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Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors

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To investigate the molecular basis for the diversity in muscarinic cholinergic function, we have isolated the genes encoding the human M1 and M2 muscarinic receptors (mAChR) as well as two previously undiscovered mAChR subtypes, designated HM3 and HM4. The amino acid sequence of each subtype reflects a structure consisting of seven, highly conserved transmembrane segments and a large intracellular region unique to each subtype, which may constitute the ligand-binding and effector-coupling domains respectively. Significant differences in affinity for muscarinic ligands were detected in individual mAChR subtypes produced by transfection of mammalian cells. Each subtype exhibited multiple affinity states for agonists; differences among subtypes in the affinities and proportions of such sites suggest the capacity of mAChR subtypes to interact differentially with the cellular effector-coupling apparatus. Subtype-specific mRNA expression was observed in the heart, pancreas and a neuronal cell line, indicating that the regulation of mAChR gene expression contributes to the differentiation of cholinergic activity.

Key words: muscarinic receptors/receptor subtypes/ligand binding/gene regulation

Introduction

Synaptic transmission by muscarinic acetylcholine receptors (mAChRs) is employed throughout the central and peripheral nervous systems to elicit a large and diverse array of neurophysiological actions. In the autonomic nervous system mAChRs regulate the force and rate of heart muscle contraction, the contraction of smooth muscle, and the secretory activity of glands which receive parasympathetic innervation (Taylor, 1985). mAChRs also constitute the majority of cholinergic synapses within the central nevous system, where they are thought to be important to such neural processes as learning and memory (Nathanson, 1987). An important aspect of mAChR functional diversity is reflected by the multitude of biochemical and electrophysiological actions evoked by acetylcholine binding to mAChRs, which include the regulation of intracellular levels of cAMP, cGMP and inositol phospholipids, and the opening or closing of the potassium, calcium and chloride ion channels found in certain tissues (Nathanson, 1987). Given its ubiquitous role in neuronal signal transduction, a major question in the study of cholinergic function relates to the molecular mechanisms which underly the biological specialization of muscarinic activity.

The potential for structural heterogeneity between mAChRs was initially discerned through the discovery of selective muscarinic agents which exhibit different affinities for receptors in various tissues (Hammer *et al.*, 1980; Birdsall and Hulme, 1983; Cohen and Sokolovsky, 1987). Studies with selective antagonists such as pirenzepine, which discriminates between the high affinity M1 receptors in certain regions of the brain and the low affinity M2 receptors of heart, have permitted tentative proposals of functional differences between subtypes in their abilities to regulate specific biochemical effector systems (Gil and Wolfe, 1985; Brown *et al.*, 1985). Molecular cloning of the porcine cerebral (M1) and atrial (M2) mAChRs has established that the major receptor subtypes of brain and heart are indeed different polypeptides encoded by distinct genes which share 38% overall amino acid homology (Kubo *et al.*, 1986a,b; Peralta *et al.*, 1987). Evidence for the existence of previously uncharacterized genes encoding novel mAChR subtypes has been provided by studies with other selective muscarinic compounds (Giraldo *et al.*, 1987; Korc *et al.*, 1987), as well as hybridization analysis of mammalian genomic DNAs which revealed additional sequences homologous to but distinct from the M1 and M2 mAChR genes (Peralta *et al.*, 1987). To provide a basis for investigating mAChR functional diversity, we have isolated the genes for the human HM1 and HM2 mAChRs, as well as two novel mAChR subtypes, herein designated HM3 and HM4. Characterization of the primary structures of these four human mAChR subtypes, their agonist and antagonist binding properties, and their patterns of expression in various tissues reveals that all four mAChRs indeed represent functional proteins encoded by distinct, actively transcribed genes, and suggests unique biological functions for each receptor subtype.

Results and discussion

Molecular cloning of human muscarinic receptors reveals two novel subtypes

To isolate the genes encoding human mAChRs, we employed a strategy based upon sequences conserved between the porcine M1 and M2 mAChR subtypes, as well as similarities with other known receptors which transduce signals through interaction with guanine nucleotide binding (G) proteins. Since the protein coding sequences of the M2 mAChR and hamster β-adrenergic receptor are each contained within a single exon (Dixon *et al.*, 1986; Peralta *et al.*, 1987), a genomic library was screened for receptor clones with the expectation that human mAChR genes would also lack introns within their coding regions. The porcine M1 and M2 mAChRs exhibit the greatest degree of amino acid identity in their first five transmembrane domains and connecting loops (Peralta *et al.*, 1987); therefore low stringency hybridizations were carried out with a fragment containing this region to identify related human sequences. In addition, the M1 and M2 mAChRs share the most similarity to each other as well as to β-adrenergic receptors and the visual rhodopsins in their first cytoplasmic loop and second transmembrane domain, a possible site of G protein interaction (Zuker *et al.*, 1985; Dixon *et al.*, 1986; Nathans *et al.*, 1986; Yarden *et al.*, 1986; Peralta *et al.*, 1987). To isolate

