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MUSCARINIC RECEPTOR SUBTYPES

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INTRODUCTION

In 1914, Sir Henry Dale provided the basis for the classical and comfortable definition of muscarinic and nicotinic acetylcholine receptors: Muscarinic receptors are selectively activated by muscarine and blocked by atropine; nicotinic receptors are activated by nicotine and blocked by curare. This definition lasted over 60 years, despite isolated reports that the picture might not be so simple. We now know, as the result of molecular biological studies, that there are multiple variants of both muscarinic and nicotinic receptors. These receptors are members of two quite separate gene superfamilies and only share the property of being activated by the same ligand, acetylcholine.

The aim of this review is to discuss the structure, function, and binding properties of the different muscarinic receptor species, attempting where possible to coordinate the diverse experimental data into a uniform picture. For a more comprehensive review on muscarinic receptors, readers are directed to a recent book by Brown (1).

Selective Antagonists

The classical approach to receptor classification has been the discovery and characterization of selective antagonists and agonists. In the case of muscarinic receptors, this has also provided the evidence for the existence of subtypes, but their relatively low selectivity, allied to the large number of subtypes, has led to some confusion as regards the definition of the receptor subtypes.

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Historically, the first cardioselective muscarinic antagonist to be discovered was gallamine (which is also a nicotinic antagonist) (2). More recently other cardioselective antagonists that lack nicotinic actions—AF-DX 116 (3), himbacine (4), and methoctramine (5)—have been described. All these antagonists are more potent on cardiac receptors than the receptors on smooth muscle that mediate contraction. The reverse selectivity is shown by 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (6) and hexahydrosiladifenidol (7) and its p-Fluoro-derivative (8). A different selectivity is shown by pirenzepine, the drug which, in the late 1970s and early 1980s, had a major role in our appreciation of the existence of mAChR subtypes. Pirenzepine binds and acts selectively on a muscarinic receptor subpopulation found in neuronal tissue while it has a lower affinity for the receptors found in heart and smooth muscle (9). Telenzepine exhibits a comparable selectivity to pirenzepine but is considerably more potent (10).

Selective Agonists

No very selective agonists have been described. Candidates include McN-A-343 (11, 12), *cis*-3-acetoxy-S-methylthiane and propargyl esters of arecoline, and arecaidine derivatives (7).

Problems arise in the definition of selectivity in functional assays and in binding assays where, aside from the possible coexistence of multiple receptor species, there are multiple states of the receptor (13), which are linked to the ability of the receptors to interact with G-proteins (14).

The Problem of the Definition and Nomenclature of Muscarinic Receptor Subtypes

Because of the current lack of highly selective muscarinic drugs, the results of binding, pharmacological, mechanistic, and molecular studies must be combined to arrive at a robust definition. The appeal of such an approach is that it may be possible to define a muscarinic receptor subtype in terms of its amino acid sequence, pharmacological profile (in binding and functional studies), and its ability to activate selectively one effector mechanism. Most of the existing data can be combined to form such a definition. However, gaps in our knowledge and experimental discrepancies do still exist.

Partly as a result of the difficulties in defining a receptor subtype, many different nomenclatures have arisen. In this review, we use as the basis, an emerging pharmacological definition of M_1, M_2, and M_3 receptors and, for structural information, an m1–m5 definition of the amino acid sequences (Table 1). These can be prefixed by a capital letter—R(at), H(uman) etc—to indicate species. At present, the available evidence (discussed later in this review) points toward M_1 receptors having the m1 sequence etc, so that it is anticipated that these two nomenclatures will merge eventually. The naming

Table 1 Muscarinic receptor nomenclature[1]

Pharmacological characterization					
Subtype	M_1	M_2	M_3	--	--
Other names used previously	$M_1\alpha$, A	$M_2\alpha$, cardiac M_2, C	M_2, $M_2\beta$, B, glandular M_2	M_2	—
Selective antagonists	Pirenzepine, (+)-Telenzepine	AF-DX 116, himbacine, methoctramine, gallamine*	p-Fluorohexahydrosiladifenidol, Hexahydrosiladifenidol,	—	—
	$M_1 > M_3 \geq M_2$	$M_2 > M_1 \geq M_3$	$M_3 > M_1 \geq M_2$		
Molecular characterization					
Sequences	m1	m2	m3	m4	m5
Other names used previously	mAChRI, M1	mAChRII, M2	mAChR III, M4	mAChRIV, M3	
Numbers of amino acids	460	466	589/590	478/479	531/532

[1] This nomenclature is identical to that recommended by the 4th Symposium on Subtypes of Muscarinic Receptors and the British Pharmacological Society Nomenclature Subcommittee, 1989.

of M_5 as a discrete pharmacological entity may be considered premature because of lack of precise knowledge of its location or function in vivo. However, there is a candidate M_4 receptor, present in the striatum, rabbit lung and some cell lines, e.g. NG108 15 cells.

MUSCARINIC RECEPTOR GENES

Cloning of Muscarinic Receptor Genes

In 1986, Numa and colleagues cloned cDNAs encoding the porcine cerebral and cardiac muscarinic receptor (the m1 and m2 receptors, respectively) (15, 16). Their cloning strategy was conventional and consisted of (*a*) purification of the receptor from cerebral cortex using affinity chromatography; (*b*) tryptic digestion of the purified receptor to yield proteolytic fragments suitable for amino acid sequence analysis; (*c*) construction of degenerate oligonucleotide probes based upon the partial amino acid sequences and (*d*) screening of cDNA libraries with the oligonucleotide probes to identify hybridizing clones. One year later, two further muscarinic receptor clones were identified (17) by a quite different strategy. Comparison of the sequences of the rat m1 receptor and the hamster β2 adrenergic receptor (18) identified a region of sequence homology in the second transmembrane domain. By constructing an oligonucleotide probe corresponding to this region and screening rat cortex cDNA libraries at low stringency, workers identified these hitherto unidentified muscarinic receptor clones (m3 and m4 receptors). Since all four muscarinic receptors lacked introns in their coding sequences, the screening of genomic libraries became an appealing strategy, because no assumptions had to be made about tissue distribution and the problem of full-length clones was obviated. This strategy led to the identification of a fifth member of the muscarinic receptor gene family (m5) (19); the same receptor was cloned subsequently from a rat brain cDNA library (20). Recently a number of G-protein–coupled receptors have been cloned that do indeed have introns in their coding sequences, and thus cDNA libraries may yet prove to be a source of further receptor clones. At the time of writing, the m1 gene has been cloned from human (19, 21, 22), rat (17), pig (15), and mouse (23); the m2 and m3 from human (19, 24, 25), rat (17, 26), and pig (15, 20, 27), the m4 and m5 from human and rat (19, 20).

The question naturally arises: How many more muscarinic receptor genes are there? Suggestions of further clones came from an analysis of genomic DNA hybridized at low stringency with a partial m1 cDNA. This revealed up to ten hybridizing bands in rat digests and up to six bands in human DNA digests (17). By rehybridizing the blots at high stringency with individual muscarinic probes, bands corresponding to the m1-m5 receptors could be identified. Whether or not the remaining bands correspond to other muscarinic receptors, pseudogenes, other receptors, or unrelated proteins must await

their cloning and expression. The potential significance of this unexpected degree of receptor heterogeneity to our understanding of neural communication is discussed in later sections.

Expression of Muscarinic Receptor Genes in the Nervous System

Prior to the advent of molecular cloning studies, the only tools available to study the pattern of muscarinic receptor distribution were tritiated receptor ligands. However, binding studies performed on recombinant muscarinic receptors have shown that these ligands do not discriminate sufficiently among the different subtypes to be useful for labelling individual subtypes (28) (see later). The earlier autoradiographic studies undoubtedly demonstrated the heterogenous localization of multiple subtypes of muscarinic receptor, but we now know that in general, specific subtypes could not be selectively labelled in an unambiguous manner. One possible exception is the use of low concentration of ^{3}H-pirenzepine, which may exclusively label M_1 sites.

