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An Arginine Residue Conserved in Most G Protein-Coupled Receptors Is Essential for the Function of the m1 Muscarinic Receptor

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SUMMARY

An exceptionally conserved sequence that is shared among most G protein-coupled neurotransmitter receptors is an aspartate-arginine-tyrosine triplet that is located at the amino terminus of the putative second cytoplasmic domain, where the arginine residue is invariant. Using the m1 subtype of muscarinic acetylcholine receptors as an example, we induced a point mutation of the arginine residue at position 123 into asparagine. This mutation resulted in a precipitous decrease in the coupling of m1 receptors to activation of phosphoinositide hydrolysis, in spite of the expression of the wild-type and mutant receptors at similar concentrations in Chinese hamster ovary cells. There were no significant effects on antagonist or partial agonist binding. In marked contrast, whereas binding of the full agonist carbachol to wild-type receptors exhibited high and low affinity components, this agonist bound to a single low affinity state in asparagine-123 mutant cells. Furthermore, agonist-induced enhancement of the specific binding of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate was not observed in membranes of cells expressing the mutant receptor. A similar mutation in the m2 muscarinic receptor resulted in a significant but smaller decrease in its coupling to inhibition of cAMP formation. On the other hand, a point mutation of tyrosine-124 in the m1 receptor sequence produced less marked changes in agonist-induced phosphoinositide hydrolysis and no effects on agonist or antagonist binding to the receptor. Taken together, our data demonstrate for the first time that this highly conserved arginine residue plays an important role in coupling of muscarinic receptors to signal transduction mechanisms.

Nearly 100 neurotransmitter receptors that are coupled to the activation of G proteins have been cloned thus far (1, 2). Hydropathy analysis of the amino acid sequences of these receptors suggests a generalized motif of seven hydrophobic stretches that transverse the cell membrane (3). These transmembrane regions are connected by loops that extend extracellularly and intracellularly (3). There is significant homology in the primary amino acid sequence of the predicted transmembrane domains among various G protein-coupled receptors, particularly in those subtypes of receptors that are activated by the same neurotransmitter. In contrast, there is less general similarity when the overall sequences of extracellular and intracellular loops are compared, especially regarding the third intracellular loop (1). Mutagenesis and chimera studies have localized amino acid residues that might be involved in the binding of neurotransmitters to their respective receptors (1, 2, 4, 5), as well as certain regions within the cytoplasmic domains that might play an important role in the coupling of receptors to G proteins (6–8). The most important of these are small stretches located in both the amino and carboxyl termini of the putative third intracellular loop (2, 9, 10). However, it has been suggested that other cytoplasmic domains work in concert with the third intracellular loop to culminate in maximal efficacy and perhaps full selectivity of coupling of receptors to G proteins (5, 11).

One of the most interesting consistencies related to the existence of homologies among various G protein-coupled receptors is the marked conservation of a triplet composed of aspartic acid-arginine-tyrosine residues located in the amino terminus of the second putative cytoplasmic domain (1). Whereas the arginine residue in this conserved triplet is invariant, there are a few instances in which the aspartate and tyrosine residues are conservatively substituted (1).

Point mutation of the aspartic acid in this triplet to asparagine in the sequence of the m1 muscarinic receptors (12) or the α_{2A}-adrenergic receptors (13) decreased the potency of agonists in stimulating PI hydrolysis or in inhibiting adenylate cyclase activity, respectively, without a change in the maximal response. In contrast, there was an almost complete loss of coupling of β_2-adrenergic receptors to activation of adenylate

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ABBREVIATIONS: PI, phosphoinositide; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

cyclase after mutation of the corresponding aspartate residue (14). Surprisingly, similar site-directed mutagenesis studies aimed at altering either the arginine or the tyrosine residues in this highly conserved triplet have not been performed with any member of this family of neurotransmitter-activated G protein-coupled receptors.

In this report we demonstrate that point mutation of arginine-123 in the sequence of rat m1 muscarinic receptors resulted in almost complete loss of receptor function. This residue belongs to the conserved aspartate-arginine-tyrosine triplet and is located at the amino terminus of the putative second cytoplasmic loop. These effects on receptor function were reflected in terms of both a large decrease in maximal agonist-induced activation of PI hydrolysis and stimulation of binding of a GTP analog after expression of similar concentrations of the wild-type and mutant receptors in CHO cells. There was also a loss of high affinity agonist binding, without an influence on antagonist binding to the receptor. A similar mutation in the corresponding arginine residue of m2 muscarinic receptors also resulted in a decrease in the coupling of these receptors to inhibition of cAMP formation. Point mutation of the tyrosine residue adjacent to arginine-123 of the m1 muscarinic receptor attenuated receptor function to a lesser extent. Therefore, our data suggest that this conserved arginine residue plays a pivotal role in the coupling of muscarinic receptors to signal transduction mechanisms.

