

Identification of a family of muscarinic acetylcholine receptor genes.

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MUSCARINIC RECEPTORS MEDIATE many of the actions of acetylcholine in the central and peripheral nervous systems (1). Two pharmacologically distinct classes of such receptors, M1 and M2, have been defined on the basis of their affinities, high and low, respectively, for the antagonist pirenzepine (2). However, a variety of heterogeneous properties of muscarinic receptors have not always correlated well with this classification, which suggests that there may be other classes. It has been unclear whether the apparently different receptors represent modifications of a single receptor or whether they are different proteins. Recently a porcine brain muscarinic receptor complementary DNA (cDNA) was cloned and expressed in *Xenopus* oocytes to produce functional receptors (3). The presence of related messenger RNA (mRNA) in tissues rich in M1 receptors and its absence in tissues rich in M2 receptors suggested that there is more than one muscarinic acetylcholine receptor gene. More recently a related porcine cardiac cDNA sequence with the tissue distribution expected of M2 receptors was reported (4). Although no expression data were presented to verify that it is a muscarinic receptor, its identity has since been confirmed by expression of the porcine gene (5) and the corresponding human gene in mammalian cells.

Muscarinic receptors are members of a large class of neurotransmitter, hormone, and light receptors which act through binding to and activation of guanosine 5-triphosphate (G) binding proteins. The activated G proteins can stimulate or inhibit adenylate cyclase, stimulate phosphatidylinositol turnover, and directly regulate ion channels, thereby effecting a variety of cellular responses (6). Other members of this class that have recently been cloned are the visual pigment proteins or opsins (7) and α -adrenergic receptors (8, 9). Hydrophobicity analysis of each of these receptor sequences suggests the presence of seven transmembrane domains. In fact, they all have substantial amino acid sequence conservation, especially in regions proposed to be transmembrane domains (3, 8). The sequence conservation among these receptors suggested that cDNAs of other receptors coupled to G proteins might be isolated by hybridization to probes derived from the highly conserved regions. Using such a probe to screen a rat cerebral cortex cDNA library,

we isolated three different clones. One of these clones is the rat homolog of the porcine brain muscarinic receptor. The other two clones are closely related to, but distinct from, the brain and cardiac muscarinic receptors; expression of these clones in mammalian cells establishes that they are also muscarinic receptors.

Our cloning strategy for the most effective identification of a variety of G protein-- coupled receptors had three major components. (i) We used a rat cerebral cortex cDNA library of 7 10^6 clones in the pcD mammalian expression vector (10), which favors production of relatively complete cDNAs and which facilitates the expression of clones in mammalian cells. (ii) We used as probe a sequence that not only was highly conserved among the known receptor sequences but also was located near the 5' end of the coding sequence so that cDNAs that hybridized were likely to contain the complete coding sequence. The sequence chosen (3-TC TGT CAG TTG TTG ATG AAG GAC GAC TCG GAC CGG ACA CGA CTG GAG TAG TAC CCG-5') was taken from the beginning of the second transmembrane region of the porcine brain cDNA (nucleotides 170 to 225 of the noncoding strand) but modified at positions 5, 38, and 53 to more accurately reflect mammalian codon usage and to better approximate the hamster α -adrenergic receptor sequence from which it differs by 14 bases. (iii) We characterized the clones before isolation by using Southern blots of portions of the library containing as many as 5 10^5 clones, a procedure that was facilitated by having a plasmid rather than a phage vector (11). Assuming that the first two components have assured that all the detectable clones corresponding to a particular message are nearly the same size, the blots allowed us to focus on representatives of the major size classes revealed with a given restriction enzyme (*Xho* I or *Bam* HI) and within the size classes to focus on the largest cDNA. Once a band of interest was identified, the pool containing the band was used to start new cultures containing tenfold fewer clones per pool and the blot analysis was repeated. Two to three cycles of subdivision allowed a pool to be produced in which the band of interest was the only hybridizing band and from which the corresponding clone was easily isolated by colony hybridization. The validity of the strategy is established by the fact that the first four clones (12) represented three different gene products and contained the complete coding sequence of two of them. The use of these clones as probes to reexamine the whole library at high stringency indicated that there were no copies of these cDNAs in the library that were substantially longer than the ones we had isolated.

