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Site-Directed Mutagenesis of m_1 Muscarinic Acetylcholine Receptors: Conserved Aspartic Acids Play Important Roles in Receptor Function

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SUMMARY

Muscarinic acetylcholine receptors contain a region encompassing the second and third transmembrane domains that is rich in conserved aspartic acid residues. To investigate the role of four conserved aspartic acids at positions 71, 99, 105, and 122 in muscarinic receptor function, point mutations in the rat m_1 muscarinic receptor gene were made that converted each Asp to Asn, and wild type or mutant genes were stably expressed in Chinese hamster ovary cells that normally lack muscarinic receptors. Substitution of Asp^{71} or Asp^{122} with Asn produced mutant receptors that displayed high affinity for carbachol but decreased efficacy and potency, respectively, in agonist-induced activation of phosphoinositide hydrolysis, suggesting that these residues may mediate receptor-GTP binding protein interactions. Substitution of Asp^{99} or Asp^{105} with Asn produced marked decreases in ligand binding affinities and/or covalent incorporation of [^{3}H]propylbenzilylcholine mustard, suggesting that these residues may be involved in receptor-ligand interactions.

Muscarinic acetylcholine receptors have been shown to be members of a large family of membrane-spanning proteins that interact with G proteins and activate a number of intracellular effector systems (1). To date, five distinct subtypes of muscarinic receptors have been identified based on gene cloning, sequencing, and expression (2–8). The extent of sequence identity among the five muscarinic receptors is approximately 35% and homology increases to greater than 50% when favored amino acid substitutions are considered (9). Among the conserved amino acid residues of the muscarinic receptor subtypes are four aspartic acids, at position 71 in the second transmembrane helix, at positions 99 and 105 in or near the third transmembrane helix, and at position 122 in or near the second intracellular loop of the rat m_1 muscarinic receptor. Aspartic acids are present at analogous positions in most members of this gene family sequenced to date (10–16), suggesting that they may play important roles in receptor structure or function. Indeed, site-directed mutagenesis data from bacteriorhodopsin (17) and β_2-adrenergic receptors (18–20) indicate that the conserved transmembrane aspartic acids affect ligand binding and/or receptor activation.

In order to investigate the role of the four conserved aspartic acids in muscarinic receptor function, we made single point mutations in the rat m_1 muscarinic receptor gene to independently convert Asp^{71}, Asp^{99}, Asp^{105}, and Asp^{122} to Asn and permanently transfected CHO cells, which lack muscarinic receptors, with each mutant gene. This approach provides clonal cell lines expressing pure receptor populations and facilitates their detailed pharmacological and biochemical characterizations. Wild type and mutant muscarinic receptors were characterized by muscarinic cholinergic ligand binding, affinity labeling with [^{3}H]PrBCM, and activation of PI hydrolysis in transfected cells. Our findings indicate a unique role for each of the four aspartic acid residues in ligand binding and/or agonist activation of muscarinic receptors.

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO Laboratories. CHO-K1 cells were from the American Type Culture Collection. [^{3}H]QNB (60–87 Ci/mmol) and [^{3}H]PrBCM (30–60 Ci/mmol) were from New England Nuclear/Dupont. Pirenzepine and AF-DX 116 were from Boehringer Ingelheim (Ridgefield, CT). Restriction endonucleases were from Bethesda Research Laboratories. pSVL was from Pharmacia. All other reagents were from Sigma.

Site-directed mutagenesis and continuous expression of re-

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ABBREVIATIONS: G protein, guanine nucleotide regulatory protein; CHO, Chinese hamster ovary; PrBCM, propylbenzilylcholine; PAGE, polyacrylamide gel electrophoresis; QNB, quinuclidinyl benzilate; PI, phosphoinositide; SDS, sodium dodecyl sulfate.

ceptors in CHO cells. The entire coding region of the rat m_1 muscarinic receptor contained within a 2.2-kilobase *TaqI-BamHI* restriction fragment of genomic clone c71 (21) was cloned into the *SmaI* site in the polylinker region of the expression vector pSVL or into M13mp18 for mutagenesis. The single base mutation (GAC to AAC) that converts Asp to Asn was introduced into the receptor at base numbers 211, 295, 313, or 364 by oligonucleotide-directed mutagenesis, as previously described by Kunkel (22). The mutant receptors were selected as described (18, 19) and authenticity of the mutations was confirmed by double-stranded dideoxy sequencing.

CHO cells lacking muscarinic receptors were cotransfected with pSVL containing the wild type or mutant m_1 muscarinic receptor gene and pMSVneo containing the dominant selectable marker, aminoglycoside phosphotransferase, using the $CaPO_4$ precipitation method (23). Cells were grown in selective medium containing Geneticin (G-418, 500 μg/ml). Colonies derived from single cells were isolated and expanded and membranes prepared from transfected cells were assayed for muscarinic receptor expression with [^{3}H]QNB.

Total RNA isolation. RNA isolation was performed according to the method of Chirgwin *et al.* (24).

Cell membrane preparation and ligand binding assays. Preparation of cell membranes and [^{3}H]QNB binding assays were performed as described (18, 25). Binding reactions, performed in triplicate, were initiated by addition of membranes and samples were incubated for 60 min at 37°. Specific binding was defined as the difference in QNB binding observed in the absence and presence of 1 μM atropine. Protein was assayed using the fluorescamine assay, with bovine serum albumin as a standard (26). Ligand binding data were analyzed using the LIGAND curve-fitting program of Munson and Rodbard (27).

