Structure of a G-protein-coupling Domain of a Muscarinic Receptor Predicted by Random Saturation Mutagenesis*

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Muscarinic acetylcholine receptors are members of a family of receptors that mediate signal transduction by coupling with G-proteins. Most members of this family display significant sequence homology, particularly within regions predicted to form seven transmembrane domains, TM1–TM7 (1–4).1 This sequence homology, particularly within regions predicted to form the G-protein-coupling domain.

The third intracellular loop (i3) plays a critical role in the coupling of many receptors to G-proteins. In muscarinic receptor subtypes, the N- and C-terminal regions (Ni3 and Ci3) of this loop are sufficient to direct appropriate G-protein coupling. The relative functional contributions of all amino acids within Ni3 was evaluated by constructing libraries of m5 muscarinic receptors containing random mutations in Ni3 and screening them using high throughput assays based on ligand-dependent transformation of NIH 3T3 cells. In receptors that retained a wild type phenotype, the pattern of functionally tolerated substitutions is consistent with the presence of three turns of an α helix extending from the transmembrane domain. All of the amino acid positions that tolerate radical substitutions face away from a conserved hydrophobic face that ends with an arginine, and helix-disrupting proline substitutions were not observed. All of the mutant receptors with significantly compromised phenotypes had amino acid substitutions in residues predicted to form the hydrophobic face. Similar data from the Ci3 region (Burstein, E. S., Spalding, T. A., Hill-Eubanks, D., and Brann, M. R. (1995) J. Biol. Chem. 270, 3141–3146) are consistent with the presence of a single helical turn extending from the transmembrane domain, with an alanine that defines G-protein affinity. Functionally critical residues of Ni3 and Ci3 are predicted to be in close proximity where they form the G-protein-coupling domain.

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1 The abbreviations used are: TM, transmembrane; PCR, polymerase chain reaction; NMS, N-methylscopolamine; R-SAT, receptor selection and amplification technology.
dom saturation mutagenesis to the study of muscarinic receptors. Both approaches stem from the observation that muscarinic receptors induce ligand-dependent transformation of NIH-3T3 cells, with a dose-response relationship that is similar to that of phospholipase C stimulation (29). In the first approach, functional receptors are cloned from a library of mutant receptors by virtue of their ability to induce foci in response to agonist treatment. This approach allows large numbers of mutant receptors to be screened simultaneously for functional clones and is especially suited to the isolation of clones with rare phenotypes. We have also found that this ligand-dependent transformation of NIH 3T3 cells can be monitored using a reporter gene. In our assay, ligands select and amplify cells that express functional receptors (receptor selection and amplification technology, R-SAT, patents pending (30, 32). This assay allows graded responses to be measured, thus permitting a quantitative evaluation of mutant receptor phenotypes. We have shown that this response can be mediated by Gq (32) and have used this approach to study the Ci3 region of the m5 receptor (30). In the present study, we used these two complementary strategies to identify residues within the Ni3 domain of the m5 muscarinic receptor that are required for signal transduction and to establish structural motifs that define receptor-G-protein interactions.

**EXPERIMENTAL PROCEDURES**

**PCR Reactions/Oligonucleotides**—The m5 receptor fragments used for library construction (Fig. 1) were prepared by PCR on a Techne PHC-3 Dry-Block Thermocycler using the GeneAmp PCR kit (Perkin-Elmer). Cycling conditions included an initial 5-min melting step at 94 °C for both wild type and mutant oligonucleotides, followed by 30 cycles of melting, annealing, and extension and a final 10-min extension at 72 °C. Specific cycling parameters for wild type primers are 30 s each at 94, 60, and 72 °C. For mutated primers, lower stringency annealing conditions were used based on the expectation of multiple mismatches between mutated primers and templates; specifically: 94 °C (30 s), annealing at 45 °C with a 2-min ramp to 72 °C (30 s). The following oligonucleotides were synthesized on a model 391 DNA synthesizer (ABI) and used as PCR primers: P1, ATCCTCTGGGCCCCAGCAATCCTCTGCTGGCAG; P2, GATGGTCATGACAGAAACAGGGATGTA-GAAGGCAGCAATCTACCGGGAAACAGAGAAGCGAACCAAGGACCTGGCTGACCTCCAG; P3, TTCAGCACTGAATTCTTCCCCTGGGCTTTCCTT. Mutations were introduced into p3 during synthesis by substituting an equimolar mixture of the four bases for the wild type base at a 15% rate (11% misincorporations). P3 was phosphorylated by treatment with polynucleotide kinase prior to gel purification.