The amino acid sequences derived from the three cDNA

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sequences (13) are compared to each other and the porcine receptors in Fig. 1. The second clone isolated encodes a 458-amino acid protein with potential glycosylation sites at residues 2 and 12. The 98% identity of this clone to the porcine m1 clone establishes that it is the rat homolog and it will accordingly be called the m1 clone (14). Of the ten amino acid substitutions, three are located near the amino terminus (Val Ala5), in transmembrane region VI (Leu Val374), and in transmembrane region VII (Val Ile411) whereas the remainder are in the large loop between regions VI and VII. By analogy with the structure of rhodopsin where the extracytoplasmic and cytoplasmic loops are identified (15), this loop is presumably cytoplasmic. The fourth clone isolated encodes a 589-amino acid protein with potential glycosylation sites at amino acids 6, 15, 41, 48, and 52 whereas the third clone isolated represents an incomplete version of this clone. Since it differs from both the m1 clones and the porcine m2 clone but will be shown below to be a muscarinic receptor, it will be referred to as the m3 receptor. The first clone isolated does not contain the complete coding sequence, but comparison with the corresponding human gene suggests that it lacked only the first five nucleotides of the coding sequence. Two additional cDNAs of approximately the same size were isolated and proved to begin at exactly the same position even though differing lengths of the G tails, which were used to prime the synthesis of the second strand of cDNA, suggested that they were independent clones. Assuming the first five nucleotides are the same as those in the human gene, this cDNA would encode a protein of 478 amino acids with potential glycosylation at residues 3, 8, and 13. This clone is called the m4 receptor.

Comparison of these sequences with those of the porcine receptors shows a high degree of conservation in the regions corresponding to postulated transmembrane regions I through V and VI through VII with much less homology in the amino and carboxyl terminal regions (preceding region I or following region VII). In addition, the large cytoplasmic loop between transmembrane regions V and VI is variable in size (157 to 203 amino acids) and shows little similarity among m1 through m4 except for a large fraction (17 to 20%) of basic residues. This loop is much smaller (approximately 30 to 50 residues) in the -adrenergic receptor and rhodopsin. With this loop and the amino and carboxyl terminals excluded from the comparison, the m3 sequence has 79% identity to the m1 sequence and the m4 sequence has 65% identity to m1. However, m4 is more closely related to the porcine m2 sequence with 85% identity. The much poorer homology in the amino terminal and large cytoplasmic loop indicates that m4 is not the rat homolog of the porcine m2. Cloning of two different human genes closely related (97 and 95% overall) to m2 and m4, respectively, confirms that they are

distinct genes. The nonconservative substitutions in the muscarinic receptor sequences are located primarily in the i3 and i4 cytoplasmic domains and in the o1 through o4 external domains. The positions at which only conservative changes occur in the muscarinic, adrenergic, and opsin sequences are clustered in the inner half of the transmembrane domains and near the inner face of the membrane.

The m1 and m3 rat genes were isolated from a partial Eco RI rat genomic library (16) and sequenced. All but 20 nucleotides of the m3 cDNA sequence are found in a single continuous stretch of 3507 bases; this result demonstrates that there are no introns in the coding or 3 untranslated sequence. However, since the cDNA and gene sequences diverge prior to base -19 (numbering from the first base of coding sequence) and since the gene sequence at this point (-40 to -20) matches the consensus splice acceptor sequence (17), there must be an intron in the 5 untranslated sequence. Similarly the entire 2528 bases of the m1 cDNA is found in a continuous stretch in the m1 gene. There is a potential splice site at -83 to -70 of the gene sequence, which has been verified by ribonuclease mapping of the cerebral cortex message (18). We also screened a partial Hae III-Alu I human genomic library (19) and obtained the human m2 and m4 genes. The human m4 sequence encodes a 478-amino acid protein that differs from the rat protein by 24 amino acid substitutions, 1 amino acid deletion, and 1 amino acid insertion. Of the 25 amino acid changes, 15 occur in the large cytoplasmic loop and 6 occur in the amino terminal extracytoplasmic tail. This gene has no introns in the coding sequence but has a potential splice acceptor site 30 to 53 bases upstream of the initiation codon. The human m2 gene encodes a 466-amino acid protein which differs from the porcine m2 receptor by 13 amino acid substitutions, 11 of which occur in the large cytoplasmic loop. It also has no introns in the coding sequence but the 5 untranslated sequence is sufficiently well conserved relative to the pig m2 cDNA sequence to allow the identification of a splice acceptor site at bases -67 to -47. Thus all four genes lack introns in the coding sequence and, as far as has been ascertained, in the 3 untranslated sequence as well. In this regard they are similar to -adrenergic receptor gene but differ from the mammalian opsins which have four introns interrupting the coding sequence. In contrast to the adrenergic receptor where no intron has as yet been identified in the 5 untranslated sequence, the muscarinic receptor genes contain either a splice site or a potential splice site less than 100 bases upstream of the initiation codon. The lack of introns in such a large coding sequence is unexpected for a multiple domain receptor in light of arguments that suggest that introns facilitate evolution of new functions by allowing reassortment of functional domains of proteins (20).