With the availability of DNA probes specific for each receptor gene, the problem of receptor subtype distribution has been reexamined using Northern blot analysis and in situ hybridization (29).

Northern blots show that m1 and m3 mRNAs are found in brain and exocrine glands, while m2 mRNA is found in cardiac and smooth muscle (21, 29, 30). m4 transcripts have a very discrete tissue distribution and are found mainly in neural tissue. Although no m5 transcripts have been detected by RNA hybridization, their presence in the rat brain can be inferred from the fact that the m5 cDNA was cloned from a rat brain cDNA library (20).

In situ hybridization has been used to examine the cellular distribution of each of the muscarinic receptor mRNAs in sections of rat brain (31, 19). These studies have demonstrated that m1, m2, m3, and m4 transcripts each have a unique distribution throughout the nervous system. m1 mRNA has a predominantly telencephalic localization and is particularly abundant in cerebral cortex, striatum, and hippocampus. m2 transcripts are very rare and are only found in significant quantities in the medial septum and pons, with lesser amounts present in the thalamus. m3 mRNA like the m1 is found predominantly in the forebrain, but in addition is found in several thalamic nuclei and brain stem nuclei. m4 transcripts are also found in cortex, striatum, and hippocampus. Recent studies have shown that very low levels of m5 transcripts are present in hippocampus, and in some brain stem nuclei (32). These generalities obscure many of the subtler differences that exist in the patterns of distribution of each subtype such as the different laminar distributions of the m1, m3, and m4 transcripts within the cerebral cortex. The significance of this heterogeneous distribution is considered in a later section.

Coexpression of mAChR Genes

Are different muscarinic receptor genes coexpressed by the same cell? The most direct demonstration of coexistence comes from in situ hybridization studies of autonomic ganglia which have shown that most autonomic ganglionic cells coexpress m1, m2, m3, and m4 genes (33). When examined purely in terms of signal transduction, this observation is difficult to explain, because both m1 and m3 receptors tend to couple to stimulation of PI metabolism while both m2 and m4 prefer to couple to inhibition of adenylyl cyclase. If these coupling selectivities also occur in vivo, then it is not immediately obvious why a cell would employ pairs of receptors that perform the same function. Several possibilities arise:

(*a*) Muscarinic receptors may couple to different effector mechanisms in vivo than when expressed in foreign host cells. Ultimately this possibility will be addressed only when specific pharmacological probes are available that act upon only one receptor subtype.
(*b*) Different subtypes of receptor may be coupled to the same effector but regulated in a different manner or in response to different signals. This would allow a cell greater flexibility in modifying its response to a particular signal.
(*c*) Different subtypes could be directed to different cellular domains such as the direction of presynaptic receptors to nerve terminals. This would presumably be reflected in differences in their interactions with the cytoskeletal apparatus of the cell.

PRIMARY SEQUENCE ANALYSIS OF THE MUSCARINIC RECEPTOR SUBTYPES

About 20 separate members of the G-coupled receptor family have now been cloned, including catecholamine receptors (34–36), serotonin receptors (37–39), peptide receptors such as the substance K receptor (40), and the *mas* oncogene (41), the rhodopsin family (42), and several receptors of undefined specificity (43). The mAChRs show greatest (ca 35%) homology with the α_2 adrenergic receptors, and least (ca 20%) with the peptide receptors and opsins.

We attempt to summarize here the main conclusions from analysis of the mAChR sequences, and their comparison with other G-coupled receptors. Figure 1 shows the Rm1–m5 mAChR sequences, aligned for maximum homology. Figure 2 shows a model, approximately to scale, of the way in which the transmembrane (TM) helixes may be disposed with respect to one another. Figure 3 shows a simplified representation of the TM sequences of the m1 mAChR projected onto a helical wheel diagram.

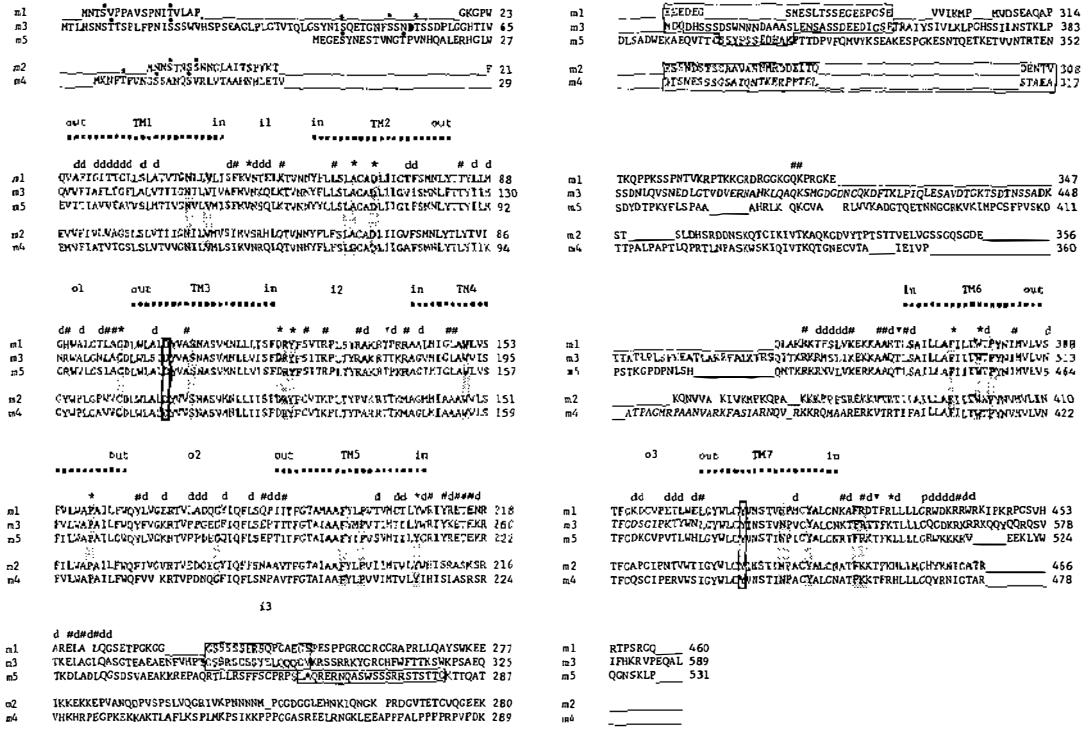

Figure 1 The five rat muscarinic receptor sequences aligned for maximum homology using the algorithm of Wilbur & Lipmann. The transmembrane helices, the intracellular (i) and extracellular (o) loops are indicated. ●, possible N-glycosylation sites, # amino acids conserved in m1/3/5 but replaced by a different residue conserved in m2/4. d, nonconservative substitutions. ▼ possible A- or C-kinase sites. p, possible palmitoylation site. Shaded residues are strongly conserved across the G-coupled receptor family. The cationic amine receptor-specific Asp and Tyr are delineated. Reasonably well-conserved Ser and Thr-rich sequences in i3 are boxed. Residues also marked with an * are not absolutely conserved.

Erratum: Site of gylcan attachment in N-glycosylation sequences NXS/T are N not S/T.

