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Discovery of an Ectopic Activation Site on the M_1 Muscarinic Receptor

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ABSTRACT

Receptors have well-conserved regions that are recognized and activated by hormones and neurotransmitters. Most drugs bind to these sites and mimic or block the action of the native ligands. Using a high-throughput functional screen, we identified a potent and selective M_1 muscarinic receptor agonist from a novel structural class. Using a series of chimeric receptors, we demonstrated that this ligand activates the receptor through a region that is not conserved among receptor subtypes, explaining its unprecedented selectivity. This region of the receptor is distinct from the conserved region that is recognized by traditional ligands. The finding that receptors for small-molecule transmitters can have multiple, structurally distinct activation sites has broad implications for the study of receptor structure/function and the potential for the discovery of novel ligands with high selectivity.

G-protein-coupled receptors that bind monoamine ligands (e.g., serotonin, adrenaline, dopamine, histamine, and acetylcholine) comprise the most intensively studied and exploited receptor family for the development of therapeutic agents by the pharmaceutical industry. The natural ligands for monoamine receptors are believed to bind a highly conserved pocket located deep within the transmembrane (TM)-spanning regions and to mediate receptor activation primarily through TM3, TM5, TM6, and TM7 (Spalding et al., 1994; Baldwin et al., 1997; Gether, 2000; Lu et al., 2001). Of the amino acids in these regions, 74% are identical in all five muscarinic receptor subtypes (Bonner et al., 1988). Potent small-molecule agonists are also believed to bind monoamine receptors through the same highly conserved regions (Strader et al., 1989, 1991; Wess et al., 1991; Page et al., 1995; Spalding et al., 1998; Ward et al., 1999; Allman et al., 2000).

The muscarinic M_1 receptor has been targeted for the discovery of therapeutics for Alzheimer's Disease, and several companies have developed M_1-selective agonists (e.g., Tecle et al., 1998; Wood et al., 1999; Bartolomeo et al., 2000; Wienrich et al., 2001). Many potent compounds came out of these programs, and several were shown to improve cognition in animals (WAY-132983 and CI-1017; Bartolomeo et al., 2000; Weiss et al., 2000) and people (Xanomeline, Bodick et al., 1997). However, many of the compounds also produced classic muscarinic side effects such as sweating, nausea and diarrhea (Bodick et al., 1997, Bartolomeo et al., 2000; Thal et al., 2000). In vitro assays have shown that these compounds activate the M_1, M_3, M_4, and M_5 muscarinic receptor subtypes at similar concentrations (Table 1 and Tecle et al., 1998; Wood et al., 1999; Bartolomeo et al., 2000; Wienrich et al., 2001). This may be a direct result of the ligands activating the receptors through regions where the amino acid sequence is almost identical. Since drug interactions with nontarget receptor subtypes are often responsible for the unwanted side effects of commercial pharmaceuticals, there is strong motivation to design more selective ligands.

We describe a novel muscarinic agonist, AC-42, and demonstrate its selectivity for the M_1 muscarinic subtype using two functional assays. We tested the hypothesis that the selectivity of AC-42 results from interactions (positive or negative) with amino acids that are not conserved between the M_1 and other muscarinic subtypes by measuring the functional activity of AC-42 on 15 chimeric receptors in which regions of the M_1 receptor were replaced by the M_5 sequence and three recombinant receptors containing mutations in the acetylcholine binding site.

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ABBREVIATIONS: AC-42, 4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; CT, C-terminal domain of the receptor; DMEM, Dulbecco's modified Eagle's medium; i1–i3, internal loops of the receptors; IBMX, 3-isobutyl-1-methylxanthine; NT, N-terminal domain of the receptor; o1–o3, external (outer) loops of the receptor; pEC_{50}, $-\log EC_{50}$; R-SAT, receptor selection and amplification technology; TM, transmembrane; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

TABLE 1
Pharmacological characterization of AC-42 and other muscarinic agonists on M_1 to M_5. Assays were carried out using R-SAT. Values represent the mean ± S.E.M. Maximum response (Max. Resp.) values are normalized relative to the maximum response of the cells to carbachol.