Materials and Methods

Site-directed mutagenesis. The rat m1 (15) and m2 (16) muscarinic receptor genes were kindly provided by Dr. Claire Fraser, Institute for Genomic Research (Gaithersburg, MD), and Drs. Josephine Lai, Henry Yamamura, and William Roeske, University of Arizona (Tucson, AZ), respectively. Point mutations in the receptor genes were induced using the Altered Sites mutagenesis system (Promega). Appropriate primers were designed and synthesized to change the arginine-123 and tyrosine-124 residues in the m1 receptor sequence into asparagine and phenylalanine, respectively, and arginine-121 in the m2 receptor sequence into asparagine. All mutations were confirmed by dideoxy chain termination DNA sequencing. The coding sequences of the wild-type and the mutated receptors were subcloned into the pCMV-3 mammalian expression vector (supplied by Dr. D. W. Russell, University of Texas Southwestern Medical Center). CHO cells were co-transfected with 20 μg of pCMV-3 and 2 μg of $pMSV_{neo}$ per 100-mm plate, using standard methods. Transfected cells were selected in the presence of 600 μg/ml G418, in Dulbecco's modified Eagle medium containing 10% bovine calf serum. Clonal cell lines were screened to select cells that expressed similar numbers of wild-type and mutant m1 receptors. Such screening was accomplished by performing complete saturation receptor binding experiments using *N*-[^{3}H]methylscopolamine as a ligand. Similar protocols were applied in the case of wild-type and mutant m2 receptors, except that the cells used originated from a single colony rather than a single mother cell. However, it was confirmed that the transfection was stable by growing cells through several generations.

Cell culture. CHO cells that stably express wild-type and mutant muscarinic receptors were grown in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum and 0.005% geneticin. Cells were incubated at 37° in an atmosphere consisting of 10% CO_2/90% humidified air and were used for experiments 4–7 days after subculture.

Radioligand binding assays. CHO cells were harvested and suspended in oxygenated Krebs-Henseleit buffer, pH 7.4, composed of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, and 1.2 mM glucose. Intact cells (~ 0.12 mg of protein) were incubated with increasing concentrations of *N*-[^{3}H]methylscopolamine (81.5 Ci/mmol; NEN DuPont Research Products, Boston, MA) (0.02–2 nM), in a final volume of 1 ml of buffer. Nonspecific binding was determined in the presence of 2 μM atropine. Bound radioactivity was separated by filtration over GF/B glass fiber filters, using a Skatron cell harvester, after 60 min at 37°. For competition binding experiments intact cells were incubated with 0.2 nM (m1) or 0.4 nM (m2) *N*-[^{3}H]methylscopolamine, in the presence or absence of increasing concentrations of the various competitors, for 1 hr at 37° and the reaction was terminated by filtration as described above. Competition curves were analyzed for single and multiple affinity states by iterative nonlinear regression using the computer program LIGAND (17).

Assay of PI hydrolysis. CHO cells were suspended in Krebs-Henseleit buffer, labeled with *myo*-[^{3}H]inositol (10 μCi/ml, 82 Ci/mmol; Amersham, Arlington Heights, IL) for 1 hr at 37°, and washed free of nonincorporated radioactivity. Labeled cells were suspended in the same buffer containing 10 mM LiCl and were incubated at 37° for 15 min. Stimulation of PI hydrolysis was induced by increasing concentrations of carbachol or McN-A-343 for 1 hr at 37°. The reaction was terminated with chloroform/methanol/concentrated HCl (2:1:0.01, v/v). Total labeled inositol phosphates were separated by ion exchange chromatography on Dowex AG1-X-8 resin, using [^{14}C]inositol-1-phosphate as a recovery standard, as described previously (18).

Assay of cAMP formation. CHO cells transfected with the wild-type or mutant m2 muscarinic receptor genes were suspended in Krebs-Henseleit buffer and labeled for 1 hr at 37° with 10 μCi/ml [^{3}H]adenine (40–60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). Cells were then washed and suspended in buffer containing 1 mM isobutylmethylxanthine. The formation of cAMP was induced by 20 μM forskolin, in the absence or in the presence of increasing concentrations of carbachol, for 10 min at 37°. [^{3}H]cAMP was separated by ion exchange chromatography, using [^{14}C]cAMP as a standard to account for variations in sample recovery, as described previously (19).

Assay of [^{35}S]GTPγS binding. The assay was performed as described by Lazareno *et al.* (20). CHO cells were harvested and suspended in buffer containing 20 mM HEPES and 10 mM EDTA, pH 7.4. Cell membranes were prepared by homogenization of the cells using a Polytron homogenizer at 23,000 rpm for 30 sec, followed by centrifugation at 1000 × *g* for 10 min. The resulting supernatant was centrifuged further at 35,000 × *g* for 30 min to obtain membranes. Fifty micrograms of cell membrane protein were incubated with 50 pM [^{35}S]GTPγS, 0.1 μM or 1 μM GDP (for m1 and m2 receptors, respectively), and increasing concentrations of carbachol in HEPES buffer containing 100 mM NaCl and 10 mM $MgCl_2$, pH 7.4, in a final volume of 1 ml. Nonspecific binding was measured in the presence of 1 μM GTPγS. After 30 min at 30° the binding reaction was terminated by filtration as described (20).

Data analysis. All data are reported as means ± standard errors. Statistical comparisons of the means were performed using Student's *t* test. Dose-response curves were fitted according to a logistic four-parameter sigmoid model using the GraphPad computer program (ISI, Philadelphia, PA).