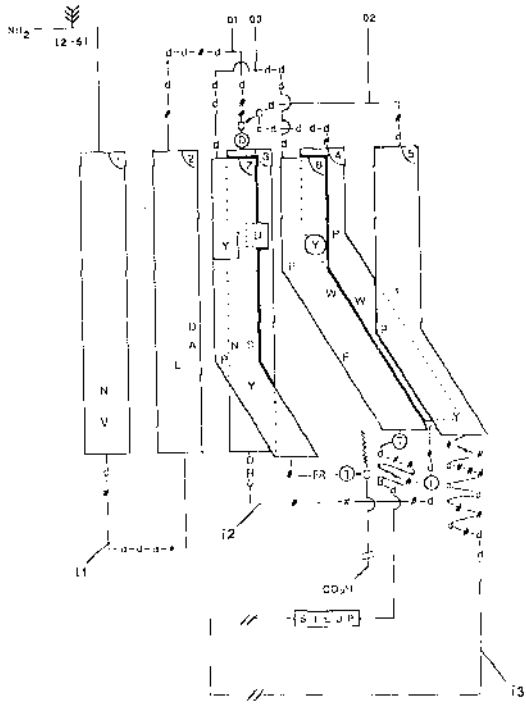

Figure 2 A hypothetical model of the mAChRs showing the disposition of the TM helices (numbered). Residues conserved across the G-coupled receptor family are shown as unenclosed capitals. The cationic amine Asp and Tyr are boxed. Some other important residues are circled. ⩛ possible N-glycosylation sites. } palmitic acid moiety. See Figure 1 for remaining symbols.

Hydropathy Analysis

Analysis using the method of Kyte & Doolittle shows that the TM regions of the mAChRs vary in their hydrophobicity. TM7 contains a particularly high proportion of hydrophilic amino acids. Plotting the sequences of the transmembrane regions on helical nets shows that TM3 and TM7 are both strongly amphiphilic, with a face consisting of hydrogen bonding residues (Tyr, Ser, Thr, Asn, Trp, Asp) running the entire length of the helix. The remainder of the helix surface is uniformly hydrophobic. Hydrophilic patches can be identified on other helixes, e.g. TM6 and TM1. In TM6, a cluster of hydrophilic residues around the proline induces a marked fluctuation in the hydropathicity plot.

Comparisons of the mAChR Sequences

A crude measure of the relatedness between different sequences can be obtained by calculating the a priori probability (P) that any pair of correspond-

ing amino acids will be identical if the observed number of overall matches is to be obtained.

On this basis, the greatest homology exists within TM2-TM7. The rank order is TM2=TM3=TM7 (P=0.96)>TM6 >TM4>TM5 (P=0.91).TM1 is much more variable, (P=0.76). i1, i2, and the o-loops show high conservation (P = ca. 0.85). There is much more overall sequence variation in the N-terminus, in i3, and in the C-terminus. However, subsections of i3 and the C-terminus are much better conserved. Comparing the N- and C-terminal 19 residues of i3, and the first 22 residues of the C-terminal tail, we obtain values of P ranging from 0.6 to 0.80.

In the mAChR sequences there are a number of instances, marked by a "#" in Figures 1 and 2, in which a residue conserved in m1, m3, and m5 is substituted by a different residue conserved in m2 and m4. When the relatedness parameter is recalculated after subgrouping the sequences, its value increases to 0.93–0.95 in i2, and to 0.69–0.86 in the N-terminal region of i3. A low level of homology now becomes detectable in the central portion of i3 (P=0.34). Relatedness is not increased in other parts of the sequence. These subcategories correlate with the ability of the mAChRs to discriminate between functionally distinct phospholipase C-specific, and adenylyl cyclase-specific G-proteins (see below).

The m1 and m3 sequences may be more closely related to one another than to m5. This is particularly apparent in i3. The m5 sequence is marginally more closely related to m3 than to m1. Genetic drift is almost all confined to the loops, with by far the largest number of changes found in i3. There are only four examples in TM2–7, of which two may be sequencing errors (G for A in m4/TM2; S for A in m3/TM3); the others are conservative substitutions. As expected, genetic drift is correlated with nonconservative differences between the subtypes. These positions are marked "d" in Figure 1 (marking of the central part of the i3 loop has been omitted). These uncorrelated variations, peculiar to a given subtype, are particularly clustered in the o loops, with 4 examples in o1, 7 in o2 and 6 in o3.

There is only very modest conservation in the middle section of i3 (see boxed sequences in Figure 1). The sequences include runs of Ser and Thr and include acidic amino acids and prolines. They are flanked by regions of greater interspecies variability, which border on much more invariant regions at the N- and C-terminal extremities of i3.

Conserved Residues and Functional Sites

Remarkably few residues are conserved across the entire G-coupled receptor family. Since one of the hardest problems in nature is the production of a stable protein fold, it may be assumed that these invariant residues articulate the essential skeleton of the molecule.

These amino acids are highlighted in Figure 1. Their approximate positions within the model structure are shown in Figure 2. Residues that vary in one or two instances, almost always in the *mas* sequence, or one of the rhodopsins, are also marked with an asterisk.

PROLINES IN THE TM HELIXES The prolines found within TM 4,5,6,7 are located 2–4 turns from the presumed extracellular termini of the helixes. Their likely effect is to introduce a bend of ca 30° (Figure 2) without disrupting the distribution of residues when projected onto a helical wheel (see Figure 3). If so, the bundle of TM helixes must contain both angled and straight members. The conjunction of the strongly hydrogen-bonding Asn residue with the Pro in TM7 may disrupt the helical structure at that point. Mutation to serine of the TM7 proline in the β_2 AR prevented correct folding and expression of the receptor (44), and a Drosophila mutant defective in a specific prolyl isomerase failed to express normal levels of rhodopsin (45).

Lefkowitz & Caron (34) have discussed several possibilities that flow from the presence of prolines in TM helixes. We would like to suggest another, namely that the articulation of the kinked against the linear TM helixes may allow a form of lever action, permitting relative movement within the helical bundle. This could transmit a conformational change across the bilayer in response to ligand binding.

CYSTEINE RESIDUES IN THE EXTRACELLULAR DOMAIN With the exception of *mas* (46), all members of the mammalian G-coupled receptors have a pair of conserved cysteine residues in the o1 and o2 loops. In the mAChRs, we have evidence from labelling, peptide mapping, and sequencing studies (47) that they form a disulfide bond. This complements site-directed mutagenesis studies on rhodopsin (48), and the β-AR (49).

The disulfide seems necessary for stabilization of the protein fold, but not necessarily for ligand binding. Reduction of a disulfide bond reduces the affinity of both antagonists and agonists for mAChRs (50, 51). Paradoxically, this potentiates the ability of the agonist-receptor complex to activate G-proteins in reconstitution experiments with both mAchRs and βARs (52, 53). These effects, if caused by reduction of the o1-o2 disulfide, show that the bond is not essential for transmission of the agonist-induced conformational change. However, it may still play a part in tuning the response by providing a constraint that biases the receptor towards the ground state.

Two cysteine residues are also found in the o3 loop of the mAChRs. Preliminary evidence suggests that they are probably not involved in disulfide bond formation. If such a bond were to exist, it would be peculiar to the mAChRs.

N-GLYCOSYLATION SITES The mAChR sequences all have between two and five potential N-glycosylation sites (Asn-X-Ser/Thr) in the N-terminal sequence. Inhibition of glycosylation with the inhibitor tunicamycin caused a specific depletion of cell-surface mAChRs in N1E-115 cells (54). However, deletion of the β-AR glycosylation sites did not affect ligand binding, although preventing receptor glycosylation (49). Likewise, glycosidase treatment, and wheat germ agglutinin had little effect on the binding of 3-quinuclidinyl benzilate to atrial mAChRs (55).

ASPARTIC ACID RESIDUES The mAChRs have five conserved acidic residues, of which two, namely the Asp embedded in TM2 (missing only in the blue cone pigment; 56) and the Asp at the C-terminus of TM3 are highly conserved across the whole G-coupled receptor family. The TM2 Asp is located in a conserved sequence incorporating the C-terminal amino acids of the i1 loop. The corresponding cDNA probe was used for cross-hybridization to identify the previously unknown m3 and m4 mAChRs (17). The TM3 Asp participates in a highly conserved triplet of amino acids, Asp-Arg-Tyr, in which the arginine is completely conserved. This sequence is probably involved in G-protein recognition (see below).