	M_1			M_2			M_3			M_4			M_5		
	Max. Resp.	pEC_{50}	*N*	Max. Resp.	pEC_{50}	*N*	Max. Resp.	pEC_{50}	*N*	Max. Resp.	pEC_{50}	*N*	Max. Resp.	pEC_{50}	*N*
	%			%			%			%			%		
AC-42	66 ± 3	6.54 ± 0.05	32	N.R.		6	N.R.		15	N.R.		13	N.R.		13
Carbachol	100	6.13 ± 0.03	47	100	6.34 ± 0.09	19	100	6.55 ± 0.05	26	100	6.54 ± 0.05	20	100	6.64 ± 0.14	18
Pilocarpine	94 ± 2	5.67 ± 0.04	32	70 ± 8	5.56 ± 0.18	7	59 ± 9	5.79 ± 0.08	15	66 ± 7	5.35 ± 0.09	10	77 ± 7	5.60 ± 0.11	12
McN-A-343	96 ± 4	6.23 ± 0.08	6	98 ± 11	4.94 ± 0.08	4	50 ± 6	5.45 ± 0.05	4	102 ± 12	6.25 ± 0.12	5	119 ± 15	6.07 ± 0.11	4
Oxotremorine-M	106 ± 5	7.41 ± 0.12	8	108 ± 4	6.28 ± 0.40	2	119 ± 21	7.04 ± 0.25	3	125 ± 3	7.51 ± 0.24	4	117 ± 21	7.07 ± 0.28	3
CI-1017	76 ± 4	6.34 ± 0.06	6	99 ± 13	5.46 ± 0.21	5	51 ± 6	5.85 ± 0.09	9	118 ± 10	6.40 ± 0.08	8	62 ± 9	5.97 ± 0.12	8
WAY-132983-A5	107 ± 8	8.34 ± 0.16	8	73 ± 1	7.44 ± 0.17	4	52 ± 3	7.96 ± 0.15	11	121 ± 4	9.01 ± 0.07	3	59 ± 12	8.18 ± 0.22	6
Xanomeline	122 ± 6	7.19 ± 0.09	16	108 ± 10	6.25 ± 0.26	6	66 ± 6	6.63 ± 0.21	8	112 ± 10	7.56 ± 0.12	4	95 ± 11	6.60 ± 0.28	4

pEC_{50}, $-\log(EC_{50})$; N.R., no response; maximum response of AC-42 was not significantly different from zero on the M_2 to M_5 receptor subtypes.

Materials and Methods

Compounds

Carbamylcholine (carbachol), atropine, forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (St. Louis, MO). 4-*n*-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride (AC-42) was synthesized by Organic Consultants Inc. (Eugene, OR). Compound structure was verified by NMR. Purity was greater than 99% measured by high-performance liquid chromatography and gas chromatography. Xanomeline was synthesized as described by Sauerberg et al. (1992). Compound structure was verified by NMR. Purity was greater than 99% measured by high-performance liquid chromatography and gas chromatography. CI-1017 was a generous gift of Pfizer (New York, NY). WAY-132983-A5 was a generous gift of Wyeth-Ayerst (Princeton, NJ). *N*-[methyl-^{3}H]scopolamine was obtained from Amersham Biosciences (Piscataway, NJ).

DNA Constructs

The human M_1 to M_5 receptors were expressed from the m1 to m5 genes cloned as described previously (Bonner et al., 1987, 1988). To construct the chimeric receptors, the m1 and m5 genes were subcloned into the pSI vector (Promega, Madison WI). All data in Fig. 2 were collected using receptors expressed from the pSI vector. Restriction sites were introduced into the M_1 and M_5 receptors at the following conserved amino acid residues by site-directed mutagenesis using Quikchange (Stratagene, La Jolla, CA) according to manufacturer's instructions: TM1, *Spe*I introduced at leucine-45 (M_1) and -50 (M_5); TM3, *Xba*I site introduced at leucine-104 (M_1) and -109 (M_5); TM4, *Apa*I (site occurs naturally in M_1) introduced at alanine-163 in M_5; TM6, *Sal*I site introduced at serine-388 (M_1) and -465 (M_5); and TM7, Mlu 1 site introduced at alanine-419 (M_1) and -496 (M_5). Constructs were sequenced to ensure that no changes were introduced in the amino acid sequence. Chimeric receptors were constructed by subcloning.