Fig. 1. Primary sequence homology between the four human muscarinic receptor subtypes. Since maximum homology occurs between HM1 and HM4 or HM3 and HM2, the sequence alignment is presented in this order. Gaps were introduced to maximize significant sequence identity and to compensate for the large size of the cytoplasmic loop 5–6 of HM4. Identities are indicated by the boxed amino acid residues. Asterisks denote potential N-linked glycosylation sites in the amino-terminal domains. Regions I–VII are putative transmembrane domains (Peralta *et al.*, 1987) and are indicated by lines over the hydrophobic sequences. The hydrophilic loops are numbered according to the transmembrane regions which they connect and their orientations are indicated with respect to the extracellular (outer, o) or cytoplasmic (inner, i) side of the plasma membrane. The amino-terminal region is indicated by H_2N- and is postulated to be extracellular, while the carboxy-terminal region is indicated by -COOH and is predicted to reside on the cytoplasmic side of the plasma membrane. Numbers at the left denote the position within each protein sequence of the first residue in each line.

clones with this highly specific local identity, candidates were then characterized for their ability to hybridize with two sets of oligonucleotide probes based upon the corresponding porcine M2 mAChR sequences (amino acids 55–62 and 64–70, detailed in Materials and methods) (Peralta *et al.*, 1987). Eighteen of 23 clones hybridizing with the M2 mAChR restriction fragment were also found positive with both sets of oligonucleotides, and could be divided into four classes based on their ability to hybridize preferentially with individual members of each pool of oligonucleotides. Since the porcine M1 and M2 mAChRs are strikingly dissimilar in their large third cytoplasmic loop (Peralta *et al.*, 1987), probes consisting of these subtype-unique sequences were used to identify one class of the human clones as the homologue of the porcine cerebral M1 mAChR, and a second class as the homologue of the porcine atrial M2 mAChR. Nucleotide sequence analysis of the M1 homologue predicts a polypeptide of 460 amino acids sharing 98.9% identity with the porcine M1 mAChR (Kubo *et al.*, 1986a), while the M2 homologue sequence reveals a protein of 466 amino acids with 97.4% identity to the porcine M2 mAChR subtype (Peralta *et al.*, 1987) (Figure 1). The virtual sequence identity between each pair of human and porcine mAChR subtypes, together with their ligand binding characteristics (see below), suggests that these clones encode the human M1 and M2 mAChR subtypes, thus designated HM1 and HM2 respectively. HM1 and HM2, like the porcine M1 and M2 mAChR subtypes, share 39% overall amino acid identity.

Sequence analysis of the remaining two classes of human clones with homology to the porcine mAChRs revealed that each class encodes a polypeptide highly homologous to, yet quite distinct from the HM1 and HM2 subtypes (Figure 1). The first of these, designated HM3, predicts a protein of 479 amino acids and M_r 53 049 sharing 40 and 55% overall amino acid identity with HM1 and HM2 respectively. The other novel class of clones, designated HM4, encodes a significantly larger protein of 590 amino acids and M_r 66 127 displaying 43, 35 and 37% overall identity with HM1, HM2 and HM3 respectively (Figure 1). Analysis of cDNA clones corresponding to each class of mAChR indicates that all four human mAChR subtypes lack introns in their respective coding regions (unpublished data). Accuracy of the initiation and termination codon assignments shown in Figure 1 is further indicated by the ability to express muscarinic ligand-binding activity utilizing these predicted signals (see below).

Each human muscarinic receptor contains a large cytoplasmic domain unique to each subtype

Hydropathicity analysis by the method of Kyte and Doolittle (1982) indicates that each of the four human mAChRs contains seven hydrophobic, potential transmembrane domains, suggesting that all four subtypes adopt a structure within the plasma membrane similar to that proposed for rhodopsin and β-adrenergic receptors, with an extracellular amino terminus and intracellular carboxy terminus (Figure 1) (Zuker *et al.*, 1985; Dixon *et al.*, 1986; Nathans *et al.*, 1986; Yarden *et al.*, 1986; Peralta *et al.*, 1987). The high degree of identity between all four subtypes within these membrane-spanning segments and the short connecting loops (65%) (Figure 1) supports our previous proposal that binding of acetylcholine may well occur in the cleft created upon insertion of these hydrophobic domains into the lipid bilayers (Peralta *et al.*, 1987). Two aspartic acid residues are conserved among all four membrane subtypes in the otherwise highly hydrophobic second and third transmembrane domains suggesting that these negatively charged residues may participate directly in the binding of the endogenous neurotransmitter acetylcholine, a quaternary amine.