Cloning/Construction of DNA Libraries—PCR products were treated with T4 DNA polymerase to create blunt ends, ligated to create concatamers, and restricted with EcoRI and Apal to release the randomly mutated Ni3/Apal/EcoRI inserts. Vector DNA was prepared from Hm5pCD plasmid DNA, which contains a single EcoRI site and two Apal sites, by digesting plasmid DNA with EcoRI followed by partial digestion with Apal. The final ligation was a forced, two-piece ligation between vector and insert with compatible cohesive ends. Libraries of mutant recombinant m5 receptors were prepared by transforming competent Escherichia coli that were either purchased (Libray Efficiency RR1; Life Technologies, Inc.) or prepared according to the method of Hanahan (33). Plasmid DNA was purified using QiaPrep 500 (pools of clones) or QiaWet (individual clones) from Qiagen Inc. according to the manufacturer's instructions.

**Cell Culture/Transfections**—NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, 4 mW
L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% calf serum. For all transfections, subconfluent monolayers were trypsinized and replated at 2–10 \( \times \) 10^5 cell/10-cm dish and transfected the following day by CaPO4 precipitation (34). After 16–24 h exposure to precipitate cells were washed and then after a further 24 h used for functional assays.

**Binding Assays— AV-12 cells (obtained from Lilly) were transfected by calcium phosphate precipitation using 20 μg of receptor DNA. Membranes were prepared and \(^{[3H]}\)NMS binding assays performed essentially as described previously (13). Membranes were prepared in binding buffer containing 25 mM sodium phosphate, 5 mM magnesium, pH 7.4, binding was allowed to proceed for 4 h at room temperature, and reactions were terminated by filtration. Nonspecific binding was assessed in the presence of 1 μM atropine. Binding data experiments were fit to the following mass action equation,

\[
A = \frac{B_{\text{max}}X/K_D}{1 + X/K_D} \quad (\text{Eq. 1})
\]

to obtain the dissociation constant \( K_D \) and the number of binding sites \( B_{\text{max}} \), where \( A = \left( ^{[3H]}\right)\text{NMS specifically bound} \) and \( X = \text{free concentration of} \left( ^{[3H]}\right)\text{NMS} \).

**Functional Screens/ Focus Assay—** A pool of \( \sim 680 \) clones was screened for the ability to transform NIH 3T3 cells in the presence of the muscarinic agonist carbachol (100 μM). Transfections were performed using the entire pool and also using three 230 cloned divisions of the pool. For each transfection, 450 ng of pooled DNA was used per 10-cm dish (triplicate dishes for each condition). Media and carbachol were changed every 3–4 days until macroscopically visible foci formed (2–3 weeks). Foci were removed from the plate using a pipette or with the aid of a dissecting microscope, total RNA extracted (35), and cDNA synthesized using random hexamers as primers (36). These cDNA templates were used to amplify 1.6-kilobase pair fragments using P4 and P5 (Fig. 1) as PCR primers. P3 is complementary to a plasmid DNA sequence that is transcribed from the pcD vector but lies upstream of the m5 receptor cDNA, preventing inadvertent amplification of genomic

