_{50} values using GraphPAD software (GraphPAD Software, San Diego, CA). Affinity values (K_{app}) were determined using the Cheng–Prusoff equation (Cheng and Prusoff, 1973) and can be considered approximations of K_i values.

2.6. Immunostaining

About 48 h after transfection, HEK/293 cells were transferred into 12 well plates containing poly-D-lysine (12 μg/ml) pre-treated glass coverslips (12 h incubation at 37°C). Twenty-four hours later cells were washed twice with PBS (Phosphate Buffered Saline) and incubated 4 times 15 min with buffer A: 137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.64 mM KH_2PO_4, 3 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES pH 7.4 and 2% bovine serum albumin. Each coverslip was then incubated for 30 min with 90 μl of a commercial anti-Flag antibody (55 μg/ml in buffer A). After washing off the unbound antibody (4 times 15 min with buffer A), cells were incubated for 30 min with a 1:100 dilution of a Cy3 conjugated goat anti-mouse IgG + IgM antibody (Jackson ImmunoResearch Laboratories). In order to eliminate non-specific labelling, the secondary antibody was preadsorbed for 30 min on non-transfected HEK/293 cells. The unbound secondary antibody was removed by washing twice with buffer A lacking Bovine Serum Albumin. The cells were then fixed with 4% formaldehyde in PBS for 10 min. After washing with PBS, the coverslips were mounted on microscope slides using a PBS/glycerol solution (1:1, v/v). All the staining procedure was performed at room temperature. Images were obtained using a fluorescence microscope (III RS, Zeiss).

Table 2
Ligand binding properties of the wt, Flwt and FlD103E receptors
Ligand binding parameters were obtained by competition binding experiments as described under experimental procedures. K_{app} values were calculated from IC_{50} values using the Cheng–Prusoff equation and can be considered as approximations of K_i values. The fold decrease in affinity for the FlD103E receptor from the Flwt construct was determined as the ratio K_{app} FlD103E/K_{app} Flwt. Data are presented as mean ± SEM for three to five independent experiments.

Agonists	wt	Flwt	FlD103E	
	K_{app}(μM)	K_{app}(μM)	K_{app}(μM)	Fold
Carbachol	21 ± 4	20 ± 6	2780 ± 670	140
Bethanechol	80 ± 20	40 ± 6	1300 ± 240	32
Oxotremorine-M	4.4 ± 1.0	3.2 ± 1.0	93 ± 22	29
Oxotremorine	0.9 ± 0.1	0.8 ± 0.2	2.0 ± 0.1	2.5
McN-A343	29 ± 3	29 ± 7	50 ± 12	1.7
Arecoline	22 ± 3	18 ± 3	153 ± 15	8.5
Pilocarpine	15 ± 2	11 ± 2	76 ± 7	6.9
Antagonists	K_{app} (nM)	K_{app} (nM)	K_{app} (nM)	Fold
Atropine	1.8 ± 0.3	1.5 ± 0.3	1.6 ± 0.1	1.1
N-methylscopolamine	0.26 ± 0.06	0.26 ± 0.03	1.5 ± 0.2	5.7
Pirenzepine	510 ± 80	530 ± 140	820 ± 60	1.5
BIBN 99	67 ± 14	66 ± 20	90 ± 10	1.4
pfHHSiD	320 ± 60	320 ± 40	360 ± 60	1.1
Methoctramine	11 ± 1	10 ± 1	32 ± 0	3.2

3. Results

We have studied the effect of mutating residues predicted to be involved in the ligand binding site of the muscarinic M_2 receptor (Fig. 1A). Each of these residues was converted into alanine. Aspartate 103 (D103) and asparagine 404 (N404) were also changed to glutamate and glutamine, respectively. Mutant receptor constructions were epitope-tagged with the Flag sequence for expression characterisation (Fig. 1B). The muscarinic M_2 receptor mutants were transiently expressed in HEK/293 cells and analysed by saturation binding with [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate and by competition binding with increasing concentrations of a variety of muscarinic agonists and antagonists.

In non-transfected HEK/293 cells, we were not able to measure specific [^{3}H] *N*-methylscopolamine or [^{3}H] quinu-

clidinyl benzilate binding (data not shown). As summarized in Table 1, expression levels of the different mutant receptors and the wt (for nomenclature, see Section 2.3) receptor ranged from 260 ± 50 fmol/mg for the FlW155A construct to 1570 ± 50 fmol/mg of membrane protein for the FlT190A mutant.

3.1. Pharmacology of the wild type receptor and wild type flag construct

To allow immunodetection of receptor protein, the octapeptide DYKDDDDK, known as Flag, was inserted after leucine 14 in the wt receptor. The binding characteristics of the wt muscarinic M_2 receptor with or without this additional peptide sequence were studied in parallel. Radioligand binding results are presented in Table 1. Binding of [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate was specific and saturable using membranes prepared from cells expressing wt or Flwt construct. As represented on Fig. 2, the Scatchard analysis is consistent with a single class of binding sites for both receptor constructs. The dissociation constants of [^{3}H *N*-methylscopolamine for the wt receptor and the Flwt construct were 260 ± 30 pM and 240 ± 30 pM, respectively. [^{3}H] quinuclidinyl benzilate bound to the wt receptor with a K_D of 180 ± 20 pM and to the Flwt construct with a K_D of 200 ± 20 pM (Table 1). Competition binding experiments with 13 different muscarinic ligands showed that there was little or no difference between the two recombinant receptors (Table 2). For both receptors, the K_{app} values were independent of the radioligand displaced (data not shown). These results show that insertion of the Flag sequence at the N-terminal part of the muscarinic M_2 receptor did not change its pharmacological characteristics. Therefore, we decided to produce all the mutations on the Flwt construct.