Functional Assays

Receptor Selection and Amplification Assays. R-SAT (ACADIA Pharmaceuticals, San Diego, CA) assays were performed with minor modifications from those procedures described previously (Bräuner-Osborne and Brann, 1996; Burstein et al., 1997).

For the preparation of Figs. 1B and 2, NIH 3T3 cells were grown in 96-well tissue culture plates to 70 to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml L-glutamine (1% penicillin/streptomycin/glutamine; Invitrogen, Carlsbad, CA) and 10% calf serum (Sigma-Aldrich). Cells were transfected for 18 h with DMEM containing 0.08 mg/ml receptor DNA, 0.08 mg/ml pCI-Gqi5 (M_2 and M_4 only; Burstein et al., 1997), 0.3 mg/ml pSI-Bgal (Promega), which is a DNA construct encoding the gene for β-galactosidase, and 0.5% v/v Superfect (Qiagen, Valencia, CA). The medium was replaced with DMEM containing 1% penicillin/streptomycin/glutamine, 0.5% calf serum, 2% cyto-sf3 (Kemp Biotechnologies, Frederick, MD), and varying concentrations of ligand. Cells were grown in a humidified atmosphere with 5% ambient CO_2 for 5 days. Medium was removed from the plates, and β-galactosidase activity was measured by the addition of *o*-nitrophenyl-*d*-galactopyranoside in phosphate-buffered saline with 5% Nonidet P-40. The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nM. Data were analyzed using Prism software (GraphPad Software, San Diego, CA).

To prepare Table 1, R-SAT was carried out using a similar method. Cells were grown in larger volumes and were harvested 1 day after transfection as described previously (Bräuner-Osborne and Brann, 1996). Transfected cells were frozen at −135° after harvesting in DMEM containing 10% calf serum and 10% dimethyl sulfoxide. Cells

