Structure/Function Relationships of a G-Protein Coupling Pocket Formed by the Third Intracellular Loop of the m5 Muscarinic Receptor†

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Received August 27, 1997; Revised Manuscript Received January 9, 1998

ABSTRACT: Using random saturation mutagenesis, we have previously identified the amino acids K439, A440, and A441 in the C-terminus of the third intracellular loop (Ci3) of the m5 muscarinic receptor as being critical for G-protein coupling [Burstein, E. S., Spalding, T. A., Hill-Eubanks, D., and Brann, M. R. (1995) *J. Biol. Chem. 270*, 3141−3146]. In the present study, we have constructed a series of point mutants at each of these residues and characterized their functional phenotypes in order to define the structure/function relationships of each of these residues for G-protein coupling. Although a wide variety of substitutions were tolerated at K439, most caused significant increases in the EC_{50} of carbachol and decreases in the maximum response (R_{max}). Only other basic residues were well tolerated (<10-fold increase in EC_{50}, >70% of wild type). Acidic substitutions had the largest effects, reducing R_{max} to under 20% of wild type. At A440, only the conservative substitution threonine was well tolerated. Substitutions by hydrophobic, polar, and basic residues caused 10−80-fold increases in EC_{50} values and in many cases also significantly reduced R_{max} (<70% of wild type). In contrast, at A441 mutations selectively affected EC_{50} but not R_{max} values. Previously we identified I216, Y217, T220, and R223 as the residues in the N-terminus of the i3 loop of m5 (Ni3) that are critical for G-protein coupling [Burstein, E. S., Spalding, T. S., and Brann, M. R. (1996) *J. Biol. Chem. 271*, 2882−2885]. To investigate whether there were additive contributions of Ni3 and Ci3 to G-protein coupling, the functional responses of two double mutants, R223E/K439E and Y217S/A441T, were evaluated. Though these mutations were tolerated individually, both double mutant receptors produced almost indetectable responses. Little or no changes in expression levels or ligand binding properties were detected, suggesting the observed effects were caused primarily by changes receptor/G-protein coupling. We conclude that K439 participates in G-protein activation through an ionic mechanism, that A440 fulfills a structural role forming part of the G-protein coupling pocket, and that A441 contributes to receptor affinity for G-proteins. We propose that the third intracellular loop forms a G-protein coupling pocket comprised of a positively charged "lip" and a hydrophobic core.

Muscarinic receptors consist of five genetically defined subtypes (m1−m5) that belong to a superfamily of seven transmembrane receptors that couple to G-proteins (*1*−*7*). The muscarinic receptor family can be subdivided into the receptors that couple to Gq-like G-proteins (m1, m3, and m5; see ref *8*) and those that couple Gi-like G-proteins (m2 and m4). The structural basis for receptor/G-protein coupling is not well understood and awaits the availability of high-resolution structures of receptors. To elucidate the structural basis of receptor activation and G-protein coupling, we have developed a novel random saturation mutagenesis protocol that circumvents many of the limitations inherent to traditional approaches (*9*). In this procedure, libraries of mutated receptors are created by polymerase chain reaction (PCR) using doped oligonucleotides that span discrete domains postulated to be important for interactions with ligands and/or G-proteins. The libraries are then screened using a high-throughput functional assay called R-SAT (receptor selection and amplification technology,[1] *10*−*12*, patent pending) to identify receptors that retain the ability to mediate signal transduction. By grouping phenotypically similar receptors and examining their associated patterns of functionally tolerated substitutions, we are able to establish structure/function relationships.

Extensive work has shown that the third cytoplasmic loop (i3) is a major determinant of G-protein coupling for receptors (*13*−*16*) and that only the N- and C-terminal regions of the i3 loop (hereafter Ni3 and Ci3) are required for function (*17*−*20*). Therefore, we have targeted these regions of the m5 muscarinic receptor for analysis by random saturation mutagenesis (*10*, *21*). On the basis of our results, both Ni3 and Ci3 are predicted to form α-helical extensions of transmembrane domains 5 and 6 (TM5 and TM6), respectively. Of the 42 amino acid residues targetted in these studies, most could be freely mutated without impairing function (*10*, *21*). Surprisingly, this included parts of highly conserved sequence motifs such as the KERK motif (residues

† This work was supported by U.S. Public Health Service Grant RO1 GM52737 from the National Institute of General Medical Sciences. E.S.B. was supported by National Research Award Fellowship F32NS09436.