3.2. Effect of the aspartate D103 residue

Binding of muscarinic ligands and more generally of all biogenic amines to their receptors is thought to be initiated by an ion–ion interaction between the positively charged ligands and the negatively charged aspartate present on the

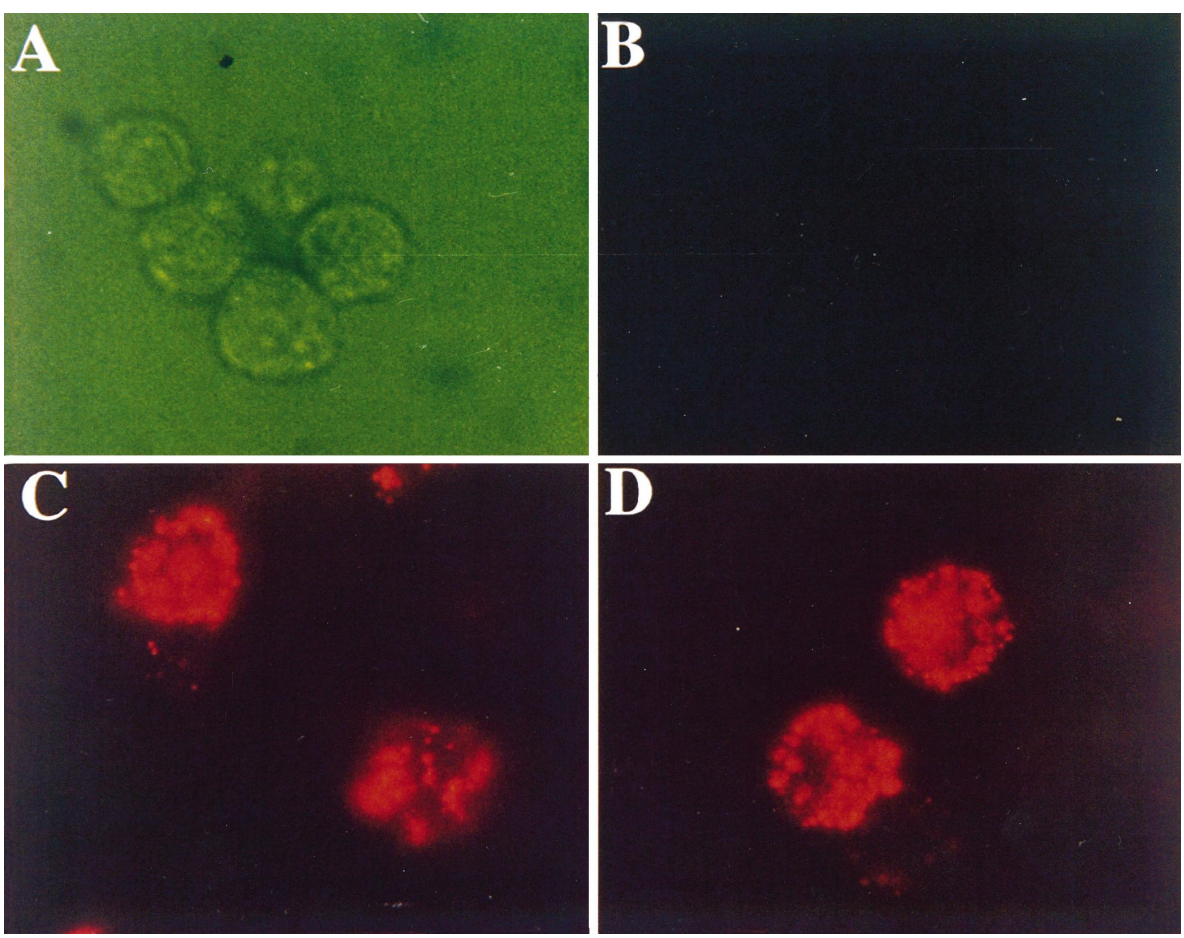

Fig. 3. Localization of epitope-tagged muscarinic M_2 receptors by immunofluorescence. HEK/293 cells were transfected with DNA constructs coding for wt muscarinic M_2 receptor (A and B) as a negative control, Flwt (C) and FlD103A (D) receptors. Immunofluorescence studies were carried out as described under experimental procedures. Image A was obtained with light microscopy while images B, C and D were obtained after epifluorescence illumination.

third transmembrane α helix. When we mutated aspartate 103 to alanine (FlD103A), we were not able to detect any specific binding either with [^{3}H] *N*-methylscopolamine or with [^{3}H] quinuclidinyl benzilate, even using concentrations of ligand up to 10 nM final, 100 μg of membrane per tube and 3 h of incubation. Immunofluorescence detection of the Flag sequence showed that the FlD103A mutant receptor was correctly expressed on the cell surface (Fig. 3) and thus the absence of binding was not due to a lack of receptor expression. In order to determine if this loss of binding was the result of the lack of a negative charge or due to a conformational change, we mutated aspartate 103 to glutamate. Results are presented in Table 1. The FlD103E mutant bound [^{3}H] *N*-methylscopolamine with a slight decrease in affinity (4-fold) whereas the binding of [^{3}H] quinuclidinyl benzilate was not affected. In competition experiments with agonists (Table 2) there were large decreases in affinities for carbachol, bethanechol and oxotremorine-M (140-, 32- and 29-fold, respectively), whereas the affinities for arecoline, pilocarpine, McN-A343 and oxotremorine differed by less than 8.5-fold. The binding of antagonists was not greatly affected by this mutation (Table 2).