Measurement of PI hydrolysis. Transfected CHO cells expressing wild type or mutant m_1 muscarinic receptors were assayed for carbachol-stimulated PI hydrolysis according to the method of Berridge (28). Cells were seeded onto 35-mm culture dishes at a density of 2.5×10^5 cells/plate in nutrient mixture F-10 supplemented with 10% fetal bovine serum. [^{3}H]myo-inositol (2.5 μCi) was added to each plate and cells were incubated for 16 hr at 37°. Before the addition of agonists, cells were treated with 10 mM LiCl for 30 min. Carbachol was then added to the cultures and agonist-induced formation of inositol phosphates was allowed to proceed for 30 min at 37°. Incubations were stopped by the addition of 10% perchloric acid, extracts were alkalinized, and total inositol phosphates were purified by chromatography on Dowex resin. Data are expressed as the fold basal stimulation of PI hydrolysis. Basal rates of PI hydrolysis in transfected CHO cells correlate directly with the extent of lipid labeling by radiolabeled inositol.[2]

Affinity labeling of muscarinic receptors with [^{3}H]PrBCM. Membranes from transfected cells were incubated with cyclized [^{3}H]PrBCM at a concentration equivalent to at least 10 times the concentration of receptor binding sites determined with [^{3}H]QNB. Alkylation reactions were carried out at 30° for 30 min. Membranes were washed twice by centrifugation to reduce the concentration of unincorporated [^{3}H]PrBCM. Membrane samples were incubated in SDS sample buffer for 2 min at 90° and labeled proteins were separated by SDS-PAGE according to the method of Laemmli (29). For detection of ^{3}H, gels were sliced into 2-mm pieces and each slice was dissolved in 250 μl of 50% H_2O_2 and assayed by scintillation counting.

Results

Ligand binding analysis to wild type and mutant m_1 muscarinic receptors. In order to examine the function of four conserved aspartic acid residues in the muscarinic receptor, site-directed mutagenesis and permanent gene expression were utilized. Aspartic acid, which carries a negative charge, was replaced with asparagine, an amino acid similar in size to aspartic acid but possessing no net charge, with the rationale that this mutation would allow for assessment of the role of the highly conserved negative charge in receptor function at each locus. Using our established protocols (18, 19, 30), several colonies of cells were isolated that expressed wild type or Asn^{71}, Asn^{99}, or Asn^{122} mutant muscarinic receptors over a range of densities from 200 to 2600 fmol of receptor/mg of membrane protein. Seventy-five to 80% of the colonies assayed from each transfection displayed high affinity, saturable binding of the radioligand [^{3}H]QNB. The calculated equilibrium dissociation constants (K_d) for QNB binding to wild type and Asn^{71}, Asn^{99}, and Asn^{122} mutant receptors were 15, 20, 62, and 25 pM, respectively (Fig. 1; Table 1), values in good agreement with those reported for m_1 receptors expressed in B-82 cells (21) and from animal studies (31, 32). The affinity of the Asn^{99} mutant receptor for QNB was approximately 4-fold lower than that determined for QNB binding to wild type m_1 muscarinic receptors expressed in CHO cells at a similar density (Fig. 1; Table 1).

Quantitation and detailed pharmacological characterization of the Asn^{105} mutant muscarinic receptor were compromised by a relative lack of binding of QNB to the mutant receptor. At high radioligand concentrations (>1 nM), membranes containing the Asn^{105} mutant receptor displayed low amounts of specific [^{3}H]QNB binding, suggesting a markedly decreased affinity of the mutant receptor for muscarinic antagonists. Based on these data alone, we could not definitively determine whether the lack of QNB binding observed was attributable to a change in the ability of the mutant receptor to bind ligands

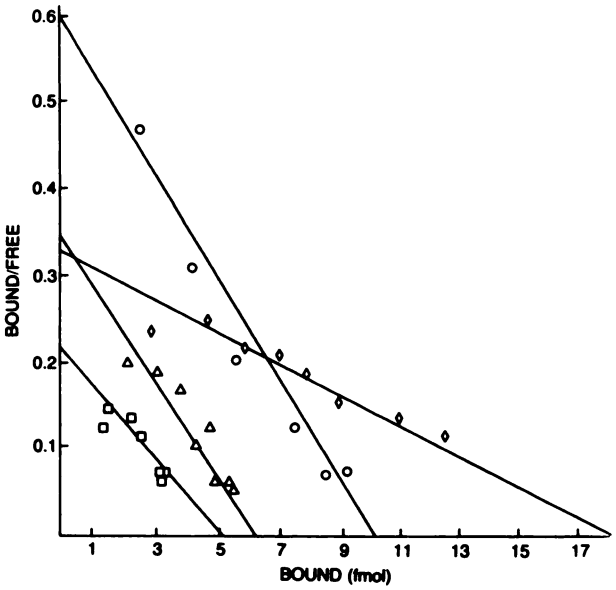

Fig. 1. Scatchard analysis of [^{3}H]QNB binding to membranes from cells expressing wild type, Asn^{71}, Asn^{99}, and Asn^{122} mutant m_1 muscarinic receptors. Membranes prepared from transfected cells expressing wild type (7.5 μg) (○), Asn^{71} mutant (17 μg) (△), Asn^{99} mutant (7.5 μg) (◇), or Asn^{122} mutant receptors (24 μg) (□) were incubated in 1 ml, final volume, of 50 mM $NaPO_4$ buffer, pH 7.4, with increasing concentrations of [^{3}H]QNB (15–250 pM) in the absence and presence of 1 μM atropine for 60 min at 37°. Samples were filtered over Whatman GF/C glass fiber filters using a Brandel M-24R cell harvester and filters were washed with 20 ml of $NaPO_4$, pH 7.4. Specific binding was calculated as the difference in QNB bound in the absence and presence of atropine. Data from saturation binding experiments were analyzed using the LIGAND curve-fitting program of Munson and Rodbard (27). Data are representative of two or three separate binding experiments performed in triplicate.

