The Second Intracellular Loop of the m5 Muscarinic Receptor Is the Switch Which Enables G-protein Coupling

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We have completed a systematic search of the intracellular loops of a muscarinic acetylcholine receptor for domains that govern G-protein coupling. A unique feature of the second intracellular (i2) loop was an ordered cluster of residues where diverse substitutions cause constitutive activation. A second group of residues in i2 was identified where mutations compromised receptor/G-protein coupling. The residues of each group alternate and are spaced three to four positions apart, suggesting an α-helical structure where these groups form opposing faces of the helix. We propose that the constitutively activating face normally constrains the receptor in the “off-state,” while the other face couples G-proteins in the “on-state.” Therefore, the i2 loop functions as the switch enabling G-protein activation.

Muscarinic acetylcholine receptors are members of a large family of receptors that mediate signal transduction by coupling with G-proteins (1–5). Most members of this family display significant sequence homology, particularly within regions predicted to form 7-transmembrane domains (TM1–7) (6). Current models of receptor structure arrange the transmembrane domains in a counterclockwise circle centered around TM3 forming a pocket where ligands could bind (7–9). Consistent with this proposed structural configuration, mutational data indicate that residues located on TM2, TM3, TM5, TM6, and TM7 are needed for ligand interactions with muscarinic receptors (10–16). In particular, a central role of Asp105 in TM3 forming a pocket where ligands could bind (7–9). Consistent with this proposed structural configuration, mutational data indicate that residues located on TM2, TM3, TM5, TM6, and TM7 are needed for ligand interactions with muscarinic receptors (10–16). In particular, a central role of Asp105 in TM3 forming a pocket where ligands could bind (7–9).

Numerous experiments have shown that receptor/G-protein coupling occurs through the intracellular loops. By preparing chimeras between subtypes from the two functional classes (e.g. chimeric m1/m2 and m3/m2 receptors), the third cytoplasmic loop (i3) has been shown to be the region that defines subtype specificity for distinct G-proteins (17–20). The central portion of the i3 loop can be deleted without impairing coupling to G-proteins, indicating that only the N- and C-terminal regions of the i3 loop (Ni3 and Ci3, adjacent to TM5 and TM6, respectively) are required for function (21–24). In contrast to results with i3, work on i2 indicates less of a role in governing G-protein coupling specificity and more of a role in governing overall strength of coupling (25, 26). In fact, the DRY motif at the junction of TM3 and i2 is one of the most highly conserved regions of G-protein-coupled receptors (6), thus it is likely that the i2 loop plays a fundamental role in G-protein coupling for all receptors.

Contemporary pharmacological theory states that G-protein coupled receptors spontaneously interconvert between active (R*) and inactive (R) states (known as the two-state model or extended ternary complex model, see Refs. 27–30). Consistent with this theory, we have observed that overexpression of compatible G-proteins constitutively activates muscarinic receptors by promoting formation of R* (31–33). This concept also explains numerous observations that receptors are capable of activating G-proteins and eliciting responses in the absence of agonists (29, 34–39). The observable levels of constitutive activity of wild-type receptors are relatively low indicating that wild-type receptors exist predominantly as R. However many mutationally activated G-protein coupled receptors have been described which have levels of constitutive activity that are significantly higher than the original wild-type receptors (40–44). In general, activating mutations are thought to alter the equilibrium between R and R* to increase the proportion of receptor that exists as R*. By inference, residues where mutations are activating therefore may play a restraining role, maintaining receptors in an inactive conformation (27, 42). Conceptually, receptor domains that undergo large conformational changes upon transitioning from R to R* are “switch regions,” and would be expected to contain many such residues which restrain the receptor in R, as well as residues that are required in R*, for example, to couple G-proteins.

In a systematic search for residues that affect G-protein coupling, both the i2 and i3 loops of the human m5 muscarinic receptor have been randomly mutated, and tested for their functional properties. Previously we reported that the functionally important residues of Ni3 and Ci3 are distributed with a periodicity of 3 to 4 positions, suggesting these regions form α-helical extensions of their adjacent transmembrane domains (45–47). Similarly, in i2 we find the distribution of functionally important residues suggests an α-helical structure extending from TM3.