3.3. Effect of the aromatic residues

According to the refined model, the ion–ion interaction between the ligand and the receptor may be stabilised by a hydrophobic cleft consisting of the three aromatic residues W99, W400 and Y403 which are capable of forming π–ion interactions. In addition, W155 may also be involved in a hydrophobic interaction with the ligands.

The saturation experiments are presented in Table 1. Mutation W99A, which forms the top of the putative hydrophobic cage, did not affect the binding of [^{3}H] *N*-methylscopolamine or [^{3}H] quinuclidinyl benzilate (Table 1). Competition binding experiments (Table 3) showed that this mutation did not change the binding parameters of the different ligands except for methoctramine. For the FlW400A, there was no specific binding using [^{3}H] *N*-methylscopolamine, whereas [^{3}H] quinuclidinyl benzilate bound with a 4-fold decrease in affinity compared to Flwt. The affinities of most of the tested ligands were also markedly decreased in the FlW400A mutant even if less dramatically than for the W155 residue (see below). McN-A343, pfHHSiD and methoctramine binding were unaffected (Table 3). The FlY403A mutant was unable to bind either [^{3}H] *N*-methylscopolamine or [^{3}H] quinuclidinyl benzilate even at concentrations of radioligand up to 10 nM final and 3 h of incubation (Table 1).

As for the FlW400A mutant, FlW155A showed no specific binding with [^{3}H] *N*-methylscopolamine (even up to 10 nM final) while [^{3}H] quinuclidinyl benzilate bound with a 15-fold decrease in affinity compared to Flwt. The competition binding experiments (Table 3 and Fig. 4) revealed that the affinity of three ligands were not or little affected: the agonist McN-A343 and the antagonists pfHHSiD and methoctramine. However, the affinity of all other ligands tested was drastically affected by this mutation. Binding of the agonists carbachol and bethanechol were not detectable, and oxotremorine-M, oxotremorine, arecoline and pilocarpine decreased by 101- to 525-fold. The affinity of the tested antagonists decreased from 26-fold for pirenzepine to 960-fold for *N*-methylscopolamine. Thus, it seems that W155 interacts with most but not all of these ligands.

Table 3
Ligand binding properties of the receptors with mutations of residues thought to be involved in aromatic ring interactions
Ligand binding parameters were obtained by competition binding experiments as described under experimental procedures. K_{app} values were calculated from IC_{50} values using the Cheng–Prusoff equation. The fold decrease in affinity for mutant receptors was determined as the ratio K_{app} mutant/K_{app} Flwt. Data are presented as mean ± SEM for three independent experiments.
ND: no detectable binding.

Agonists	Flwt	FlW99A		FlW400A		FlW155A	
	K_{app} (μM)	K_{app} (μM)	Fold	K_{app} (μM)	Fold	K_{app} (μM)	Fold
Carbachol	20 ± 6	80 ± 15	4	335 ± 60	17	ND	
Bethanechol	40 ± 6	190 ± 60	4.8	510 ± 50	13	ND	
Oxotremorine-M	3.2 ± 1.0	18 ± 6	5.6	54 ± 11	17	1680 ± 600	525
Oxotremorine	0.8 ± 0.2	2.3 ± 0.6	2.9	30 ± 6	37	170 ± 30	212
McN-A343	29 ± 7	7.6 ± 1.5	0.3	21 ± 2	0.7	18 ± 6	0.6
Arecoline	18 ± 3	12 ± 3	0.7	87 ± 6	4.8	1820 ± 390	101
Pilocarpine	11 ± 2	18 ± 5	1.6	160 ± 20	15	2880 ± 1000	260
Antagonists	K_{app} (nM)	K_{app} (nM)	Fold	K_{app} (nM)	Fold	K_{app} (nM)	Fold
Atropine	1.5 ± 0.3	1.6 ± 0.2	1.1	47 ± 10	31	280 ± 10	186
N-methylscopolamine	0.26 ± 0.03	0.41 ± 0.03	1.6	38 ± 5	146	250 ± 40	960
Pirenzepine	530 ± 140	880 ± 70	1.7	12 140 ± 1400	23	13 840 ± 1200	26
BIBN 99	66 ± 20	53 ± 22	0.8	18 700 ± 2700	280	4500 ± 1000	68
PfHHSiD	320 ± 40	140 ± 50	0.4	1800 ± 240	5.6	2050 ± 660	6.4
Methoctramine	10 ± 1	125 ± 30	13	16 ± 2	1.7	49 ± 22	5.1