[2] C. M. Fraser, unpublished observation.

TABLE 1

Wild type and mutant muscarinic receptors: ligand binding affinities and PI hydrolysis

B_{max}, maximum number of binding sites.

	B_{max}	Ligand binding, K_i[a]						PI hydrolysis, carbachol	
		[^{3}H]QNB	Pirenzepine	AF-DX 116	Atropine	Carbachol	Oxotremorine	EC_{50}	Fold basal response
	fmol/mg[b]	pM	nM	μM	nM	μM	μM	μM	
Wild type	1385 (200)[c]	15 (3)[d]	28 ± 2.2 (2)	0.73 ± 0.17 (2)	1.71 ± 0.21 (3)	252 ± 20 (3)	2.1 ± 0.2 (3)	5 (4)	23 (4)
Asn^{71}	360	20 (2)	37 ± 6.7 (2)	0.53 ± 0.06 (2)	0.71 ± 0.13 (3)	46 ± 3 (3)	1.7 ± 0.6 (2)	ND[e]	2.1 (3)
Asn^{99}	2666	62 (2)	122 ± 11 (2)	1.10 ± 0.11 (2)	5.50 ± 0.70 (2)	1377 ± 908 (2)	7.3 ± 0.4 (2)	120 (3)	17.5 (3)
Asn^{122}	210	25 (2)	19.6 ± 2.4 (2)	0.62 ± 0.08 (2)	1.20 ± 0.33 (3)	130 ± 9.1 (3)	2.7 ± 0.6 (2)	53 (4)	19 (4)

[a] Affinity constant (K_d) for the radioligand [^{3}H]QNB was determined directly in equilibrium binding studies. K_i values for competing cholinergic ligands were calculated using the LIGAND curve-fitting program of Munson and Rodbard (27). Data are presented as mean ± standard error.

[b] Calculated from Scatchard analysis of saturation isotherms.

[c] Cells expressing 200 fmol/mg wild type receptor used as a control for PI turnover studies with Asn^{71} and Asn^{122} mutant receptors. Cells expressing similar densities of wild type receptor as those expressing Asn^{99} mutant receptors were used as a control in determinations of PI turnover.

[d] Number in parentheses represents number of experiments performed in triplicate for ligand binding and duplicate for PI hydrolysis.

[e] ND, value not determined due to low maximal response.

or to anomalies in mutant receptor gene transcription and/or mutant receptor processing. To address this question in part, Northern blot analysis of total RNA isolated from CHO cells transfected with the gene encoding wild type or Asn^{105} mutant muscarinic receptors was performed. As illustrated in Fig. 2, receptor-specific mRNA was detected in cells transfected with the Asn^{105} mutant receptor gene. The mRNA transcript of the mutant receptor was the same size as that for the wild type receptor (2.2 kilobases; Fig. 2), indicating that this mutation did not interfere with the transcription of a full length receptor-specific mRNA. Thus, altered receptor gene transcription does not appear to be responsible for the low levels of [^{3}H]QNB binding observed in cells transfected with the Asn^{105} mutant receptor gene. In the absence of receptor quantitation by other means (immunoblotting experiments, for example), we cannot at present determine whether the mutant receptor is correctly processed and inserted into the membrane. Strader *et al.* (33) demonstrated that the identical amino acid substitution at the corresponding locus in the β-receptor had no effect on receptor processing but reduced the affinity of the mutant receptor for ligands by 10,000-fold. Our data on the Asn^{105} mutant m_1 muscarinic receptor are not inconsistent with this mutation in the muscarinic receptor producing a similar phenotype as the same mutation in the β-receptor.

Antagonist binding to wild type or Asn^{71}, Asn^{99}, and Asn^{122} mutant receptors was further characterized in competition displacement studies with the nonselective muscarinic receptor antagonist atropine, the m_1-selective antagonist pirenzepine, and the m_2-selective antagonist AF-DX 116. In each case, the rank order of potency of these agents was consistent with the pharmacology of an m_1 receptor subtype: QNB > atropine > pirenzepine > AF-DX 116 (Fig. 3A; Table 1). The affinities of the Asn^{71} and Asn^{122} mutant muscarinic receptors for atropine (K_i = 0.71 and 1.2 nM), pirenzepine (K_i = 37 and 19.6 nM), and AF-DX 116 (K_i = 0.53 and 0.62 μM) were essentially the same as those of the wild type receptor (atropine K_i = 1.71 nM; pirenzepine K_i = 28 nM; and AF-DX 116 K_i = 0.73 μM). It was noted that the affinities of the Asn^{99} mutant receptor for atropine (K_i = 5.5 nM) and pirenzepine (K_i = 122 nM) were 3.2- and 4.3-fold lower, respectively, than the affinities of the wild type m_1 muscarinic receptor for these agents (Table 1). Replacement of Asp^{99} with Asn had little or no effect on the affinity of the mutant receptor for AF-DX 116 (K_i = 0.73 and 1.1 μM, for wild type and mutant receptors, respectively).