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[1] Abbreviations: TM5 and TM6, transmembrane regions 5 and 6; Ni3 and Ci3, N- and C-terminal regions of intracellular loop 3; R-SAT, receptor selection and amplification technology.

S0006-2960(97)02132-6 CCC: $15.00 © 1998 American Chemical Society
Published on Web 03/05/1998

	Ni3															Ci3									
helix	V	V	V																				VI	VI	VI
m5 number	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	436	437	438	439	440	441	442	443	444	445
m2 (Gi)	L	Y	W	H	I	S	R	A	S	K	S	R	I	K	K	R	E	K	K	V	T	R	T	I	L
m4 (Gi)	L	Y	I	H	I	S	L	A	S	R	S	R	V	H	K	R	E	R	K	V	T	R	T	I	F
m1 (Gq)	L	Y	W	R	I	Y	R	E	T	E	N	R	A	R	E	K	E	K	K	A	A	R	T	L	S
m3 (Gq)	L	Y	W	R	I	Y	K	E	T	E	K	R	T	K	E	K	E	K	K	A	A	Q	T	L	S
m5 (Gq)	L	Y	C	R	I	Y	R	E	T	E	K	R	T	K	D	K	E	R	K	A	A	Q	T	L	S
Consensus	**L**	**Y**	*	*	**I**	**Y**	*	*	**T**	*	*	**R**	*	*	*	*	*	*	**K**	**A**	**A**	*	*	**L**	*

FIGURE 1: Amino acid sequences of the N- and C-termini of the third intracellular loop (Ni3 and Ci3, respectively) of the five muscarinic receptor subtypes. The consensus sequence indicates residues that are critical for G-protein coupling (see refs *10* and *21*). Residues in the consensus denoted with an asterisk could be freely mutated without disrupting function. Roman numerals V and VI denote residues proposed to reside in transmembrane domains 5 and 6, respectively.

436−439 in m5) and the BBXXB motif of the m1 receptor (38, residues 438−442 in m5). Only seven residues (four in Ni3 and three in Ci3) were identified as critical for G-protein coupling. Generally these residues were hydrophobic. Charged residues could be freely mutated with the exception of a single arginine in Ni3 (*21*) and a single lysine in Ci3 (10) (see Figure 1). Thus, receptor/G-protein coupling occurs through a very limited subset of amino acid residues.

Although random mutagenesis is extremely useful as an initial step for identifying key residues, to derive structure/function relationships, it is necessary to construct point mutations, first to test the substitutions in the absence of a background of other substitutions, and second to thoroughly examine the effects of a variety of amino acid side chains at a given position. An extended analysis of the Ni3 region in m5 was performed in which a series of point mutants were constructed and functionally characterized for each of the residues (I216, Y217, T220, and R223) previously identified by random mutagenesis as critical for G-protein coupling (*22*). Clear differences were seen both in the pattern of tolerated mutations of each residue and in the phenotypic effect of those mutations. From these results it was possible to distinguish between likely roles for each residue in G-protein coupling. In the present study, we have similarly analyzed the functionally important residues in Ci3. We have also constructed and analyzed two double mutant receptors, one in which both conserved basic residues were changed to acidic residues, and one in which both residues that are predictive of G-protein coupling selectivity were changed to the analogous residues in the m2/m4 muscarinic receptors. A model is presented in which the third intracellular loop forms a G-protein coupling pocket comprised of a positively charged "lip" and a hydrophobic core.

EXPERIMENTAL PROCEDURES

Library Construction. Libraries containing randomly introduced mutations at K439 and A440 were constructed as described (*10, 22*) except that cassettes containing point mutations were ligated into compatible cohesive ends using an existing unique *Eco*RI site and an *Afl*II site created by introducing silent mutations into Thr 443 (ACA → AC*C*) and Leu 444 (CTG → *T*T*A*) of the coding sequence for the human m5 receptor (pCDE3-m5) rather than blunt-end ligated as previously described. Oligonucleotides were doped with an equimolar mixture of the four bases at each individual codon rather than at a 15% rate over multiple codons as previously described for random saturation mutagenesis (*10, 21, 23*). *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 characterization in the functional assays so that, in essence, the functional studies were done blindly. Over half of the identified amino acid substitutions were represented in two or more individual clones.