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To establish that the clones are actually muscarinic receptors we have expressed them in mammalian cells by making use of the SV40 promoter incorporated into the pcD vector. Because the m1 and m3 rat cDNAs contained complete coding sequences, these clones could be used directly for transfection. However, because the m4 cDNA lacked an initiation codon, we have substituted human m4 5 untranslated and coding sequence for the rat coding sequence of the m4 cDNA clone, thereby making a hybrid DNA containing human coding and rat 3 untranslated sequences. The two sequences were joined at an Avr II restriction site that occurs at the beginning of the 3 untranslated sequence of both clones. Because there was no convenient restriction site in the 30 bases between the initiation codon and the possible splice acceptor site with which to join the coding sequence to the SV40 promoter, we used a Sac I site located 506 to 511 bases upstream and ligated it to the Bam HI site in the SV40 intron of the pcD vector, using the Bam HI-Sac I portion of the pUC18 multiple cloning site as a linker. This construct removes the 19S splice acceptor as well as the SV40 19S initiation codon, forcing the use of an initiation codon in the human sequence and potentially using the putative human splice acceptor site. A similar construct was created with the human m2 gene and the rat m4 cDNA for expression of the human m2 receptor in which the 5 end of the human sequence is defined by a Dra I site (-43) and the 3 end by an Avr II site (1556 to 1561) in the 3 untranslated region. The 5 end was ligated to the pcD vector at the Pst I site which normally defines the 5 end of a cDNA using a Pst I-Hinc II linker from pUC18. The Avr II site was ligated to the Avr II site of the rat m4 cDNA so that most of the 3 untranslated sequence and the polyadenylated tail are derived from the m4 cDNA. The m1, m3, and hybrid m2 and m4 DNAs were transfected into mouse fibroblast (A9) or COS-7 cells. To assay the transfectants for the presence of muscarinic receptor, membranes were prepared from the transfectants 48 hours after transfection and assayed for their ability to bind specifically the muscarinic antagonist [3H]quinuclidinyl benzilate (3H-QNB). Transfected COS-7 cells expressed 2×10^4 to 2×10^5 receptors per cell in different experiments while transfected A9 cells expressed 1×10^4 to 2×10^4 receptors per cell. Neither host cell expressed any muscarinic receptors before transfection. Displacement of 3H-QNB binding with the selective antagonist pirenzepine (Fig. 2) demonstrated that m1, m3, and m4 transfectants had a high affinity for pirenzepine. Thus the m1, m3, and m4 receptors would probably have been previously classified as M1 receptors. Human m2 transfectants had a low affinity for pirenzepine, consistent with its M2 classification and the results with the porcine m2 clone (5). Displacement of 3H-QNB binding by the muscarinic agonist carbachol also displayed inhibition constant (IC₅₀) values in the micromolar range.

To determine how many muscarinic genes are in the genome, we have examined a genomic blot of rat and human DNA with a 516-base probe corresponding to transmembrane regions II to V of the rat m1 gene (Fig. 3). Hybridization at moderate stringency reveals nine to ten bands in each rat digest and five or six bands in each human digest. Comparison of the restriction map of the rat m1 and m3 genes and the human m2 and m4 genes as well as high-stringency hybridization of the four genes allowed the identification of one and only one band in each digest as corresponding to each of the four known genes. Thus there are one or two additional members of the muscarinic gene family in the human genome and potentially as many as five additional members in the rat genome. The difference between the number of bands detected in rat and human suggests that some of these genes may be pseudogenes, but determination of their functionality will require their cloning and sequencing and, possibly, their expression.

As a first step in a detailed mapping of the distribution of the muscarinic receptors in the rat, we have performed *in situ* hybridization to mRNA with oligonucleotides (21) derived from the amino terminal coding sequence of the rat m1, m3, and m4 sequences. These probes are highly specific for the individual receptors since they have essentially no sequence homology to each other. On RNA blots they detected mRNA species of approximately 3000, 5200, and 3100 nucleotides for m1, m3, and m4, respectively. All of these mRNA species were more abundant in brain than in heart. *In situ* hybridization (Fig. 4) indicates that m1, m3, and m4 mRNAs are abundant in cerebral cortex, with m1 and m4 uniformly distributed but m3 localized primarily in the inner and outer layers. The m1 and m3 mRNAs are highly abundant in hippocampus but m1 is much more abundant than m3 in the dentate gyrus; m4 mRNA is only moderately abundant in hippocampus and of low abundance in dentate gyrus. Thus M1 receptors previously characterized in brain are a mixture of m1, m3, and m4 receptors.