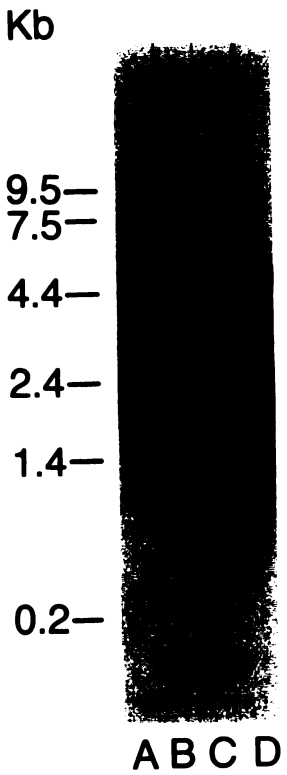

Fig. 2. Northern blot analysis of total RNA isolated from CHO cells transfected with wild type or Asn^{105} mutant muscarinic acetylcholine receptors. Total RNA was isolated from three confluent 150-mm plates of transfected CHO cells according to the method of Chirgwin (24). Twenty micrograms of total RNA from the indicated cell lines were separated on a formaldehyde-agarose gel, as described by Maniatis *et al.* (39), and transferred to a Hy-Bond N nylon membrane (Amersham). The Northern blot was hybridized with a random-primed ^{32}P-labeled rat m_1 muscarinic receptor cDNA clone (2 × 10^6 dpm/ml of hybridization solution) for 16 hr at 42°. The membrane was washed twice in 2× standard saline citrate at room temperature, twice in 2× standard saline citrate plus 1% SDS for 30 min at 56°, and once with 0.1× standard saline citrate at room temperature, dried, and exposed to X-ray film for 30 h at −70°. *Lanes A–D* contain 20 μg of total RNA from control CHO cells, CHO cells expressing 200 fmol of wild type receptor/mg of protein, CHO cells expressing 1385 fmol of wild type receptor/mg of protein, and CHO cells expressing Asn^{105} mutant muscarinic receptor, respectively. *Numbers on the left of the figure* indicate kilobases as determined from RNA size markers.

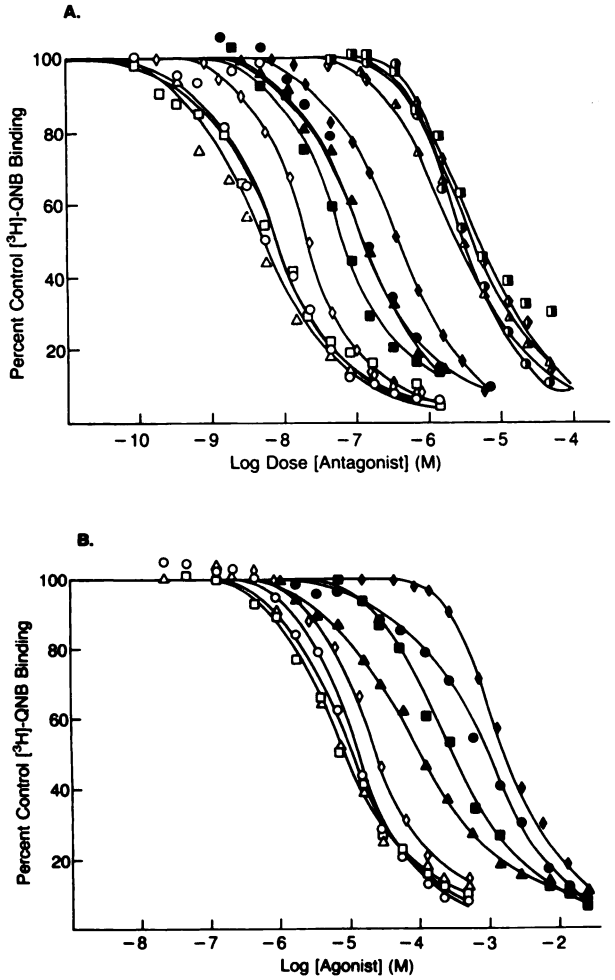

Fig. 3. Muscarinic cholinergic ligand binding to wild type and mutant m_1 muscarinic receptors. Membranes from transfected CHO cells expressing wild type (*circles*) or Asn^{71}, (*triangles*), Asn^{99} (*diamonds*), or Asn^{122} (*squares*) mutant receptors were incubated in 50 mM $NaPO_4$ buffer, pH 7.4, containing an approximate K_d concentration of QNB and the indicated concentrations of muscarinic agents for 60 min at 37°. Samples were filtered as described in the legend to Fig. 2. A, Competition displacement experiments with the muscarinic antagonists atropine (*open symbols*), pirenzepine (*filled symbols*), and AF-DX 116 (*half-filled symbols*). B, Competition displacement experiments with the muscarinic agonists oxotremorine (*open symbols*) or carbachol (*filled symbols*). Data represent the average of two or three separate experiments performed in triplicate. Data were analyzed using the LIGAND curve-fitting program of Munson and Rodbard (27).

As illustrated in Fig. 3B and Table 1, the K_i for the binding of the partial agonist oxotremorine to the wild type m_1 muscarinic receptor was determined to be 2.1 μM. Whereas the affinities of the Asn^{71} and Asn^{122} mutant receptors for oxotremorine were essentially unchanged, as compared with the wild type receptor (K_i = 1.7 and 2.7 μM, respectively), the K_i of the Asn^{99} mutant receptor for oxotremorine was increased 3.5-fold to 7.3 μM. In contrast to our findings with oxotremorine, the affinities of the Asn^{71} (K_i = 46 μM) and Asn^{122} (K_i = 130 μM) mutant muscarinic receptors for the full agonist carbachol were significantly increased as compared with the wild type receptor (K_i = 252 μM) (Fig. 3B; Table 1). The affinity of the Asn^{99} mutant receptor for carbachol (K_i = 1377 μM) was decreased 5.4-fold as compared with the wild type receptor (Fig. 3B; Table 1).