Cell Culture. NIH 3T3 cells (ATCC CRL 1658) and TSA cells were incubated at 37 °C in a humidified atmosphere (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 4500 mg/L glucose, 4 nM 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 for TSA cells (Gibco).

*Transfection Procedure and Functional Assays. R*eceptor selection and *a*mplification *t*echnology (R-SAT) assays were performed as described using 0.2 μg of receptor, 0.5 μg of p-SI-β-galactosidase (Promega, Madison WI), and 2 μg of salmon sperm DNA transfected into 2×10^5 cells/well of a six-well rack (*10, 21, 23*) except that cells were combined with ligands in DMEM supplemented with 2% cyto-SF3 synthetic supplement (Kemp Laboratories) instead of calf serum. After 16 h the 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

$$R = A + B[x/(x + c)]$$

where A = minimum response, B = maximum response − minimum response, $c = EC_{50}$, R = response, and x = concentration of ligand. Curves were generated by least-squares fits using the program KaleidaGraph (Abelbeck Software). The background absorbance of the plate was subtracted from all values. The maximum response to m5 was assigned a value of 100% and was typically 0.5 absorbance unit over a background of 0.1 unit. Maximum response values from separate transfections were normalized according to an internal standard using the response of endogenously expressed prostanoid FP receptors to the

prostanoid agonist cloprostanol (Kayman Chemicals) as described previously (*22*).

Binding studies were performed as described (*22, 24*). TSA 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 GppNHp immediately before use.

RESULTS

We used a PCR-based protocol to construct libraries of receptors containing randomly introduced mutations at K439 and A440, residues within Ci3 previously shown to be functionally important (*10*; see Figure 1). We identified functional receptors based on their ability to amplify NIH 3T3 cells in a ligand-dependent manner using an assay called *r*eceptor *s*election and *a*mplification *t*echnology or R-SAT (*10*−*12*, patent pending). In our assay technology, ligands select and amplify cells that express functional receptors. These same cells also express a reporter gene; thus receptor activity is assayed as a change in enzyme levels. Functional receptors were identified in a primary screen, using a single dose (100 μM) of carbachol, and then subjected to a detailed concentration/response analysis, using doses of carbachol ranging from 200 pM to 500 μM. The results of this analysis are shown in Table 1 and Figure 2.

At lysine 439 only the conservative substitution arginine was well tolerated (<10-fold increase in EC_{50}, R_{max} > 70% of wild type) (Table 1, Figure 2A). The EC_{50} values of carbachol were increased significantly (>10-fold wild-type EC_{50}) at all of the other mutant receptors, which included hydrophobic, polar, and acidic substitutions. Over half of these substitutions also reduced R_{max} below 70% of wild type. Substitutions by the smallest residues (Ser, Ala, and Gly) tended to cause more adverse affects. Substitution by glutamate was poorly tolerated, reducing R_{max} to below 20% of wild type. These results indicate that a basic residue is required at position 439 and suggest that it makes an ionic interaction which is necessary for G-protein activation.

At alanine 440 only replacement by threonine was well tolerated (Table 1, Figure 2B), in agreement with data from random mutagenesis experiments (*10*). Basic, neutral, and hydrophobic residues were tolerated, but all increased the EC_{50} values more than 10-fold and most reduced R_{max}. Hydrophobic residues were not especially favored over charged substitutions of similar size. The acidic residue glutamate was not tolerated. Although the A440E receptor gave a slight response in the initial screen (which is reproducibly more sensitive than a full concentration/response analysis; Burstein and Spalding, unpublished observations), no measurable response was seen in further assays. These results suggest that there are structural constraints at position 440, and thus threonine may be well tolerated because it is of similar size to alanine.

All mutations at alanine 441 caused significant increases in EC_{50} without affecting R_{max} (Table 1), suggesting that the primary effect of mutations at this residue is to reduce receptor affinity for G-proteins (*10*). In contrast to the results for A440, threonine caused the most adverse functional effects at A441, whereas the very dissimilar amino acids lysine and cysteine had relatively minor effects on function. The three most poorly tolerated substitutions at A441 are similar in size to alanine, and thus it is unlikely that they impair function by sterically altering the architecture of the G-protein coupling pocket. Proline could alter the orientation of the putative helical extension of Ci3, while serine or threonine probably disrupts specific amino acid side-chain interactions involved in G-protein coupling. Overall these data suggest that, rather than a strict requirement for alanine at position 441, certain amino acids such as serine and threonine are excluded from this position.