Although there have been only two pharmacologically well-defined classes of muscarinic receptors, we have established that there are four distinct muscarinic receptor genes. Why are there so many if only a single subunit is required to make a functional receptor? A formal possibility is that the receptors are composed of heterogeneous subunits but that receptors formed from a single subunit will function *in vitro*. There is a precedent for such a proposal in the ability of the nicotinic acetylcholine receptor lacking either the α or β subunits to function, albeit poorly (22). However, the available data on mRNA distribution indicate that there are large variations in the stoichiometry of the receptors, making this possibility seem less probable. The possibility of differential regulation of

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the mRNAs as indicated by their tissue specificity provides a partial justification for the number of different receptors. However, we suspect that the tissue specificity may in turn reflect an underlying functional variation. Cultured 1321N1 astrocytoma cells have a muscarinic response characterized by a stimulation of phosphatidylinositol turnover but an absence of any adenylate cyclase inhibition whereas NG108-15 neuroblastoma-glioma hybrid cells show a muscarinic inhibition of adenylate cyclase but no stimulation of phosphatidylinositol turnover. These differences reflect a difference in the receptors rather than in the competence of the cells for the particular response when stimulated with other ligands (23). Thus the variety of receptor genes may well reflect specialization of different forms of the receptor for different coupling mechanisms. Since different types of muscarinic receptor are often mixed in a given tissue, cell lines stably expressing a single receptor will be essential in characterizing the different receptors (24).

A detailed study of the relation between the structure and function of the receptors should also be possible, with the similarities and dissimilarities in amino acid sequence of the four receptors providing valuable clues to structurally important features. Studies involving deletions of the extracytoplasmic loops of the α -adrenergic receptor (25) have already indicated that binding of ligand is not determined primarily by the extracytoplasmic domains, thereby implying that binding occurs to the transmembrane regions. The high degree of conservation of the i2 cytoplasmic loop suggests that it might be involved in the more general ability to bind G proteins. Because deletion from the carboxyl terminal of the i3 cytoplasmic loop eliminates adenylate cyclase stimulation in the receptor, this region may provide the specificity in cellular effector systems. Although this loop is highly variable among the muscarinic receptor types, it is nevertheless well conserved between species.

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12. Complementary DNA clones were identified by hybridization to blots made from plasmid minipreparations of pools of clones in the cDNA library. The blots were hybridized in 6 standard saline citrate (SSC) (0.9M NaCl, 0.09M sodium citrate, pH 7.0) at 60°C to the 56-nucleotide oligonucleotide that had been labeled with ^{32}P by using T4 polynucleotide kinase. Blots were washed under the same conditions as hybridized. The three cDNAs were identified as Bam HI and Xho I bands of the following sizes (in kilobases): m1, 1.8 and 2.9; m3, 2.8 and 3.9; and m4, 2.4 and 1.05. The 0.51-kb Sac I-Pst I fragment corresponding to amino acids 55 to 224 of the m1 cDNA was cloned into the pSP64 plasmid to make clone cRm1p5, which was used as probe to screen genomic libraries by hybridization in 3 SSC at 60°C.
13. To conserve space the 8.7 kb of cDNA sequence and 10.5 kb of sequence from the two rat genes and the two human genes described below are not shown but have been deposited in the GenBank database (accession numbers M16404 to M16409). The m1, m3, and m4 cDNAs contain 1085, 1769, and 1145 bases of 3 untranslated and 62, 40, and 0 bases of 5 untranslated sequence, respectively. The sequences homologous to the oligonucleotide probe differ from the probe by 1, 7, and 9 nucleotides, respectively.
14. We propose that the porcine brain clone and its homologs from other species be called the m1 receptors, not to signify the M1 receptor classification but to represent the first muscarinic receptor of defined primary structure. The porcine cardiac receptor thus becomes the m2 receptor as the second receptor to be sequenced, and the two new receptors that we have identified become the m3 and m4 receptors.
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Photo: Fig. 1. Comparison of the deduced amino acid sequences of receptors m1 through m4. The names of the sequences are prefixed with P (pig), R (rat), or H (human) to identify the species. Dashes signify gaps introduced to align the sequences. The transmembrane domains of the proteins are delineated by dashes above the sequence and are numbered I through VII. The proposed outer and inner domains are labeled, respectively, o1 through o4 and i1 through i4. Asterisks immediately above the aligned sequences indicate positions at which all the muscarinic sequences are identical. Open circles below the sequences indicate positions at which there are nonconservative substitutions in at least one muscarinic sequence where conservative substitutions are defined as