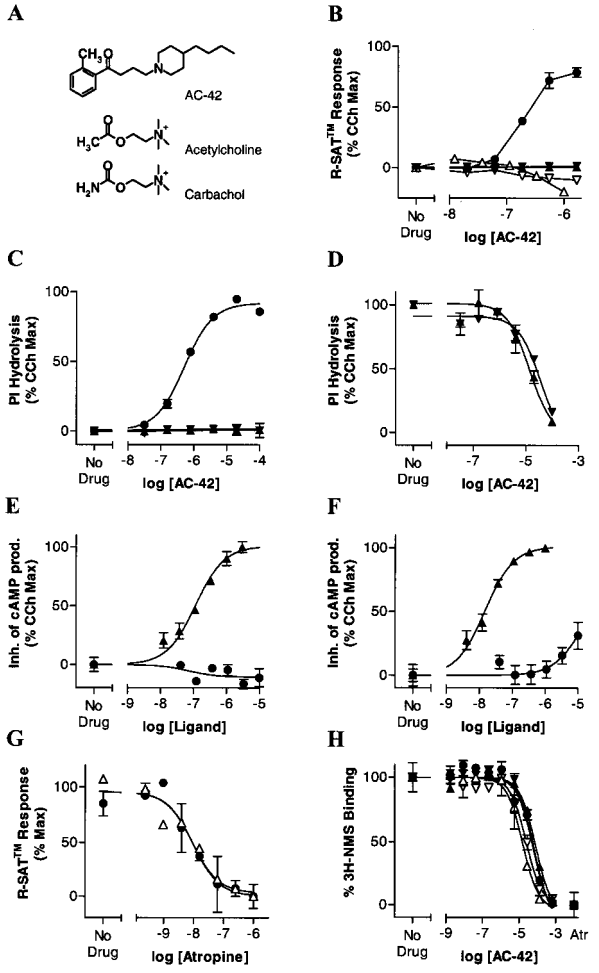

Fig. 1. Pharmacological characterization of AC-42. A, chemical structures of AC-42, carbachol (CCh), and acetylcholine. B, agonist activity of AC-42 on the M_1 (●), M_2 (△), M_3 (▲), M_4 (▽), and M_5 (▼) muscarinic receptor subtypes. Assays were carried out using R-SAT. Response values were normalized relative to the maximum response of the cells to carbachol. Points represent the mean ± S.E.M. of six determinations. pEC_{50} values for carbachol in this experiment were: M_1, 6.3; M_2, 7.0; M_3, 6.4; M_4, 6.7; and M_5, 6.7. Data are typical of four experiments. C, stimulation of phosphatidyl-inositol hydrolysis by AC-42 in CHO cells expressing M_1 (●), M_3 (▲), and M_5 (▼) muscarinic receptors (Buckley et al., 1989). Points represent the mean ± S.E.M. of duplicate determinations. pEC_{50} values for carbachol in this experiment were: M_1, 5.9; M_3, 6.1; and M_5, 5.9. Data shown are typical of two experiments. D, inhibition of phosphatidyl-inositol hydrolysis stimulated by 5000 nM carbachol by AC-42 in CHO cells expressing M_3 (▲) and M_5 (▼) receptors. The EC_{50} of carbachol in assays of this type was typically 1000 nM. Points represent the mean ± S.E.M. of duplicate determinations. Inhibitory responses were normalized relative to the maximum inhibition by the antagonist atropine. Data are typical of two experiments. E, inhibition of cAMP production by AC-42 (●) and carbachol (▲) in CHO cells expressing the M_2 receptor. Points represent the mean ± S.E.M. of triplicate determinations. Data are typical of three experiments. F, inhibition of cAMP production by AC-42 (●) and carbachol (▲) in CHO cells expressing the M_4 receptor. Points represent the mean ± S.E.M. of five determinations. Data are typical of three experiments. G, inhibition of agonist activity stimulated by 2000 nM carbachol (△) and 1000 nM AC-42 (●) by the muscarinic antagonist atropine. Typically, the EC_{50} for carbachol on M_1 is 700 nM and the EC_{50} of AC-42 on M_1 is 260 nM. Assays were carried out using R-SAT. Points represent the mean ± S.E.M. of triplicate determinations. Data are typical of two experiments. H, inhibition of binding of 1 nM [^{3}H]*N*-methylscopolamine by AC-42. Radioligand binding assays were carried out using the human embryonic kidney cell line tsA 201 (Chahine et al., 1994) transiently expressing the M_1 (●), M_2 (△), M_3 (▲), M_4 (▽), and M_5 (▼) muscarinic receptor subtypes (Wess et al., 1991; Spalding et al., 1998). Points represent the mean ± S.E.M. of duplicate determinations. Atr represents binding in the presence of 1 μM atropine. Data are typical of two experiments.

were thawed and added directly to ligands at a density of 20,000 cells per well of a half-area 96-well plate.

Phosphatidyl Inositol Hydrolysis Assays. Phosphatidyl inositol hydrolysis assays were carried out as described by Jensen et al. (2000).

cAMP Assays. cAMP assays were carried out using the Biotrak cAMP enzymeimmunoassay procedure (Amersham). CHO m2 and CHO m4 cells (Buckley et al., 1989) were seeded at 20,000 cells per well of a 96-well plate the day before the assay was carried out. The cells were equilibrated in DMEM containing 0.1% bovine serum albumin (BSA) and 0.5 mM IBMX (Sigma-Aldrich) for 90 min. Ligands were added in DMEM plus 0.1% BSA, 0.5 mM IBMX, and 500 nM forskolin (Sigma-Aldrich), and the cells were incubated for 30 min at 37°C. The reaction was halted by the removal of the ligand followed by the addition of 200 μl of 0.25% dodecyltrimethylammonium bromide in 50 mM sodium acetate, 0.02% BSA, and 0.01% preservative. Intracellular cAMP was then measured according to the manufacturer's instructions.