Besides the three residues described in this paper, we have previously identified four residues in Ni3 that were critical for G-protein coupling (*21, 22*). These included R223, where

Table 1: Functional Phenotypes of Mutant Receptors[a]

position	substitution	EC_{50}/EC_{50} (WT)	maximum response (% WT)	EC_{50} (μM)
K439	lysine (WT)	1	100 ± 23	0.3 ± 0.0
	arginine	4	83 ± 14	1.2 ± 0.4
	leucine	12	58 ± 2	3.8 ± 0.5
	valine	18	48	5.8
	glutamine	21	93 ± 19	6.7 ± 0.9
	methionine	23	54 ± 8	7.3 ± 6.1
	threonine	23	65	7.4
	isoleucine	30	57	9.6
	cysteine	37	91 ± 35	11.7 ± 7.6
	serine	39	52 ± 15	12.5 ± 5.4
	glutamate	41	16 ± 7	8.8 ± 18
	alanine	49	87 ± 16	15.6 ± 4.8
	glycine	62	66	19.8
A440	alanine (WT)	1	100 ± 13	0.10 ± 0.05
	threonine	3	78 ± 28	0.32 ± 0.1
	lysine	12	83 ± 4	1.2 ± 0.5
	isoleucine	13	99 ± 10	1.3 ± 0.3
	proline	13	113 ± 38	1.3 ± 0.4
	glycine	14	65 ± 7	1.4 ± 0.5
	arginine	15	69 ± 1	1.5 ± 1.7
	asparagine	17	59	1.7
	leucine	25	66 ± 22	2.5 ± 1.4
	histidine	37	111 ± 8	3.7 ± 4.8
	glutamine	82	46	8.2
	glutamate	NR[b]	NR	NR
A441	alanine (WT)	1	100 ± 5	0.18 ± 0.05
	cysteine	3	100 ± 4	0.5 ± 0.1
	lysine	4	110	0.7
	leucine	6	86	1.0
	isoleucine	11	130 ± 20	2.0 ± 1.2
	valine	11	96	2.0
	arginine	12	96 ± 17	2.3 ± 0.7
	glutamate	12	115 ± 18	2.1 ± 1.1
	tyrosine	13	130 ± 12	2.5 ± 0.7
	glycine	15	71	2.7
	serine	20	120 ± 5	3.7 ± 1.3
	proline	44	100 ± 21	7.9 ± 1.6
	threonine	60	76 ± 13	10.9 ± 6.7
	wild type	1	100 ± 7	0.22 ± 0.08
K439	glutamate	41	16 ± 7	8.8 ± 18
R223	glutamate	82	72 ± 10	17.7 ± 6.4
R223/K439	Glu/Glu	NR	NR	NR
	wild type	1	100 ± 13	0.3 ± 0.2
A441	threonine	108	96 ± 18	38 ± 28
Y217	serine	20	36 ± 11	6.8 ± 10
Y217/A441	Ser/Thr	0.3	12 ± 5	0.1 ± 0.2

[a] WT denotes the wild-type residues at that position in m5. The EC_{50}s for functional assays were calculated as described under Experimental Procedures. Data are the means ±SE of 2−3 independent experiments. Data for position 441 were taken from ref *17*. The EC_{50} values for each group of mutants were normalized to the wild-type value in that group. Representatives from each group were subsequently reassayed together to confirm their relative rankings. [b] NR, no response.