In contrast to the virtual identity between the human and porcine homologues of the M1 or M2 mAChRs, which suggests severe functional constraints on the structures of these pairs of subtypes, all four human subtypes diverge dramatically in the large loop connecting transmembrane domains 5 and 6 (Figure 1). The length of this region differs significantly between the four mAChR subtypes, consisting of 156, 181, 184 and 241 residues in HM1, HM2, HM3 and HM4 respectively. By analogy with rhodopsin, the 5–6 loop region should reside on the cytoplasmic side of the plasma membrane, suggesting that the exceptional degree of divergence between mAChR subtypes in this region may be related to their capacity for differential coupling to distinct biochemical effectors or ion channels (Nathanson, 1987). Limited homology occurs in the 5–6 loop region adjacent to the transmembrane domains, as well as the serine, threonine and acidic residue-rich middle region of the loop, between HM1 and HM4 (22%) and between HM2 and HM3 (21%), suggesting greater functional similarity between these pairs of mAChR subtypes (Figure 1). Southern blot analysis of genomic DNA utilizing probes derived from the 5–6 loop of each subtype indicates that the human genome does not encode other sequences highly homologous to these unique mAChR domains (data not shown). Limited amino acid conservation is also evident in the amino-terminal regions of the four subtypes, which vary in length from 22 to 67 residues. However, a conserved feature of the amino terminus of all four subtypes is the presence of two to five potential sites for N-linked glycosylation (Figure 1), a feature common to rhodopsins and the β-adrenergic receptor (Zuker *et al.*, 1985; Dixon *et al.*, 1986; Nathans *et al.*, 1986; Yarden *et al.*, 1986). The high degree of native mAChR glycosylation (Nathanson, 1987), as well as an absence of such sites in the other predicted extracellular regions of each subtype, suggest the functional similarity of these otherwise unrelated amino-terminal sequences.

The four human muscarinic receptor subtypes exhibit distinct antagonist binding properties

To confirm that the molecular clones isolated in this study encode functional mAChRs, and to determine whether different human mAChR subtypes exhibit distinct pharmacologic characteristics when presented in a similar cellular context, a transient mammalian expression system (Eaton *et al.*, 1986) was utilized to study the ability of each subtype to bind muscarinic compounds. Saturation binding experiments carried out with intact transfected cells or cell homogenates using the muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB) revealed that all four human mAChR genes encode polypeptides which specifically bind this radioligand with high affinity (Table I). The apparent dissociation constants (K_D) determined by Scatchard analysis of the saturation binding data ranged from 22.8 to 112.2 pM for intact cells and 16.6 to 173.5 pM for cell homogenates (Table I). The affinity of the HM4 subtype for [^{3}H]QNB was notably lower in both whole cells and homogenates than that of the other human mAChR subtypes (Table I), whereas less variation is typically seen in the binding of this compound by receptors expressed in a variety of animal tissues (Nathanson, 1987). Non-transfected cells expressed <200 endogenous mAChRs/cell, while transfected cells expressed 7000–90 000 receptors, depending upon the specific subtype and particular transfection.

Competition binding experiments using [^{3}H]QNB as the displaceable ligand were carried out to determine the affinity of additional anti-muscarinic drugs for each of the recombinant mAChR subtypes (Figure 2; Table I). The classic muscarinic antagonist atropine bound with high affinity to all four human

Table I. Apparent dissociation constants (K_D) for the binding of antagonists and agonists to the four human mAChR subtypes

		HM1	HM2	HM3	HM4
Antagonist-binding					
[^{3}H]QNB					
homogenate	K_D (pM)	16.6	26.5	37.5	173
cells	K_D (pM)	22.8	83.3	39.1	112
Atropine	K_D (nM)	3.3	16.6	1.2	1.1
Pirenzepine	K_D (μM)	0.5	12.5	2.7	1.2
AF-DX116	K_H[a] (nM)	50.0	20.0	–	300
	K_L[b] (μM)	6.8	0.8	2.6	1.1
	%H[c]	13.5	40.7	–	19.5
Agonist-binding					
Carbachol	K_H[a] (μM)	7.3	0.1	–	8.1
	K_L[b] (μM)	470	130	200	560
	%H[c]	30.1	28.4	–	6.5
Oxotremorine	K_H[a] (nM)	–	8.0	22.0	–
	K_L[b] (μM)	4.1	5.3	4.1	4.0
	%H[c]	–	20.0	5.1	–

The K_D values for [^{3}H]QNB were determined independently by Scatchard analysis of saturation binding curves obtained from the same population of intact cells (n = 4) or cell homogenates (n = 2) that were utilized for antagonist or agonist binding studies respectively. The statistical variation of each value and the mean receptor numbers determined from Scatchard analysis, expressed as [^{3}H]QNB binding sites/cell and fmol/mg homogenate protein are described in Materials and methods. All competition displacement data were fitted by non linear least squares regression with one or two site models and the best fit was selected by analysis of variance (Munson and Rodbard, 1980).