PI hydrolysis in cells expressing wild type and mutant m_1 muscarinic receptors. Rat m_1 muscarinic receptors expressed in murine B-82 cells and in CHO cells display agonist-mediated dose-dependent increases in PI hydrolysis (21, 34, 35). Addition of carbachol or oxotremorine to CHO cells expressing wild type m_1 receptors produced a linear increase in the formation of radiolabeled inositol phosphates between 0 and 40 min (not shown). The EC_{50} value and the maximum level of PI hydrolysis was found to be dependent on receptor density (not shown). In cells expressing 200 fmol of wild type receptor/mg of membrane protein (a density equal to or less than that in CHO cells expressing the Asn^{71} and Asn^{122} mutant receptors), carbachol and oxotremorine evoked 23-fold and 6-fold maximal increases, respectively, in the basal rate of formation of inositol phosphates (Fig. 4A). The EC_{50} values for carbachol- and oxotremorine-stimulated PI hydrolysis were 5 and 6 μM, respectively. Neither agent elicited any change in PI hydrolysis in control CHO cells (not shown).

PI hydrolysis in response to either carbachol or oxotremorine was markedly attenuated with the Asn^{71} mutant muscarinic receptor (Fig. 4A). Maximum levels of agonist-stimulated PI hydrolysis in cells expressing Asn^{71} mutant receptors were 2.1-fold and 1.1-fold, respectively, the basal rate of formation of inositol phosphates.

In cells expressing Asn^{122} mutant receptors, carbachol and oxotremorine produced a maximal stimulation of PI hydrolysis similar to that in cells expressing wild type receptors; however, the EC_{50} for carbachol-stimulated PI hydrolysis was shifted from 5 μM to 53 μM (Fig. 4A).

Addition of increasing concentrations of carbachol or oxotremorine to cells expressing similar densities of wild type or Asn^{99} mutant receptors produced dose-dependent increases in the rate of formation of inositol phosphates (Fig. 4B). Carbachol and oxotremorine produced maximal increases in PI hydrolysis in cells expressing the Asn^{99} mutant receptor essentially identical to those observed in CHO cells expressing wild type m_1 muscarinic receptor at a similar density (Fig. 4B; Table 1). However, the EC_{50} value for carbachol-stimulated PI hydrolysis in cells expressing Asn^{99} mutant receptors was 120 μM, a value significantly greater than that determined for cells expressing wild type receptors. The decreased potency of carbachol for stimulation of PI hydrolysis in cells expressing the Asn^{99} mutant receptor most likely reflected the decrease in mutant receptor affinity for carbachol.

Affinity labeling of wild type and mutant m_1 muscarinic receptors. Affinity labeling of wild type m_1 muscarinic receptor in transfected CHO membranes (250 fmol of QNB binding sites) with 2 nM PrBCM and analysis of the labeled proteins by SDS-PAGE revealed a single specifically labeled protein that migrated with a mobility corresponding to a molecular weight of 84,000 (Fig. 5). The size of the PrBCM-labeled m_1 muscarinic receptor expressed in CHO cells is in agreement with previously reported values for membrane-associated muscarinic receptors from a number of species and tissues (25). PrBCM labeling of CHO cell membranes containing an identical concentration of Asn^{99} mutant receptor binding sites also yielded a specifically labeled protein that migrated with the same mobility as the wild type receptor; however, the amount of specific PrBCM-labeled protein present was only 10–15% of that observed with the wild type receptor (Fig. 5). It was possible that the marked reduction in PrBCM labeling of the