Uniquely, a cluster of constitutively activating residues was identified in i2 which are also distributed in a pattern consistent with an α-helix, and which alternate with residues required for G-protein activation. We propose that the i2 loop has two protein faces, one comprised of residues important for attaining the active state of the receptor, and the other comprised of residues important for maintaining the inactive state of the receptor, and that the i2 loop operates as a conformational switch enabling G-protein coupling.

MATERIALS AND METHODS

Library Construction—Mutations were randomly introduced into the i2 loop from residues 127 to 151 using oligonucleotides doped at a 15%
rate with an equimolar mixture of the four bases as described previously (45) except that cassettes containing point mutations were ligated into compatible cohesive ends using an existing unique EcoRI site, and an SpeI site created by introducing silent mutations into Leu132 (CTT → CTA) and Leu132 (CTG → CTA) of the coding sequence for the human m5 receptor (pCDE2-m5) rather than blunt-end ligated. A second library containing mutations from 129 to 143 was constructed based upon results with the first library including the observations that positions 144–149 could be freely mutated without affecting the functional properties of the receptor, whereas mutations at residues 127 and 128 severely affect receptor function. The lower mutation rate at residues 150 and 151 was due to inefficient priming of the 3' end of the oligonucleotide in the polymerase chain reaction (47). Therefore, the oligonucleotide used for the second library contained wild-type sequence at the 3' end (codons 144 and 145) to eliminate polymerase chain reaction artifacts. Point mutations at each of four residues, Phe130, Arg134, Tyr138, and Arg142 were constructed as described (46) using oligonucleotides doped with an equimolar mixture of the four bases at each individual codon rather than at a 15% rate over multiple codons as described above for random saturation mutagenesis of the i2 loop. *Escherichia coli* (DH5α) were transformed, and individual transformants were picked, amplified, and plasmid DNA isolated for all subsequent studies. Clones were sequenced after positive identification and characterizations in the functional assays so that in essence, functional studies were done blindly.

Cell Culture—NIH 3T3 cells (ATCC number CRL 1658) and tsA cells (human embryonic kidney cell line tsA 201, Ref. 48) were incubated at 37 °C in a humidified atmosphere (5% CO2) in Dulbecco’s modified Eagle’s medium supplemented with 4500 mg/liter glucose, 4 mM l-glutamine, 50 units/ml penicillin G, 50 units/ml streptomycin (A.B.I.) and 10% calf serum for 3T3 cells or 10% fetal bovine serum (Life Technologies, Inc.) for tsA cells.

**Transfection Procedure and Functional Assays—**Receptor selection and amplification technology (R-SAT) assays were performed as described (45) using 0.2 µg of receptor and 0.5 µg of p-SL-jpgalactosidase (Promega, Madison WI) transfected into 2 × 105 cells per well of a 6-well rack for dose-response analysis, except that cells were combined with ligands in Dulbecco’s modified Eagle’s medium supplemented with 2% cyto-SF3 synthetic supplement (Kemp Laboratories) instead of calf serum. For screening, R-SAT assays were performed entirely in 96-well plates, using proportionately less DNA. Clones were screened in the presence of 100 µM carbachol, 2 µM atropine, or no drug. Plates were read at 420 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices). Data from R-SAT assays were fit to the equation: \( r = A + Bx/(x + c) \), where \( A \) = minimum response, \( B \) = maximum response minus minimum response, \( c = EC_{50} \), \( r \) = response, and \( x \) = concentration of ligand. Curves were generated by least-squares fits using the program KaleidaGraph28 (Abelbeck Software). Maximum response values were normalized according to an internal standard based on the fact that NIH 3T3 cells endogenously express prostanoid FP receptors which produce a robust R-SAT response when exposed to the prostanoid agonist cloprostanol (Kayman Chemicals). We assayed each batch of transfected cells with 10 nM cloprostanol (EC50 = 1 nM) + 1 µM atropine (to suppress any constitutive muscarinic activity), and used the resulting response to normalize the dose-response data obtained using carbachol and atropine.