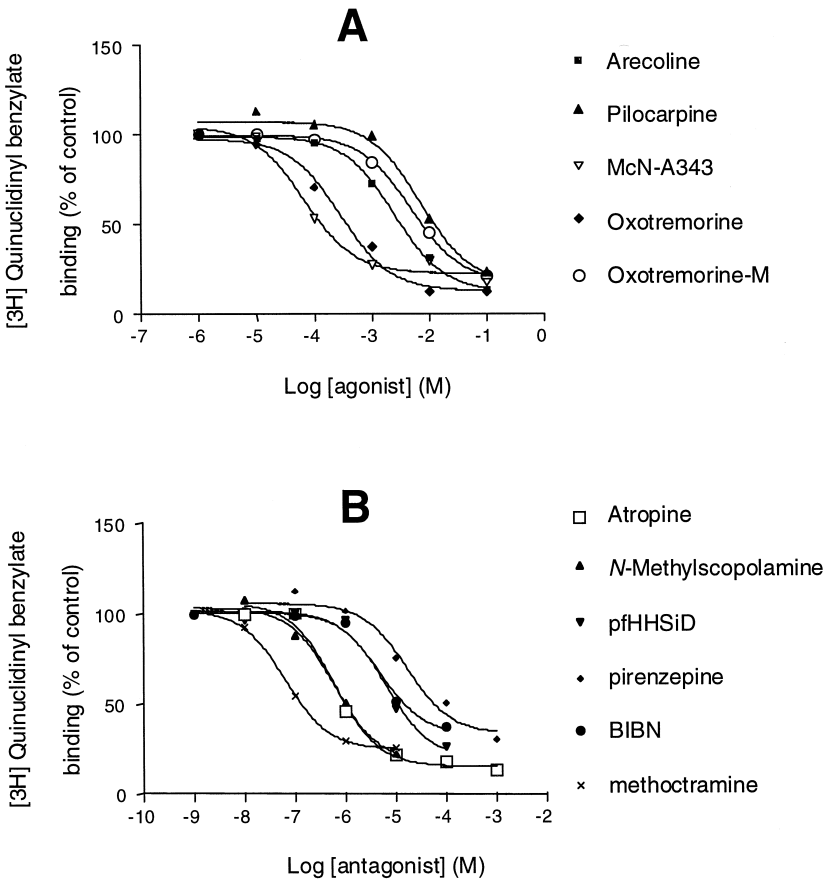

Fig. 4. Inhibition of [^{3}H] quinuclidinyl benzylate binding on the W155A receptor by different muscarinic agonists (A) and antagonists (B). [^{3}H] quinuclidinyl benzylate binding was measured in the presence of various concentrations of the indicated agonists (A) and antagonists (B). The concentration [^{3}H] quinuclidinyl benzylate was about 3 μM. Data shown are from one experiment, representative of three to five.

3.4. Effect of Q179, T187, T190 and N404 residues

Q179, T187, T190 and N404 residues are predicted to be involved in hydrogen bonding with the ligands. Results after mutation of these amino acids to alanine are presented in Tables 1 and 4. Both the saturation and the competition binding experiments showed that the Q179A mutant did not decrease the affinity of the tested ligands. On the contrary, most of the ligands tested seemed to bind to the receptor with a slight increase in affinity, especially the agonists. In contrast, the N404A mutation abolished the binding of both [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate. When this residue was mutated to glutamine, we were able to detect specific binding of [^{3}H] quinuclidinyl benzilate but not [^{3}H] *N*-methylscopolamine (Table 1). The competition experiments (Table 4) showed that all the antagonists tested as well as carbachol, oxotremorine-M, bethanechol and oxotremorine bound with a decrease in affinity. Arecoline, pilocarpine and McN-A343 binding was not affected by this mutation.

Mutating T187 and T190 to alanine slightly decreased the affinity for [^{3}H] *N*-methylscopolamine (about 3-fold) with no change in [^{3}H] quinuclidinyl benzilate binding compared to the Flwt receptor (Table 1). The competition binding showed that neither mutation greatly affected antagonist binding affinities as shown by differences from Flwt of less than 6-fold. However, there were large decreases of a subgroup of agonists. Carbachol, bethanechol and oxotremorine-M were much more affected than were oxotremorine, arecoline and pilocarpine. McN-A343 binding was unchanged. When both threonines were mutated simultaneously, there was little or no effect on [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate binding nor was antagonist binding affected except pirenzepine (11-fold). Again, as observed with the single mutants T187A and T190A, changes in agonist affinities fell into two groups: carbachol, bethanechol and oxotremorine-M whose affinities fell by 21-fold or more and the other four agonists whose affinities decreased by 7-fold or less.

3.5. Immunofluorescence detection

To confirm the cell surface expression of mutant receptors, particularly when binding was abolished, we inserted the Flag sequence at the N-terminus. Comparison of the wt muscarinic M_2 receptor with the Flwt construct showed that the presence of the tag sequence did not change the binding properties. As shown on Fig. 3, receptors containing this tag were detectable by immunofluorescence. The

Table 4
Ligand binding properties of receptors with mutations expected to perturb hydrogen bonding
Ligand binding parameters were obtained by competition binding experiments as described under experimental procedures. K_{app} values were calculated from IC_{50} values using the Cheng–Prusoff equation. The fold decrease in affinity for mutant receptors construct was determined as the ratio K_{app} mutant/K_{app} Flwt. Data are presented as mean ± range or SEM for two to three independent experiments.