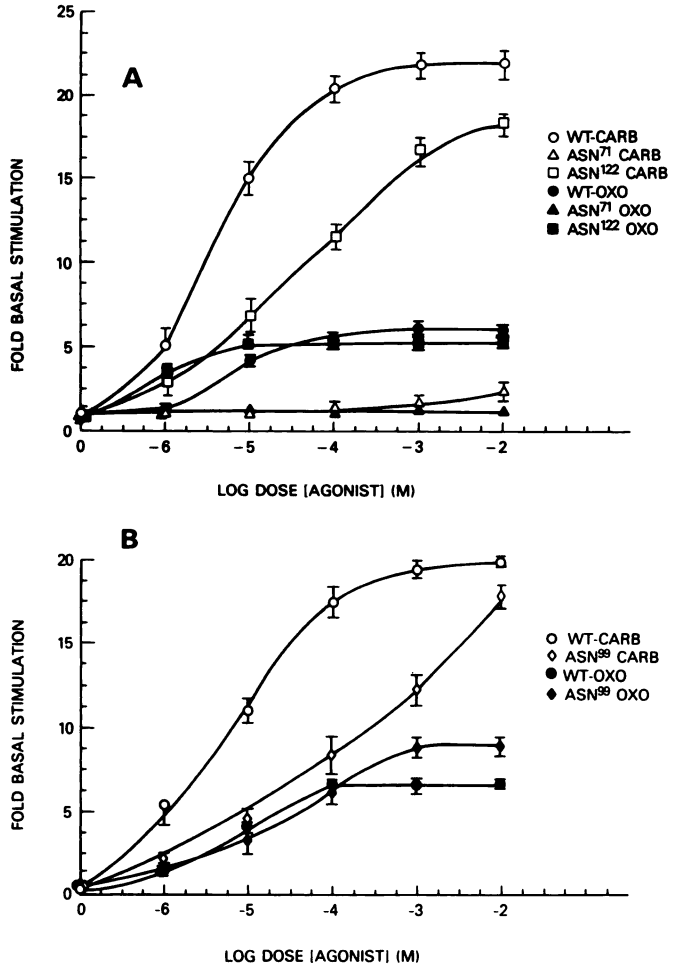

Fig. 4. Measurement of PI hydrolysis in CHO cells transfected with wild type and mutant muscarinic receptors. A, CHO cells expressing wild type (200 fmol of receptor/mg of membrane protein), Asn^{71} mutant (360 fmol of receptor/mg of membrane protein), or Asn^{122} mutant receptors (210 fmol of receptor/mg of membrane protein) were assayed for carbachol- and oxotremorine-stimulated PI hydrolysis according to the method of Berridge (28). Total inositol phosphates were measured in these experiments. Data are expressed as fold basal stimulation of PI hydrolysis. The basal levels of PI accumulation in transfected cells expressing wild type or Asn^{71} or Asn^{122} mutant receptors were 8,752 ± 1,063 dpm, 10,476 ± 1,253 dpm, and 5,534 ± 786 dpm, respectively. These values represented 4.1%, 4.5%, and 4.2%, respectively, of the total labeled phosphoinositides incorporated into the transfected cells. Data represent the mean of three or four separate experiments performed in duplicate. B, CHO cells expressing wild type (1385 fmol of receptor/mg of membrane protein) or Asn^{99} mutant receptors (2660 fmol of receptor/mg of membrane protein) were assayed for carbachol- and oxotremorine-stimulated PI hydrolysis, as described in Experimental Procedures. Data are expressed as the fold basal stimulation of PI hydrolysis. The basal levels of PI accumulation in transfected cells expressing wild type and Asn^{99} mutant receptors were 2377 ± 856 dpm and 2721 ± 745 dpm, respectively. Data represent the mean of five separate experiments performed in duplicate. *CARB*, carbachol; *OXO*, oxotremorine.

Asn^{99} mutant receptor was due to a change in the affinity of the receptor for this compound. In order to assess this question, PrBCM binding to wild type and Asn^{99} mutant receptors was assayed by membrane filtration over glass fiber filters. As illustrated in Figure 5, *inset A*, we observed a dose-dependent increase in specific PrBCM binding to the wild type m_1 receptor in CHO cell membranes; the maximum number of binding sites determined with PrBCM was similar to that determined with

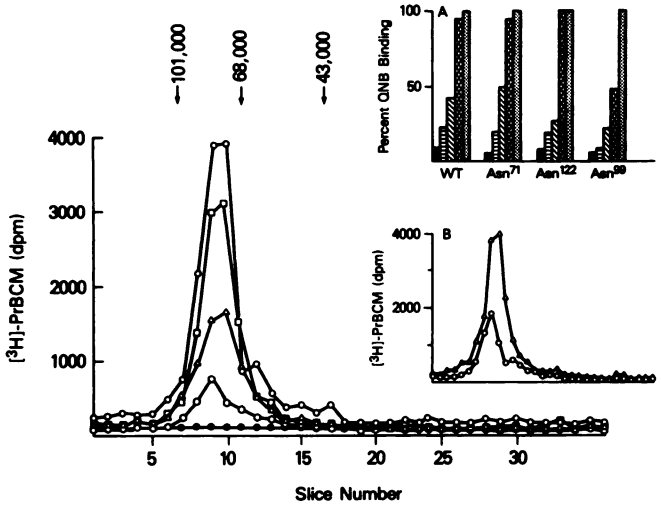

Fig. 5. [^{3}H]PrBCM labeling of wild type and mutant m_1 muscarinic receptors. Membranes from transfected CHO cells expressing wild type (○) or Asn^{71} (□), Asn^{122} (△), or Asn^{99} (○) mutant receptors containing 250 fmol of QNB binding sites were labeled with 2 nM cyclized PrBCM, as described in Experimental Procedures, and analyzed on a 10% SDS-polyacrylamide gel. Gels were sliced and counted for radioactivity. ●, PrBCM binding to membranes labeled in the presence of 1 μM atropine. The mobility of molecular weight markers used for SDS-PAGE calibration is indicated by the *arrows* at the top of the figure. *Inset A*, dose-response curves for PrBCM binding to wild type and mutant receptors. Membranes containing 30 fmol of QNB binding sites prepared from the indicated transfected cell lines were incubated with increasing concentrations of PrBCM under identical conditions used for irreversible labeling. The extent of PrBCM (reversible and irreversible) binding to the muscarinic receptors was assessed by filtration over Whatman glass fiber filters, as described in the legend to Fig. 1. The concentrations of PrBCM used for binding studies were 0.2 (■), 0.5 (■), 1.0 (▨), 2.5 (■), and 5 (▨) nM. Data are representative of two experiments performed in triplicate. *Inset B*, Covalent labeling of Asn^{122} and Asn^{99} mutant muscarinic receptors with 5 nM PrBCM. Membranes from cells expressing Asn^{122} (△) or Asn^{99} (○) mutant receptor were labeled with PrBCM and analyzed by SDS-PAGE as described above, with the exception that the concentration of PrBCM used was increased to 5 nM.

