Mapping the Ligand Binding Pocket of the Human Muscarinic Cholinergic Receptor Hm1: Contribution of Tyrosine-82

Volkmar Drübbisch,[1] Jelveh Lameh,[1] Mohan Philip,[2] Yogesh K. Sharma,[1] and Wolfgang Sadée[1,3]

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The ligand binding pocket of many G protein-coupled receptors is thought to be located within the core formed by their seven transmembrane domains (TMDs). Previous results suggested that muscarinic antagonists bind to a pocket located toward the extracellular region of the TMDs, primarily at TMDs 2, 3, 6, and 7. Tyrosine-82 (Y82) is located in TMD2 only one helical turn from the presumed membrane surface of Hm1, whereas a phenylalanine (F124) is found in the equivalent position of the closely related Hm3. In order to determine the contribution of Y82 to Hm1 ligand binding and selectivity versus Hm3, we constructed the point mutation Y82 F of Hm1 and measured binding affinities of various ligands, with ^{3}H-*N*-methylscopolamine (^{3}H-NMS) as the tracer. The Hm1 wild-type receptor and the Y82F mutant were transfected into human embryonic kidney U293 cells. Whereas the affinities of NMS, carbachol, and atropine were either unchanged (carbachol) or enhanced by less than twofold (atropine and NMS), the affinity of the Hm1-selective pirenzepine was reduced threefold by the Y82 F mutation. These changes parallel affinity differences of Hm1 and Hm3, indicating that the Y82 F mutation affects the binding pocket and that Y82 contributes to the binding selectivity among closely related muscarinic receptors.

KEY WORDS: muscarinic cholinergic Hm1 receptor; site-directed mutagenesis; receptor binding pocket.

INTRODUCTION

Molecular modeling of the putative structure of G protein-coupled receptors (GPCRs) suggests a circular arrangement of seven transmembrane domains, with the binding pocket embedded in its core (1–3). The molecular cloning of five distinct muscarinic cholinergic receptor genes (m1–5) (4–6) has prompted detailed studies on the location of the ligand binding pocket with the use of genetic mutations and binding assay. In analogy to results with the β_2 adrenergic receptor (e.g., Refs. 7 and 8), analysis of point mutations of m1 and m3 (9,10) and m2/m3 chimeras (11) suggested that the binding pocket is formed by portions of the TMDs toward the extracellular side of the membrane, with primary importance of TMDs 2, 3, 6, and 7 in defining binding selectivity (3). Specifically, antagonist binding is thought to occur in the outermost portion of the TMDs, with D-105 in TMD3 of m1 playing a major role as the negatively charged counterion (10). Further, serine-124 in the outer portion of TMD2 and tyrosine 529 in an equivalent location of TMD7 of m3 were shown to be important in antagonist binding (9). In contrast, the primary binding pocket for agonists may be more deeply embedded in the transmembrane domains (9).

Sequence comparisons indicate a close similarity between m1 and m3, particularly in TMDs 2, 3, 6, and 7 which contribute to binding selectivity (Fig. 1). There are only a few amino acid changes (highlighted in Fig. 1), and of particular interest to antagonist binding is the substitution of tyrosine-82 (Y82) in m1 to penylalanine-124 (F124) in m3 at the top of TMD2. Whereas many ligands have similar affinities to m1 and m3, the m1 selective antagonist pirenzepine binds significantly less tightly to m3 (range, 3 to 15-fold) (4–6,9–11). We therefore constructed the Y82F point mutation of the human muscarinic Hm1 receptor to test whether this amino acid contributes to the binding pocket and affects the binding selectivity of m1.

MATERIALS AND METHODS

^{3}H-*N*-Methylscopolamine (^{3}H-NMS; sp act, 80 Ci/mmol) was obtained from Amersham, Arlington, IL. Atropine sulfate was purchased from Merck, Inc., Rahway, NY, and carbachol and pirenzepine were from Sigma Chemicals, St. Louis, MO.

Y82F Point Mutation

The gene encoding Hm1 was obtained from a human placental genomic library in vector EMBL3 and transferred to pSG5, using *Bam* HI/*Eco* RI (12). For the point mutation, Hm1 was transferred to M13 mp18, using *Bam*HI/*Eco*RI, and single-stranded DNA incorporating dUTP was made (Kunkel method; for general protocols, see Ref. 13). The Y82F mutation was then introduced with a 24-mer oligonucleotide by changing the 82 codon from TAT (tyrosine) to TTT (phenylalanine). Introduction of the point mutation was verified by single-stranded DNA sequencing (13), and the Y82F mutant Hm1 gene was transferred back into pSG5 with *Bam*HI/*Eco*RI. Plasmid DNA was purified by LiCl/isopropanol and PEG 8000 precipitation (13).