Radioligand Binding Assays. [^{3}H]*N*-methylscopolamine radioligand binding assays were carried out as described by Wess et al. (1991). Receptor expression levels and [^{3}H]*N*-methylscopolamine affinity for M_1, M_5, and four chimeric receptors (C1, L1, NC3, and NC4) were quantified in NIH 3T3 cells 2 days after transfection using 12 [^{3}H]*N*-methylscopolamine concentrations between 2 and 0.002 nM in the presence or absence of 10 μM atropine. The affinities of the chimeric receptors for [^{3}H]*N*-methylscopolamine were similar and ranged between the values for M_1 and M_5 (pK_d values, mean ± S.E.M.: M_1, 10.3 ± 0.3; M_5, 9.6 ± 0.2, C1, 10.0 ± 0.4; L1, 10.2 ± 0.2; NC3, 10.2 ± 0.2; and NC4, 10.2 ± 0.2; $n = 2$).

Results and Discussion

We screened a library of 145,000 structurally diverse small organic chemicals for agonist activity on the M_1, M_3, and M_5 muscarinic receptors using a cell-based functional assay (Bräuner-Osborne and Brann, 1996). Four compounds were identified that showed significant agonist activity on the M_1 receptor. The most potent of these was AC-42 (Fig. 1A). Figure 1B shows a comparison of AC-42 activity on the M_1, M_2, M_3, M_4, and M_5 subtypes measured using this assay. AC-42 showed potent and efficacious agonist activity on the M_1 muscarinic subtype, but it showed no detectable agonist activity on the M_2, M_3, M_4, or M_5 subtypes. Figure 1C shows AC-42 activity measured using a phosphatidyl inositol hydrolysis assay (M_1, M_3, and M_5 only). As in Fig. 1B, AC-42 showed agonist activity on the M_1 subtype only. At high concentrations, AC-42 inhibited carbachol-stimulated activity on M_3 and M_5 receptors; thus, it is a low-potency antagonist at these subtypes (Fig. 1D). AC-42 did not significantly inhibit forskolin-stimulated cAMP production in CHO cells expressing the M_2 receptor (Fig. 1E). At high concentrations, AC-42 inhibited forskolin-stimulated cAMP production in CHO cells expressing the M_4 receptor (Fig. 1F); however, it showed no significant activity at doses in which we observe robust activity on M_1 (e.g., 1 μM). The M_1 response to AC-42 was blocked by atropine at the same concentrations that atropine blocked the cellular response to carbachol, confirming that this response is mediated by muscarinic receptors (Fig. 1G). AC-42 inhibited [^{3}H]*N*-methylscopolamine binding with low potency on all five subtypes (Fig. 1H).

AC-42 shows unprecedented selectivity for the M_1 muscarinic receptor over the other muscarinic subtypes compared with other functionally selective M_1 agonists (Table 1). We hypothesized that this unprecedented selectivity may be caused by interactions involving amino acid residues that are

not conserved between the M_1 receptor and the other subtypes, and we devised a study using chimeric receptors to identify the regions involved. The amino acid sequences of the five muscarinic receptor subtypes are extremely similar, but the M_5 subtype is most similar to M_1 (Bonner et al., 1988); therefore we constructed a series of chimeras between M_1 and M_5 (Fig. 2). Figure 2A shows the gross structure of the receptors, with seven transmembrane domains (TM1–7) linked by three intracellular and three extracellular loops (i1–3 and o1–3), an extracellular N-terminal domain (NT), and an intracellular C-terminal domain (CT) as indicated. Residue numbering is given in Fig. 2B. Figure 2C shows the distribution of nonidentical residues in the M_1 and M_5 receptors. Transmembrane domains TM2, TM3, and TM6 contain the smallest numbers of differences; TM1, 4, 5, and 7 and the short loop domains i1, i2, o2, and o3 contain more; and the largest numbers of differences are in the i3 loop and the NT and CT domains.