| A | Pre-screen | B | Foci |
|---|---|---|---|
| Clone | Sequence | Clone | Sequence |
| Hm5 | LCRYRTEKRTDADLQ | Hm5 | LCRYRTEKRTDADLQ |
| 1 | LCRVRTDEKRTDADLQ | F25 | LCRVRTDEKRTDADLQ |
| 2 | LCRMREKRTDADLQ | F44 | LCRVRTDEKRTDADLQ |
| 3 | LCRVTREKRTDADLQ | F80 | LCRVRTDEKRTDADLQ |
| 4 | LCRVTDEKRTDADLQ | F87 | LCRVRTDEKRTDADLQ |
| 5 | LCRVTREKRTDADLQ | F97 | LCRVRTDEKRTDADLQ |
| 6 | LCRVTREKRTDADLQ | F98 | LCRVRTDEKRTDADLQ |
| 7 | LCRVTREKRTDADLQ | F101 | LCRVRTDEKRTDADLQ |
| 8 | LCRVTREKRTDADLQ | F102 | LCRVRTDEKRTDADLQ |
| 9 | LCRVTREKRTDADLQ | F108 | LCRVRTDEKRTDADLQ |
| 10 | LCRVTREKRTDADLQ | F109 | LCRVRTDEKRTDADLQ |
| 11 | LCRVTREKRTDADLQ | F111 | LCRVRTDEKRTDADLQ |
| 12 | LCRVTREKRTDADLQ | F119 | LCRVRTDEKRTDADLQ |
| 13 | LCRVTREKRTDADLQ | F126 | LCRVRTDEKRTDADLQ |
| 14 | LCRVTREKRTDADLQ | F132 | LCRVRTDEKRTDADLQ |
| 15 | LCRVTREKRTDADLQ | F133 | LCRVRTDEKRTDADLQ |
| 16 | LCRVTREKRTDADLQ | F156 | LCRVRTDEKRTDADLQ |
| 17 | LCRVTREKRTDADLQ | F163 | LCRVRTDEKRTDADLQ |
| 18 | LCRVTREKRTDADLQ | F165 | LCRVRTDEKRTDADLQ |
| 19 | LCRVTREKRTDADLQ | F222 | LCRVRTDEKRTDADLQ |
| 20 | LCRVTREKRTDADLQ | F225 | LCRVRTDEKRTDADLQ |
| 21 | LCRVTREKRTDADLQ | F254 | LCRVRTDEKRTDADLQ |
| 22 | LCRVTREKRTDADLQ | F255 | LCRVRTDEKRTDADLQ |

**Table 1**

**Sequences of randomly mutated m5 receptors**

| Clone | Sequence |
|-------|----------|
| A, prescreen. Mutated receptors were selected at random, prior to any functional tests, from a library of transformed E. coli. B, foci. Sequences of randomly mutated receptors that form foci in response to carbachol. Positions that tolerate radical substitutions are indicated by a dash. Consensus indicates amino acid positions where either no substitutions or only closely related amino acid substitutions (conserved) are tolerated. 1 indicates positions where only hydrophobic residues are tolerated, and 2 indicates positions that had a lower rate of mutagenesis based on the sequences of the randomly selected clones shown in (consensus in A).

| Consensus | Consensus (Conserved) |
|-----------|-----------------------|
| Ly--iy--t--r--l--v1 m a 22 | Ly--iy--t--r--l--v1 m a 22 |
m5 DNA that may be present as a minor contaminant in total RNA preparations. The PCR products were directly sequenced using P1 as a primer and Taq polymerase using a cycle-sequencing protocol (Life Technologies, Inc.).

Functional Screens/R-SAT—R-SAT was performed as described in detail elsewhere (30, 32). Briefly, aliquots of plasmid DNA from 350 individual mutant receptor clones were cotransfected with plasmid DNA encoding β-galactosidase. Transfected cells were trypsinized and replated in 96-well plates and cultured in the presence or absence of carbachol for 4–5 days. After washing with PBS, β-galactosidase activity was determined using o-nitrophenyl galactopyranoside (ONPG, Sigma) as substrate and quantified colorimetrically as absorbance at 405 nm. Plasmid DNA from functional clones was sequenced using Sequenase 2.0 as described by the manufacturer (U. S. Biochemicals Corp.). Data from R-SAT assays were fit to the four parameter equation (as follows),

\[
R = \frac{D + (A - D)/(1 + (X/E_{50}))}{1 + (X/E_{50})} \quad (\text{Eq}. \, 2)
\]

to obtain minimum response (A), maximum response (D), and \(E_{50}\) (where \(R\) is absorbance at \(A_{405}\) at concentrations X of carbachol). Curves were generated by nonlinear least squares regression using the graphics/analysis program KaleidaGraph™ (Abelbech).