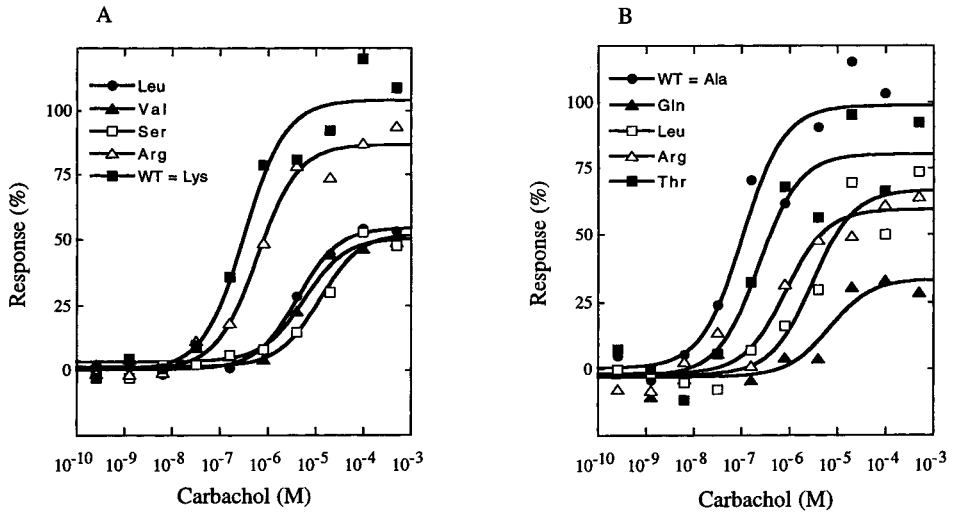

FIGURE 2: Concentration/response curves of receptors containing point mutations in residues of the Ci3 domain of the m5 muscarinic receptor that are needed for G-protein coupling. (A) Position 439: lysine (wild-type), ■; arginine, △; serine, □; valine, ▲; leucine, ●. (B) Position 440: alanine (wild-type), ●; arginine, △; leucine, □; glutamine, ▲; threonine, ■.

Table 2: Ligand Binding Properties of Mutant Receptors[a]

position	substitution	B_{max}	K_d ([^{3}H]NMS)	IC_{50} (Cch)
	m5	2.8 ± 0.2	71 ± 20	53 ± 46
K439	glutamate	1.7 ± 0.1	98 ± 21	147 ± 89
	leucine	1.9 ± 0.1	121 ± 12	nd[b]
	glycine	2.7 ± 0.2	184 ± 26	156 ± 122
	glutamine	2.6 ± 0.1	179 ± 27	nd
	alanine	3.0 ± 0.1	149 ± 20	nd
A440	glutamine	5.8 ± 0.4	263 ± 44	97 ± 33
	glutamate	2.8 ± 0.2	318 ± 43	200 ± 68
	leucine	2.1 ± 0.1	142 ± 12	nd
	arginine	0.5 ± 0.1	104 ± 16	nd
	Y217S/A441T	1.2 ± 0.2	119 ± 26	nd
	R223E/K439E	2.0 ± 0.1	172 ± 10	271 ± 97

[a] The K_d (picomolar) and B_{max} (picomoles per milligram) values for [^{3}H]NMS were determined as described under Experimental Procedures. The IC_{50} (micromolar) values for carbachol were obtained in the presence of 500 pM [^{3}H]NMS. Hill numbers were as follows: m5, 0.63; K439E, 0.88; K439G, 0.68; A440Q, 0.98; A440E, 0.91; and R223E/K439E, 1.07. Results are means ± error of duplicate determinations. [b] nd, not determined.

a loss of positive charge was correlated with dramatic increases in the EC_{50} values of carbachol, and Y217, where mutations reduced both maximum response and carbachol potency. We therefore constructed two double mutants, each containing a mutation in Ni3 and Ci3, to examine whether there were additive contributions of these domains to G-protein coupling. K439 and R223 were both changed to acidic residues (R223E/K439E). Individually, these mutations were tolerated but impaired function. However, a receptor with both mutations had almost no detectable functional response (Table 2, Figure 3A), demonstrating that there is a cumulative effect of losing positive charge at each of these positions. Similarly, mutation of both residues proposed to regulate G-protein coupling specificity [Y217 in Ni3 (see ref *22*) and A441 in Ci3 (this paper and ref *10*)] to the corresponding residues conserved in the Gαi-coupled muscarinic receptors (Y217S/A441T) significantly impaired function more than either mutation alone (Table 2, Figure 3A).

The [^{3}H]NMS and carbachol binding properties of 10 mutants that spanned the observed phenotypic range were evaluated to determine if the phenotypic changes could be accounted for by altered expression levels or ligand affinities (Table 2). There were little or no changes in the binding properties of these receptors, implying that the effects of these mutations were mainly on interactions with G-proteins.

DISCUSSION

In this paper the structure/function relationships of K439, A440, and A441 in G-protein coupling have been defined by constructing a series of point mutations at each residue and testing the mutants for function. By examining the effects of a variety of amino acid side chains at each position, clear differences become apparent both in the pattern of tolerated mutations of each residue and in the phenotypic effect of those mutations. We have shown that K439 participates in G-protein activation through an ionic mechanism, that A440 fulfills a structural role forming part of the G-protein coupling pocket, and that A441 contributes to receptor affinity for G-proteins. Previously we similarly analyzed the key residues in Ni3 (I216, Y217, T220, and R223, see ref *22*). Below is a comparison of the data for Ni3 and Ci3, followed by a proposal of how a G-protein coupling pocket composed of these residues might operate.