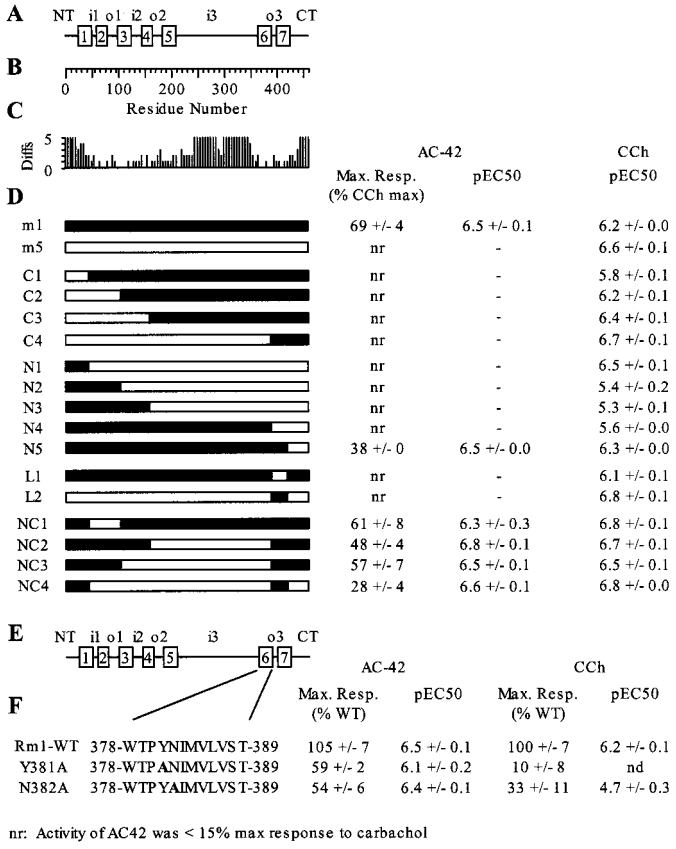

Fig. 2. Determination of the AC-42 binding site using chimeric receptors. A, schematic diagram of M_1 muscarinic receptor showing the position of the extracellular NT, seven transmembrane domains (□, marked 1–7), three intracellular loops (i1–i3), three extracellular loops (o1–o3), and intracellular CT. B, residue numbering in the human M_1 receptor; C, distribution of nonconserved residues in M_1 and M_5. The human M_1 and M_5 receptors were aligned as shown by Bonner et al. (1988). The M_1 sequence was divided into five amino acid segments, and each segment was scored from 1 to 5 to indicate the number of nonidentical amino acids. Where fewer than two residues in a 10-residue stretch were identical (e.g., in the i3 loop), any occurrences of identical residues were considered to be an artifact of the alignment and the segment was scored as 5. The graph shows the number of differences in each five–amino acid segment. D, structure and activity of chimeric receptors containing M_1 and M_5 sequence. Filled sections represent M_1 sequence, and open sections represent M_5 sequence. Functional assays were carried out using R-SAT. Maximum response data are normalized relative to the maximum response to carbachol for each mutant. The constitutive activity of all constructs was low; none of the constructs showed an atropine response that was greater than 10% of the maximum response to carbachol (data not shown). pEC_{50} represents the −log of the EC_{50} (concentration of ligand giving a half-maximal response) in molar units. Values show the mean ± S.E.M. of at least two determinations. E, schematic diagram showing the position of point mutations made in the rat M_1 receptor (Ward et al., 1999). F, effect of single point mutations on agonist activity induced by AC-42 and carbachol. Data are normalized relative to the maximum response of the wild-type rat M_1 receptor. Values show the mean ± S.E.M. of five determinations.

Regions of the M_1 receptor were replaced with the M_5 sequence, and the resultant chimeric receptors were tested for their ability to be activated by AC-42 and carbachol (an acetylcholine analog, Fig. 1A). Figure 2D shows the chimera constructs and the results of the agonist experiments. Chimeras C1 to C4, in which progressively smaller C-terminal portions of M_1 were paired with the M_5 sequence at the N terminus, displayed essentially wild-type responses to carbachol; however, these chimeras showed no response to AC-42. Chimera C1, in which only 45 amino acids in the N terminus and TM1 were replaced with M_5 sequence, was studied in more detail. Chimera C1 was expressed at concentrations similar to those of M_1 (1.5 and 1.8 pmol/mg, respectively), confirming that it is expressed and correctly folded. The chimera also responded to carbachol at concentrations similar to those of the wild-type receptor (pEC_{50} = 5.8 and 6.2, respectively), confirming that it is functional. In contrast, C1 gave an almost undetectable response to AC-42, whereas wild-type M_1 gave a robust response with a maximum response of 69% relative to carbachol and a pEC_{50} of 6.5. We concluded that the first 45 amino acids at the N terminus of the M_1 receptor are necessary for AC-42 to induce high-potency agonist activity.