RESULTS

Our strategy for preparing a library of m5 muscarinic receptors, saturated with mutations in the N-terminal 20 amino acids of the third intracellular loop (N3 domain), is illustrated in Fig. 1 and described under “Experimental Procedures.” Incorporation of mutations directly into PCR primers used in the construction of recombinant receptors allows precise control of the rate of base misincorporation and insures a random distribution of substitutions. The protocol used in the synthesis of PCR primers in these studies was expected to yield a nucleotide substitution rate of 11%. In 21 recombinants selected at random from an E. coli library of ~680 clones, the observed nucleotide substitution rate was 10%, with an average of 4.9 amino acid substitutions per recombinant receptor (Table IA). Nine of the sequenced clones (43%) contained stop codons and thus would be unlikely to express functional receptors. With the exception of the extreme 3' end, mutations were randomly distributed throughout the sequenced fragment. The lower rate of mutations in the extreme 3' end was not unexpected as oligonucleotides with these substitutions would be inefficient PCR primers and thus would be selected against during construction of the library of mutants. Also as expected from the relatively low rate of base misincorporation that was used, the vast majority of amino acid changes were caused by single base changes. In fact, only 15% of the amino acid changes were due to two simultaneous base changes in a codon.

The same pool of 680 mutant receptors were expressed in AV-12 cells and tested for their ability to bind the muscarinic antagonist [\(^3\)H]NMS. No detectable [\(^3\)H]NMS binding is observed in AV-12 cells prior to transfection. Using equal amounts of DNA for transfection, the pool of mutant receptor DNAs expressed fewer binding sites (54 ± 16 fmol/mg) than did wild type m5 receptor DNA (220 ± 28 fmol/mg). The affinity of the pool of mutant receptors (\(K_{D}\) of 104 ± 8 pm) was similar to that of wild type (140 ± 10 pm). These data suggest that ~25% of the mutant receptors bind [\(^3\)H]NMS. The presence of stop codons explains the inactivity of the majority of mutant receptors (see above). Assuming the ~170 receptors that are active within the pool have an average of 4.9 amino acid substitutions per receptor, it can be estimated that more than 800 individual amino acid substitutions (averaging ~40 substitutions at each position) were functionally screened in our study.

To identify receptors that couple to G-proteins and induce functional responses, we screened mutant receptors using two high throughput assays that are based on the ability of \(G_{15}\) coupled receptors to transform NIH 3T3 cells in response to the agonist treatment. Fig. 2 illustrates the dose-response relationships of carbachol in the two assays. Carbachol induces focus formation with an \(E_{50}\) of 1,960 ± 280 nm and R-SAT amplification responses with an \(E_{50}\) of 123 ± 22 nm. The greater potency of carbachol in the R-SAT assay may be related to the shorter time course of this assay (4 days) versus the focus assay (14 days). In the shorter assay it is likely that high levels of transiently expressed receptors are present, while in the focus assays lower levels of stably expressed receptors are likely to predominate. Using focus assays large numbers of mutant receptors can be screened for function (several hundred recombinants per plate) and thus are practical for identification of rare phenotypes.

We screened the pool of 680 mutants for receptors that induce formation of foci. No foci were observed in the absence of the muscarinic agonist carbachol. In the presence of carbachol, a total of 23 independent mutant receptor sequences were recovered (3.4% of the total library), suggesting that ~14% of

Fig. 2. Carbachol dose-response relationships for receptors transiently expressed in NIH 3T3 cells. Functional responses were measured using focus formation (open triangles) and R-SAT (filled squares). Focus data represents the number of macroscopic foci per 10-cm² dish (mean of data from two plates) after 14 days of treatment with carbachol. R-SAT data represent the absorbance at 405 nm (mean of duplicate determinations) after 4 days of treatment with carbachol. Lines are fits of the data to the mass action relationship described under “Experimental Procedures.”