The N-terminal part of TM3 contains two Asp residues, one proximal and one distal to the disulfide-bonded cysteine residue. The proximal Asp (circled in Figure 2), which is not particularly well-conserved across the G-coupled receptor family, is located at or near the membrane surface. The distal Asp (boxed in Figures 1 and 2) is thought to be located within the outer leaflet of the bilayer. It is characteristically present in the cationic amine receptors, but absent from the rhodopsins and peptide receptors. There is strong evidence that this residue interacts directly with the headgroup of amine ligands in muscarinic and other cationic amine receptors.

A CATIONIC AMINE RECEPTOR-SPECIFIC TYROSINE A tyrosine residue in TM7, boxed in Figures 1 and 2, is particularly interesting in that its presence is correlated with the distal Asp of TM3, occurring only in the cationic amine binding subclass of G-coupled receptors. Provocatively, this Tyr residue is situated precisely the same number of amino acid residues from the conserved Asn-Pro sequence in TM7 as the retinal attachment lysine in the rhodopsin family. In rhodopsin, a Glu occupying a position very similar to that of the TM3 distal Asp in the mAChR is postulated to act as a counter-ion to the protonated retinal Schiffs base (42), suggesting a close association between TM3 and TM7. We shall argue that the conjunction between the TM3 Asp and the TM7 Tyr has a homologous role in the cationic amine receptors and may be crucial in ligand binding and receptor activation.

PUTATIVE PALMITOYLATION SITE TM7 is characteristically capped off by an aromatic-basic doublet, very often Phe-Arg. In many G-coupled receptors, this is followed after about 9 residues by one or more cysteines, which in both rhodopsin and the β-adrenergic receptors are palmitoylated (57, 58). The same is likely to be true in the mAChR sequences, and by analogy with the βARs, such a modification, if present, is likely to be important for G-protein coupling (see below).

POSSIBLE SITES FOR PHOSPHORYLATION AND OTHER FEATURES The mAChR sequences contain a number of possible phosphorylation sites. These are discussed in more detail below.

The mAChR sequences have other features characteristic of transmembrane proteins, namely:

(*a*) Conserved aromatic residues both within and at the boundaries of TM helixes, likely to fulfill specific structural or hydrogen-bonding requirements.

(*b*) Systematic compositional differences between the o and i loops, which may be related to the mechanisms of polypeptide insertion (59). The basic residues in the i loops may act as stop-insertion signals, interacting with the negatively charged phospholipid head-groups.

(*c*) Several other very highly conserved residues occur in the G-coupled receptor sequences (Figures 1 and 2). Their function can only be guessed at.

A Helical Wheel Model of the mAChRs

If the TM sequences of the mAChRs are drawn on helical wheel projections, it is possible to arrange them so that most of the conserved residues are on the inside of the structure (or at helix boundaries), while most of the nonconserved residues are on the outside, facing the lipid bilayer (Figure 3). It is then found that the majority (ca 30 out of 47) of the potential hydrogen-bonding

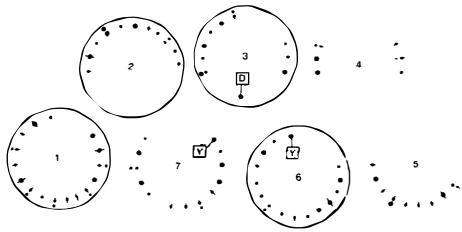

Figure 3 A helical wheel model of the Rm1 mAChR. ● potential hydrogen bonding residues, • non-polar residues. A line through the symbol indicates nonconservation in the m1–m5 sequences.

side chains face one another around a central pore-like cavity. Several of the apparently outward-facing hydrogen-bonding residues are at or near helix boundaries, where they may be able to interact with the aqueous phase.

Tyr 7 in TM7 and Asp 6 in TM3 can be brought into close proximity, in accord with the hypothesis that the two residues may interact in the cationic amine receptor sequences. There are scarcely any nonconserved residues in the central pore-like region. (Val 1, Thr 8 and Ser 12 in TM1, Ala 9 in TM3, Val 17 in TM5 and Leu 1 in TM6). If this model is accurate, the mAChRs enclose a highly conserved, hydrophilic cavity. We shall show that this is involved in ligand binding. Most of the variation in the TM regions occurs in hydrophobic portions of the structure facing the lipid bilayer, where they are not subject to the necessities of interhelix hydrogen bonding, or packing constraints.

RESIDUES INVOLVED IN G-PROTEIN COUPLING

Coupling Specificity and Functional Subtypes

In general, m1, m3, and m5 mAChRs are coupled to triphosphoinositide breakdown in a variety of expression systems, while m2 and m4 are linked to adenylyl cyclase inhibition. The evidence is discussed in more detail below. A caveat is that at least one important m2-mAChR response, the opening of K channels gated by a pertussis-toxin sensitive G-protein (60), has not yet been demonstrated using cloned expressed mAChRs, although the coupling of m2 mAChRs to the opening of a relatively nonselective Na/K conductance has been observed in oocyte membranes (61). Thus, the selectivities of the different cloned sequences for this response cannot be assessed. Nonetheless, there is a crude correlation between biochemical selectivity and sequence similarity, in that both criteria subclassify the mAChR sequences in the same way.

Determinants of Coupling Specificity

THE I3 LOOP Critical determinants of G-protein specificity reside in the i3 loop. The interchange of i3, and the C-terminal seven amino acids of TM5 between m1 and m2 mAChRs by the construction of chimeric receptors changed the coupling specificity without changing the pharmacology (62). Recently, PI coupling specificity has been localized more accurately to the N-terminal 17 amino acids of i3 of the m3 mAChR (63). Here the sequences of m1, m3 and m5 show the most obvious correlated divergence from those of m2 and m4 (Figures 1, 2), particularly a number of substitutions which give m2/4 a higher local positive charge density than m1/3/5.

Experiments using α_2/β_2 chimeras (64) or βAR deletion or substitution mutations (65, 44) have demonstrated similar conclusions, although the

substitution of a short sequence at the N-terminus of the i3 loop of the βAR with one from the α_2 AR did not abolish cyclase activation (64). These studies have localized another important determinant of G-protein coupling to the C-terminal 7 residues of i3.

The N- and C-termini of i3 are thought to form amphiphilic helical extensions of TM5 and TM6, which may well lie close together on the cytoplasmic surface of the receptor (Figure 2). This structure may be simulated by the simple amphiphilic peptide mastoparan, which activates G-proteins in a manner resembling agonist-occupied receptors (66), showing greater efficacy against G_i and G_o (to which mAChRs couple) than against G_s. The mastoparans may be useful tools for studying receptor-G-protein interactions, and they may be able selectively to mimic or antagonize specific receptor-G-protein interactions. The basic requirements for G-protein recognition may be quite simple.

G-coupling is not the only function subserved by the i3 loop. It contains probable substrate sites for kinases (see below). It also influences receptor expression. Thus in the chimeric receptor studies of Kubo et al (62), the i3 loop from m1 depressed the expression of m2, which was otherwise good, while the i3 loop from m2 enhanced the expression of m1, which was otherwise poor. In the βAR, deletion or splicing of i3 sequences bordering the TM helix boundaries frequently prevented receptor expression, reinforcing the suggestion that these regions may also act as stop-transfer signals.

THE I1 AND I2 LOOPS Other residues in the cytoplasmic domain may participate in G-protein recognition and coupling, as well as influencing receptor folding. In m1 mutation of the conserved i2 Asp to Asn raised the carbachol affinity of the receptor (in CHO cells), but reduced the efficacy of coupling to PI breakdown (66a). There was no effect on the binding of reversible antagonists. In the β_2AR mutation of the conserved i2 Pro (also found in i2 of the mAChRs) to Thr reduced isoproterenol potency and efficacy in stimulating adenylyl cyclase (65). We reiterate that i2, like i3, provides a basis for classifying mAChRs into PI or cyclase couplers, as well as differentiating the G_i and G_o coupled mAChR sequences from the G_s-coupled βAR sequences. Interestingly, the i2 loop becomes susceptible to cleavage in photobleached rhodopsin and is thus affected by the agonist-induced conformational change (67).