QNB. The concentration of PrBCM required for half-maximal saturation of the wild type receptor was 1 nM. The Asn^{99} mutant receptor also specifically bound PrBCM in a dose-dependent manner; however, the concentration of PrBCM required for half-maximal saturation was increased approximately 2-fold (Fig. 5). The decrease in the affinity of the Asn^{99} mutant receptor for PrBCM was consistent with that observed for other muscarinic antagonists (Table 1). To compensate for the decreased affinity of the Asn^{99} mutant receptor for PrBCM, affinity labeling was repeated with 5 nM PrBCM, a concentration sufficient to saturate all of the receptor binding sites. SDS-PAGE analysis of Asn^{99} mutant muscarinic receptors labeled under these conditions revealed the presence of a specifically labeled protein with molecular weight 84,000; however, the extent of mustard labeling of the mutant receptor was still only 40% of the maximal level observed with the wild type receptor Fig. 5, *inset B*.

It was of interest to determine whether substitution of Asp with Asn at other loci in the muscarinic receptor affected PrBCM labeling. Asn^{71} and Asn^{122} mutant m_1 muscarinic receptors were labeled with PrBCM under conditions identical to those used with the wild type receptor and the extent of labeling was assessed in membrane filtration assays and by SDS-PAGE. As illustrated in Fig. 5 (*inset A*), PrBCM specifi-

cally labeled each mutant receptor in a dose-dependent manner. The affinity of the Asn^{71} receptor for PrBCM was identical to that observed with the wild type receptor when assayed by filtration or SDS-PAGE. In contrast, the affinity of the Asn^{122} mutant receptor for PrBCM was reduced approximately 1.5-fold, as compared with the wild type receptor (Fig. 5, *inset A*); SDS-PAGE of the Asn^{122} mutant receptor labeled with 2 nM PrBCM revealed a corresponding 50% decrease in covalent labeling of the mutant receptor (Fig. 5). When the Asn^{122} mutant receptor was incubated with 5 nM PrBCM, labeling equivalent to the maximal level observed with the wild type receptor was achieved (Fig. 5, *inset B*).

Discussion

In the present study, the roles of conserved aspartic acids located in or near the second and third transmembrane domains of the rat m_1 muscarinic receptor were examined by site-directed mutagenesis and permanent expression in CHO cells. The aspartic acid residues were replaced with asparagine, which eliminates the negative charge at each position in the receptor. Previous mutagenesis studies have implicated a role for the corresponding transmembrane aspartic acids in ligand binding and agonist-induced activation of the β_2-adrenergic receptor (18–20) and in proton translocation in bacteriorhodopsin (17), suggesting that they play important functional roles in these proteins.

Mutation of Asn^{71}, in the second transmembrane domain of the muscarinic receptor, produced a receptor that had normal antagonist and partial agonist binding and a 5.5-fold higher affinity for carbachol than the wild type receptor. These data are similar to those derived from site-directed mutagenesis of Asp^{79} in the β_2-adrenergic receptor (18, 20), in that antagonist binding was not affected by the mutation whereas agonist binding was changed, and suggest that different coordinates may exist for the binding of agonists and antagonists in both β-adrenergic and muscarinic acetylcholine receptors. Unlike the corresponding mutation in the β_2-adrenergic receptor, which decreased the affinity of the receptor for agonists (18, 20), substitution of Asp^{71} with Asn in the muscarinic receptor increased the affinity of the receptor for full agonists. These data suggest that, if this Asp residue is directly involved in agonist binding in both β-adrenergic and muscarinic receptors, then this locus may differentially interact with unique structural determinants in adrenergic and muscarinic ligands to produce opposing changes in receptor affinities. However, it is also possible that this conserved aspartic acid residue does not participate directly in agonist binding; the opposing changes in the affinity of the analogous β-adrenergic and muscarinic acetylcholine receptor mutants at this locus may reflect altered receptor conformations that affect the free energy of receptor-ligand interactions.

Despite the higher affinity of the Asn^{71} mutant muscarinic receptor for agonists, this receptor induced only minimal stimulation of PI hydrolysis in transfected cells. The discrepancy between the increase in receptor affinity for carbachol and the decreased efficacy of full and partial agonists to induce formation of inositol phosphates suggests a loss in the ability of agonists to activate the mutant receptor. In this respect, Asn^{71} mutant muscarinic receptors are similar to Asn^{79} mutant β_2-adrenergic receptors, which were unable to mediate agonist-induced activation of adenylate cyclase in B-82 cells (18). These data suggest that the conserved Asp found in the second transmembrane domain of all members of this gene family may be involved in agonist-induced receptor activation.

Substitution of Asp^{122} in the third transmembrane domain of the m_1 muscarinic receptor with Asn produced a mutant receptor that had normal antagonist and partial agonist affinity but a 1.9-fold higher affinity for carbachol. These findings are similar to those described for the analogous Asn^{130} mutant β_2-adrenergic receptor, which exhibited normal antagonist binding and higher affinity agonist binding than the wild type β-receptor (19). Both carbachol and oxotremorine were able to stimulate PI hydrolysis in cells expressing the Asn^{122} receptor; however, the EC_{50} for maximal carbachol stimulation of PI hydrolysis was shifted from 5 μM in cells expressing wild type receptor to 53 μM in cells expressing Asn^{122} mutant receptor. The increase in receptor affinity for carbachol and the rightward shift in the dose-response curve for carbachol-induced PI turnover implies a change in the efficiency of receptor-effector coupling and suggests a possible role for Asp^{122} in receptor-G protein interactions. The corresponding mutation in the conserved aspartic acid residue at position 130 in the human β_2-adrenergic receptor had a similar effect on agonist binding, as well as affecting receptor-G protein coupling (19), which suggests that this locus may be critical for normal receptor-G protein alignment.