**Binding Studies—**The studies were performed as described (46). Cells were harvested 72 h after transfection and stored at −80 °C. Membranes were prepared in binding buffer containing 25 mM sodium phosphate (pH 7.4), 5 mM magnesium, and 50 µM Gpp(NH)p immediately before use.

## RESULTS

The second intracellular loop (i2) is highly conserved, with 13 of 25 amino acids being identical among the muscarinic subfamily (1). To investigate structure-function relationships of m5 receptor/G protein coupling in the i2 loop we employed random-saturation mutagenesis in combination with a high throughput functional assay (R-SAT™ see Refs. 45, 49, and 50). Randomly doped oligonucleotides were used in a polymerase chain reaction based construction method (see “Materials and Methods”) to generate libraries of mutant m5 muscarinic receptors. The libraries were screened for functional receptors using R-SAT™, and these receptors were sequenced and subjected to concentration-response analysis.

Overall, 63 functional receptors were isolated from libraries containing over 900 unique receptors. Three basic phenotypes of receptors were observed (see Fig. 1) including activated receptors (constitutive activity ≥ 15%), wild-type like receptors (EC50 ≤ 10-fold, EC50 WT and max response ≥ 70% WT), and compromised receptors (EC50 > 10-fold EC50 WT or max response < 70% WT or both). To facilitate comparison of the molecular differences between activated, wild-type like, and compromised receptors, the associated mutations were tabulated by position and compiled into lists (see Table I).

**Random Mutagenesis Identifies a Cluster of Constitutively Activating Residues—**A striking finding was the large number of constitutively activated receptors identified in the i2 library (Table I and Fig. 1A). These receptors mediated ligand

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2 T. Messier and M. Brann, personal communication.
independent responses which could be reversed by the negative antagonist atropine. Maximum response was defined as the difference between the maximum response to carbachol and minimum response to atropine. Maximum response was normalized to the maximal response for wild-type m5. Constitutive activity and constitutive activity values are rounded to the nearest 5%. Substitutions are denoted using the single letter amino acid code. Periods indicate wild-type sequence at those positions. A, activated receptors. Receptors with constitutive activity > 15%. Bold text: residues with activating substitutions in multiple clones and in point mutants. B, wild-type like receptors. Receptors with EC50 ≤ 10-fold EC50 wild-type and max response > 70% wild-type. Bold text: residues which were either never, or only conservatively mutated in wild-type or activated mutants. Italics: residues which tolerate limited subsets of amino acid substitutions. Phenylalanine and cysteine are naturally occurring variants at the analogous position to Tyr129 in other G-protein coupled receptors and are well tolerated substitutions in the m1 muscarinic receptor (51). Bulky, hydrophobic substitutions are observed at Leu136 as previously discussed (52). R159W is a conservative substitution based on the PAM250 scoring matrix which compares sequences of proteins with known homology (53). Positions that tolerate radical substitutions are indicated by a dash. l indicates positions with a lower rate of mutagenesis (see Methods). C, compromised receptors. Receptors with EC50 > 10-fold wild-type EC50, or max response < 70% of wild-type. Bold text: as for B except these residues were also never or only conservatively mutated in compromised receptors.

A Constitutively Activated Mutants

| m5 | EC50 | Maximum | Constitutive | Rate | Response (%) | Activity (%) |
|----|------|---------|--------------|------|--------------|--------------|
| I 149 Y | V | S | 0.2 | 120 | 100 | 5 |
| 253 G | 0.5 | 120 | 15 |
| II 11 L | G | N | 0.02 | 120 | 50 |
| 4 C | 0.1 | 135 | 30 |
| 234 Q | 0.1 | 115 | 20 |
| 14 L | 0.2 | 120 | 25 |
| 265 R | 0.5 | 120 | 25 |
| 266 R | 0.5 | 120 | 25 |
| 147 C | 0.4 | 110 | 20 |
| 189 I | 0.1 | 110 | 15 |
| 6 V | 2 | 120 | 15 |

- F - R - T - Y - R - Constitutively Activating Residues

independent responses which could be reversed by the negative antagonist atropine. In general, the EC50 values of carbachol at the activated receptors were lower than the value for wild-type m5. Although mutations were introduced randomly into i2, mutations associated with constitutive activity were not randomly distributed, but were instead clustered at Phe130, Arg134, Thr137, Tyr138, and Arg142. In contrast, analysis of the i3 loop by random mutagenesis identified very few constitutively activated receptors (45–47), suggesting that mutations in the i2 loop have a greater propensity to cause constitutive activity.