In both studies, residues could be categorized into those where mutations caused selective effects on EC_{50} values and those where R_{max} values were also affected. Assuming the receptor has to first bind the G-protein and then catalyzes G-protein activation (see ref *40*), a selective increase in EC_{50} would be expected if a mutation decreased receptor affinity for G-proteins. Theoretically, a selective increase in EC_{50} can be overcome by increasing receptor occupancy to produce the same R_{max}, although eventually a point is reached where the binding affinity is reduced such that even at full receptor occupancy R_{max} is reduced. Conversely, when R_{max} is reduced, there is a clear indication that the ability to activate G-proteins is impaired. We therefore suggest that residues where only mutations selectively affect EC_{50} values are "affinity" residues involved in binding G-proteins, but residues where mutations frequently reduced R_{max} were likely to also be involved in G-protein activation. Residues could also be categorized by the patterns of tolerated substitutions. In some cases particular amino acid side chains were required

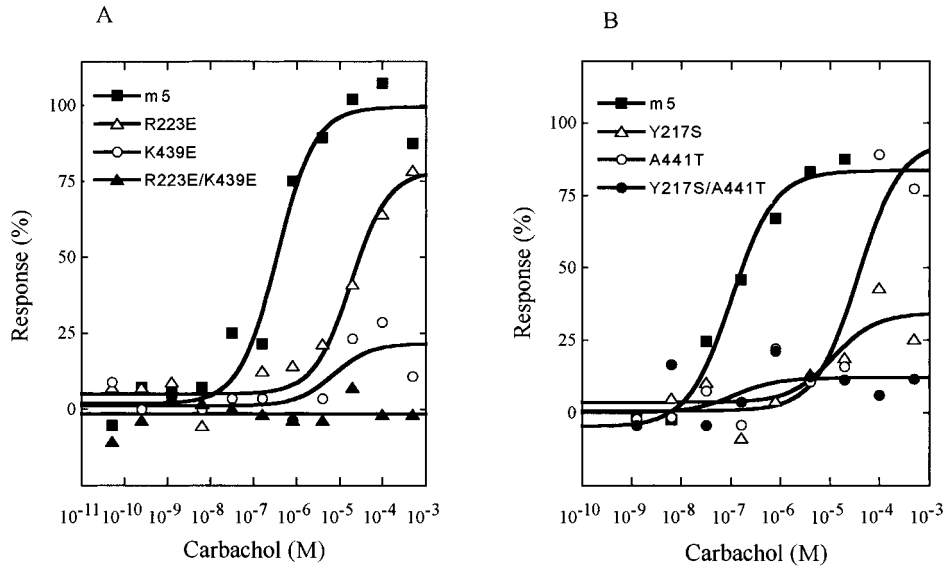

FIGURE 3: Concentration/response curves of receptors containing point mutations in residues of the Ci3 domain, Ni3 domain, or both domains of the m5 muscarinic receptor that are needed for G-protein coupling. (A) Mutation of functionally conserved basic residues to acidic residues: wild-type, ■; R223E, △; K439E, ○; R223E/K439E, ▲. (B) Mutation of functionally conserved residues predictive of G-protein coupling selectivity to the analagous residues in muscarinic receptors coupled to pertussis toxin-sensitive G-proteins: wild-type, ■; Y217S, △; A441T, ○; Y217S/A441T, ●.

and in other cases size or structural class was the critical parameter. The former suggests specific protein−protein interactions, while the latter suggests roles in forming the architecture of the G-protein coupling pocket.

For example, although basic residues were strongly favored at both R223 (*22*) and K439, all other substitutions at R223 selectively increased EC_{50} values without impairing R_{max}, whereas many substitutions at K439 also significantly impaired R_{max}. Glutamate was not phenotypically distinguishable from most other substitutions at R223, while glutamate markedly impaired function more than any other substitution at K439. It has been proposed that this lysine anchors the cytoplasmic end of TM6 to the membrane (*39*) and therefore the effect of the glutamate substitution could also be to disrupt the positioning of TM6. However, a global effect of K439E on receptor structure is unlikely as this mutant binds carbachol normally (see Table 2), and therefore this hypothesis seems less likely than a direct effect on G-protein coupling. We suggest that the different phenotypic effects of substitutions at K439 and R223 reflect their different roles in G-protein coupling; K439 is required for G-protein activation while R223 is required to efficiently bind, but not to activate, G-proteins.

