Amino Acid Side Chains That Define Muscarinic Receptor/G-protein Coupling

STUDIES OF THE THIRD INTRACELLULAR LOOP*

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Amino acids in the third intracellular loops of receptors play pivotal roles in G-protein coupling. To define their structural requirements, we have subjected the N- and C-terminal regions of this loop (Ni3 and Ci3, respectively) of the m5 muscarinic receptor to random saturation mutagenesis. (see Burstein, E. S., Spalding, T. A., Hill-Eubanks, D., and Brann, M. R. (1995) J. Biol. Chem. 270, 3141-3146 and Hill-Eubanks, D., Burstein, E. S., Spalding, T. A., Bräuner-Osborne, H., and Brann, M. R. (1996) J. Biol. Chem. 271, 3058-3065). In the present study, we have extended our analysis of Ni3 by constructing libraries of receptors containing mutations randomly distributed throughout Ni3 and Ci3. We then used a functional assay we have developed called R-SAT (receptor selection and amplification technology (Refs. 17-20), patent pending) to screen these libraries and identify the mutant receptors that retained the ability to transduce proliferative signals. We grouped phenotypically similar receptors and compiled lists of their associated substitutions, and based on these mutation patterns, we were able to ascertain structure/function relationships. Both regions were predicted to form α-helical extensions by our analysis and generally showed conservation of hydrophobic residues and little conservation of charged residues, with the exception of an invariant arginine in Ni3 (18) and an invariant lysine in Ci3 (17). In the present study, we have extended our analysis of Ni3 by constructing point mutant libraries of residues we previously identified as functionally important and characterizing their functional phenotypes allowing us to distinguish their specific roles.

EXPERIMENTAL PROCEDURES

Library Construction—Point mutation libraries at each of four residues: Ile216, Thr220, and Arg223 were constructed as described (17), except that oligonucleotides were doped with an equimolar mixture of the 4 bases at each individual codon rather than at a 15% rate over multiple codons as described previously for random saturation mutagenesis (17-19). 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 number CRL 1658) and TSA cells 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 (ABI) and 10% calf serum for 3T3 cells or 10% fetal bovine

Muscarinic receptors consist of five genetically defined subtypes (m1-m5), which belong to a superfamily of seven transmembrane receptors that couple to G-proteins (1-7). Although the crystal structures have been solved for two G-protein α-subunits in both the active and inactive conformations (8), to date, the structural basis for receptor/G-protein coupling is not well understood and awaits the availability of high resolution structures of receptors. Most of our current knowledge of the structural basis of receptor/G-protein coupling was determined from molecular experiments in which receptor domains have been systematically deleted, exchanged, and/or mutagenized. 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 (9-12). 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 are required for function (13-16).

To define the structural requirements of Ni3 and Ci3, we have subjected these regions of the m5 muscarinic receptor to random saturation mutagenesis (17, 18). In these studies we constructed libraries of receptors containing mutations randomly distributed throughout Ni3 and Ci3. We then used a functional assay we have developed called R-SAT (receptor selection and amplification technology (Refs. 17-20), patent pending) to screen these libraries and identify the mutant receptors that retained the ability to transduce proliferative signals. We grouped phenotypically similar receptors and compiled lists of their associated substitutions, and based on these mutation patterns, we were able to ascertain structure/function relationships. Both regions were predicted to form α-helical extensions by our analysis and generally showed conservation of hydrophobic residues and little conservation of charged residues, with the exception of an invariant arginine in Ni3 (18) and an invariant lysine in Ci3 (17). In the present study, we have extended our analysis of Ni3 by constructing point mutant libraries of residues we previously identified as functionally important and characterizing their functional phenotypes allowing us to distinguish their specific roles.

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serum for TSA cells (Life Technologies, Inc.).

Transfection Procedure and Functional Assays—R-SAT assays were performed as described using 0.2 µg of receptor, 0.5 µg of p-SI-jigalactosidase (Promega, Madison, WI), and 2 µg of salmon sperm DNA transfected into 2 × 10⁶ cells/well of a six-well rack (17-19) except that cells were combined with ligands in Dulbecco’s modified Eagle’s medium supplemented with 2% cyt-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 the equation: $R = D + (A - D)(1/(1 + x(c)))$, where $R$ = minimum response, $D$ = maximum response, and $c = EC_{50}$ ($R$ = response, $x$ = concentration of ligand). Curves were generated by least squares fits using the program KaleidaGraph™ (Abelbeck Software). Maximum response values were normalized according to an internal standard based on the fact that NIH 3T3 cells endogenously express prostanoid FP receptor that produce a robust R-SAT response when exposed to the prostanoid agonist cloprostanol (Cayman Chemical). We assayed each batch of transfected cells with 10 nM cloprostanol ($EC_{50} = 1$ 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—Binding studies were performed as described (17-19, 24), except TSA cells were used instead of COS7 cells. Cells were harvested 72 h after transfection and stored at ~80 °C. Membranes were prepared in buffer containing 25 mM sodium phosphate (pH 7.4), 5 mM magnesium, and 50 µM GppNHp as described (32) immediately before use.