Random Mutagenesis Identifies a Cluster of Functionally Conserved Residues—Receptors which were phenotypically similar to wild-type m5 also contained a non-random pattern of mutations (see Table I and Fig. 1B). While the majority of residues in i2 tolerated multiple radical substitutions, eight residues were either never mutated or were substituted infrequently and only with conservative amino acids. No mutations were observed at Asp127, Arg128, or Phe135 in wild-type like or activated receptors. Since the random mutagenesis procedure certainly produced receptors containing mutations to these residues (46), mutations at these residues probably disrupt receptor function. Several other residues were also identified where non-conservative mutations may disrupt receptor function. Tyr129 was only mutated to phenylalanine and cysteine which were found at the analogous position in other G-protein coupled receptors and are well tolerated substitutions in the m1 muscarinic receptor (51). Ile136 and Thr142 were only conservatively mutated to valine and alanine, respectively. Only bulky hydrophobic substitutions were observed at Leu136, a structural requirement previously predicted for this residue (52).

3 E. S. Burstein, T. A. Spalding, and M. R. Brann, unpublished data.
PAM250 matrix (53). Overall these data define a consensus of residues which are likely to be crucial for receptor/G-protein coupling and are therefore important for the active state of the receptor.

Receptors with compromised phenotypes (EC$_{50}$ > 10-fold EC$_{50}$ WT or max response < 70% WT or both) are shown in Table I. These receptors had a more random distribution of mutations than the other groups of receptors, and most of the compromised receptors (14 out of 21) contain mutations at residues which were never mutated in the fully functional receptors (see Table I), providing more evidence for the functional importance of these residues. Functional receptors containing mutations at Arg$_{128}$^ref were never observed, consistent with its pivotal role in G-protein coupling (see Ref. 54).

Mutations at Constitutively Activating Residues Disrupt an Inactive State of m5—Receptors appear to function by switching between active and inactive conformations (27–30). The effect of constitutively activating mutations is to increase the preference of the receptor for the active state and this can be achieved either by disrupting interactions important for maintaining the inactive state, or by strengthening interactions needed for active state. To distinguish between these possibilities, libraries of receptors were constructed which contained point mutations at each of four residues associated with the activated phenotype. The libraries were screened for constitutive active receptors which were then sequenced and subjected to concentration-response analysis with carbachol and atropine. As shown in Table II and Fig. 2, at each position diverse substitutions representing most of the major classes of amino acids were constitutively activating. Given this diversity, it is likely that the primary effect of these mutations is to disrupt interactions needed to maintain the inactive state rather than to strengthen interactions needed in the active state.

Ligand binding studies revealed that receptors having constitutively activated, wild-type, and compromised phenotypes were expressed at similar levels, thus these diverse phenotypes are unlikely to be due to changes in expression levels (Table III). Two compromised receptors (clones 19 and 151, see Table I for functional phenotypes) bound carbachol with similar affinity as wild-type m5, thus the reduced potency of carbachol seen in functional assays is almost certainly because of impaired G-protein coupling. As in the functional assay, carbachol affinity was greater for activated receptors than for wild-type m5 and tended to be greatest at the most constitutively activated receptors, a predicted consequence of constitutive activity (27, 30). In contrast, antagonist affinities (and potencies, see Table II) were largely unaffected. Since the i2 loop resides in the cytoplasm, it is unlikely that the observed increases in carbachol affinity are due to direct changes in the ligand-binding site but probably result from allosteric effects.

**DISCUSSION**

The i2 Loop Has Two Protein Faces Which Function as a Conformational Switch—We have shown that the i2 domain contains a group of residues necessary for holding the receptor in an inactive conformation (the off-state) and another group necessary for G-protein coupling (the on-state). These residues occur with a periodicity of 3 to 4 positions and when mapped on a perfect e-helix, form opposing faces, offset approximately 100° (see Fig. 3). Given the clear division between constitutively activating residues and functionally conserved residues, we speculate that i2 switches conformation to alternatively prevent or promote G-protein coupling through these two groups. The two putative protein faces formed by these groups could engage/disengage G-proteins, other accessory proteins, or other domains of the receptor.