[a]K_D for the high affinity binding state.

[b]K_D for the low affinity binding state.

[c]The amount of high affinity receptors as a percentage of the total number of receptors.

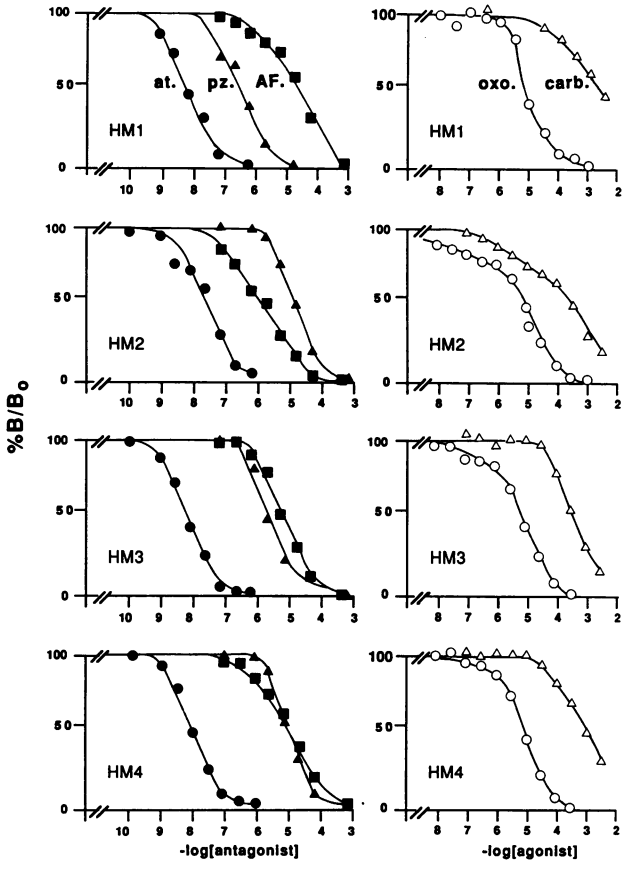

Fig. 2. Ligand binding properties of human mAChR subtypes expressed by transfection of mammalian cells. Competition displacement of [^{3}H]QNB binding by muscarinic antagonists (**left panel**) was studied in intact cells following transfection with each of the four muscarinic receptor clones; agonist binding (**right panel**) was determined with cell homogenates prepared as described in Materials and methods. The antagonists studied were atropine (●), pirenzepine (▲) and AF-DX116 (■). The agonists studied were carbachol (△) and oxotremorine (○). The apparent dissociation constants (K_D) of the different ligands are summarized in Table I.

subtypes, with HM1, HM3 and HM4 displaying similar K_D values (K_D = 3.3, 1.2 and 1.1 nM respectively). In contrast to the small regional variations in atropine affinity reported for various tissues (Birdsall and Hulme, 1983), the HM2 subtype bound atropine with significantly lower affinity (K_D = 16.6 nM) than the other three human mAChR subtypes as well as the natural porcine atrial M2 mAChR (K_D = 1.0 nM) (Schimerlik and Searles, 1980) or a recombinant porcine M2 mAChR (K_D = 4.1 nM) (Peralta *et al.*, 1987). Substantial differences were also detected in the abilities of the four human mAChRs to bind the selective muscarinic antagonist pirenzepine, which distinguishes between M1 and M2 mAChR subtypes (Hammer *et al.*, 1980), and the cardioselective antagonist AF-DX116 (Giraldo *et al.*, 1987). The affinity of pirenzepine for HM1 was 25-fold higher than for the HM2 subtype, a difference comparable with that observed for M1 receptors of rat cerebral cortex and hippocampus and M2 receptors of rat atria, confirming the assignment of the HM1 and HM2 subtypes (Table I). Notably, the affinity of HM3 and HM4 for pirenzepine is much closer to that of HM1 than HM2 (Table I), suggesting that these mAChR subtypes may have been characterized as M1 receptors by previous investigators.

Of the four recombinant human mAChRs, only HM3 exhibited homogeneous binding to the antagonist AF-DX116 (Figure 2). Binding of AF-DX116 to the other three mAChR subtypes was heterogeneous and best fit to computer models assuming two classes of sites, indicating that this drug distinguishes between high and low affinity states for each of these receptors. The value calculated for the single affinity state of the HM3 subtype (K_D = 2.6 μM) was comparable with the low affinity state of the HM2 and HM3 subtypes (K_D = 0.8 and 1.1 μM respectively), whereas the low affinity state of HM1 was significantly lower (K_D = 6.8 μM). HM2 displayed the largest proportion of high affinity AF-DX116 binding sites (40.7% of the total receptor sites) as well as the highest affinity (K_D = 20 nM) of the four human mAChRs for this ligand (Table I). Thus, the HM2 subtype is most similar to M2 receptors of porcine and rat atria in displaying the highest overall affinity for AF-DX116 (Giraldo *et al.*, 1987; Korc *et al.*, 1987).