Results

Effects of mutagenesis of arginine-123 on the function of m1 muscarinic receptors. In this work, we induced a mutation of the basic arginine residue at position 123 of the rat m1 muscarinic acetylcholine receptor into the uncharged amino acid asparagine. The genes encoding both the wild-type and mutant receptors were stably transfected into CHO cells. There was no general trend that suggests an effect of this particular mutation on the level of receptor expression in the various cell lines that were screened. Cell lines derived from single cells were selected on the basis of expression of a similar number of receptors. Thus, maximal specific binding of *N*-[^{3}H]methylscopolamine averaged 893 ± 125 and 981 ± 97 fmol/mg of protein in cells containing wild-type and mutant receptors,

respectively, with corresponding K_d values of 287 ± 22 and 183 ± 18 pM. Thus, the two groups of cells expressed 1.4 ± 0.2 × 10^5 and 1.7 ± 0.2 × 10^5 receptors/cell, respectively. Activation of muscarinic receptors by the full agonist carbachol mediated a concentration-dependent stimulation of PI hydrolysis at wild-type m1 receptors, with a maximal response of a 38-fold increase over basal levels and an EC_{50} value of 1.4 ± 0.2 μM. In contrast, carbachol maximally increased PI hydrolysis at the asparagine-123 receptor by only 3-fold, indicating a precipitous loss of receptor function (Fig. 1A). An even more deleterious effect of this mutation was observed regarding the PI response to the partial m1 receptor agonist McN-A-343 (18); its maximal 28-fold stimulation of PI hydrolysis (EC_{50} = 2.4 ± 0.1 μM) at wild-type receptors completely disappeared at the mutant receptors (Fig. 1B).

Effects of mutagenesis of arginine-123 on binding of ligands to m1 receptors. There was no significant difference in the binding of the muscarinic receptor subtype-selective antagonists pirenzepine and AF-DX 116 (4) to the wild-type receptor or the asparagine-123 receptor (Fig. 2A). Both antagonists interacted with the wild-type or mutant receptors with a single affinity. The K_d values of pirenzepine at the wild-type and mutant receptors were 42 ± 4 and 38 ± 3 nM, respectively, with corresponding K_d values of AF-DX 116 of 0.9 ± 0.1 and 1 ± 0.1 μM. A similar finding was observed in the case of the partial agonist McN-A-343, which also interacted with a single receptor conformation, with K_d values of 8.3 ± 0.9 and 9.6 ± 0.8 μM at the wild-type and asparagine-123 mutant receptors, respectively (Fig. 2B). There were no significant differences

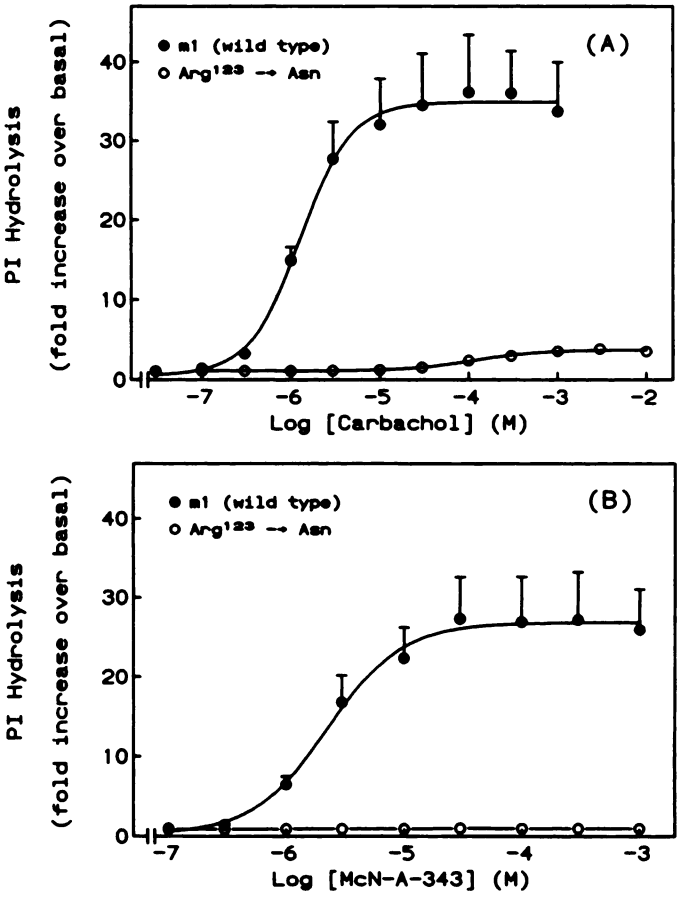

Fig. 1. Effects of site-directed mutagenesis of arginine-123 on PI hydrolysis at m1 muscarinic receptors. CHO cells expressing the wild-type or asparagine-123 mutant receptors were grown for 5 days, collected, and labeled with *myo*-[^{3}H]inositol as described in Materials and Methods. Accumulation of total labeled inositol phosphates in response to receptor activation by carbachol (A) or McN-A-343 (B) was measured after 1 hr in the presence of 10 mM LiCl. Data are shown as the means + standard errors from five or six experiments performed in triplicate. Incorporation of *myo*-[^{3}H]inositol averaged 132,000 ± 14,000 and 146,000 ± 21,000 dpm/10^6 cells in CHO cells expressing wild-type and mutant receptors, respectively, with corresponding basal levels of inositol phosphates of 2000 ± 500 and 3400 ± 1100 dpm/10^6 cells. Arg^{123}, arginine-123; Asn, asparagine.