In the mAChRs, no direct information is available about the role of the i1 loop although in the βAR its integrity is critical for correct folding and expression (49, 65). A conserved Leu residue, also present in the mAChRs, has been implicated in coupling βARs to G_s.

The conserved Asp residue in TM2 (Asp 71 in the Rm1 sequence) is

important. Mutation to Ala increased the affinity of the m1 mAChR for carbachol tenfold, attenuated the GTP inhibition of agonist binding, and virtually obliterated coupling to PI breakdown (66a). This contrasts with the effect of the same mutation in the βAR (68), which was to decrease agonist affinity, independent of the efficiency of coupling, and to reduce efficacy. Antagonist binding was unaffected in either case. These observations disfavor involvement of the TM2 Asp in ligand binding and suggest a role in maintaining a conformation of the receptor capable of coupling to G-proteins, rather than a direct role in G-protein binding. The strong conservation of sequence in this area argues for a role which is independent of the minutiae of G-protein recognition.

THE C-TERMINUS In the βAR, substitution of the palmitoylated C-terminal domain Cys by Gly greatly impairs the efficacy of the receptor in activating adenylyl cyclase (58). The maximum response is reduced by 60%, and the formation of a high-affinity GTP-sensitive agonist binding state is abolished. It will be of great interest to extend these observations to the mAChRs and, if possible, to look at turnover of the putative palmitate residue in response to functional activation. It has been suggested that membrane binding of a palmitic acid residue could, in effect, create an i4 loop, which might cluster with the N- and C-termini of i3 to help form a G-protein binding surface.

The Origin of Selective Coupling

A large proportion of the cytoplasmic surface of the mAChRs may participate in the interaction with G-proteins, the conserved sequences engaging with conserved regions of the G-proteins, for instance, the virtually invariant β-subunits, which must be present for catalysis of GTP-GDP exchange at the α-subunit (52). However, only a small number of residues at the N-terminus of the i3 loop are necessary to dictate specificity. In like fashion, the receptor-recognition sequences of the G-proteins are believed to reside in an amphipathic helix at the C-terminus of the α-subunit, in the vicinity of the pertussis toxin-modifiable Cys residue that is found in the Gi and Go families (69). It is possible that direct contact occurs between this helix and the specificity helix at the N-terminus of i3.

An apparent paradox is that reconstitution experiments performed with purified mAChRs and G-proteins from both brain and heart have shown little selectivity. The G-proteins in question, designated G_o, G_i, and G_N (70, 71), probably correspond to the G_o, G_{i1}, and G_{i2} sequences identified by cDNA cloning.

Several factors may contribute to the resolution of this anomaly:

(*a*) The phosphoinositide responses of mAChRs are almost always mediated by pertussis-toxin-insensitive G-proteins (72). However, the appropriate G-protein has not yet been purified and used in reconstitution studies.
(*b*) The selectivity of the recombinant mAChRs for the G-protein-mediated K conductance has not been assessed.
(*c*) The purified mAChR preparations from brain contain more than one receptor subtype and have probably been in an undefined state of sulfhydryl oxidation.
(*d*) The state of palmitoylation of the purified mAChRs is unknown but is likely to be influential.
(*e*) The role of lipid composition of the vesicles in which reconstitution is performed remains to be systematically evaluated. The inclusion of cholesterol is important for the success of reconstitution procedures (52). Cholesterol may also be important for ligand-binding selectivity (72a).

For these reasons, we cannot yet decide whether the ability to differentiate between different G-proteins is completely encoded in the receptor subtype sequences, or whether additional cell-biological factors involving selective addressing of different receptor subtypes and G-proteins to discrete parts of the cell surface are also important. To decide this, it will be necessary to reconstitute the pure recombinant proteins, in a known state of posttranslational modification, in vesicles of defined lipid composition.

RESIDUES INVOLVED IN LIGAND BINDING

Transmembrane Helix 3: the Aspartic Acid Residues

Two findings show that the distal intra-membrane Asp residue in TM3 of the mAChR sequences participates in binding the amine headgroup of muscarinic ligands.

(*a*) Peptide mapping and sequencing studies pinpoint the distal Asp as the major site of alkylation of purified forebrain mAChRs by ^{3}H-propylbenzilylcholine mustard (^{3}H-PrBCM), a benzilylcholine analog in which the onium headgroup is replaced by a chemically reactive aziridinium moiety (73, 74).
(*b*) Mutation to Asn of the distal Asp in the m1 receptor greatly decreased antagonist affinity and reduced the phosphoinositide response of the receptor in CHO cells to less than 1% of the wild-type response (66a). A 1,000–10,000-fold reduction in both antagonist and agonist affinity also followed the mutation of the corresponding Asp in the βAR to Asn, or Glu (68).

That the mutation affects the affinities of both agonists and antagonists argues that the distal Asp binds the amine headgroup of both classes of ligand. Interestingly, the mutant βARs still activated adenylyl cyclase with low affinity, and kept their β_2-type specificity. Although the distal asp is necessary for high-affinity ligand binding, the interactions which mediate the actual conformational change may occur elsewhere in the receptor. Alternatively, the replacement of Asp by Asn or Glu may preserve a critical interaction necessary for receptor activation. This possibility is discussed further.

In contrast, mutation of the proximal Asp to Asn only slightly reduced the affinities of atropine, carbachol, and pirenzepine for m1 mAChRs expressed in CHO cells (66a). The mutant was capable of stimulating PI breakdown, although agonist potency was reduced. Similarly, mutation of the proximal Glu to Ala had little effect on ligand binding to the βAR (44). This indicates a secondary role in ligand binding.

Surprisingly, we have found that ^{3}H-PrBCM appears to be able to label the proximal Asp to some degree. This may explain why PrBCM alkylates the proximal Asp to Asn mutant in m1 inefficiently, even though reversible binding of the ligand is only slightly inhibited (66a).

The proximal Asp may be alkylated en passant, so that differences in the kinetics of binding could be influential. Our observations provide evidence for sequence-specific constraints on the mAChR binding site and suggest that certain ligands may have a choice of aspartate residues with which to interact.

Transmembrane Helixes 6 and 7. Tyrosine Residues

The unexpected correlated conservation of the distal TM3 Asp and the TM7 Tyr would be explained if the two residues were to interact directly by hydrogen-bonding. Because of its aromatic character, Tyr is often found in the interior of protein molecules. In most proteins, one or two Tyr residues make important internal cross-linking hydrogen bonds (75).

A hydrogen bond between TM3 and TM7 in the mAChRs and other cationic amine receptors could constrain the geometry much as an electrostatic interaction between the protonated retinal Schiffs base and the TM3 Glu residue in the mammalian opsin family would do (42). Its disruption by the charged headgroup of an agonist could trigger a conformational change, possibly homologous to that induced by the photoisomerization of *cis*-retinal in the rhodopsins. This could explain the ability of even a molecule as simple as tetramethylammonium to activate mAChRs, admittedly with relatively low efficacy. Perhaps coincidentally, the spacing between the carbonyl group and the onium headgroup of acetylcholine is very similar to that between the isomerizable 11-*cis* bond and the protonated Schiffs base nitrogen of the retinal chromophore.

Some deep cuts with Occam's razor dissect out the most conservative

hypothesis, namely that the position of the headgroups, the orientation of the ligands with respect to the TM helixes, and the nature of the conformational change may be extremely similar in the cationic amine receptors and the rhodopsins. While one effect of a Tyr-Asp hydrogen bond might be to stabilize the carboxylate anion in the binding site, another could be to polarize the Tyr OH group, allowing a negative charge to develop on the oxygen and creating the possibility of a direct interaction of the TM7 Tyr with the ligand headgroup. This could extend the analogy.