Structure-Function Properties of G-protein Coupling Domains in the m5 Receptor—G-protein coupling to muscarinic receptors occurs mainly through the i2 and i3 loops (55), therefore with this and previous studies (45–47, 56), we have completed analysis of the major G-protein coupling domains of the m5 muscarinic receptor by random mutagenesis (see Fig. 3). Comparison of these results reveals several patterns. G-protein coupling occurs primarily at the transmembrane/cytoplasmic borders between TM3/i2, TM5/i3, and TM6/i3, respectively. All of the functionally important residues in i2 and i3 identified by random mutagenesis are restricted to segments adjacent to these transmembrane domains whereas the remaining residues can be freely mutated without affecting function. The functionally critical residues in each domain are predominately distributed with a periodicity of 3 to 4 positions, consistent with the hypothesis that these regions form e-helical extensions of the adjacent transmembrane domains (also see Refs. 57–59).

There is generally conservation of hydrophobic residues within i2 and i3 (45, 47). These hydrophobic residues likely
fulfill structural roles in forming a G-protein coupling pocket (46, 56). When TM5 and TM6 are aligned such that residues known to be vital for receptor-ligand interactions face into the core of the receptor (10–12), the hydrophobic residues in Ni3 and Ci3 converge where they could form a G-protein coupling pocket through protein-protein interactions (56). This model is consistent with the idea that in a soluble protein, hydrophobic residues generally face inward. As TM3 is thought to face TM5 and TM6 (7), it is plausible that the conserved hydrophobic residues identified in i2 interact with those identified in Ni3 and Ci3.

Finally, there is functional conservation of basic residues in i2 and i3, in agreement with theories that receptors couple G-proteins through interactions with electrostatically negative regions of the heterotrimeric complex (60). The helical domains of Ni3 and Ci3 each terminate in basic residues (45–47), and mutation of both residues to glutamate abolishes G-protein coupling (56). We have now identified four arginine residues in i2 which are critical for receptor function, consistent with the idea that i2 plays a major role in receptor/G-protein coupling, and that basic residues are crucial to this process.

**FIG. 2.** Concentration-response relationships of receptors containing point mutations to residues Phe130, Arg134, Tyr138, and Arg142. Open symbols represent responses to the agonist carbachol. Filled symbols represent responses to the antagonist atropine. A, F130X. Circles, glutamine; squares, lysine; triangles, wild-type. B, R134X. Circles, glutamate; squares, threonine. C, Y138X. Circles, cysteine; squares, alanine; triangles, glycine. D, R142X. Circles, leucine; squares, asparagine.

**TABLE III**

Ligand binding parameters of mutant receptors

|        | $K_{D,NMS}$ ($\times 10^{-6}$ M) | $B_{max}$ (pmol/mg) | $IC_{50}$ (Cch) ($\times 10^{-6}$ M) | Fold change |
|--------|-------------------------------|---------------------|-----------------------------------|-------------|
| m5     | 2.0                           | 7.4                 | 177                               | 1           |
| R142H  | 1.7                           | 7.5                 | 9                                 | 21          |
| R142N  | 2.4                           | 9.3                 | 10                                | 18          |
| F130Q  | 1.8                           | 6.6                 | 15                                | 12          |
| Y138T  | 3.1                           | 3.3                 | 31                                | 6           |
| F130K  | 1.4                           | 3.7                 | 37                                | 5           |
| F130M  | 2.7                           | 7.5                 | 63                                | 3           |
| F130C  | 2.0                           | 6.1                 | 59                                | 3           |
| R134E  | 1.5                           | 4.3                 | 72                                | 2           |
| Clone 19 | 0.8                         | 4.7                 | 39                                | 4           |
| Clone 151 | 1.0                        | 5.5                 | 146                               | 1           |