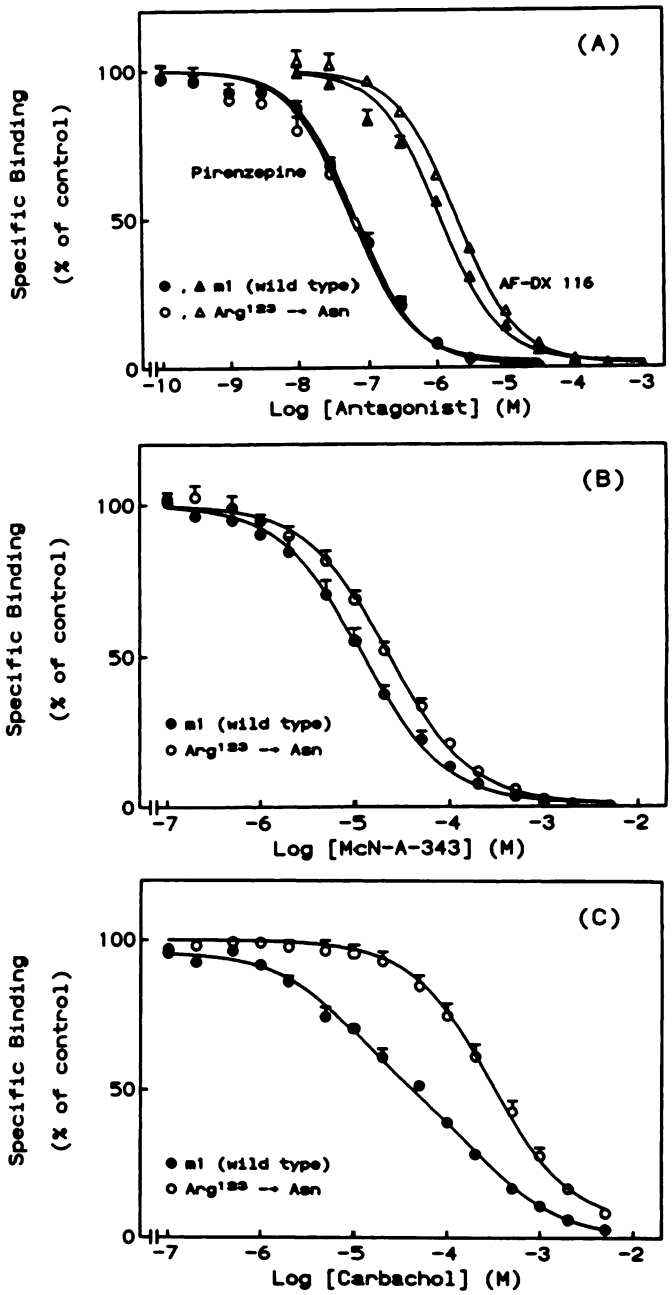

Fig. 2. Effects of mutagenesis of arginine-123 on agonist and antagonist binding to m1 receptors. Intact CHO cells expressing wild-type or asparagine-123 mutant m1 receptors were incubated with 0.2 nM *N*-[^{3}H]methylscopolamine, in the absence or in the presence of increasing concentrations of the antagonists pirenzepine or AF-DX 116 (A), the partial agonist McN-A-343 (B), or the full agonist carbachol (C), for 60 min at 37°. Data are shown as the means + standard errors from five or six experiments performed in triplicate.

between the affinities of pirenzepine, AF-DX 116, or McN-A-343 at the two receptors. In sharp contrast, this mutation resulted in a marked apparent decrease in the affinity of carbachol at the receptor (Fig. 2C). Carbachol interacted with both high and low affinity receptor conformations of the wild-type receptor, with equilibrium dissociation constants of 5 ± 1.6 μM (50% of receptors) and 120 ± 11 μM, respectively. Such binding data for receptor agonists have been interpreted according to a ternary ligand-receptor-G protein model, where the high affinity binding conformation represents the receptor state that is coupled to G protein and therefore to activation of signal transduction (21). On the other hand, the curve of carbachol binding to the asparagine-123 mutant receptor conformed to a single low affinity receptor state. The agonist-receptor dissociation constant at this receptor conformation was 148 ± 19 μM, which was not statistically different from that of the low affinity binding conformation at the wild-type m1 receptor.

Effects of mutagenesis of arginine-123 on coupling of m1 receptors to activation of G proteins. The observed diminished receptor coupling to PI hydrolysis and the disappearance of agonist high affinity binding suggest that mutation of arginine-123 might prohibit receptor-G protein interaction. To test this hypothesis, we used the enhancement of the specific binding of a radiolabeled GTP analog upon activation of muscarinic receptors as an indicator of the formation of a receptor-G protein complex. Results of these experiments supported such an interpretation. Whereas carbachol induced a concentration-dependent increase in the binding of [^{35}S]GTPγS at wild-type m1 receptors, it had virtually no effect at asparagine-123 receptors (Fig. 3).