A structural role for the TM3 Asp affects the interpretation of mutagenesis experiments. The replacement of Asp by a hydrogen-bonding residue such as Asn might preserve a structurally important hydrogen bond, while abolishing the charge interaction. However, mutation of the TM7 Tyr or the TM3 Asp to nonhydrogen bonding residues would be expected seriously to disrupt the binding site. The construction of such mutations would allow the hypothesis to be tested.

Protein modification implicates tyrosine residues in the binding site of the mAChRs. Nitration with tetranitromethane increased affinity for agonists (76). Aromatic sulfonyl fluorides blocked the binding site of both forebrain and cardiac receptors, a process subject to protection by both antagonists and agonists (77). In principle, the residues modified in this way can be localized.

The construction of receptor chimeras (64, 78), site-directed mutagenesis (44), and affinity labelling (70) also support a role for TM7 in ligand binding to adrenergic receptors.

The working hypothesis is that the amine functions of agonists and antagonists are bound in much the same way, but that bonds to other moieties are different. If this is true, the side chains which ligate the ester function in acetylcholine, and in antagonists such as atropine and benzilylcholine will be found on the TM helixes that cluster around TM3. Apart from the residues on the N-terminal side of the Pro in TM7, we may consider the cluster of hydrophilic side-chains, including another Tyr hydroxyl, around the conserved Pro in TM6 (Figure 1).

The locations of the TM6 and TM7 tyrosines are modelled in Figures 2 and 3. Stacking of such residues with the aromatic rings characteristic of muscarinic antagonists might be responsible for their high binding affinities. It will be interesting to test the credibility of these speculations by 3-dimensional model building, as well as experimentally, by the construction of appropriate mutations, and new affinity-labelling reagents.

Equilibrium Binding of Selective Antagonists

We have few clues to the determinants of antagonist selectivity in the mAChRs. Some experimental evidence is provided by the study of Kubo et al (62), in which TM1–5 of the m1 mAChR was ligated to TM6–7 of the m2

mAChR, and vice versa, and the resultant chimeras expressed in Xenopus oocytes. The results show that major determinants of the m2-selectivity of the pirenzepine analog AFDX-116 reside in the first five TM helices, while TM 6 and 7 also contribute an important determinant to the m1-selectivity of pirenzepine.

Another clue comes from recent work in our own laboratory, which reinforces the idea that ligands may have a choice of negatively charged residues with which to interact, and that constraints restricting this choice influence selectivity (80).

The binding of antagonists (including PrBCM) to mAChRs is inhibited by the protonation of groups in the free receptors with pKs in the range of 5.5–6.8. The pK measured is dependent on the antagonist used. For cardiac mAChRs, the pKs governing the binding of the m2-selective AFDX-116 (81) and of the allosteric cardioselective ligand gallamine (140, 141) are 6.7, approximately 1 pH unit higher than the pK of ca 5.7 which governs the binding of the nonselective antagonists N-methylscopolamine and atropine. The study of m1 mAChRs in the cerebral cortex, and m3 receptors in the lacrimal gland indicates that the binding of selective ligands may generally be regulated by a group with a higher pK than that controlling the binding of the nonselective analogs. Polycationic molecules such as methoctramine (82) may bridge the two residues.

It is tempting to identify these groups with the proximal and distal Asp residues labelled by PrBCM. pKs of 5.5–6.8 are high for carboxylates in an aqueous environment but are possible for COOH side chains in a hydrophobic environment, or if the ionization is modified by an adjacent carboxylate, as in maleic acid (although the pK of the second carboxylate group is lowered). Linkage between the energetics of the receptor conformational change and the ionization of a binding site Asp might also generate an abnormal pK. The presence of multiple amino functions in molecules such as methoctramine may enable them to make a second bridging interaction.

Obviously, these ideas are speculative. However, they suggest experiments. In particular, we may try to identify the groups with the observed pKs, and to see whether selective ligands differentially inhibit the alkylation of the TM3 aspartates. Mutation of the Asp residues would be expected to change the pKs governing the ligand interactions.

A fundamental obstacle to detailed understanding is that the observed differences in antagonist affinity are small (at most, 100-fold) and are capable of being accounted for by small perturbations of dispersion interactions (10-fold selectivity is equivalent to ca 1.4 Kcal/mole, which is equivalent to the Van der Waals energy of one methylene group).

The absence of great antagonist selectivity is comprehensible if, as suggested, virtually all of the residues lining the central pore of the receptor are

conserved (Figure 3), while nonconserved residues face the lipid phase. This could explain why detergent solubilization can alter or nullify antagonist selectivity; for example, pirenzepine discrimination between forebrain and heart mAChRs is lost in digitonin solution, owing to an increase in affinity of the M_2 mAChRs, while the distinction between these subtypes and the lacrimal M_3 receptors is amplified (83). Rigid molecules such as pirenzepine are more likely to be affected by minor perturbations of TM helix conformation than are their more flexible analogs.

With purified forebrain and cardiac mAChRs, the reconstitution of pirenzepine affinities to be like those in native membranes requires an approximation to the native membrane environment. Cholesterol is important for this (72a). Interestingly, the cardioselectivity of AFDX-116 is maintained after solubilization, supporting different primary determinants of selectivity for these two closely related ligands (84).

Equilibrium Binding of Agonists

The differences between agonist binding states present other problems. The major finding is that the receptor-G-protein complex has a much higher affinity than the free receptor for potent agonists. The ratio of the affinities is correlated with the pharmacological efficacy of the agonist and can be as much as 30,000-fold for acetylcholine, or oxotremorine M (85, 52). The equilibrium binding of antagonists to the G-coupled and uncoupled states is usually not affected.

A 30,000-fold difference in affinity corresponds to a free energy of ca 6.2 KCal/mole, approximately that of a single hydrogen bond. The implication is that a new interaction comes into play in the agonist-receptor-G-protein complex (which has the character of a transition state in the pathway of G-protein activation) not available in the ground state. Ex-hypotheso, this has to involve a part of the molecule other than the cationic amine headgroup. In the case of ACh, this probably implies hydrogen bond(s) to the ester function. The efficacy of an agonist may reflect its ability to exploit these new interactions, which may also dictate the primary pharmacological differences between the cationic amine receptors.

The localization of the amino acid side-chains involved in agonist binding is a high priority and will involve the synthesis and use of new irreversible ligands, as well as a search for mutations which affect excited-state but not ground-state binding. A generic difficulty in the mutagenic approach will be to distinguish direct effects on agonist binding in the excited state from alterations of the energetics of the conformational change.

A fundamentally important, unanswered question is whether subtype-related differences exist between the structure-activity relationships for equilibrium binding of agonists to either the ground state or the excited state of the

mAChRs. m3 mAChRs expressed in oocytes have a higher affinity than m1 mAChRs for carbachol (86), but differences in coupling may account for this.

Residues on the Extracellular Surface of the Receptor. Ligand Binding Kinetics

Many sequence differences are localized in the o-loops (Figure 2). That the o-loops are not primarily involved in ligand binding is suggested by deletion mutagenesis studies on the βAR (49). Although reduction of the presumptive o2-o3 disulfide bond gives a significant reduction in the affinities of both antagonists and agonists for solubilized mAChRs (50, 51), its limited magnitude suggests a fairly minor perturbation of the binding site. It is again notable that the binding of the selective ligand pirenzepine is more affected than that of nonselective ligands, while the binding of AFDX-116 is relatively insensitive, consistent with a different binding mechanism.

Even for nonselective ligands, the rate constants for binding to the different mAChR subtypes are very variable. In rat brain membranes, the dissociation rate constant of N-methylscopolamine varies up to 17-fold between subtypes, with M_3 slowest, and M_2 fastest (87). Figure 2 suggests that sequence differences in the o-loops, are clustered around the entrance to the binding site. Residues in this position are strategically placed to gate the entry or exit of ligands. This may explain the existence of kinetic variations.