**FIG. 3.** a-Helical projections of i2, Ni3, and Ci3, showing the predicted distribution of functionally conserved and constitutively activating residues. Data for Ni3 and Ci3 are taken from Refs. 45–47, and 56. A, side view: the functionally conserved portions of i2, Ni3, and Ci3 are shown forming direct helical extensions of TM3, TM5, and TM6, respectively, with residues spaced 100° apart. The nonconserved portions of the loops are shown as flexible structures. The membra
cytotoplasmic border is shown as gray shading. Key: large black circles, residues where a wide variety of substitutions were shown to cause constitutive activity. White circles, functionally conserved residues. Bold text denotes residues that did not tolerate nonconservative mutations. Italics denote conserved residues where limited subsets of substitutions were allowed (see text, Table I, and Ref. 46). White triangles, functionally conserved residues which are predictive of G-protein coupling specificity (see Refs. 45, 46, and 56). Small black circles, residues which could be freely mutated without affecting the functional properties of the receptor. B, Top view: i2, Ni3, and Ci3 are assumed to be direct helical extensions of TM3, TM5, and TM6, and are arranged counterclockwise (see Ref. 7). The helices are oriented such that residues in TM3, TM5, and TM6 which make protein-protein or protein-
ligand interactions will face into the center of the receptor. These residues include for TM3, Trp106 and Asp110; for TM5, Thr194 and Thr197; and for TM6, Trp455, Tyr458, and Asn459 (see Refs. 10–16, 56, and 67). TM3 and TM5 are oriented as suggested (8). TM6 is oriented such that the functionally conserved face predicted by Spalding et al. (67) would face TM3 and TM5.

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[Diagram of receptor structure and ligand binding parameters]

This model is consistent with the idea that in a soluble protein, hydrophobic residues generally face inward. As TM3 is thought to face TM5 and TM6 (7), it is plausible that the conserved hydrophobic residues identified in i2 interact with those identified in Ni3 and Ci3.

Finally, there is functional conservation of basic residues in i2 and i3, in agreement with theories that receptors couple G-proteins through interactions with electrostatically negative regions of the heterotrimeric complex (60). The helical domains of Ni3 and Ci3 each terminate in basic residues (45–47), and mutation of both residues to glutamate abolishes G-protein coupling (56). We have now identified four arginine residues in i2 which are critical for receptor function, consistent with the idea that i2 plays a major role in receptor/G-protein coupling, and that basic residues are crucial to this process.
The i3 Loop Governs G-protein Coupling Specificity—Despite the similarities between i2 and i3 described above, it is clear that these domains perform distinct roles in receptor/G-protein coupling. Previous work identified Tyr217 in Nt3 (46) and Ala41 in C3 (45) as the major determinants of G-protein coupling specificity, and mutation of both of these residues to the analogous residues in the Gα-connected m2 and m4 muscarinic receptors abolishes signaling by m5 (56). Although four residues in i2 predict G-protein coupling specificity based on sequence conservation (1), none of these residues was identified as being functionally critical. Thus coupling specificity is governed mainly by the i3 loop, in agreement with earlier findings using chimeric m2/m5 receptors (55).

Receptor Activation by Movement of the Transmembrane Domains Is Propagated through the i2 Loop—Ample structural (7–9) and functional (14–16) data point to a central role for TM3 in receptor-ligand interactions. Conformational changes occurring due to ligand interactions with TM3 are likely to be propagated into i2 to enable receptor/G-protein activation. Experiments on rhodopsin show that i2 moves as a rigid body following changes in chromophore interactions with TM3 (61–63). We have reported that a series of mutations at Ser465 (located at the extracellular end of TM6) constitutively activate m5, probably by causing movement of TM6 relative to the other transmembrane domains (30, 40). More recently we have observed that m5 is activated by mutations in multiple positions across TM6, similar to results for i2, and thus TM6 may function as a ligand-operated switch region of m5. (67). Recent reports on rhodopsin (64, 65) and the β2 adrenoreceptor (66) indicate that TM5/2 and TM6 move relative to each other upon receptor activation. Overall our results indicate that the ultimate goal of these changes is to reposition i2, exposing the catalytic surface of the receptor, enabling G-protein activation.

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