Effects of mutagenesis of the corresponding arginine residue of m2 muscarinic receptors on receptor function and on agonist-receptor interactions. Point mutation of the corresponding arginine residue of the m2 muscarinic receptor (located at position 121) was performed and the wild-type and mutant genes were transfected into CHO cells. There was no marked effect of this mutation on the level of receptor expression, which was in general significantly lower than that of m1 receptors. The number of expressed wild-type and mutant m2 muscarinic receptors in the cells chosen for the studies was 45 ± 5 and 38 ± 6 fmol/mg of protein, respectively, with corresponding K_d values for *N*-[^{3}H]methylscopolamine of 222 ± 7 and 172 ± 15 pM. These receptor densities translate to 0.7 ± 0.01 × 10^4 and 0.6 ± 0.01 × 10^4 receptors/cell, respectively. Similar to m1 receptors, this point mutation in the conserved arginine-121 of m2 receptors resulted in a marked, albeit smaller, attenuation of receptor function in terms of inhibiting cAMP formation (Fig. 4). Thus, whereas carbachol maximally inhibited cAMP formation by 38 ± 5% at wild-type m2 receptors, it produced only 21 ± 4% inhibition at the mutant receptors ($p < 0.05$). On the other hand, there was no significant change in the EC_{50} of the agonist (2 ± 0.5 and 2.5 ± 0.6 μM at wild-type and mutant receptors, respectively). Unfortunately, we could not detect significant agonist-induced increases, in the presence of carbachol (10 nM to 10 mM), in the binding of [^{35}S]GTPγS to membranes prepared from CHO cells expressing the wild-type m2 receptor (five experiments; data not shown). This might be due to the low level of receptor expression. Successful measurement of such a response would have offered a more direct assessment of the effects of this mutation on the interactions of m2 receptors with G proteins. Furthermore, under conditions similar to those used to detect heterogeneity of agonist binding at the wild-type m1 receptor, carbachol interacted with a single receptor conformation (K_d = 10.1 ± 1.2 μM) in intact CHO cells expressing wild-type m2 receptors (Fig. 5). Identification of the molecular mechanisms underlying such clear differences in the properties of agonist binding at the two receptor subtypes requires further careful investigation. Car-

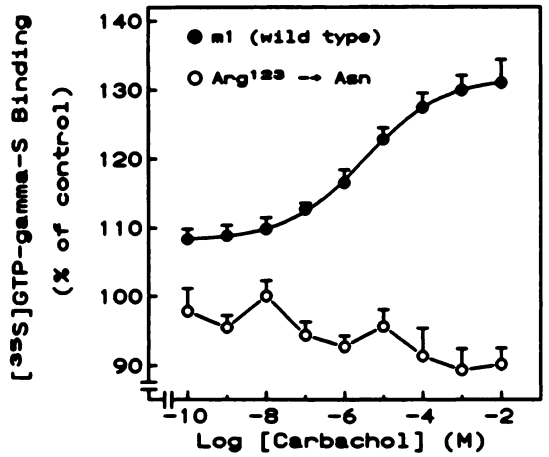

Fig. 3. Agonist-induced enhancement of [^{35}S]GTPγS binding at wild-type m1 receptors, but not at asparagine-123 mutant receptors. The specific binding of [^{35}S]GTPγS to membranes of CHO cells expressing wild-type or asparagine-123 mutant m1 muscarinic receptors was studied in the presence of increasing concentrations of carbachol. Each *point* is the mean + standard error from five experiments performed in quadruplicate. Total [^{35}S]GTPγS binding in the absence of carbachol averaged 60 ± 4 and 68 ± 4 fmol/mg of protein at the wild-type and mutant receptors, respectively, with corresponding values of 7 ± 0.6 and 10 ± 1 fmol/mg of protein for nonspecific binding.

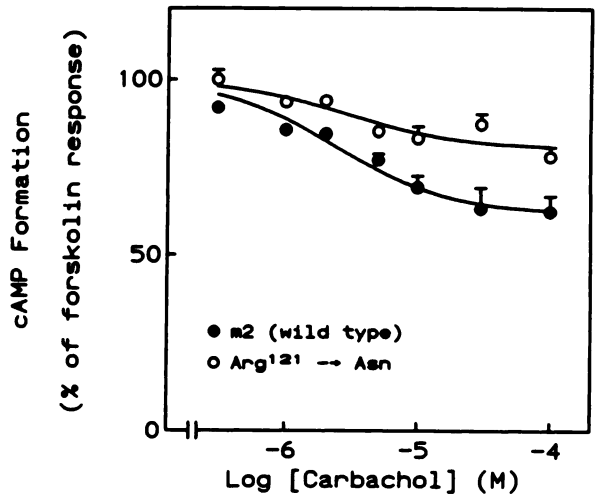

Fig. 4. Effects of point mutation of arginine-121 on the function of m2 muscarinic receptors. A single point mutation was induced in the sequence of the gene encoding the m2 muscarinic receptor, to transform arginine-121 into asparagine. The wild-type and mutant receptor genes were transfected into CHO cells and the ability of carbachol to inhibit cAMP formation induced by 20 μM forskolin was studied as detailed in Materials and Methods. The results are presented as the means + standard error of five independent experiments. Uptake of [^{3}H]adenine averaged 520,000 ± 85,000 and 420,000 ± 65,000 dpm/10^6 cells in CHO cells expressing the wild-type and mutant receptors, respectively. Basal [^{3}H]cAMP levels were 1400 ± 200 and 1000 ± 200 dpm/10^6 cells at the wild-type and mutant receptors, respectively, with corresponding levels of 15,000 ± 1,600 and 11,800 ± 2,200 dpm/10^6 cells in presence of forskolin.