The existence and importance of differences in the rates of binding of ligands—both agonists and antagonists—to the cloned mAChR subtypes is unexplored territory. They could be important for understanding the function of mAChRs in their in vivo, nonequilibrium context. They may regulate the lifetime of the agonist-receptor complex and thus its catalytic efficiency. It may be possible to target these processes with novel allosteric drugs.

REGULATION OF mAChR NUMBERS ON THE CELL SURFACE

Several reports implicate protein kinase activities in the control of mAChR concentration and function on the cell surface. Unfortunately, few structural clues to their action are available.

A promising finding is that a cyclase-linked mAChR (probably m4) purified from the chick heart is phosphorylated by β-adrenergic receptor kinase (βARK). Phosphorylation is agonist-dependent and incorporates up to 8 moles of phosphate per mole receptor into Ser and Thr residues (88).

The specificity of the enzyme is similar to that of casein kinase 2. It prefers runs of Ser and Thr residues interspersed with acidic amino acids. Potential substrate sites are found in the i3 loop of the mAChRs (Figure 1). Similar sites are found in the i3 loop of the α_2 receptor, which is a good substrate for

βARK (89), although it lacks the residues in the C-terminal tail which are phosphorylated in the β_2AR and rhodopsin (for a review, see 90).

βARK mediates short-term homologous desensitization of the βARs and the chick heart mAChR, i.e. desensitization contingent upon stimulation by the cognate agonist. In the case of cardiac mAChRs, agonist affinity is reduced with a relatively rapid time course (10 min) in parallel with the agonist-stimulated incorporation of phosphate into the receptor (91). There is no evidence for the involvement of A-kinase or C-kinases.

βARK does not phosphorylate the PI-linked α_1-adrenergic receptor (89), and it is not known whether it phosphorylates the m1 and m3 receptors from forebrain. The mAChR phosphoinositide response is often notable for its longevity and resistance to desensitization (92). It is possible that a separate family of enzymes will be identified which act on the PI-linked receptors.

βARK appears to be translocated from the cytosol to the inner surface of the cell membrane when specific recognition sites on the cytoplasmic surface of the receptor are exposed. The agonist-dependence argues for a large conformational change, probably affecting the i3 loop, and probably not requiring the presence of G-proteins (88). It may provide a useful in vitro test of agonism.

An unanswered question concerns the possible participation of an arrestin-like molecule, capable of recognizing the phosphorylated but not the non-phosphorylated form of the receptor (90). Such proteins probably mediate short-term desensitization and are significant for understanding the regulation of the in vivo pharmacological response. Both the kinases and the regulatory proteins provide novel targets for drugs.

The short-term desensitization of receptor responses is partially reversible. Longer-term exposure to agonists leads to processes of sequestration and down-regulation. Sequestration of the βARs is slowed or eliminated by mutations that interfere with receptor coupling to G_s. Again, this implies linkage to an agonist-induced conformational change (93). Factors distinct from βARK-mediated phosphorylation are probably involved because deletion of the phosphorylation sites by specific mutation slows but does not abolish sequestration, and eventual down-regulation. The latter involves proteolytic degradation of the receptors.

Second-messenger–mediated heterologous desensitization probably follows a different mechanism. In the case of the βARs, A-kinase participates in this process (90).

The purified cardiac mAChR can be phosphorylated by A-kinase, but not by C-kinase, C-GMP-dependent kinase, or phosphorylase kinase. The stoichiometry of phosphate incorporation increases after reconstitution of the receptor with cardiac G-proteins, and becomes agonist sensitive. Phosphorylation is reversed by calcineurin. It alters the total concentration of

binding sites in a reversible manner before, but not after, reconstitution of the receptors into phospholipid vesicles (94).

It has not been shown whether A-kinase phosphorylates m1 and m3 mAChRs, although C-kinase has been reported to do so (95). Intriguingly, C-kinase does not phosphorylate cardiac mAChRs, suggesting a possible linkage between the second messenger system, and the ability of the cognate second-messenger–sensitive kinase to phosphorylate the receptor.

Inspection of the mAChR sequences shows the presence of three conserved Thr residues (Figure 1), situated near clusters of basic residues close to the boundary of i2 with TM4, i3 with TM6 and the C-terminal tail with TM7 (figure 2). These could be A- or C-kinase sites strategically placed to affect G-protein coupling. In addition, there are a number of other potential kinase sites unique to given subtypes in the i3 loops of all the mAChR sequences. Whether any of these sites are used is unknown.

SIGNAL TRANSDUCTION MECHANISMS OF MUSCARINIC RECEPTORS

Of the many intracellular responses coupled to muscarinic receptor activation, the inhibition of adenylyl cyclase and the stimulation of a phosphatidylinositol bisphosphate (PIP_2)-phospholipase C appear to be mediated directly via members of the family of G-proteins. In the case of the inhibition of adenylyl cyclase, the G-protein is one of the G_i proteins that is sensitive to ADP-ribosylation by pertussis toxin, whereas at least two G-proteins are coupled to the PIP_2-phospholipase C (96, 97). These latter G-proteins exhibit different sensitivities to ADP-ribosylation by pertussis toxin.

Another response which is closely linked to muscarinic receptor activation is the opening of a certain class of K^+ channel found in the heart and in the CNS. The original and surprising suggestion (98) was that these channels were opened by the $\beta\gamma$ subunits of G-proteins, the conventional wisdom being that most if not all of the actions of G-proteins were mediated via the α-subunits, with the $\beta\gamma$ subunits inhibiting the activation processes by forming the $\alpha\beta\gamma$ heterotrimer. More recent evidence indicates that the α subunits of three G_i proteins are capable of opening the K^+ channel at 100–1000 times lower concentrations than reported for $\beta\gamma$ (99), and therefore the α subunits may be the "physiological" activators. However, Kim et al (100) provided a further twist to the story; they have evidence that $\beta\gamma$ subunits, again at relatively high concentrations, activate a membrane-bound phospholipase A_2 to produce arachidonic acid, which is converted via lipoxygenase to products which then open the K^+ channels activated by muscarinic agonists. This pathway might appear to be somewhat indirect, but one for which evidence exists from studies in other systems (101).

Another K^+ channel, the M-current, is suppressed by muscarinic agonists. This channel was thought to be regulated via muscarinic receptors (and other receptors) acting via the PIP_2-phospholipase C pathway and a pertussis toxin insensitive G-protein (102), but there is divergent evidence as to whether diacylglycerol or inositol 1,4,5-trisphosphate are the mediators. Pfaffinger and coworkers (103) suggest that in frog sympathetic neurons a further mechanism is also operating. Other effector pathways operated upon by activation of muscarinic receptors include inhibition of phosphodiesterase, release of arachidonic acid and opening of Cl^- channels.

The conclusion from these and other studies (e.g. on voltage-dependent Ca^{++} channels) is that often G-proteins may activate important effector pathways in several ways. The speed of onset and the duration of the responses operating via the different mechanisms may be of physiological importance.

Signal Transduction Mechanisms Activated by the Different Subtypes

There have been numerous attempts to rationalize the diverse array of responses transduced by muscarinic receptor activation in terms of receptor heterogeneity, with each subtype linked to a particular effector mechanism. A substantial body of data indicated that many (but not all) phosphoinositide responses were transduced by activation of receptors with a high affinity for pirenzepine (M_1) while inhibition of adenylyl cyclase occurred by activation of receptors with a low affinity for pirenzepine (M_2 old nomenclature). Thus the idea evolved that receptors with a high affinity for pirenzepine were coupled via a PTX insensitive G-protein to phospholipase C. Similarly, receptors with a low affinity for pirenzepine were thought to be coupled via a PTX sensitive G-protein to adenylyl cyclase.