Multiple agonist binding affinity states are a property of each muscarinic receptor subtype

In contrast to the single class of binding sites recognized by most muscarinic antagonists, agonist binding generally reveals the existence of multiple affinity binding states (Nathanson, 1987). The proportion of high and low agonist affinity sites can be altered by guanine nucleotides suggesting that heterogeneous agonist binding reflects conformation states of receptor which arise through interactions with G proteins (Nathanson, 1987). However, since individual subtypes may bind a given agonist with different affinities, and respond differently to guanine nucleotide addition, the presence of multiple mAChR subtypes within a single tissue could possibly give the appearance of heterogeneous agonist binding independent of the effects of G proteins. To determine the agonist binding properties of individual human mAChR

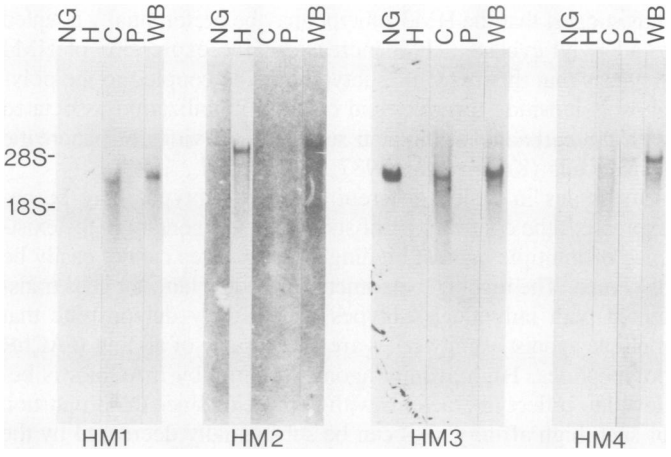

Fig. 3. Tissue specificity of mAChR subtype gene expression. Polyadenylated mRNA was isolated from the indicated rat tissues, denatured, separated by electrophoresis through a 1.1% agarose–formaldehyde gel (Maniatis *et al.*, 1982), transferred to nitrocellulose and hybridized with uniformly ^{32}P-labeled restriction fragments specific for each subtype. mRNA samples include: NG108 (NG), heart (H), cerebral cortex (C), pancreas (P) and whole brain (WB); each lane contained 5 μg of each type of mRNA, except cerebral cortex samples which contained 3 μg/lane. The positions of 28S and 18S ribosomal RNA are indicated. Hybridization of the filter with a ^{32}P-end-labeled oligonucleotide encoding a portion of the mouse alpha-actin coding region (Alonso *et al.*, 1986) was performed to confirm that equivalent amounts of each type of mRNA were present in the corresponding lanes (not shown).

subtypes, [^{3}H]QNB competition binding analysis was carried out with the muscarinic agonists oxotremorine and carbachol, a close structural relative of the natural neurotransmitter acetylcholine. As seen in Figure 2, heterogeneous binding was readily apparent for HM1, HM2 and HM4 with carbachol, and for HM2 and HM3 with oxotremorine. In each case, computer analysis of the binding data gave a statistically better fit with a two site than a one site model (Table I). Oxotremorine exhibited substantially higher affinity than carbachol for both classes of sites for a given human mAChR subtype (Table I), consistent with binding studies performed with tissue preparations (Schimerlik and Searles, 1980; Peterson and Schimerlik, 1984). The low affinity binding states calculated for either agonist were similar for each of the four mAChR subtypes, with K_D values ranging from 130 to 560 μM for carbachol, and 4.0 to 5.3 μM for oxotremorine. The high affinity carbachol binding state was notably higher for HM2 (K_D = 0.1 μM) than for HM1 and HM4 (K_D = 7.3 and 8.1 μM respectively), and in the range of the high affinity carbachol sites reported for rat cerebral M1 and porcine atrial M2 mAChRs (K_D = 1.41 and 0.83 μM respectively) (Fisher *et al.*, 1983; Peterson and Schimerlik, 1984; Collins and Crankshaw, 1986). The high affinity binding states of HM2 and HM3 for oxotremorine were comparable (K_D = 8.0 and 22.0 nM respectively) and similar to the porcine atrial M2 mAChR (K_D = 2.3 nM) (Peterson and Schimerlik, 1984). Significantly, the appearance of multiple affinity states for either oxotremorine or carbachol upon transfection of each human mAChR subtype cannot be attributed to endogenous mAChRs since their level (<200 receptors/cell) cannot account for even the smallest population of high affinity agonist binding sites observed in these experiments. Furthermore, the variable proportion of high and low affinity (or absence of high affinity) binding sites detected for each human mAChR subtype suggests that the four subtypes may differ in their abilities to couple efficiently with the G proteins present within these cells.