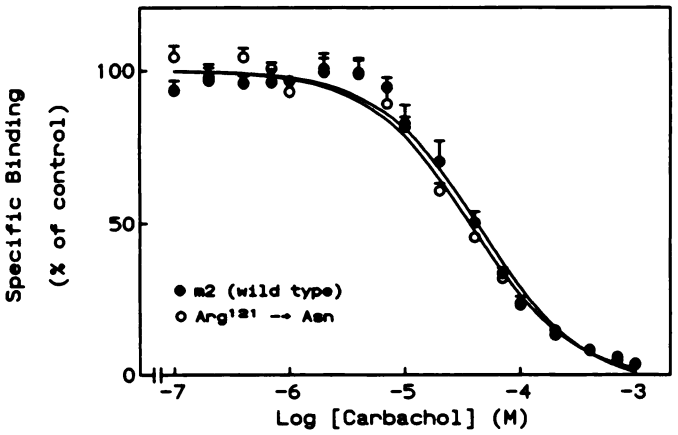

Fig. 5. Carbachol binding to the wild-type and asparagine-121 mutant m2 muscarinic receptors. Intact CHO cells expressing the wild-type receptor or the asparagine-121 m2 muscarinic receptor mutant were incubated with 0.4 nM *N*-[^{3}H]methylscopolamine in the absence or in the presence of increasing concentrations of carbachol. Incubations were for 60 min at 37°. Data shown are the means + standard errors of six independent experiments performed in triplicate.

bachol also interacted with a single state of the receptor, with a similar affinity (K_d = 8.2 ± 0.5 μM), in intact cells expressing the mutant m2 receptor (Fig. 5).

Effects of mutagenesis of the conserved tyrosine-124 residue on the function of m1 muscarinic receptors and on ligand-receptor interactions. We also studied the effects of point mutation of the conserved tyrosine-124 residue in the sequence of m1 muscarinic receptors, to phenylalanine on receptor function and the interaction of agonist and antagonist ligands with the receptor. There was a consistent precipitous decrease in the level of expression of the mutant receptor in different CHO cell lines that were screened, in comparison with the majority of the cell lines that express the wild-type receptor. The possibility that the tyrosine-124 residue in the m1 receptor sequence is essential for receptor processing should be investigated in future experiments. CHO cell lines chosen for the studies expressed the wild-type and mutant receptors at 236 ± 14 and 175 ± 16 fmol/mg of protein, respectively, with corresponding K_d values for *N*-[^{3}H]methylscopolamine binding of 261 ± 18 and 249 ± 21 pM. Thus, the two groups of cells expressed 0.4 ± 0.02 × 10^5 and 0.3 ± 0.02 × 10^5 receptors/cell, respectively. Carbachol stimulated PI hydrolysis 26 ± 2-fold at wild-type receptors, with an EC_{50} of 4.3 ± 0.5 μM. Mutation of tyrosine-124 did not result in a significant change in the maximal response (26 ± 4 times basal levels), whereas there was a significant increase ($p < 0.05$) in the EC_{50} value, to 12.8 ± 0.9 μM (Fig. 6A). In contrast, this mutation resulted in a significant decrease in the maximal stimulation of PI hydrolysis by the partial agonist McN-A-343, from 15 ± 1- to 6 ± 1-fold over basal levels (Fig. 6B). EC_{50} values of McN-A-343 at the wild-type and phenylalanine-124 mutant m1 receptors averaged 5 ± 0.5 and 8 ± 0.6 μM, respectively, and were significantly different ($p < 0.05$).

Point mutation of tyrosine-124 did not result in any significant changes in the affinity of binding of either antagonist or agonist ligands to m1 muscarinic receptors. Thus, pirenzepine interacted with a single affinity state, with K_d values of 48 ± 8 and 59 ± 11 nM at wild-type and mutants receptors, respectively (Fig. 7A). Similarly, the corresponding K_d values of AF-DX 116 were 1.1 ± 0.03 and 1.1 ± 0.05 μM at the two receptor types