Agonists	Flwt	FlQ179A		FlN404Q		FlT187A		FlT190A		FlT187 + 190A	
	K_{app} (μM)	K_{app} (μM)	Fold	K_{app} (μM)	Fold	K_{app} (μM)	Fold	K_{app} (μM)	Fold	K_{app} (μM)	Fold
Carbachol	20 ± 6	6.1 ± 1.8	0.3	460 ± 60	23	2080 ± 900	104	2950 ± 960	148	1930 ± 160	97
Bethanechol	40 ± 6	37 ± 7	0.9	3054 ± 90	76	780 ± 150	20	1180 ± 150	30	820 ± 70	21
Oxotremorine-M	3.2 ± 1.0	0.6 ± 0.1	0.2	140 ± 80	44	120 ± 8	37	150 ± 30	47	210 ± 10	65
Oxotremorine	0.8 ± 0.2	0.3 ± 0.06	0.4	23 ± 3	29	6.7 ± 0.8	8.4	9.3 ± 2	11	5.9 ± 0.8	7.4
McN-A343	29 ± 7	11 ± 0.2	0.4	75 ± 40	2.6	54 ± 4	1.9	45 ± 11	1.6	39 ± 9	1.3
Arecoline	18 ± 3	7.8 ± 1.5	0.4	52 ± 6	2.9	94 ± 11	5.2	73 ± 10	4.1	82 ± 11	4.6
Pilocarpine	11 ± 2	5.8 ± 0.2	0.5	11 ± 0.8	1.0	35 ± 5	3.1	54 ± 8	4.9	55 ± 5	5.0
Antagonists	K_{app} (nM)	K_{app} (nM)	Fold	K_{app} (nM)	Fold	K_{app} (nM)	Fold	K_{app} (nM)	Fold	K_{app} (nM)	Fold
Atropine	1.5 ± 0.3	0.7 ± 0.1	0.5	76 ± 2	51	2.3 ± 0.4	1.5	3.1 ± 0.3	2.1	2.3 ± 0.2	1.5
N-methylscopolamine	0.26 ± 0.03	0.22 ± 0.03	0.9	28 ± 3	108	0.65 ± 0.1	2.5	1.1 ± 0.1	4.2	1.3 ± 0.07	5
Pirenzepine	530 ± 140	330 ± 10	0.6	62 800 ± 2000	118	3000 ± 700	5.7	1550 ± 260	2.9	5810 ± 420	11
BIBN 99	66 ± 20	49 ± 20	0.7	6455 ± 100	98	96 ± 3	1.5	210 ± 40	3.2	220 ± 11	3.3
pfHHSiD	320 ± 40	180 ± 60	0.6	3630 ± 90	11	780 ± 6	2.4	590 ± 40	1.8	1120 ± 110	3.5
Methoctramine	10 ± 1	8.5 ± 0.5	0.9	100 ± 15	10	15 ± 3	1.6	10 ± 1	1.1	11 ± 3	1.2

anti-Flag antibody only detected receptors in which the N-terminal was expressed extracellularly as cells were not permeabilised before the immunoreaction. As a negative control, we performed the experiment on cells expressing the wt receptor, and no fluorescence was detectable (Fig. 3b). In contrast, when cells were transfected with a receptor harbouring the Flag sequence, fluorescence was present as shown on Fig. 3c and d. Similar images were obtained for all Flag constructs.

4. Discussion

Based on high resolution structures of bacteriorhodopsin (Findlay and Pappin, 1986), the putative neurotransmitter binding site of several G protein-coupled receptors was proposed (Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992). In this study, we have used a theoretical three-dimensional model of the acetylcholine binding site (Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992) to direct our mutagenesis strategy in order to characterise the ligand recognition site of the muscarinic M_2 receptor. In order to test the predictions of the model, we mutated residues predicted by the molecular model to affect binding properties of muscarinic agonists and antagonists. We successfully expressed the muscarinic M_2 receptor in HEK/293 cells and found that the recombinant receptor had all the characteristics of the pharmacologically defined M_2 receptor. Insertion of the Flag sequence was performed in order to confirm expression and correct membrane orientation of the different mutant receptors. The presence of the Flag sequence at the N-terminal part of the muscarinic M_2 receptor did not change its pharmacology; both the saturation and the competition binding experiments showed that the wt receptor and the Flwt constructs behaved the same with the different ligands tested. Using an immunofluorescence technique, we were able to verify the appropriate cell surface expression of the different mutant receptors. This was especially informative when binding with [^{3}H *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate was completely abolished as for the FlD103A, FlY403A and FlN404A mutant receptors, confirming that the absence of binding was not due to improper cell surface receptor expression.

Extensive studies have already shown the importance of the aspartate residue located on the third α helix. These studies include affinity labelling on muscarinic M_1 receptors (Curtis et al., 1989) and site-directed mutagenesis of β-adrenoceptor (Strader et al., 1988), and muscarinic M_1 and M_2 receptors (Fraser et al., 1989; Page et al., 1995; Schwarz et al., 1995). The contribution of the D103 residue to the binding site of muscarinic agonists and antagonists has been confirmed in the present study. The suppression of the negative charge of D103 completely abolished [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate binding. When this residue was mutated to glutamate, restoring the negative charge, there was a slight decrease in affinity for a subgroup of agonists (oxotremorine, arecoline pilocarpine and McN-A343) whereas the decreases for carbachol, bethanechol and oxotremorine-M were much larger, especially for carbachol (140-fold). Analogous effects have been reported for agonist binding to muscarinic M_2 receptor transiently expressed in COS-7 (Monkey kidney, SV40 transformed) cells (Page et al., 1995; Schwarz et al., 1995). In addition, we have shown that the D103E mutant had little or no effect on antagonist binding. Taken together, these results confirm the pivotal role for

bioamine binding receptors of a negatively charged aspartate residue in the third α helix.

The contribution of the three residues W99, W400 and Y403, constituting the putative aromatic cluster around the cationic head of the ligand, has been studied. The mutation W99A did not affect binding properties of the ligands tested, with the possible exception of Methoctramine, which suggests that W99 does not play a major role in ligand binding to the human muscarinic M_2 receptor. Similar studies on the muscarinic M_1 receptor transiently expressed in HEK/293 or COS-7 cells showed that the mutation W101F did not change M_1 binding characteristics. Nevertheless, the W101A mutation decreased the binding affinities of antagonists such as [^{3}H] *N*-methylscopolamine (about 10-fold), 4-DAMP and pirenzepine and some of the agonists tested (Hibert et al., 1995; Matsui et al., 1995; Lu and Hulme, 1999). Thus, whereas an aromatic ring at position 101 seems to be important for high-affinity binding to the muscarinic M_1 receptor, this residue in the muscarinic M_2 receptor does not interact with the ligands tested.