Tissue-specific expression of muscarinic receptors

Binding studies with selective muscarinic antagonists have suggested that mAChR subtypes are differentially expressed in various mammalian tissues (Hammer *et al.*, 1980; Birdsall and Hulme, 1983; Giraldo *et al.*, 1987). To directly investigate the expression of mAChRs in tissues in which mAChR subtypes have been pharmacologically defined, RNA isolated from rat heart, whole brain, cerebral cortex, pancreas and NG108-15 rat/mouse neuroblastoma × glioma cells was subjected to Northern blot analysis under stringent hybridization conditions utilizing probes derived from the subtype-specific sequences contained in the 5–6 loop region of each human mAChR. Each probe detected a discrete species of mRNA within a subset of the tissues or cells analyzed (Figure 3); the estimated sizes of the mRNAs corresponding to each subtype were 3.1 kb for HM1, 5.2 kb for HM2, 3.3 kb for HM3 and 4.5 kb for HM4. Notably, expression of each rat mAChR subtype mRNA was detected in whole brain, providing definitive evidence for the transcription of the novel HM3 and HM4 genes, and suggesting a role for each subtype in central nevous system function. In contrast, subtype-specific expression was observed in other tissues. Heart mRNA was found to hybridize only with the HM2 probe, supporting the M2 subtype assignment of this human mAChR clone. With pancreatic RNA significant hybridization was observed only with the HM4-specific probe, suggesting that this subtype corresponds to the 'glandular' mAChR subtype described in earlier studies (Figure 3) (Giraldo *et al.*, 1987; Korc *et al.*, 1987). This observation is consistent with the significantly larger size previously detected for the mAChR found in pancreatic acinar cell preparations, as expected for HM4 (Hootman *et al.*, 1985). Interestingly, expression of mAChR RNA in NG108-15 cells is restricted to HM3, suggesting that biochemical events mediated by muscarinic agonists in this neuron-derived cell line may largely reflect binding to this subtype.

Biological role of muscarinic receptor subtype diversity

The studies presented here demonstrate that the human genome contains a family of at least four evolutionarily conserved mAChRs which differ strikingly in their primary structures, their ability to bind various ligands, and in their pattern of tissue-specific expression, revealing a heretofore unappreciated degree of specialization in muscarinic cholinergic function. Despite the substantial amino acid divergence between the four mAChR subtypes, 151 of 241 residues (65%) contained within the seven predicted membrane-spanning domains and their short connecting loops are identical in each. The majority of amino acid differences among the four receptor subtypes within these regions correspond to conservative substitutions, supporting the notion that these sequences constitute the receptor ligand-binding domain. The most unique structural feature of each subtype is the large loop between the fifth and sixth transmembrane regions, which is predicted to reside on the cytoplasmic side of the plasma membrane, and is thus likely to play an important role in specifying which effector system(s) will couple to a given subtype. This possibility is supported by studies with β-adrenergic receptor deletion mutants showing that deletions of the corresponding domain of this receptor abolish its ability to regulate adenylyl cyclase activity without impairing ligand binding (Dixon *et al.*, 1987). A greater degree of similarity within the 5–6 loop region is evident between the HM1 and HM4 subtypes, and the HM2 and HM3 subtypes, suggesting that both of these pairs of subtypes share greater functional similarity.

The ligand binding properties of human mAChR subtypes ex-

pressed by transfected mammalian cells, as well as the pattern of gene expression detected for the homologous rat mRNAs, provide important clues to the relationship between the subtypes characterized here and those identified by earlier investigators. Northern blot analysis of mRNA from rat tissues revealed that only the HM2 and HM4 homologues are expressed in the heart and pancreas, respectively, suggesting that these subtypes correspond to the 'cardiac' and 'glandular' human mAChR subtypes. The assignment of the HM2 subtype is also supported by ligand binding analysis demonstrating that the recombinant HM2 polypeptide exhibits low affinity for pirenzepine, and a high proportion of high affinity sites for AF-DX116, two distinguishing characteristics of M2 receptors in mammalian atria (Hammer *et al.*, 1980; Giraldo *et al.*, 1987). In addition, HM2 shares virtual amino acid identity with the M2 mAChR protein purified from porcine atria (Peralta *et al.*, 1987). The identity of HM1 is indicated by its high affinity for pirenzepine and expression in cerebral cortex. Furthermore, HM1 exhibits virtual identity with porcine M1 mAChR clones corresponding to the major subtype purified from porcine cerebral cortex (Kubo *et al.*, 1986a). While HM1 exhibits the highest pirenzepine affinity of the four human mAChRs, the ability of the HM3 and HM4 subtypes to bind pirenzepine with relatively high affinity, and their comparable abundance to HM1 in brain, suggests that previous studies of M1 brain mAChRs displaying high pirenzepine affinity may have failed to discriminate between these three mAChR subtypes.