Of the seven residues in Ni3 and Ci3 identified as critical for G-protein coupling, four are conserved across the entire muscarinic receptor family and three (Y217, A440, and A441) are predictive of G-protein selectivity, being conserved only among muscarinic subtypes coupled to Gαq (see Figure 1). Of these three residues, the data for A441 and Y217 are most consistent with their proposed roles in governing coupling selectivity. In both cases, the worst observed substitution corresponded to the amino acid conserved in the muscarinic subtypes that couple Gαi. Y217 and A441 could be further distinguished on the basis the fact that substitutions at A441 selectively increased EC_{50} values without affecting maximum responses, while substitutions at Y217 impaired both maximum response and EC_{50} values (*22*). Thus the mutation patterns at A441 and Y217 are both consistent with their proposed roles in governing G-protein coupling specificity, but A441 probably functions as a G-protein contact site that allows for efficient receptor recognition of G-proteins while Y217 also participates in activation of G-proteins. The contributions of each residue were additive, supporting the hypothesis that no single domain or residue completely accounts for coupling specificity.

We propose that two residues in Ni3 (I216 and T220) and one in Ci3 (A440) fulfill mainly structural roles in forming a G-protein coupling pocket based on the observations that the most favored substitutions at these positions were of a similar structural class or of similar size to the wild-type residues. However, A440 differs from I216 and T220 in that few other types of substitutions were even tolerated at I216 and T220 (*22*), whereas many other substitutions were observed at A440. This greater stringency in Ni3 suggests a more highly ordered tertiary structure compared to Ci3. Phenotypically, A440 is similar to K439, where mutations tended to affect both EC_{50} and maximum response values; thus A440 could also be considered necessary for G-protein activation. We favor a structural role for A440 on the basis of the fact that there is mostly a size requirement at that position rather than a requirement for a specific functional group.

On the basis of studies using chimeric G-proteins and chimeric receptors, it has been proposed that the C-termini of Gα-subunits contact muscarinic receptors in the Ci3 regions (*29, 30*). The discovery that overexpression of G-proteins can constitutively activate receptors (*31, 32*) provides an assay to directly test for contact sites between G-proteins and receptors. We have found that overexpression of Gαq restores the EC_{50}s of compromised receptors containing mutations at A441 back to wild-type values (Burstein et al., unpublished observations), further supporting the idea that the major effect of these mutations is to reduce receptor affinity for Gαq. However, a chimeric G-protein, Gαqi5 (*28*), which preferentially couples the Gαi-coupled m2 muscarinic receptor (*33*), is not significantly better than