Transfection of Human Kidney Cells, U293

U293 cells were transfected by the calcium phosphate precipitation method (13). Briefly, 1.5×10^6 exponentially growing U293 cells were plated onto 10-cm tissue culture dishes. After 24 hr, a precipitate of 6 μg of DNA with calcium phosphate was added to freshly fed cells. After 4 hr of incubation at 37°C and a dimethyl sulfoxide (DMSO) shock, the cells were allowed to recover, and the binding experiments were performed after 48 hr.

Receptor Binding Assay

Cells were harvested 48 hr after transfection, by mechanical detachment, with phosphate-buffered saline (PBS). The whole-cell suspension (0.35 ml containing ~1.5×10^6

[1] Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143.

[2] Neurex Corporation, 3760 Haven Avenue, Menlo Park, California 94025.

[3] To whom correspondence should be addressed at School of Pharmacy, University of California, San Francisco, California 94143–0446.

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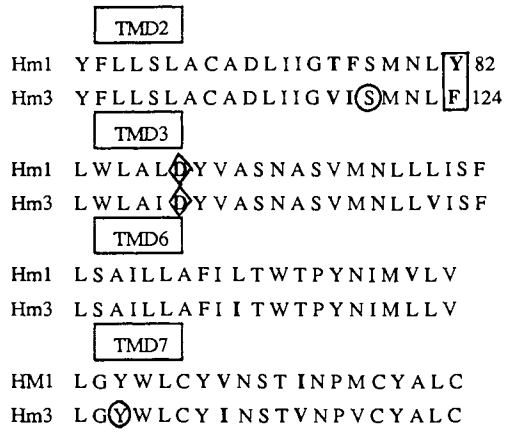

Fig. 1. Sequence comparison between putative TMDs 2, 3, 6, and 7 for Hm1 and Hm3. The amino acid changes are indicated by boldface letters. Y82/F124 in Hm1/Hm3 are boxed. Point mutations strongly affecting antagonist binding (9) in Hm3 are circled (S120,Y529). The aspartate (D) important to ligand binding in TMD3 (10) is shown in a diamond (D105 in Hm1 and D147 in Hm3).

cells per tube) was then incubated at room temperature for 90 min with ^{3}H-NMS tracer and selected drugs, in a total volume of 0.5 ml. The binding reaction was terminated by rapid filtration over glass-fiber filters, presoaked in PBS, carbachol (10 m*M*), and atropine (10 μ*M*). The filters were washed three times with 4 ml of ice-cold PBS, soaked in ScintiVerse II (Fisher Scientific, Fair Lawn, NJ), and counted in a Beckmann scintillation counter. Nonspecific binding was defined as the radioactivity bound in the presence of 10 μ*M* atropine. For saturation curves, ^{3}H-NMS concentrations from 0.1 to 20 n*M* were used. For binding competition curves with unlabeled drugs, ^{3}H-NMS concentrations between 0.1 to 0.3 n*M* were used.

Data Analysis

The competition binding curves were analyzed with the use of the logistic function, $B = B_{max} - B_{min} * L^n/(IC_{50}{}^n + L^n)$ + NSB, where B is the tracer bound (in dpm), L is the ligand concentration, and NSB is the nonspecific binding. ^{3}H-NMS saturation curves were fitted to the equation $B = (B_{max} * T)/(K_d + T)$, where T is the tracer concentration and K_d is the tracer dissociation constant. Nonspecific binding in the presence of 10 μ*M* atropine was subtracted for each tracer concentration. The curves were fitted using nonlinear regression analysis of the unweighted data, with the curve-fitting program Minim 1.2 (E. O. Purves, Department of Pharmacology, School of Medicine, University of Otago, Dunedin, New Zealand). Estimates of n for the competition curves were usually between 0.7 and 1, and fixing the value of n to unity did not significantly affect the IC_{50} estimates. Therefore, all data are reported with $n = 1$.