![Graph](image.png)
Table II

Sequences and carbachol dose/response parameters of randomly mutated m5 receptors

For all receptors functional responses were observed in at least two independent experiments. EC50 and maximum responses of mutant receptors are normalized relative to wild type receptors evaluated in the same assay. All of the illustrated data are from a single experiment where all of the doses were evaluated in parallel. The indicated parameters were estimated by nonlinear regression of data from nine doses of carbachol, each measured in duplicate. The errors are also estimated from the fits of the data. Amino acid substitutions are underlined. The receptors were ranked according to the robustness of their functional responses and divided into two groups. Group 1 receptors have essentially wild type functional phenotypes. Group 2 receptors have increased EC50 and/or decreased maximal response. Positions that tolerate radical substitutions are indicated by a dash. Consensus indicates amino acid positions where either no substitutions or only closely related amino acid substitutions (conserved) are observed. 1 indicates positions where only hydrophobic residues are tolerated, and 2 indicates positions that had a lower rate of mutagenesis based on the sequences of the randomly selected clones shown in (consensus Table I A). Group 2 receptors with mutations in amino acid positions from the consensus of Group 1 are marked (*). Receptors with "borderline" phenotypes that are difficult to statistically group are also marked (‡). Receptors with "borderline" phenotypes that are difficult to statistically group are also marked (‡) for emphasis.

| Clone  | Sequence                  | EC50/EC50% | Max         |
|--------|---------------------------|------------|-------------|
| Hm5    | LVYKRYYERTRKMLDDLQDQ     | 1.0/100    |             |
| R246   | LVYKRYYERTRKMLDDLQDQ     | 0.2 ± 0.1  | 85 ± 5      |
| R47    | LVYKRYYERTRKMLDDLQDQ     | 0.4 ± 0.1  | 115 ± 5     |
| R341   | LVYKRYYERTRKMLDDLQDQ     | 0.6 ± 0.1  | 114 ± 4     |
| R269   | LVYKRYYERTRKMLDDLQDQ     | 1.0 ± 0.2  | 71 ± 2      |
| R237   | LVYKRYYERTRKMLDDLQDQ     | 1.1 ± 0.2  | 135 ± 3     |
| R347   | LVYKRYYERTRKMLDDLQDQ     | 1.1 ± 0.3  | 74 ± 4      |
| R295   | LVYKRYYERTRKMLDDLQDQ     | 1.3 ± 0.3  | 85 ± 3      |
| R332   | LVYKRYYERTRKMLDDLQDQ     | 1.3 ± 0.4  | 100 ± 5     |
| R346   | LVYKRYYERTRKMLDDLQDQ     | 2.5 ± 0.6  | 92 ± 4      |
| R11    | LVYKRYYERTRKMLDDLQDQ     | 3.0 ± 0.7  | 120 ± 4     |
| *R236  | LVYKRYYERTRKMLDDLQDQ     | 5.8 ± 1.3  | 79 ± 3      |
| *R263  | LVYKRYYERTRKMLDDLQDQ     | 6.5 ± 2.1  | 82 ± 5      |
| *R380  | LVYKRYYERTRKMLDDLQDQ     | 6.7 ± 2.3  | 77 ± 5      |
| *R45   | LVYKRYYERTRKMLDDLQDQ     | 7.7 ± 2.5  | 72 ± 4      |
| Consensus Group I | LVY---Y---T---R---L---A---L   | 22         |             |
| G262   | LVYKRYYERTRKMLDDLQDQ     | 2.9 ± 1.2  | 49 ± 3      |
| G284   | LVYKRYYERTRKMLDDLQDQ     | 3.2 ± 1.3  | 52 ± 2      |
| G361   | LVYKRYYERTRKMLDDLQDQ     | 4.6 ± 1.7  | 54 ± 3      |
| *G255  | LVYKRYYERTRKMLDDLQDQ     | 9.5 ± 3.4  | 83 ± 5      |
| G373   | LVYKRYYERTRKMLDDLQDQ     | 17 ± 9.5   | 56 ± 6      |
| G242   | LVYKRYYERTRKMLDDLQDQ     | 23 ± 3.1   | 106 ± 3     |
| G355   | LVYKRYYERTRKMLDDLQDQ     | 38 ± 15    | 75 ± 7      |
| G340   | LVYKRYYERTRKMLDDLQDQ     | 39 ± 16    | 47 ± 4      |
| G3213  | LVYKRYYERTRKMLDDLQDQ     | 42 ± 29    | 30 ± 5      |
| G91    | LVYKRYYERTRKMLDDLQDQ     | 42 ± 7.4   | 78 ± 3      |
| G353   | LVYKRYYERTRKMLDDLQDQ     | 48 ± 23    | 32 ± 3      |
| G272   | LVYKRYYERTRKMLDDLQDQ     | 75 ± 19    | 77 ± 5      |
| G70    | LVYKRYYERTRKMLDDLQDQ     | 90 ± 26    | 63 ± 5      |
| Consensus Group I & II | L---------R---------L   | 22         |             |