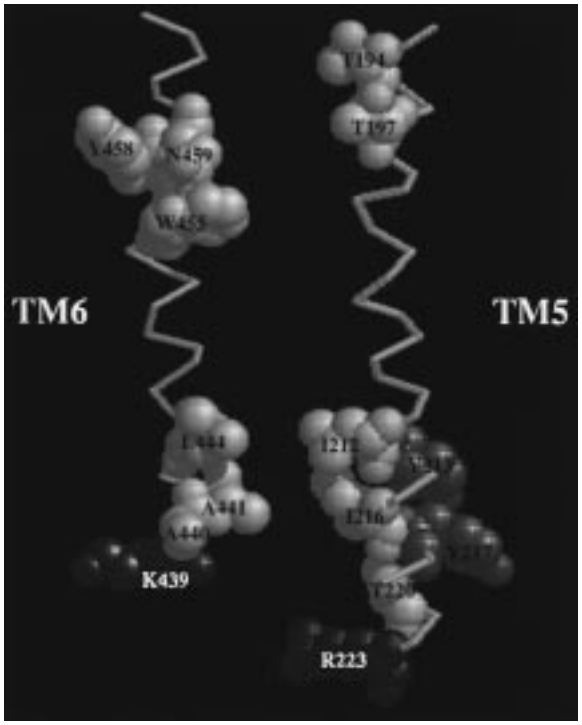

FIGURE 4: TM5/Ni3 and TM6/Ci3 were independently modeled as energy-minimized α-helices using the "Sybyl" software package to resolve clashes between side chains and intramolecular stresses caused by proline residues in the helices. Helices were aligned such that residues T194, T197, W455, Y458, and N459 [which have been implicated in ligand interactions (*24*, *41*, *42*; Spalding et al., submitted)] would face toward other helices or the center of the helical bundle. Ni3 and Ci3 domains were assumed to form α-helical extensions of TM5 and TM6, respectively (see text). The TM5/Ni3 and TM6/Ci3 domains are shown in this representation as ribbon backbones. The residues identified as critical for G-protein coupling (*10*, *21*, *22*, this paper), as well as the residues critical for ligand interactions, are shown using space-filling models. Color code: R223 and K439, blue; Y213 and Y217, green; T194, T197, L212, I216, T220, A440, A441, L444, W455, Y458, and N459, yellow.

Gαq at rescuing A441T or the double mutant Y217S/A441T (Burstein et al., unpublished observations). Thus receptor recognition of G-proteins is probably a complex interaction involving contributions from multiple epitopes besides Ci3 such as Ni3 and the i2 loop (*34*−*36*).

Many observations indicate that the transmembrane domains of G-protein coupled receptors are α-helical and that the Ni3 and Ci3 regions of muscarinic receptors form α-helical extensions of the TM5 and TM6 domains, respectively (*10*, *21*, *22*, *25*−*27*). Thus we constructed a spatial representation of a putative G-protein coupling site within the m5 muscarinic receptor (see Figure 4) with the TM5/Ni3 and TM6Ci3 domains modeled as α-helices. Each domain was modeled independently with energy minimization using the Sybyl software package to resolve clashes between side chains and intramolecular stresses caused by proline residues in the helices (T. A. Spalding, I. T. Christensen, and F. S. Joergensen, unpublished work). The vertical and rotational orientation of TM5/Ni3 and TM6/Ci3 with respect to each other was chosen on the basis of the following criteria: In random mutagenesis studies, charged substitutions were not tolerated N-terminal of Y213 and C-terminal of T443, suggesting that the TM5/Ni3 and TM6/Ci3 domains leave the cell membrane and enter the cytoplasm at these residues (*10*, *21*); thus these positions were aligned vertically. Mutations to residues T194, T197, W455, Y458, and N459 (or the analogous positions in other muscarinic receptors) are known to have major effects on ligand interactions (*24*, *41*, *42*; Spalding et al., manuscript submitted for publication) and in random mutagenesis studies, residues predicted to face away from these residues and into the lipid bilayer tolerate multiple, nonconservative substitutions (Spalding et al., manuscript submitted for publication). Thus the relative orientation of TM5 and TM6 was chosen such that T194, T197, W455, Y458, and N459 would face into the center of the receptor.

As illustrated in Figure 4, we suggest that the residues which we have identified as important in G-protein coupling lie in close proximity, where they form a putative G-protein coupling pocket. It is not possible to determine exact distances between residues on either side of the coupling pocket in this representation. The answer to this question awaits a high-resolution three-dimensional structure of m5. Included in Figure 4 are L212, Y213 (see ref *21*) and L444 (see ref *10*), which reside at the TM5/Ni3 and TM6/Ci3 junctions, respectively (discussed above). K439 and R223 (shown in blue) are located on the edges of the putative binding pocket where they are available for making high-affinity, ionic interactions with G-proteins. Since TM6 is postulated to undergo conformational changes upon receptor activation (*23*, *43*, *44*; Spalding, Burstein, and Brann, unpublished observations), K439 may link G-proteins to the receptor-mediated activation event. Y213 and Y217 (shown in green) face away from the core of the putative binding pocket formed by the other residues and therefore may be important for interacting with G-proteins upon receptor activation, when the receptor presumably undergoes conformational changes. All of the other residues (L212, I216, T220, A440, A441, and L444, shown in yellow) face each other in this representation, where they could interact hydrophobically with G-proteins and/or with each other. Since charged substitutions were rarely seen in these residues (this paper and ref *22*), and when seen they were usually poorly tolerated, we suggest that such mutations destroy the architecture of this putative G-protein coupling pocket. Thus we propose that the third intracellular loop forms a G-protein coupling pocket composed of a positively charged "lip" and a hydrophobic core.

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BI972132J