In contrast to the preferential expression of the HM2 and HM4 subtypes in heart and pancreas respectively, all four mAChR subtypes are expressed to similar extents in whole rat brain. This difference may reflect the complex nature of synaptic signalling that occurs in the central nevous system compared with the peripheral nervous system, or may indicate a physiological requirement for multiple receptor subtypes which may regulate different effector systems. Subtype-specific expression of mAChRs within the central nervous system is suggested by previous studies, which indicate that M1 and M2 mAChRs occupy postsynaptic and presynaptic locations respectively within cholinergic nerve terminals of the cerebral cortex (Mash *et al.*, 1985). Moreover, the effects of acetylcholine on ion conductances within the central nervous system have been used to correlate mAChR subtypes with specific electrophysiological functions; M2 mAChRs appear to mediate an increase in potassium conductance within brain cells, while M1 mAChRs are associated with an inhibition of potassium permeability (Egan and North, 1986). The predominant expression of a single mAChR subtype, HM3, in NG108-15 neuronal cells (Matsuzawa and Nirenberg, 1975) suggests the interesting possibility that regulation of mAChR subtype expression may be highly differentiated at the level of the individual neuron as well as in gross regions of the brain.

Earlier studies have revealed correlations between the occupation of a given receptor subtype which has been defined by ligand specificity, and the activation of a specific cellular effector. For example, preferential coupling of the M1 subtype to polyphosphoinositide turnover in rat brain and mouse anterior pituitary cells, and of the M2 subtype to inhibition of adenylyl cyclase in rat heart or mouse anterior pituitary cells and activation of the inward potassium channel of myocardium have been reported (Gil and Wolfe, 1985; Pfaffinger *et al.*, 1985; Watson *et al.*, 1986). The pattern of expression observed for the HM3 and HM4 mAChRs suggests specific roles in effector coupling for these subtypes as well. Carbachol stimulation of NG108-15 cells results in adenylyl cyclase inhibition, with no apparent effect on phosphoinositide hydrolysis (Hughes and Harden, 1987); our findings thus suggest that the HM3 subtype may be preferentially coupled to adenylyl cyclase. The pancreas-specific expression of HM4 suggests that this mAChR subtype may be coupled to the polyphosphoinositide turnover and calcium mobilization associated with the carbachol stimulated secretory activities of pancreatic acinar cells (Korc *et al.*, 1987; Sekar *et al.*, 1987).

In tissues in which different mAChR subtypes may be co-expressed, the contribution of subtype heterogeneity to the existence of multiple agonist binding affinity states cannot easily be discerned. The binding experiments presented here for cells transfected with individual subtypes conclusively demonstrate that multiple agonist affinity states are the property of all four mAChR polypeptides. High affinity agonist binding by mAChRs is believed to reflect interactions with G proteins since the proportion of such high affinity sites can be substantially decreased by the addition of guanine nucleotides or by pertussis toxin-catalyzed ADP-ribosylation, treatments which uncouple receptors from G proteins (Stryer and Bourne, 1986). The differences in the affinity and proportion of the multiple agonist affinity states detected for individual mAChR subtypes expressed in a single cell type may reflect the capacity of individual subtypes to interact differentially with G proteins, and thus to couple specifically with the various biochemical effector systems present.

In conclusion, our results demonstrate that the human mAChR gene family is composed of at least four members; specific structural features distinguish each receptor and are manifested in the distinct ligand-binding properties displayed by individual subtypes expressed by transfection of mammalian cells. This strategy will be further employed to study the biochemical properties of each mAChR subtype, and thereby continue the investigation of the physiological significance of receptor subtype multiplicity.

Materials and methods

Isolation of molecular clones

A human genomic library (Lawn *et al.*, 1978) was screened under low stringency hybridization conditions with a 680-bp uniformly ^{32}P-labeled restriction fragment encoding the first five transmembrane domains of the porcine M2 muscarinic receptor as described (Peralta *et al.*, 1987). Positive clones were screened with ^{32}P-end-labeled oligonucleotide pools, each containing 64 degeneracies, encoding either amino acids 55–62 (Gln-Thr-Val-Asn-Asn-Tyr-Phe-Leu) or 64–70 (Ser-Leu-Ala-Cys-Ala-Asp-Leu) of the porcine atrial M2 muscarinic receptor (Peralta *et al.*, 1987) and with oligonucleotides encoding unique regions of the porcine M1 (amino acids 259–286) and M2 (amino acids 240–263) mAChRs. Restriction fragments from each genomic clone which hybridized with the oligonucleotide probes described above were subcloned into M13 vectors and sequenced by the chain termination method (Sanger *et al.*, 1980; Messing *et al.*, 1981). Nucleotide sequence data will be submitted to the EMBL/GenBank Data Libraries.