The major limitation of these screens is that formation of a foci is an all or none event that is difficult to quantitate. Because R-SAT assays are much less labor intensive than focus assays, it is practical to screen individual clones. Detailed phenotypic studies are simplified by the readily availability of the cloned plasmid DNA, as no labor-intensive rescue or subcloning steps are required. The pharmacologies of many reference muscarinic ligands at cloned receptors as assayed using R-SAT have been described in detail. Overall, the pharmacologies of both agonist and antagonist ligands are similar when assayed using R-SAT and more traditional functional assays, and R-SAT assays allow a precise discrimination of full and partial agonists (30).

Using the R-SAT assay, an additional 380 clones were selected at random and individually assayed in the presence and absence of carbachol (100 μM). As in the focus assay, no receptors with agonist-independent activity were identified. Receptors that responded to carbachol were then tested for their carbachol dose-response relationships. These functional receptors were ranked according to the robustness of their functional responses and divided into two groups. It should be emphasized that the receptors represented a continuum of phenotypes, and thus the placement of a few clones into one group or the other may not be statistically significant. These clones with only slightly compromised function are marked with an asterisk. Group 1 receptors (n = 14 or 4%) displayed essentially wild type responses to carbachol, whereas Group 2 receptors (n = 13) are compromised with respect to EC50, percent maximum response, or both (Table II). The carbachol dose-response relationships for representative Group 1 and Group 2 receptors are shown in Fig. 3. Group 1 receptors averaged significantly fewer substitutions (2.9) than Group 2 (3.9). The pattern of amino acid substitutions in Group 1 receptors were not random, with many residues that were either not mutated or which only tolerated highly conservative substitutions. The rates and patterns of substitutions observed in receptors identified in foci and Group 1 receptors identified by R-SAT are nearly identical (Tables I B and Table II). These data suggest that only receptors with essentially wild type responses to carbachol were able to form foci and that the two screening procedures sampled relatively comprehensive sets of mutations that are tolerated for the wild type phenotype.

The sequences of receptors that form foci and robust responses in R-SAT both predict the same amino acids as being required for function. The positions of these critical residues are presented in Table III. The first two amino acids only tolerate a few hydrophobic substitutions; consistent with these residues forming the last turn of the TM5 α helix. The next two residues (Cys214) tolerate radical substitutions to all amino acid classes, including several charged substitutions. Arginine 215 tolerates a hydrophobic substitution and change of charge. Isoleucine 216 only tolerates substitution with hydrophobic residues of similar size, and Tyr217 was not mutated. The next two positions tolerated many radical substitutions, and only the highly conserved alanine was substituted for Thr220. The last two positions tolerated a diversity of radical substitutions, and Arg223 was not mutated. In the last 8 amino acid positions of the sequence, all of the positions tolerated multiple nonconserved substitutions. The only exceptions were the 3' end, where lack of substitutions may have been due to a low mutation rate that was an artifact of the library construction method (see above).

Proline substitutions were not observed in the 12 amino acids preceding Arg223. In seven of these positions mutation to proline would have required two base changes, making these highly conserved amino acids were allowed (Table I B).

The receptors that bind radioligand also couple to G-proteins and transduce the functional response that yields foci. Sequences isolated from these foci had an average of 2.5 amino acid substitutions per receptor, far fewer than observed in the randomly selected clones. In contrast to the clones selected at random, amino acid substitutions in the active muscarinic receptors expressed in foci were not randomly distributed. At some positions, multiple nonconserved substitutions were observed, while at other positions, either no substitutions or only
substitutions rare. On the other hand, at the other five positions substitution with proline should have been a common event. Proline substitutions were tolerated in the more cytoplasmic end of the sequence.