RESULTS

We used a PCR-based protocol (see Ref. 17) to construct point mutation libraries at each of four residues within Ni3 previously shown to be functionally important (Ref. 18; see Fig. 1). We identified functional receptors based on their ability to amplify NIH 3T3 cells in a ligand-dependent manner using an assay called R-SAT (Refs. 17-20, patent pending). We screened each library in the presence or absence of a single dose (100 µM) of carbachol to identify functional receptors. The positive clones were then sequenced and subjected to a detailed dose-response analysis (see Table I and Fig. 2).

At position 220 the observed amino acid substitutions were predominantly hydrophobic, and all were well tolerated (Table I). In fact, substitution by valine and cysteine each resulted in a constitutively active receptor with a decreased the $EC_{50}$ for carbachol (Table I, Fig. 2). The constitutive activity could be completely reversed by the negative antagonist atropine (Fig. 2). Substitutions with large hydrophobic residues (leucine and phenylalanine) or with glycine were also well tolerated, increasing $EC_{50}$ values only 6-fold or less, although the maximum response was only 70% for phenylalanine. We observed no charged substitutions, suggesting these amino acids are not permitted at position 220.

We observed a similar pattern at position 216 where hydrophobic and medium sized polar substitutions were best tolerated, causing little or no phenotypic changes from wild-type m5 (Table I). Again, most of the allowed substitutions were well tolerated. However the large aromatic residues phenylalanine and tyrosine, and the small residue glycine increased the $EC_{50}$ values 11-, 26-, and 84-fold, respectively, and had maximum responses of only 23, 64, and 67% of wild type, suggesting that amino acid size was also an important structural constraint. At position 217, again both polar and hydrophobic, but not charged, substitutions were observed, but in contrast to the results described above, almost every observed mutation at 217 increased $EC_{50}$ 10-fold or greater and decreased maximum response 20% or more (Table I). Only the phenylalanine mutant retained a wild-type phenotype. Surprisingly, glycine was well tolerated. Possibly other residues compensate the void created by glycine. These data indicate there are more strin-

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gent constraints on the particular amino acid side chain at position 217 than at positions 216 or 220. Arg223 permitted the widest range of mutations of the residues tested, including polar, nonpolar, basic, and acidic substitutions, but only basic residues were well tolerated (Table I). All other mutations increased $EC_{50}$ values 25- to over 100-fold. However the maximum responses were almost all ≤80% of wild type, in contrast to the results for Tyr217 where maximum responses of less than 60% were typical. These data indicate that loss of positive charge at position 223 reduces the efficiency with which receptors couple to G-proteins, although it does not prevent full activation from occurring.

We evaluated the $[^3H]$NMS and carbachol binding properties of a number of the Ni3 mutants described above to determine if the observed phenotypic changes could be accounted for by changes in expression levels or ligand affinities. As shown in Table II, there were little or no differences in the binding properties of these receptors. Therefore, the effects of the introduced mutations were primarily upon the abilities of these mutant receptors to couple to G-proteins.

DISCUSSION

Our results strongly support our initial findings (18) that Ni3 forms an α-helix with a conserved hydrophobic face, terminating with an invariant arginine. That study identified Ile216, Tyr217, Thr220, and Arg223 as the functionally important residues in Ni3. In the present study we have extended our analysis by analyzing libraries of receptors with all possible amino acid substitutions at these residues. Significantly, charged substitutions were not permitted at Ile216, Tyr217, or Thr220, but basic residues were strongly favored at Arg223. Furthermore, proline substitutions, which can disrupt α-helices, were never observed. These results are entirely consistent with our earlier work. Comparison of the proportion of well tolerated to poorly tolerated substitutions at each residue allows one to make inferences about their functional roles (see Table III). Many different hydrophobic and medium sized polar amino acids were well tolerated at positions 216 and 220, but few other substitutions were observed. If the function of residues 216 and 220 is to form the backbone of the G-protein-coupling pocket, then substitutions that do not disrupt the architecture of this domain should be fully compatible, while amino acids which distort this domain would not be functionally permitted. This would explain why a large proportion of the observed amino acid substitutions at these positions are well tolerated. Similar
mutations were allowed at position 217; however, most had deleterious effects on receptor function. This suggests that Tyr217 engages in more specific amino acid side chain interactions with G-proteins than either Ile216 or Thr220 (discussed below). Finally, only basic residues were well tolerated at position 223, but many radical substitutions were allowed, indicating that positive charge at this position is critical for G-protein coupling. The tolerance of Arg223 to radical substitutions suggests that these mutations do not destroy the overall structure of the G-protein-coupling site.