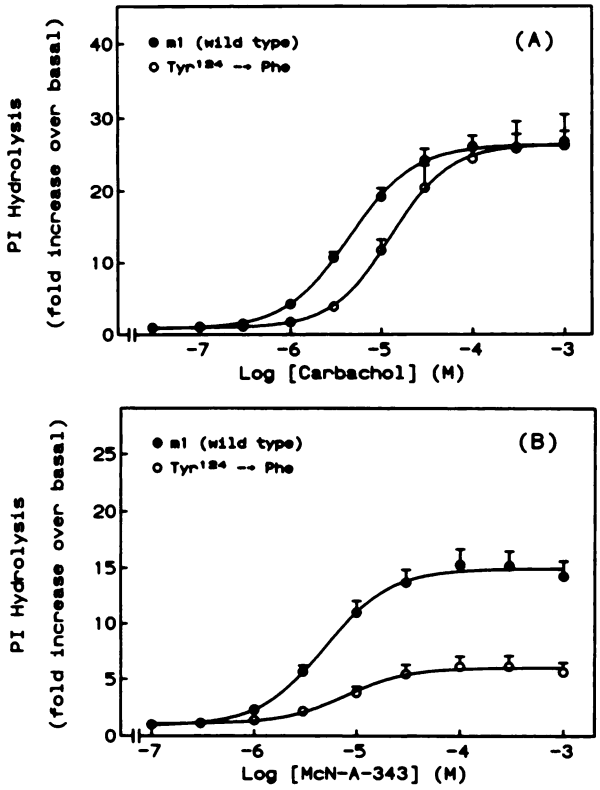

Fig. 6. Effects of mutagenesis of tyrosine-124 in the m1 muscarinic receptor sequence into phenylalanine on coupling of the receptor to PI hydrolysis. CHO cells expressing the wild-type or mutant receptors were prepared for the assay as described in Materials and Methods. Total accumulation of inositol phosphates in response to carbachol (A) or McN-A-343 (B) was measured. Incorporation of *myo*-[^{3}H]inositol averaged 60,000 ± 7,000 and 65,000 ± 3,000 dpm/10^6 cells in CHO cells expressing the wild-type receptor or the mutant m1 receptor, respectively, with corresponding basal levels of inositol phosphates of 1200 ± 100 and 1400 ± 80 dpm/10^6 cells. Data are presented as the means + standard errors of five independent experiments performed in triplicate. Tyr^{124}, tyrosine-124; Phe, phenylalanine.

(Fig. 7A). There was also no change in the K_d value of the partial agonist McN-A-343 upon mutation of tyrosine-124 (14 ± 2 and 14 ± 1 μM at wild-type and mutant receptors, respectively) (Fig. 7B). The full agonist carbachol interacted with two receptor conformations at both the wild-type and mutant receptors. At the wild-type m1 receptor, carbachol bound to a high affinity receptor conformation (45 ± 6% of total receptors) with a K_d of 14 ± 3 μM and a low affinity conformation with a K_d of 175 ± 19 μM. The K_d values of carbachol at the phenylalanine-124 receptor mutant were 11 ± 3 μM (48 ± 7% of total receptors) and 158 ± 21 μM (Fig. 7C). There were no statistically significant differences between any of the parameters obtained in CHO cells expressing wild-type or mutant receptors. Thus, differently from the situation with the asparagine-123 mutant, the K_d values for interaction of carbachol with the two affinity conformations of the receptor and the distribution of their proportions were maintained upon mutation of tyrosine-124.

Discussion

Our present data demonstrate that point mutation of the highly conserved arginine residue at position 123 of the sequence of m1 muscarinic receptors results in significant dampening of receptor function. Thus, the mutant receptor showed

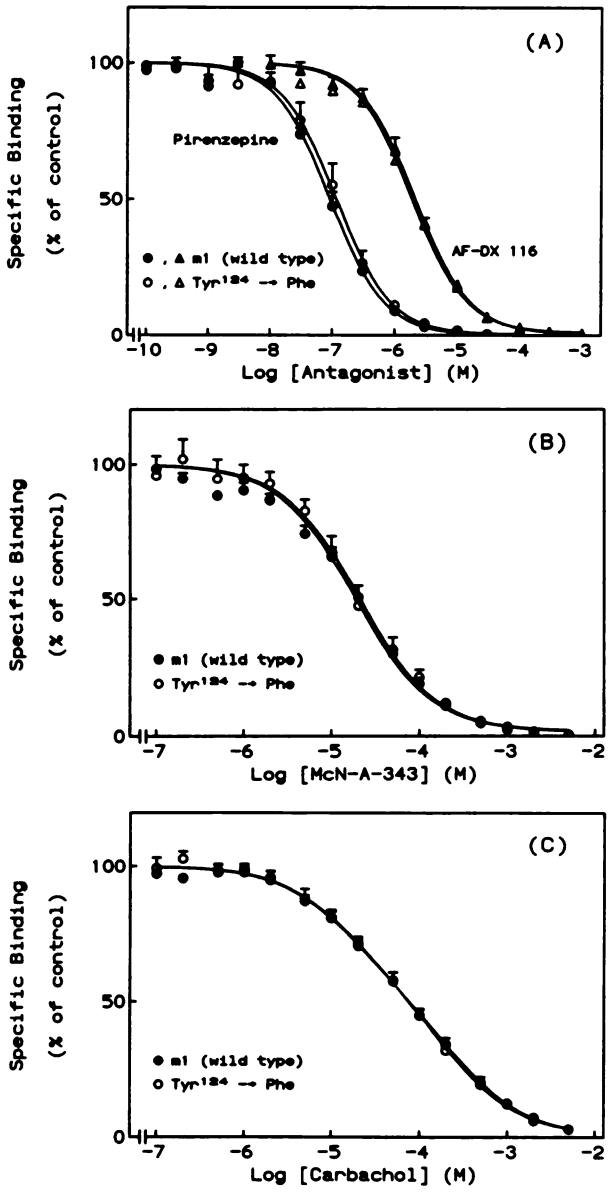

Fig. 7. Lack of effects of mutagenesis of tyrosine-124 in the m1 muscarinic receptor sequence on binding of agonists or antagonists to the receptor. CHO cells expressing the wild-type or phenylalanine-124 mutant m1 muscarinic receptors were incubated with 0.2 nM *N*-[^{3}H]methylscopolamine in the presence of pirenzepine or AF-DX 116 (A), McN-A-343 (B), or carbachol (C). Assay conditions were described in Materials and Methods. The data are shown as the means + standard errors obtained in three to seven independent experiments performed in triplicate.