Examination of the sequences of Group 2 receptors indicates that all but one of these receptors have amino acid substitutions in residues that were conserved in the Group 1 receptors (see Table II), thus confirming the functional importance of these residues. The one exception was Arg242 which had a wild type maximum response and only a modest increase in EC50. Receptors R262, R284, R361, R255, and R373 all had essentially wild type EC50 values, but these receptors had decreased maximum responses. All of these receptors had substitutions in Tyr213, a residue which was not mutated in the Group 1 receptors. All of the remaining receptors had significant increases in EC50, and all of these receptors with the exception of Arg340 had substitutions in the highly conserved Ile216 and/or Tyr217. Arg340 and Arg272 had radical substitutions in the highly conserved Thr220. Arg222 was not mutated in any of the receptors that responded to carbachol.

During the course of these studies, no receptors with substantially decreased EC50 values for carbachol or much greater then wild type maximal responses were isolated, nor were receptors that responded in the absence of added agonist. It is possible that such “activating” mutations would have been masked by the fact that most of our mutant receptors had multiple amino acid substitutions. This possibility seems unlikely as we have isolated many receptors with multiple amino

### Table III

Compilation of consensus sequences

Data are compiled from the individual sequences shown in Tables I and II. Positions that tolerate radical substitutions are indicated by a dash. Amino acid positions where only conservative substitutions are tolerated are marked. Sequences of the N13 domains for the other four muscarinic receptor subtypes are shown for comparison. Shaded residues are invariant among all muscarinic receptor subtypes. Residues predictive of G-protein coupling specificity (m1, m3, m5: Gq versus m2, m4: Gi) are denoted by u. Numbering corresponds to that of the human m5 receptor. "All" is the consensus from all three of the groups.

| Amino Acid # | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |
|--------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Hm2 (Gi)     | L  | Y  | W  | H  | I  | S  | R  | A  | S  | K  | S  | R  | I  | K  | K  | D  | K  | K  | E  | P  |  |
| Hm4 (Gi)     | L  | Y  | I  | H  | T  | S  | L  | A  | S  | R  | S  | R  | V  | H  | K  | H  | R  | P  | E  | G  |  |
| Hm1 (Gq)     | L  | Y  | W  | R  | I  | Y  | R  | E  | T  | E  | N  | K  | A  | R  | E  | L  | A  | A  | L  | Q  |  |
| Hm3 (Gq)     | L  | Y  | W  | R  | I  | Y  | K  | E  | T  | K  | R  | T  | K  | E  | L  | A  | G  | L  | Q  |  |
| Hm5 (Gq)     | L  | Y  | C  | R  | I  | Y  | R  | E  | T  | K  | D  | L  | A  | D  | L  | Q  |  |
| Pre-screen   | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Foci         | L  | Y  | -  | I  | Y  | -  | T  | -  | R  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Group 1      | L  | Y  | -  | I  | Y  | -  | T  | -  | R  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| All          | L  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

### Fig. 3

Carbachol dose-response relationships of randomly mutated m5 receptors. A, group 1 receptors with essentially wild type functional phenotypes, and B, group 2 receptors with functionally compromised phenotypes. R-SAT responses were normalized to a wild type receptor evaluated in the same experiment. Lines are fits of the data to the mass action relationship described under “Experimental Procedures.”
muscarinic receptors has been one of the most intensively studied regions using site-directed mutagenesis, and of the functionally critical residues that we have now identified have been tested in the earlier studies of the closely related m1 and m3 receptors. In the m1 receptor, a large portion of the i3 loop after the arginine analogous to Arg223 can be deleted without altering receptor activation of phosphatidylinositol metabolism (24), and in the m3 receptor, the analogous arginine has been implicated in coupling (38–40). Also in the m1 receptor, three of the charged residues that were predicted to be involved in G-protein coupling, based on sequence comparisons, can be mutated without altering function (24). In the present study, the analogous residues tolerate multiple non-conserved substitutions. Finally, in m3 receptors the residue analogous to Tyr217 has been shown to be a major determinant of G-protein selectivity (41).