We tabulated the observed substitution patterns at each tested position in m5 with the residues found at the analogous positions in the biogenic amine receptor family (Table III). In general, the well tolerated substitutions we identified in m5 are represented at these positions in other receptors, indicating that there is an evolutionary as well as functional conservation of these residues. In particular, this relationship holds for Ile216 and Thr220, where all but one residue (Ser in the G-coupled m2 and m4 receptors) is represented as a well tolerated residue in m5. It is surprising that there is so little conservation of basic residues at the position analogous to Arg223 in the other monoamine receptors (Table III) considering how strongly favored this property was in our analysis. This apparent discrepancy can be resolved when one considers that alignments of receptor sequences are currently based on homology and undoubtedly become less reliable further from the transmembrane/cytoplasmic border. Thus, assumptions about what constitutes an analogous residue to Arg223 in other receptors may be wrong in some cases. It is noteworthy that in all of the monoamine receptors that do not have a basic residue at the position analogous to Arg223, there is a basic residue located either one position before, and/or three positions after it (2, 26–28), which might perform the same function. Therefore it is reasonable to speculate that there is a general requirement for positive charge in this domain in all monoamine receptors, although this remains to be proven for other receptors (31). We propose that Arg223 functions to recruit G-proteins to the receptor by an ionic mechanism. Based on our earlier results (18), Arg223 is situated at the end of an α-helical extension into the cytoplasm, appropriately located to fulfill this function.

The analogous (to Tyr217) tyrosine in the m3 muscarinic receptor has been proposed to be a key determinant of G-protein-coupling specificity (25). This residue is conserved in all the muscarinic subtypes that couple to phosphoinositide turnover, but is replaced by a serine in the subtypes that couple to inhibition of adenyl cyclase (see Ref. 7). Significantly, we found that substitution of serine caused the greatest decrease
in receptor function of all mutations observed at Tyr\(^{217}\). Thus, within the muscarinic receptor family, Tyr\(^{217}\) may well be a major determinant of G-protein-coupling specificity. However examination of all the residues found at that position in the monoamine receptors indicates that this model is too simplistic to explain how receptor-G-protein specificity. For example, tyrosine is found in many G\(_s\)- and G\(_i\)-coupled receptors, and we saw that phenylalanine, which is also found in numerous G\(_i\)-coupled receptors, was well tolerated at Tyr\(^{217}\) (see Table III). Furthermore other epitopes, notably the i2 loop (29, 30), contribute to coupling specificity, so the exact requirements remain obscure. Possibly, specificity is determined by cooperation between multiple epitopes.

We isolated two constitutively activated receptors, T220V and T220C (see Fig. 2; Table I). We found that the m5 receptor can also be constitutively activated by mutations at position 465 in TM6.\(^3\) In that study we observed that every observed substitution (11 total) activated the receptor, which led us to speculate that residue 465 stabilizes the inactive state of the receptor. A similar interpretation was used to explain activation of the \(\alpha 1B\)-adrenergic receptor (22). In contrast to those results, most substitutions at position 220 were not activating. This may indicate that Thr\(^{220}\) forms the G-protein-coupling pocket and is involved in the structural transition between active and inactive states of the receptor, explaining why mutations cause either gain or loss of function.

The potential for Ni3 to form an amphipathic \(\alpha\)-helix has been appreciated for several years (32), based on the observation that mastoparan, a peptide toxin from wasp venom, directly activates G-proteins and adopts an amphipathic \(\alpha\)-helical structure (33). Despite intensive study of this working hypothesis, the critical determinants for G-protein coupling in Ni3 had been unresolved. Subsequent mutagenesis studies on this domain have alternatively implicated the hydrophobic residues (15, 21, 31, 34), the hydrophilic residues (16), or the amphiphatic nature of Ni3 (35). These apparent contradictions can now be resolved with our data which elucidate for the first time the precise identities and functional roles of the residues in Ni3 critical for G-protein coupling. Together our results for Ni3 (Ref. 18 and this paper) and C13 (17) indicate both domains form \(\alpha\)-helices terminating with functionally conserved basic residues. Thus we propose that G-proteins are recruited to receptors by an ionic mechanism and that protein/protein interactions occur through hydrophobic interactions.

\(^3\) T. A. Spalding, E. S. Burstein and M. R. Brann, submitted for publication.

### Table III

| Position | Biogenic receptors | m5 receptor mutations |
|----------|--------------------|-----------------------|
|          | G\(_s\)-coupled     | G\(_i\)-coupled        | Well tolerated | Poorly tolerated |
| I216     | I, V, T             | I, T                  | I, V          | V, L, I, C, A, (T) | F, Y, G |
| Y217     | Y, I                | F, Y, W, S            | F, Y, L       | Y, F, (G)          | A, C, T, O, L, V, S |
| T220     | T, L, A, V          | S, L, A               | A            | V, C, A, T, G, L, (F) |
| R223     | R, Q, T, E          | R, L, W               | Q, S, I       | R, H, K, (S)       | N, G, V, Y, E, L, M |

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