a markedly decreased ability to respond to agonist stimulation by increased PI hydrolysis or binding of a labeled GTP analog. This was accompanied by a loss of the agonist high affinity binding conformation but no changes in the affinity of partial agonists or antagonists in their binding to the receptor. Mutation of the corresponding arginine residue in the m2 muscarinic receptor also resulted in diminished coupling of the receptor to inhibition of cAMP formation, suggesting a more generalized role of this particular residue in the function of various subtypes of muscarinic receptors. However, there were quantitative differences in the magnitudes of the effects of this mutation on the function of m1 and m2 receptors. In contrast, mutation of tyrosine-124 of the m1 receptor resulted in a smaller decrease in receptor coupling to stimulation of PI hydrolysis, compared with the precipitous effects of mutation of its neighboring arginine-123. Mutation of this tyrosine also did not result in significant changes in the binding of either agonist or antagonist ligands to the m1 receptor.

Therefore, several pieces of evidence point to an important role of arginine-123 (m1) or arginine-121 (m2) in the ability of muscarinic receptors to couple to intracellular transduction mechanisms. It is not likely that the observed effects are due to a more generalized detrimental influence on receptor conformation resulting from mutagenesis of this residue, because synthetic peptides that match the sequence of the second intracellular loop of α_{2A}-adrenergic receptors (22) or β_1-adrenergic receptors (23) also suppress receptor function. It is noteworthy that similar peptides that lack the aspartate-arginine-tyrosine triplet sequence do not exhibit such inhibition (23). A peptide with a sequence identical to that of the second intracellular loop of mammalian rhodopsin inhibits the ability of rhodopsin to bind to transducin, and this effect is potentiated in the presence of peptides similar to other intracellular loops (24).

One explanation for the less pronounced effects of this mutation on the function of m2 receptors, compared with that of m1 receptors, is the known existence of a generally higher proportion of spare receptors for the coupling of the m2 receptor subtype to signal transduction mechanisms (18, 19, 25, 26). It should be noted, however, that differences in receptor spareness measured at m1 receptors (18) and m2 receptors (25) expressed in CHO cells are rather small. Alternatively, other residues in the m2 receptor sequence that are also involved in receptor coupling to G proteins might play a compensatory functional role in the absence of this arginine residue. Such discrepant consequences of a single point mutation regarding the functions of m1 and m2 muscarinic receptors should be valuable in addressing future questions related to possible different structural requirements for coupling of the two receptors to signaling mechanisms.

The profound effects of mutagenesis of arginine-123 on the function of m1 muscarinic receptors are in sharp contrast to the less pronounced effects of mutation of the neighboring conserved aspartate residue at position 122, with which there was only a 10-fold decrease in agonist potency in increasing PI hydrolysis, without a change in the maximal response (12). The influence of the latter mutation is qualitatively similar to that of replacement of the tyrosine residue at position 124 by phenylalanine that has been demonstrated in the present work. Thus, it is possible that mutagenesis of these moieties that are immediately adjacent to arginine-123 impairs the ability of m1 receptors to activate G proteins through indirect effects on the central arginine residue.

It remains to be investigated whether the role of this arginine in receptor coupling is dependent on its charge. It is likely that this is the case, because replacement of this residue with another basic amino acid such as lysine actually resulted in an increase in the affinity of agonist binding to m1 receptors (27), indicating an enhancement of receptor-G protein coupling (21). Unfortunately, direct measurements of receptor function were not obtained in the latter study. It has also been shown that polyanions such as heparin suppress the function of m2 muscarinic (28) and β_2-adrenergic (29) receptors. This effect might

be due, at least in part, to neutralization of the charge on important conserved residues that correspond to arginine-123 in the m1 receptor. Furthermore, basic peptides such as mastoparan compete with β-adrenergic receptors in their ability to activate G proteins (30).

In summary, our results show profound detrimental effects of site-directed mutagenesis of the arginine residue in the second cytoplasmic loop on agonist binding to and activation of m1 muscarinic receptors, suggesting an important role of this residue in receptor function. A similar role of analogous residues in signal transduction mediated by other G protein-coupled receptors might exist, due to the highly conserved nature of this amino acid in almost all members of this receptor family (1). In support of this notion, it has been demonstrated recently that point mutation of the cognate arginine residue in mammalian rhodopsin (31) or reversal of the position of this residue with the preceding glutamate (32) abolishes the ability of rhodopsin to activate transducin. In fact, a mutation of this arginine residue in human rhodopsin has recently been identified in patients suffering from retinitis pigmentosa (33). It is noteworthy, however, that sequence analysis of some recently cloned G protein-coupled receptors (e.g., secretin, glucagon, calcitonin, and parathyroid hormone receptors) indicates the absence of an aspartate-arginine-tyrosine triplet in their second cytoplasmic loops (2). Although these receptors display the usual seven putative transmembrane domains, they do not share sequence similarities with other receptors, and it has therefore been suggested that they might belong to a specialized subfamily of G protein-coupled receptors (2). These sequence comparisons of various subfamilies of G protein-coupled receptors suggest the intriguing possibility of alternate coupling mechanisms. An understanding of such complexity and intricacy of receptor coupling to G proteins requires further research.

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