In contrast to W99, the two other aromatic amino acids, W400 and Y403, hypothesized to interact with the amino head group of the muscarinic ligands, were crucial for proper ligand binding. For the Y403A mutant, we could not detect specific binding with either [^{3}H] *N*-methylscopolamine or [^{3}H] quinuclidinyl benzilate, even at high radioligand concentrations and up to 3 h of incubation. Matsui et al. (1995) reported similar results with the Y381A muscarinic M_1 mutant receptor which strongly diminished [^{3}H *N*-methylscopolamine binding but had little effect on [^{3}H] quinuclidinyl benzilate binding. Mutation of this residue to Phenylalanine, either in the muscarinic M_2 (Vogel et al., 1997) or in the muscarinic M_3 receptors (Wess et al., 1991) induced an affinity decrease for the agonists, whereas binding of the tested antagonists, [^{3}H] *N*-methylscopolamine, [^{3}H] quinuclidinyl benzilate and trihexylphenidyl, was only slightly affected. Taken together, these results suggest that the aromatic ring of tyrosine is important for high affinity binding of muscarinic antagonists and that tyrosine 403 is actually located in or at least in the vicinity of the binding site.

The binding results obtained with the third aromatic residue of the hydrophobic cleft, W400, suggest that it plays an important role in ligand binding as affinities of most of the ligands tested were decreased 5-fold or more with the exception of methoctramine and McN-A343. Wess et al. (1993) previously reported for the rat muscarinic M_3 receptor that mutation of the corresponding residue to phenylalanine (W503F), led to a decrease in affinity for carbachol (8-fold), acetylcholine (18-fold), *N*-methylscopolamine (5-fold) and 4-DAMP (24-fold). This suggests, as predicted from the model, that W400 is indeed located in the muscarinic ligand binding site. As for the Y403 residue, since mutations to phenylalanine are more conservative than alanine mutations, it seems that the aromatic side chain of the amino acid is crucial for high affinity binding suggesting hydrophobic interactions between W400, Y403 and muscarinic ligands although site directed mutagenesis does not allow precise identification of the ligand-receptor interactions.

The results of mutations directed at the three aromatic residues predicted to form a hydrophobic cleft surrounding the amine moiety of muscarinic ligands indicate that for the muscarinic M_2 receptor only two out of three of these residues (W400 and Y403) clearly contribute to the primary ligand binding site. In contrast to the muscarinic M_1 receptor where W101 plays a supporting role, W99 is not involved in the muscarinic M_2 receptor binding site, suggesting a different mode for ligand binding between the two receptor subtypes.

The three-dimensional model of the muscarinic agonist binding site suggests that W155 should be in close contact with agonists. Mutation W155A leads to a drastic decrease in binding affinity for almost all the ligands tested, suggesting that most muscarinic ligands have a strong interaction with W155. Interestingly, this residue is located on transmembrane region IV, two residues before proline 157. For the muscarinic M_3 receptor mutation of P201, corresponding to P157A of the muscarinic M_2 receptor, has been shown to affect ligand binding (Wess et al., 1993) similarly to the effect we observed with W155 mutation. The consequences of the P157A mutation were attributed to an indirect effect of a conformational change in the binding site since P157 was thought to point toward the outside of the receptor and not into the binding cleft. Our results suggest that this indirect effect might correspond to a perturbation of the interaction between the ligand and W155.

The putative amino acids responsible for interaction between muscarinic receptors and the ester function of acetylcholine remained unknown. The three-dimensional models of the agonist binding site (Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992) have led to the hypothesis that an asparagine residue, N404, on helix 6, conserved in the five muscarinic receptor subtypes, is the main anchoring point for acetylcholine and related agonists. It has been postulated that there is hydrogen bonding interaction between the amide function of N404 and the ester function of acetylcholine. We report here that N404A dramatically decreases the affinity for both [^{3}H] *N*-methylscopolamine and [^{3}H] quinuclidinyl benzilate since we were unable to detect any specific binding with these radiolabeled antagonists. In contrast, N404Q, which retains potential hydrogen bonding, is still able to bind [^{3}H] quinuclidinyl benzilate (with a 4-fold decrease in affinity), although the mutation reduces the binding of all the antagonists (10- to 118-fold) and a subgroup of the agonists tested (23- to 76-fold), McN-A343, arecoline and pilocarpine being unaffected. This could be due to the steric constraint introduced by the longer side chain of glutamine compared to asparagine. These results indicate that hydrogen bonding is important

for the interaction of the receptor with antagonists. However, a proper steric conformation is of crucial importance for correct ligand binding. Since we were not able to calculate binding constants for agonists with the N404A mutant, it was not possible to determine if hydrogen-bonding actually occurs between agonists and this residue on the muscarinic M_2 receptor. In a study on the rat muscarinic M_3 receptor, Blüml et al. (1994) have reported that the corresponding mutation (N507A) had only minor effects on agonist binding, but had a dramatic effect on antagonist binding with a 6730-fold decrease in the affinity for scopolamine, suggesting that this residue is at least implicated in antagonist binding. Moreover, Eberlein et al. (1988) described that the endocyclic amide bond of pirenzepine, which can participate in hydrogen bonding, is very important for correct interaction with muscarinic receptors. More recently, Murgolo et al. (1996) presented a model in which the oxygen atom of the amide group of pirenzepine is ideally placed to interact with the asparagine residue on the muscarinic M_1 receptor.

The binding results obtained on the FlQ179A receptor show clearly that this amino acid does not contribute to ligand binding as neither agonist nor antagonist binding were significantly affected by this substitution.