RESULTS AND DISCUSSION

Expression of Hm1 and the Y82F Mutant Receptor

Transfection in U293 cells with the mammalian expression plasmid pSG5 gave good yields of receptor expression for both Hm1 wild-type and Y82F. The transfection yield of Hm1 for the saturation binding experiments was 792 ± 347 fmol/mg protein (n = 23; using a single tracer concentration of 1.5 n*M* ^{3}H-NMS) over a series of previous experiments. Yields for Y82F were lower (268 ± 25 fmol/mg protein; n = 3), which could have resulted from individual differences among DNA preparations or a somewhat lower transfection or expression efficiency of the Y82F mutant. U293 cells also express some native muscarinic cholinergic receptors, but only at a level of 32 ± 1 fmol/mg protein; therefore, tracer binding occurs primarily at the large excess of transfected receptors in either case. Similar IC_{50} values obtained for carbachol and atropine, when using ^{3}H-NMS tracer concentrations varying between 0.1 to 0.3 n*M* (Table I), further supporting the notion that the small number of native receptors could not have affected the results, even if the native receptor were to have higher affinities to ^{3}H-NMS than the transfected receptor. Binding assays were performed in transiently transfected U293 cells, rather than stably transfected clonal cell lines, because we plan on analyzing numerous additional mutants. Even though results are more variable when using transient transfection, this protocol allows us to screen rapidly multiple mutants. The binding assay was done with whole-cell suspension, rather than cell homogenates, because of loss of ^{3}H-NMS sites upon homogenization. Further, binding sites are observed under more physiological conditions, but comparison to literature results must reflect the various experimental protocols used in receptor binding studies.

Ligand Binding to Hm1 and the Y82F Mutant

A typical saturation binding curve with ^{3}H-NMS is shown in Fig. 2, and the resultant K_d values are provided in Table I. A K_d value of 0.83 to 0.85 n*M* for Hm1 is relatively high, as most NMS literature values, obtained with cell membrane homogenates, are below 0.1 n*M* for both m1 and m3 receptors (5,6,9,11,14,15). However, in a whole-cell assay with human neuroblastoma cells, SK-N-SH, which contains largely Hm3 but also Hm1 receptors, the K_d of ^{3}H-NMS was 1.2 n*M* (16), in close agreement with our results. While previously reported NMS binding to m3 tends to be tighter than to m1, differences are small. Similarly, the Y82F mutation caused a small increase in NMS affinity, to give a K_d of ~0.5 n*M* (Table I).

We then selected three additional ligands for displacement curves, namely, carbachol, atropine, and pirenzepine. To allow better comparison between affinities of these agents at Hm1 and the Y82F mutant, IC_{50} values obtained from ^{3}H-NMS displacement curves were converted to K_d values with the use of the Cheng–Prusoff estimation and the experimental K_d values for NMS at Hm1 and Y82F (Table I). Whereas carbachol and atropine were previously shown to display similar or slightly lower affinities for m1 compared to m3 (4,6,10,11,14,15,17,18), pirenzepine has a marked preference for m1 over m3 (4–6,10,15,17–19) (Fig. 3). For example, carbachol K_d values (0.2 n*M* ^{3}H-NMS) for m1 were reported to be twofold higher than those for the m3 receptors (stably transfected in CHO cells) (14). Similarly, we find an average value of 0.34 n*M* for Hm1 and 0.17 m*M* for Y82F (Table I). Differences among Hm1 and Y82F were significant ($P < 0.05$). Atropine was previously found to display rather

Table I. IC_{50} or K_d Values (±SD) for Hm1 Wild-Type and the Tyrosine-82 → Phenylalanine-82 (Y82F) Mutant Receptor[a]

	K_d, Hm1 (n*M*)		K_d, Y82F (n*M*)	
^{3}H-NMS	0.83 ± 0.07 0.85 ± 0.11		0.46 ± 0.05 0.54 ± 0.08	
	IC_{50}, Hm1	Estimated K_d[b]	IC_{50}, Y82F	Estimated K_d[b]
Carbachol	0.41 ± 0.06 m*M*[c]	0.37 ± 0.05 m*M*	0.28 ± 0.05 m*M*[c]	0.23 ± 0.04 m*M*
	0.41 ± 0.08 m*M*[d]	0.30 ± 0.06 m*M*	0.22 ± 0.05 m*M*[d]	0.14 ± 0.03 m*M*
	0.43 ± 0.11 m*M*[d]	0.32 ± 0.08 m*M*	0.23 ± 0.05 m*M*[d]	0.14 ± 0.03 m*M*
	0.44 ± 0.06 m*M*[c]	0.39 ± 0.05 m*M*		
Atropine	0.44 ± 0.08 n*M*[c]	0.39 ± 0.07 n*M*	0.42 ± 0.05 n*M*[c]	0.35 ± 0.04 n*M*
	0.28 ± 0.03 n*M*[d]	0.21 ± 0.02 n*M*	0.30 ± 0.07 n*M*[d]	0.19 ± 0.04 n*M*
			0.38 ± 0.04 n*M*[d]	0.24 ± 0.02 n*M*
Pirenzepine	43 ± 6 n*M*[d]	32 ± 4 n*M*	129 ± 19 n*M*[d]	81 ± 12 n*M*
	57 ± 14 n*M*[d]	42 ± 10 n*M*	146 ± 30 n*M*[d]	91 ± 19 n*M*

[a] Each value represent an individual experiment done under identical conditions, except for the tracer concentration as indicated.