changes between members of one of the following groups: S (Ser), T (Thr), P (Pro), A (Ala), and G (Gly); N (Asn), D (Asp), E (Glu), and Q (Gln); H (His), K (Lys), and R (Arg); M (Met), I (Ile), L (Leu), and V (Val); F (Phe), Y (Tyr), and W (Trp); or C (Cys) (26). Dark dots below the sequence identify positions at which there are only conservative changes when this group is compared to the rhodopsin and -adrenergic receptor sequences. Most of the amino acids in i3 have not been aligned since they show almost no sequence identity between different receptors. A schematic diagram of the receptors is shown at the bottom with dark circles representing the residues conserved in rhodopsin and -adrenergic receptor and open circles or boxes representing positions at which there are nonconservative changes within the muscarinic sequences.

Photo: Fig. 2. Pharmacological characterization of muscarinic receptor clones. COS-7 cells (2 $\times 10^6$ per 10-cm plate) were cultured overnight and transfected with 20 μ g of plasmid DNA by calcium phosphate precipitation (27). Fresh medium was applied after 24 hours and cultures were incubated for an additional 24 hours, at which point cells were scraped from the dish, homogenized, and pelleted. The membranes were suspended in binding buffer (10 mM HEPES, pH 7.4, 5 mM MgCl₂) at a protein concentration of 50 μ g/ml. Aliquots (25 μ g) were incubated for 2 hours at room temperature in a total volume of 1 ml containing 100 pM 3H-QNB [adapted from (28)]. Nonspecific binding was defined with 1 M atropine. Data were analyzed by nonlinear regression with the following function

$$\% \text{ bound} = 100 - 100(X/IC_{50})/(1 + X/IC_{50})$$

where IC₅₀ is the inhibition constant, and X is the ligand concentration. Lines are computer-generated fits to the data: m1, IC₅₀ = 35.3 nM; m2, 1340

190 nM; m3, 156.38 nM; and m4, 36.8 nM. The affinity constants (K(D)) for 3H-QNB for the m1 (32 pM), m2 (59 pM), m3 (14 pM), and m4 (81 pM) transfectants were similarly determined with 2 to 200 pM 3H-QNB.

Photo: Fig. 3. Genomic blot of rat and human DNA. About 2 μ g of rat or human DNA was digested with Eco RI or Pst I, electrophoresed in a 0.7% agarose gel, and blotted onto nitrocellulose. The blot was hybridized to cRm1p5 probe in 3 SSC at 60°C and washed under the same conditions. The bands (rat Eco RI, rat Pst I, human Eco RI, human Pst I; in kilobases) corresponding to individual receptor genes as identified by high-stringency hybridizations are: m1 (4.8, 2.7, 3.8, 3.5), m2 (8.5, 2.8, 6.2, 11), m3 (14, 6.4, 7.5, 2.7), and m4 (20, 1.7, 18, 0.8).

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Photo: Fig. 4. Localization of muscarinicreceptor mRNA in a coronal section of rat brain: 48- or 45-base oligonucleotides complementary to sequence encoding amino acids 2 to 17 (m3: GCT GAT GTT GGG AAA CAA AGG CGA GGT TGT ACT GTT ACT GTG CAA GGT), 3 to 18 (m1: TGG TGC CAA GAC AGT GAT GTT GGG ACT GAC AGC AGG GGG CAC TGA GGT), or 3 to 17 (m4: GCG CAC AGA CTG ATT GGC TGA GCT GCC ATT GAC AGG CGT GAA GTT) of the rat receptors were ³⁵S-labeled and hybridized as described (21). The hybridized probe was visualized by exposure to x-ray film for 35 days. Regions rich in mRNA appear light, and nonspecific hybridization, as defined with similar probes to mRNAs not expressed in brain (transducin, rhodopsin), was homogeneous and equivalent to the darkest areas. Heavily labeled structures include the hippocampus (H; m1 and m3), and dentate gyrus (DG; m1). Cortex (CX) is moderately labeled with all three probes.