In the 3D model of the acetylcholine binding site proposed in 1991, helix V was oriented in such a way that the side chain of alanine 191 and alanine 195 pointed towards bound acetylcholine, while threonine 187 and threonine 190 side chains were located at the interface between helix IV and helix V, and thus unable to contribute to acetylcholine binding. Wess et al. (1991) have shown that mutation of those two threonines to alanine (T231A and T234A) in the rat muscarinic M_3 receptor decreased acetylcholine affinity, suggesting that hydrogen bonds could exist between these threonines and agonists. They found that affinities of carbachol and acetylcholine were decreased by about 10- and 35-fold on the T231A and T234A receptors, respectively, while the binding of the tested antagonists was unaffected. From these results, they suggested that these side chains most probably form a hydrogen bond with the carbachol carbamate function or the acetylcholine ester function. The original model of the muscarinic receptor was optimised to account for these experimental results (Nordvall and Hacksell, 1993). Helix V had to be rotated by about 60° to allow the hydrogen bonding interactions. We report here the effect of substitutions of residues T187 and T190 to alanine in the human muscarinic M_2 receptor. Both residues contributed to agonist binding as shown by the dramatic decrease in carbachol affinity (about 100-fold) and to a lesser extent for oxotremorine-M and bethanechol (about 40- and 25-fold, respectively). In contrast, binding of antagonists was essentially unaffected by these mutations. Surprisingly, both residues affected binding of all of the ligands in the same manner, suggesting that one can substitute for the other with a cooperativity effect. It seems that the smaller the molecules, the more they are affected by the threonine mutations. An explanation would be that the binding is not stable once a molecule is in the binding cleft. For example, carbachol may be anchored by its quaternary amine group to the D103 residue and could flip from one putative hydrogen bond to the other. In order to check this hypothesis, the double mutant T187A–T190A was constructed. Mutating both threonines simultaneously had similar effects on agonist and antagonist binding as did either single mutant. These results show that there is no cooperativity between the two threonine residues. Therefore, these residues may be only indirectly involved in high affinity agonist binding. Two other hypothesis may account for the non-cooperativity of the two Threonine residues. Firstly, Wess et al. (1992) proposed that these threonine residues together with tyrosine residues on the third and seventh transmembrane helices create a hydrophilic «hydroxyl-rich» environment within the ligand pocket that allows the efficient interaction of the ester moieties of ligands with the receptor protein. Secondly, Page et al. (1995) proposed that an agonist binding in a ''productive'' mode can switch to a ''non-productive'' mode following a mutation. This new mode of binding will not be affected by any further mutation within the productive binding site. Whether these residues are directly or only indirectly involved in ligand binding remains to be elucidated.

An interesting feature revealed by this study is that the binding of two structurally similar molecules like oxotremorine and oxotremorine-M can be differently affected by the same mutation. For example, with the FlD103E mutant receptor, oxotremorine-M underwent a 30-fold affinity decrease, whereas oxotremorine binding was unaffected. These results indicate that each ligand-receptor complex must be considered as a specific entity. Similar observations were recently reported using pharmacological evaluation of muscarinic and opioid ligand-receptor interactions (Richards and Van Giersbergen, 1995; Befort et al., 1996). Thus, the interactions between a receptor and ligands cannot be generalised to a chemical or a functional class of related ligands.

We have also highlighted some differences in ligand binding between muscarinic M_1, M_2 and M_3 receptor mutants. These variations could be due to different experimental procedures in membrane preparation, ligand binding or could reflect differences between cell lines used. However, muscarinic M_1 W101A and M_1 W101F receptors expressed in HEK/293 (Hibert et al., 1995) and COS-7 (Matsui et al., 1995) cells as well as D103A and D103E muscarinic M_2 mutant receptors expressed in COS-7 cells (Page et al., 1995; Schwarz et al., 1995) or HEK/293 cells (present study) produced similar results suggesting that the variations reported for the different muscarinic receptor subtypes are most probably due to real structural differences. Due to the very high homology in the predicted binding pocket, it is likely that selectivity between receptor subtypes is due to conformational differences rather than

to single amino acid residues. Assessment of natural receptor conformation is beginning to be addressed by different groups working on muscarinic receptors (Liu et al., 1995).

In conclusion, we have clearly established the involvement of a number of residues predicted to be involved in the primary ligand binding site. In addition to providing evidence for the ion pair interaction between ligands and aspartate residue located in the third transmembrane helix, we have demonstrated the importance of two of three aromatic residues proposed to form a hydrophobic cluster around the amine head group of muscarinic ligands. The refined model allowed us to identify a Tryptophan residue (W155) which plays an important role in both agonist and antagonist binding. We have also confirmed the involvement of residues N404, T187 and T190 in the binding of some of the ligands tested. Our results also suggest that the two threonine residues in α helix V might not be directly implicated in hydrogen bonding although this is not unequivoqual evidence. These binding data show both that one receptor interacts differently with highly structurally related ligands, and reciprocally, that highly homologous receptor subtypes interact in a different way with a same ligand. Taken together, our experimental results show the strength of site directed mutagenesis to evaluate the value of a G protein-coupled receptor three-dimensional model.

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References

Befort, K., Tabbara, L., Kling, D., Maigret, B., Kieffer, B.L., 1996. Role of aromatic transmembrane residues of the δ-opioid receptor in ligand recognition. J. Biol. Chem. 271, 10161–10168.

Blüml, K., Mutschler, E., Wess, J., 1994. Functional role in ligand binding and receptor activation of an asparagine residue present in the sixth transmembrane domain of all muscarinic acetylcholine receptors. J. Biol. Chem. 269, 18870–18876.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Caufield, M.P., 1993. Muscarinic receptors characterization, coupling and function. Pharmacol. Ther. 58, 319–379.