[b] K_d values were estimated from the IC_{50} values with the use of the Cheng–Prusoff estimation (see Ref. 20): $K_d = (IC_{50})/(1 + [L]/K_{dL})$, where L is the tracer concentration and K_{dL} its dissociation constant, taken from the values given here for ^{3}H-NMS.

[c] Tracer ^{3}H-NMS concentration, 0.1 n*M*.

[d] Tracer ^{3}H-NMS concentration, 0.3 n*M*.

similar affinities for m1 and m3 receptors (4,6,10,17,18), and our results (average K_d of 0.30 and 0.26 n*M* for Hm1 and Y82F, respectively) did not show significant differences among the two receptors.

In contrast to carbachol and atropine, pirenzepine's affinity is 3- to 15-fold greater for m1 than m3 (4–6,10,15–19). For example, Bonner *et al.* (19) reported pirenzepine IC_{50} values of 35 ± 3 n*M* for m1 and 156 ± 38 n*M* for m3. The average pirenzepine K_d values obtained in this study were 37 n*M* for Hm1 and 86 n*M* for Y82F, which account for part of the affinity shift between m1 and m3 (Table I).

The combined results indicate that residue Y82 in m1 and the equivalent F124 in m3 play a role in determining the ligand selectivity between these two receptors. Further, exceedingly small changes in the receptor protein, such as the Y82F transition, can affect the receptor binding profile and contribute to pharmacological differences among receptor subtypes.

Our results do not differentiate between the hypothesis that Y82 participates directly in the ligand binding pocket and the hypothesis that Y82 indirectly affects the binding pocket by changing overall receptor configuration. For example, Y82 could interact with S78 by hydrogen bonding across one helical turn, and the equivalent S120 in m3 has been shown by site-directed mutagenesis to affect strongly antagonist binding to m3 (9). Further mutational analysis and modeling studies are required to resolve these questions.

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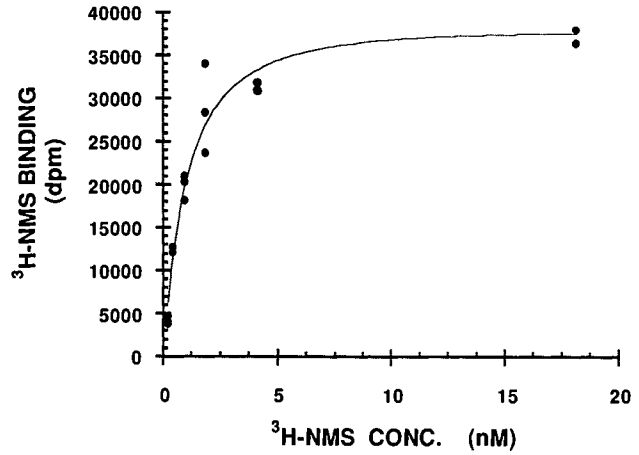

Fig. 2. Example of a ^{3}H-NMS saturation curve for the Hm1 wild-type receptor. The curve represents the fitted line, with nonspecific binding subtracted. Each point represents an individual measurement.

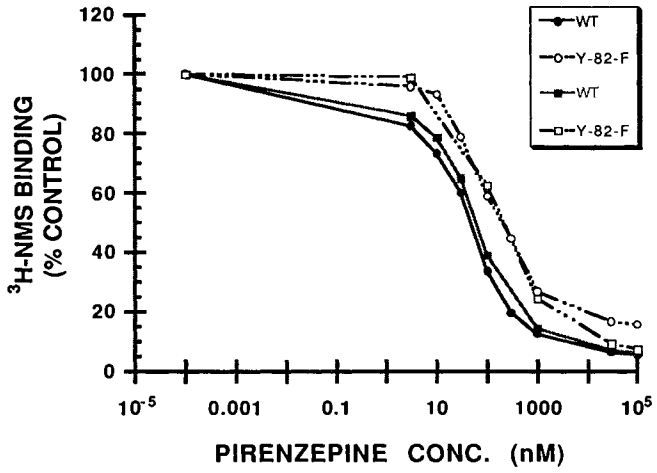

Fig. 3. Pirenzepine displacement curves with 0.3 n*M* ^{3}H-NMS. The lines connect the mean values of three measurements each.

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