Chimeric receptors in which N-terminal portions of the M_1 receptor were paired with M_5 sequence in the C terminus (N1–N5) were also tested for their responsiveness to carbachol and AC-42. Chimera N1, in which 50 amino acids in the N terminus and TM1 of M_5 were replaced with M_1 sequence, responded to carbachol but did not respond to AC-42, indicating that this region alone is not sufficient to confer high-potency agonist activity from AC-42.

Chimeras N1 to N5 responded to carbachol with pEC_{50} values within 0.9 log units of the pEC_{50} of the wild-type M_1 (i.e., the EC_{50} is within 8-fold). Four of these chimeras were unable to respond to AC-42; however, construct N5, in which only the lower part of TM7 and the CT domain (residues 418–460) were replaced with the M_5 sequence, could be activated by AC-42, suggesting that key determinants for interaction with AC-42 lay within the region spanning extracellular loop 3 (o3) and the top of TM7. This region of the M_1 receptor was replaced with the M_5 sequence (chimera L1). The resultant chimera was expressed at higher concentrations than M_1 (3.8 pmol/mg) and responded well to carbachol, but produced an almost undetectable response to AC-42. The reciprocal construct, in which this region of M_5 was replaced with the M_1 sequence similarly responded to carbachol but not to AC-42. We concluded that residues 418 to 460 are also necessary but are not sufficient for receptor activation by AC-42.

From these results, we hypothesized that residues close to both TM1 and TM7 of M_1 were involved in receptor activation by AC-42. We constructed a series of chimeras in which the middle of the M_1 receptor was replaced by the M_5 sequence (NC1–NC4). Strikingly, chimera NC3, in which residues 105

to 387 comprising part of TM4, TM5, and TM6 were replaced with the M_5 sequence, was virtually identical to full-length M_1 in its response to AC-42. This chimera is expressed at slightly lower concentrations than M_1 (0.9 pmol/mg). The construct that contained the least M_1 sequence but was still potently activated by AC-42 was NC4, in which only residues 1 to 45 and 388 to 418 contained the M_1 sequence. NC4 is expressed at 3-fold higher concentrations that M_1 (4.8 pmol/mg). NC4 gave a robust response to AC-42 (pEC_{50} = 6.6). Chimeras N1 and L2, in which only one of these regions in M_5 was replaced with M_1, did not respond to AC-42. We therefore concluded that residues 1 to 45 (NT-TM1) and 388 to 418 (o3-TM7) of the M_1 receptor act synergistically and play the primary role in its activation by AC-42.

Receptor binding and activation by classic agonists such as carbachol is mediated largely through interactions with TM6, and two highly conserved residues, tyrosine-381 and asparagine-382, are central to this process (Spalding et al., 1998; Ward et al., 1999; Lu et al., 2001). We tested AC-42 on rat M_1 receptors obtained from Dr. Ed Hulme (National Institute for Medical Research, London, England), in which tyrosine-381 and asparagine-382 in TM6 were mutated to alanine (Fig. 2, E and F). These receptors are expressed at levels approximately 20 and 10% of that of the wild-type receptor (Ward et al., 1999). Consistent with Dr. Hulme's observations, neither mutant responded well to carbachol (Ward et al., 1999). Mutation of tyrosine-381 resulted in a 90% decrease in the maximum response of the receptor to carbachol, and mutation of asparagine-382 resulted a 30-fold decrease (1.5 log units) in the pEC_{50} of carbachol. In contrast, AC-42 still produced a robust agonist response, with a maximum response greater than 50% of that of the wild-type receptor and almost no change in pEC_{50}. This suggests that unlike carbachol, AC-42 does not interact with either tyrosine-381 or asparagine-382 in the wild-type M_1 receptor, implying that the ligand binding sites of these two agonists are physically distinct.