Our data identifies two additional residues, Ile216 and Thr220, that together with Tyr217 form a hydrophilic face of a helical extension of TM5. Based on these predictions, all of the mutant receptors with significantly compromised phenotypes had substitutions in residues that can be assigned within a structural context. The receptors that had wild type EC50 values for carbachol and substantially decreased maximal responses had substitutions in Tyr213, a residue predicted to be within the TM domain. Receptors with substantially increased EC50 values for carbachol had all substitutions in residues predicted to form the hydrophilic face of N3. Because most of the receptors have multiple amino acid substitutions, the assignment of individual residues to specific mutant phenotypes is preliminary. In an extension of the present study, we have now defined the precise functional roles of all of these residues by site-directed mutagenesis (43).

In a parallel study of the Ci3 region, we have shown that this region also has helical structure that is a critical determinant of G-protein coupling (30). However, that region was far more tolerant of mutation then the N3, due to only a single turn of an α helix being essential for function. Alanine 441, which is positioned in the last turn of that helical extension of TM6, was found to be a major determinant of receptor affinity for Gαi. If one assumes that G-protein-coupled receptors consist of seven TM domains that are arranged counter-clockwise (when viewed from the extracellular space) sequentially from N- to C-terminal in a bundle around a ligand binding site, then the location of the N3 region with respect to the rest of the receptor can be approximated (3, 4, 42). In this orientation, highly conserved residues in the N3 and Ci3 regions (Tyr217, Thr220, Ala440, Ala441) that we have now implicated in G-protein coupling are in close proximity to one another (Fig. 4). It should be noted that these are the only functionally required amino acids within the i3 loop that are predictive of G-protein-coupling selectivity (Gαi versus Gαq) of muscarinic receptor subtypes (shaded residues of Fig. 4). Taken together, these data suggest that G-protein coupling involves a domain formed by N3 and Ci3. Additional evidence for the cooperation between the N- and C-terminal regions of the i3 loop is provided by recent studies in which co-expression of isolated N- and C-terminal regions of the i3 loops of various receptors inhibit signal transduction (31).

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Fig. 4. Proposed locations of functionally critical residues in Ni3 and Ci3. A, viewed from the intracellular surface toward the interior of the cell, with lighter and wider lines nearer the viewer. Transmembrane (TM) domains are arranged sequentially (TM1–TM7) in the preferred counterclockwise orientation, as proposed based on comparison with the structure of rhodopsin (as discussed in recent reviews; Refs. 40 and 41). Ni3 and Ci3 are drawn as a continuation of TM5 and TM6, respectively. The beginning of the intracellular domain is indicated by the first residues (C and Q, respectively) that tolerate mutation to charges. The circled residues do not tolerate radical substitutions, and the darker shading indicates residues that are also predictive of the Gαi versus Gαq coupling selectivity of muscarinic receptor subtypes. The extent of the helical structure is predicted by the periodicity in tolerated substitutions and the lack of tolerance of helix disrupting proline substitutions. B, linear view of the Ni3 and Ci3 regions. Upper box indicates proposed TM domains where charged substitutions are not tolerated. Shaded (also in bold) residues do not tolerate radical substitutions. Darker shading is as in A.

A growing body of evidence suggests that the Ni3 regions of G-protein-coupled receptors exist as amphipathic α helices (21–23, 38, 39). Our data provides compelling evidence that the Ni3 region of the m5 receptor forms an α helical structure and identifies all of the functionally critical residues within this structure (Fig. 4). First, no charged substitutions were tolerated in receptors with wild type phenotypes until Cys214, consistent with this residue marking the beginning of the more hydrophilic Ni3 region. Second in a helical representation, all of the conserved, largely hydrophobic, amino acids are clustered on one face of the helix which extends approximately 10 residues (~3 turns of a helix) into the intracellular space to the completely conserved arginine (Arg223). The opposing face of this helix, where most of the charged residues are located, tolerates multiple, nonconservative substitutions. And third, the lack of helix-disrupting proline substitutions between the TM domain and Arg223 provides additional evidence that this region may form a helical structure with functional importance. Proline substitutions are tolerated after Arg223 (20). Finally, a comparison of this Ni3 domain with the corresponding domains of the three α1 receptor subtypes, which also couple with the G-protein Gαq and form foci, reveals a similar pattern of hydrophobic residue conservation, suggesting that this may be a general feature among these functionally related receptors.

The Ni3 region of muscarinic receptors has been one of the most intensively studied regions using site-directed mutagenesis, and of the functionally critical residues that we have now identified have been tested in the earlier studies of the
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