Cheng, Y.C., Prusoff, W.H., 1973. The relation ship between inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Curtis, C.A.M., Wheatley, M., Bansal, S., Birdsall, N.J.M., Eveleigh, P., Pedder, E.K., Poyner, D., Hulme, E.C., 1989. Propylbenzilylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor. J. Biol. Chem. 264, 489–495.

Dixon, R.A.F., Sigal, I.S., Candelore, M.R., Register, R.B., Scattergood, W., Rands, E., Strader, C.D., 1987a. Structural features required for ligand binding to the β-adrenergic receptor. EMBO J. 6, 3269–3275.

Dixon, R.A.F., Sigal, I.S., Rands, E., Register, R.B., Candelore, M.R., Blake, A.D., Strader, C.D., 1987b. Ligand binding to the beta-adrenergic receptor involves its rhodopsin-like core. Nature 326, 73–77.

Eberlein, W.G., Engel, W.W., Trummlitz, G., Schmidt, G., Hammer, R., 1988. Tricyclic compounds as selective antimuscarinics: 2. Structure–activity relationships of m1-selective antimuscarinics related to pirenzepine. J. Med. Chem. 31, 1169–1174.

Findlay, J.B.C., Pappin, D.J.C., 1986. The opsin family of proteins. Biochem. J. 238, 625–642.

Fraser, C.M., Wang, C.D., Robinson, D.A., Gocayne, J.D., Venter, J.C., 1989. Site directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. Mol. Pharmacol. 36, 840–847.

Heitz, F., McClue, S.J., Harris, B.A., Guenet, C., 1995. Expression of human m2 muscarinic receptors in Sf9 cells: characterisation and reconstitution with G-proteins. J. Recept. Signal Transduction Res. 15, 55–70.

Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E., Downing, K.H., 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. J. Mol. Biol. 213, 899–929.

Hibert, M.F., Trumpp-Kallmeyer, S., Bruinvels, A., Hoflack, J., 1991. Three-dimensional models of neurotransmitter G-binding protein-coupled receptors. Mol. Pharmacol. 40, 8–15.

Hibert, M.F., Hoflack, J., Trumpp-Kallmeyer, S., Paquet, J.-L., Leppik, R., 1995. 3D models of hormone receptors: experimental validation. In: Sanz, F. (Ed.), Trends in QSAR and Molecular Modelling, pp. 508–513.

Higuchi, R., Krummel, B., Saiki, R.K., 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. 16, 7351–7367.

Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L., Conlon, P.J., 1988. A short polypeptide marker sequence useful for recombinant protein identification and purification. Biotechnology 6, 1204–1210.

Liu, J., Schöneberg, T., van Rhee, M., Wess, J., 1995. Mutational analysis of the relative orientation of transmembrane helices I and VII in G protein-coupled receptors. J. Biol. Chem. 270, 19532–19539.

Lu, Z.-L., Hulme, E.C., 1999. The functional topography of transmembrane domain 3 of the m1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. J. Biol. Chem. 274, 7309–7315.

Matsui, H., Lazareno, S., Birdsall, N.J.M., 1995. Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. Mol. Pharmacol. 47, 88–98.

Murgolo, N.J., Kozlowski, J., Tice, M.A.B., Hollinger, F.P., Brown, J.E., Zhou, G., Taylor, L.A., McQuade, R.D., 1996. The N4 nitrogen of pirenzepine is responsible for selective binding of the M_1 subtype human muscarinic receptor. Bioorg. Med. Chem. Lett. 6, 785–788.

Nordvall, G., Hacksell, U., 1993. Binding-site modeling of the muscarinic m1 receptor: a combination of homology based and indirect approaches. J. Med. Chem. 36, 967–976.

Page, K.M., Curtis, C.A., Jones, P.G., Hulme, E.C., 1995. The functional role of the binding site aspartate in muscarinic acetylcholine receptors, probed by site-directed mutagenesis. Eur. J. Pharmacol. 289, 429–437.

Richards, M.H., Van Giersbergen, P.L.M., 1995. Differences in agonist potency ratios at human m1 muscarinic receptors expressed in A9L and CHO cells. Life Sci. 57, 397–402.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Part 3, 16.34.

Schwarz, R.D., Spencer, C.J., Jaen, J.C., Mirzadegan, T., Moreland, D., Tecle, H., Thomas, A.J., 1995. Mutations of aspartate 103 in the Hm2 receptor and alterations in receptor binding properties of muscarinic agonists. Life Sci. 56, 923–929.

Shertler, G.F.X., Villa, C., Henderson, R., 1993. Projection structure of rhodopsin. Nature 362, 770–772.

Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E.H., Hill, N.S., Dixon, R.A.F., 1988. Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have diffrent roles in receptor function. J. Biol. Chem. 263, 10267–10271.

Trumpp-Kallmeyer, S., Hoflack, J., Bruinvels, A., Hibert, M.F., 1992. Modeling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. J. Med. Chem. 35, 3448–3462.

Vogel, W.K., Sheehan, D.M., Schimerlik, M.I., 1997. Site-derected mutagenesis on the m2 muscarinic acetylcholine receptor: the significance of Tyr403 in the binding of agonists and functional coupling. Mol. Pharmacol. 52, 1087–1094.

Wess, J., Gdula, D., Brann, M.R., 1991. Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. EMBO J. 10, 3729–3734.

Wess, J., Maggio, R., Palmer, J.R., Vogel, Z., 1992. Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. A study with m3 muscarinic receptor point mutants. J. Biol. Chem. 267, 19313–19319.

Wess, J., Nanavati, S., Vogel, Z., Maggio, R., 1993. Functional role of proline and tryptophan residues highly conserved among G protein coupled receptors studied by mutational analysis of the m3 muscarinic receptor. EMBO J. 13, 331–338.