AC-42 is an agonist with unprecedented selectivity for the M_1 muscarinic receptor subtype. Its agonist activity is mediated at least in part by synergistic activity of the N terminus and the upper part of TM1, and the third extracellular loop and the upper part of TM7. By analogy with the recently published crystal structure of rhodopsin (Palczewski et al., 2000), the upper portions of TM1 and TM7 are expected to interact in the 3-dimensional structure of the muscarinic receptor (Fig. 3), and we propose that they form an ectopic (outer) selectivity domain that mediates the agonist activity of AC-42. AC-42 may have additional interactions with other parts of the receptor (e.g., with the conserved residue aspartate-105 in TM3), but these are insufficient to confer agonist activity. The unprecedented selectivity of AC-42 is explained because the amino acid sequences of the N terminus and TM1 regions of the receptor are highly divergent within the five muscarinic subtypes. Thus, the ectopic activation site is not present on the other receptors.

The existence of multiple ligand-binding sites on the muscarinic receptor is well known. The allosteric modulators gallamine and alcuronium are known to bind muscarinic receptors simultaneously with carbachol (Lazareno et al., 2000), and mutations outside the carbachol-binding site affect their binding (Matsui et al., 1995; Ellis and Seidenberg, 2000). These ligands have extremely weak partial agonist activity (Jakubik et al., 1996) and block muscarinic cholinergic transmission in vivo (Clark and Mitchelson, 1976; Lee and El-Fakahany, 1991). In contrast, AC-42 is a potent agonist in the absence of other ligands.

We demonstrated that a potent and efficacious agonist, AC-42, activates the M_1 receptor through regions of the receptor that are distinct from those used by carbachol. This finding overturns the common belief that potent agonists binding to monoamine receptors must interact with a conserved ligand-binding domain located within the transmembrane domains of the receptor. We predict that medicinal chemistry based on agonists that bind through ectopic ligand-binding sites will prove to be an extremely fruitful avenue of drug discovery.

Acknowledgments

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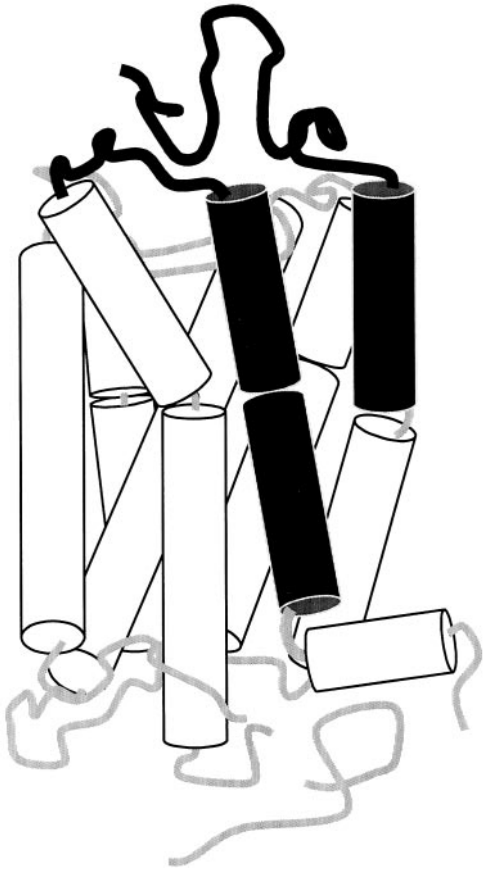

Fig. 3. Representation of the 3-dimensional structure of the muscarinic receptor (Spalding and Burstein, 2001), modeled after the crystal structure of rhodopsin (Palczewski et al., 2000). Cylinders represent α helices. Lines represent connecting loops. The extracellular surface is at the top of the page. TM1 is on the right, and the helices proceed in a counterclockwise direction. Filled cylinders and lines represent M_1 sequence in chimera NC4, which contained M_1 sequence in only the N terminus, TM1, the o3 loop, and TM7. Chimera NC4 gave a robust agonist response to AC-42.

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