Protein sequence homologies

Sequence comparisons were first determined by separate alignment of each pair of receptors (Needleman and Wunsch, 1970; Fitch and Smith, 1983). This analysis revealed a high degree of identity within the predicted transmembrane domains and short hydrophilic loops of each pair of mAChR and thus allowed the alignment of all four sequences by visual inspection. The large cytoplasmic domain joining the putative fifth and sixth transmembrane domains was analyzed separately for both amino acid and nucleotide homology. The scoring parameters are those of Dayhoff *et al.* (1983) with a deletion penalty identical to that of Lipman and Pearson (1985).

Expression of human mAChRs

Each mAChR coding sequence was inserted into a derivative of pF8CIS9080 containing *Eco*RI, *Sma*I and *Bam*HI linker sites replacing the Factor VIII coding region (Eaton *et al.*, 1986). The following coordinates indicate the nucleotide sequences of each receptor that were inserted into the expression vector: positions −66 to +1418, HM1; bp −42 to +1467, HM2; bp −81 to +1598, HM3; bp −7 to +1795, HM4 (Figure 2). For transient expression of mAChRs, 60-mm plates of human embryonic kidney cells (50% confluent) were transfected as previously described (Eaton *et al.*, 1986) by the calcium phosphate method (Wigler *et al.*, 1979). Sixty hours after transfection cells were harvested in PBS containing 5 mM EDTA and tested in ligand-binding assays.

Ligand-binding analysis

Binding studies were carried out with intact cells or with cell homogenates, as described earlier (Peralta *et al.*, 1987). Incubations were 75 min at 37°C for intact cells and 60 min at 20–22°C for cell homogenates. Non-specific binding was determined in the presence of 10 μM atropine and was always <10% of the total [^{3}H]QNB binding. The mean K_D values for [^{3}H]QNB binding and receptor numbers expressed per transfected cell were determined by Scatchard analysis of [^{3}H]QNB saturation binding by intact cells (n = 4) utilizing the LIGAND program (Munson and Rodbard, 1980) and are as follows: HM1, 22 ± 8 pM, 9000 ± 2100 sites/cell; HM2, 83 ± 41 pM, 48 600 ± 1400; HM3, 39 ± 6 pM, 27 400 ± 7600; HM4, 112 ± 55 pM, 89 600 ± 21 700. Similar results for [^{3}H]QNB-binding were obtained for cell homogenates (n = 2) with the range of values as follows: HM1, 17 pM, 13 fmol/mg homogenate protein (n = 1); HM2, 26 ± 10 pM, 155 ± 31 fmol/mg; HM3, 37 ± 29 pM, 44 ± 20 fmol/mg; HM4, 172 ± 73 pM, 332 ± 241 fmol/mg. Antagonist competition displacement experiments were performed with a subset of the transfections described above and the mean K_D values for [^{3}H]QNB binding and receptor numbers are: HM1, 19.5 ± 1 pM, 7100 ± 3300 sites/cell (n = 2); HM2, 95 ± 60 pM, 58 100 ± 13 800 (n = 3); HM3, 45 ± 9 pM, 16 400 ± 3200 (n = 3); HM4, 112 ± 83 pM, 82 900 ± 28 800 (n = 3). The agonist competition displacement experiments were performed with cells from three transfections and the K_D values for [^{3}H]QNB binding and receptor numbers for one representative experiment are as follows: HM1, 42 pM, 13 250 sites/cell; HM2, 57 pM, 78 000; HM3, 27 pM, 34 200; HM4, 16 pM, 133 000. The K_D values for competing antagonists and agonists were determined from three transfections and displacement experiments performed in duplicate (with the exception of cholinergic binding to HM1 which was analyzed in duplicate in one experiment); equivalent results were obtained and the values of one representative experiment are presented in Table I. Untransfected cells expressed <200 muscarinic receptors/cell (<1.3 fmol/mg homogenate protein).

Northern hybridization analysis

Subtype-specific restriction fragment probes encoded the following unique regions of the large cytoplasmic domain of each receptor: HM1, amino acids 265–363; HM2, amino acids 268–381; HM3, amino acids 265–394; HM4, amino acids 292–390. Each fragment was uniformly radiolabeled with [α-^{32}P]dCTP (Maniatis *et al.*, 1982). Stringent hybridization conditions for Southern and Northern filters are as previously described (Peralta *et al.*, 1987).

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Note added in proof

Following the original submission of this manuscript Bonner *et al.* (Science, **235**, 527–532, 1987) reported the cloning and partial sequence analysis of three mAChR subtypes. Rm1 (rat), Rm3 (rat) and Hm4 (human). Comparison of their subtypes with the mAChRs reported here indicates that Hm4 is equivalent to HM3, and suggests that Rm1 and Rm3 are rat homologues of HM1 and HM4 respectively. Assignment of the rat subtypes warrants further investigation since Bonner *et al.* showed by Southern analysis that there may be five additional uncharacterized rat muscarinic genes.