As summarized in Table 1, substitution of Asp^{99} in or near the second extracellular domain of the m_1 muscarinic receptor with Asn produced a mutant receptor that displayed reduced affinity for the muscarinic antagonists QNB, atropine, pirenzepine, and PrBCM and the agonists carbachol and oxotremorine. The loss in affinity for each of these ligands was of similar magnitude (2- to 5.4-fold) and was consistently observed in several experiments. The decrease in Asn^{99} mutant receptor affinity for carbachol was accompanied by an increase in the K_{act} for carbachol-stimulated PI hydrolysis in transfected cells. Asp^{99}, which carries a negative charge in the native receptor, could potentially serve as a site of electrostatic interaction with the protonated amine group of muscarinic ligands; the change in mutant receptor affinity for agonists and antagonists following substitution of Asp^{99} with Asn suggests that this residue may participate in ligand binding.

It has been well established that the muscarinic receptor can be covalently labeled, with a high degree of specificity and efficiency, with PrBCM (36). The reactive form of PrBCM is the aziridinium ion, which is an analogue of the ammonium head group found in both muscarinic agonists and antagonists (36). Chemical evidence suggests that the nucleophile that attacks the PrBCM aziridinium ion is a carboxylate moiety (37, 38). Based on protein chemical and peptide mapping studies of purified, PrBCM-labeled, muscarinic receptors from rat brain, Curtis *et al.* (38) suggested that the site of alkylation is an acidic amino acid in the sequence of the m_1 muscarinic receptor between methionine residues at positions 88 and 114. Within this domain of the muscarinic receptor are two conserved aspartic acids at positions 99 and 105 that could serve as potential sites for PrBCM alkylation. The irreversible alkylation reaction is regulated by a group on the receptor with a pK of 6.2, consistent with its location in a hydrophobic environment (38).

Our site-directed mutagenesis studies suggested that one site of PrBCM alkylation of the m_1 muscarinic receptor may be

Asp^{99}. PrBCM was shown to reversibly bind to the Asn^{99} mutant m_1 muscarinic receptor expressed in CHO cell membranes, but the amount of protein irreversibly labeled by the mustard was only 10–15% of that observed with the wild type receptor. Although the apparent affinity of the Asn^{99} mutant receptor for PrBCM was approximately 2-fold lower than the affinity of the wild type receptor, the decrease in PrBCM-labeled mutant receptor on SDS-PAGE could not be accounted for solely on the basis of a change in receptor affinity for PrBCM. The observation that submaximal levels of covalent receptor labeling were observed at high ligand concentrations implies that more than one nucleophilic residue on the receptor may attack the reactive species of PrBCM. An obvious candidate for this alternate site of incorporation would be Asp^{105}. Indeed, recent peptide sequencing data from Hulme[3] have identified two sites of PrBCM alkylation in the rat m_1 muscarinic receptor, at Asp^{99} and Asp^{105}. Thus, data from site-directed mutagenesis studies and peptide sequencing suggest that both Asp^{99} and Asp^{105} participate in the binding of the positively charged amine groups of muscarinic ligands and perhaps imply that both residues are necessary for the formation of a stable ligand binding conformation.

It was somewhat surprising to observe that mutation of Asp^{122}, presumably located in or near the cytoplasmic side of the third putative transmembrane helix of the muscarinic receptor, reduced the affinity of the mutant receptor for PrBCM and similarly increased the concentration of PrBCM required for maximal covalent incorporation. Pharmacological characterization of the Asn^{122} mutant receptor indicated that the binding of other muscarinic antagonists was unchanged, suggesting that Asp^{122} did not participate directly in antagonist binding. However, it is possible that substitution of Asp^{122} with Asn may alter receptor conformation in such a manner as to influence the affinity of the receptor for PrBCM or, alternatively, to alter the inherent nucleophilicity of labeled Asp residues, thereby reducing the efficiency of alkylation.

In summary, all members of the G protein-coupled family of receptors share a number of common structural features, the most notable of which is the presence of a core of seven amphipathic transmembrane helices connected by alternating intracellular and extracellular loops. Contained within the transmembrane domains are negatively charged aspartic acid residues that are conserved in all members of this gene family. Numerous investigators have speculated that the aspartic acids may be likely sites of ligand interaction in adrenergic and muscarinic receptors (16, 20, 38). Our site-directed mutagenesis data from muscarinic (present study) and adrenergic (18, 19) receptors, together with data from Khorana and co-workers on bacteriorhodopsin (17), suggest a more universal role for two of the aspartic acids in the second and third transmembrane domains of these proteins in the process of receptor activation.

The conserved aspartic acids in or near the second extracellular loop and the proximal portion of the third transmembrane domain of the muscarinic receptor appear to be involved in ligand binding interactions with the positively charged onium groups of muscarinic ligands. Our mutagenesis study, together with data from Hulme,[3] allow for preliminary assignment of a part of the ligand binding domain of the muscarinic receptor to a region rich in conserved aspartic acid residues that has been shown by previous mutagenesis experiments to influence agonist binding and receptor activation in other related systems (17, 20).

The use of site-directed mutagenesis to examine the relationship between receptor structure and function has shed new light on the role of specific amino acid residues in receptor mechanisms and complements biochemical studies performed with native receptor proteins. Pharmacological and biochemical studies of other site-directed receptor mutants permanently expressed in culture cells will extend our knowledge on the structural determinants of receptor-ligand interactions and the molecular basis of receptor function.

[3] E. C. Hulme, personal communication.

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