There were already enough exceptions to this rule to undermine its simplicity, but when molecular cloning studies demonstrated the existence of five different receptors the idea became even less tenable, because there are now more receptors than known signal transduction mechanisms. Either there are other signal transduction mechanisms that we are unaware of, or the reasons for such a large degree of receptor heterogeneity are not founded purely upon considerations of specific coupling to particular signal-transduction pathways.

However, it was at least possible to test this "selective coupling" hypothesis rigorously because transformed cell lines could be generated that each expressed individual muscarinic receptor genes. Signal transducers and effectors activated by individual receptors could thus be investigated. A number of expression systems have been used to investigate signal-transduction mechanisms used by cloned muscarinic receptors; the results are summarized in Table 2. An inspection of Table 2 reveals both broad generalizations and

some notable exceptions. Generally, m1, m3 and m5 receptors couple to a stimulation of phosphoinositide hydrolysis (19, 61–63, 97, 104–109) while activation of m2 and m4 receptors leads to an inhibition of adenylyl cyclase (96, 97, 105). Depending upon the cell type examined, activation of m1 and m3 receptors can lead to an increase in the levels of cAMP (19, 104, 105, 107), release of arachidonic acid (110), activation of K^+ and Cl^- channels (61, 104, 109, 111–113) and an inhibition of the M-current (109). Many of these responses have been shown to be dependent upon a rise in intracellular Ca^{2+} and are mediated via a PTX-insensitive G-protein and are thus probably secondary to the stimulation of phosphoinositide hydrolysis. An interesting anomaly is the observation that m1, but not m3, receptors also couple to an inhibiton of adenylyl cyclase via a PTX-sensitive G-protein when expressed in RAT-1 cells (107, 108). This observation serves to underline two fundamental points; (*a*) A single muscarinic receptor gene product can couple to more than one G-protein and (*b*) m1 and m3 receptors, when expressed in the same host cell at comparable receptor densities, can couple to different sets of G-proteins. This ties in with the earlier observations that m2 and m4 receptors can also couple to stimulation of phosphoinositide hydrolysis via different G-protein(s) than those used to couple to inhibition of adenylyl cyclase (96, 97). However, in this case, coupling to PI hydrolysis is only evident when the m2 or m4 receptors are expressed at high densities. Both sets of experiments illustrate the potential for one receptor to couple to a multiplicity of G-proteins and effectors but do not address whether this promiscuity is also seen with endogenous muscarinic receptors. To answer this question we need highly specific probes for all the components of the receptor/G-protein/effector cascade and a knowledge of their stoichiometry within the cell. Another unresolved contradiction is the seemingly opposite effects upon mitogenesis that stimulation of m1 and m3 receptors in transformed CHO cells causes: In one study, activation of m1 and m3 receptors led to an inhibition of thymidine uptake (110) whereas in another study activation of the same receptors caused an increase in thymidine uptake (114). In these cases, the receptor densities were comparable, and the explanation remains unclear.

A receptor may be coupled to a singular signal transducer, but the effector response subsequently activated may vary according to the nature or state of the particular cell type. A recent example is provided by the observation that muscarinic agonists can act as mitogens on glia isolated from developing rat brain (114). However, their mitogenic potential varies as a function of the developmental age of the glia and is maximally effective on glia derived from neonatal rats. In a commentary upon these data, Hanley suggests that the mitogenicity of a neurotransmitter depends upon the cell being in a permissive state (115). It is conceivable that this permissive state and the molecular basis of it, vary as a function of developmental age.

Table 2 Effector Mechanism[1]

Receptor	Expression system	Stimulation of PI hydrolysis	Inhibition of adenylyl cyclase	cAMP stimulation	Release of arachidonic acid	Increase in intracellular Ca^{2+}	Activation of K^+ channels	Opening of Cl^- channels	Inhibition of M current	Inhibition of mitogenesis	Stimulation of mitogenesis
m1	CHO	***									***
	A9	***		***	***		***	***		***	
	Kidney cells	***		***							
	Oocyte							***			
	NG108	***				***	***		***		
	RAT-1	***	***								
	B82	***									
m2	CHO	***	***								***
	A9		***								
	Kidney cells	***	***								
	Oocyte							***			
m3	CHO	***		***			***			***	***
	A9	***		***	***		***	***		***	
	Kidney cells	***		***							
	Oocyte							***			
	NG108	***				***	***		***		
	RAT-1	***									
m4	CHO		***							***	***
	A9		***								
	Kidney cells	***	***								
m5	CHO	***		***	***		***			***	

[1] A summary of effector mechanisms activated by stimulation of cloned muscarinic receptors expressed in a variety of cell types. Data are taken from refs. 19, 20, 23, 24, 61, 62, 86, 96, 97, 104–114

These mitogenic and trophic actions of acetylcholine and other neurotransmitters are poorly understood at present but promise to offer a novel understanding of neurotransmitter action and possibly receptor heterogeneity.

Possible Functions of Muscarinic Receptor Subtypes in the Nervous System

What is the significance of the heterogeneous distribution of muscarinic receptor genes discussed earlier? Definitive answers to this question will be provided only when specific pharmacological probes are found that can be used to perturb the function of each receptor subtype. However, predictions can be made by comparing the pharmacology of endogenous CNS muscarinic receptors with the pharmacology of recombinant muscarinic receptors expressed in foreign host cells. In cerebral cortex, activation of muscarinic receptors causes a stimulation of PI hydrolysis and an inhibition of the M-current, both of which are mediated by a receptor expressing a high affinity for pirenzepine (116, 117). Both m1 and m3 receptors can stimulate PI hydrolysis and inhibit M-currents, but only m1 receptors have a high affinity for pirenzepine (118). Stimulation of PI hydrolysis in hippocampus is also mediated by a similar receptor (118). A number of other muscarinic-receptor mediated–responses occur in hippocampus including inhibition of M-current, inhibition of a Ca^{2+}-activated K^+ conductance and reduction of a Ca^{2+} current, but none of these responses has been characterized pharmacologically. In striatum, activation of muscarinic receptors causes an inhibition of adenylyl cyclase and an augmentation of dopamine release, the responses being mediated by receptors with low (118) and high (119, 120) affinities for pirenzepine. Since m1 and m4 receptor mRNAs are the dominant transcripts in striatum (29), the implication is that the m4 receptor mediates the inhibition of adenylyl cyclase, because this receptor expresses a low affinity for pirenzepine (28) and inhibits adenylyl cyclase when expressed in a number of different cell types (96, 97, 105). Some of the strengths and ambiguities of these comparisons are provided by a consideration of the possible function of the m2 receptor in the medial septum and pons. In the pons, activation of a receptor with low affinity for pirenzepine leads to activation of a hyperpolarizing K^+ conductance (121), similar to that seen in atria, and also to a stimulation of PI hydrolysis (122). The existence of m2 receptors in atria indicates their possible involvement in opening K^+ channels, but it remains unclear whether the same receptor is responsible for the stimulation of PI hydrolysis because m2 receptors couple to PI hydrolysis only when the receptor is expressed at high density (see earlier). In the medial septum, the m2 receptor probably subserves an entirely different function: The medial septum contains the major forebrain cholinergic projection nuclei that provide

the cholinergic innervation for the cerebral cortex and hippocampus. Release of acetylcholine from cholinergic nerve terminals is inhibited by muscarinic autoreceptors with a low affinity for pirenzepine (119, 123, 124), and the possibility arises that the m2 mRNA present in the medial septum and Diagonal Band of Broca encode presynaptic receptors that are then transported to nerve terminals. The signal transduction mechanism underlying control of neurotransmitter release is not known, but most of the major pathways used by activated muscarinic receptors (raising intracellular Ca^{2+}) or hyperpolarization caused by an increase in K^+ conductance) could potentially perform this function.

THE PHARMACOLOGICAL PROPERTIES OF NATIVE MUSCARINIC RECEPTORS

Antagonists

One important component in a coordinated definition of muscarinic receptor subtypes is the pharmacological profile of the different receptors when expressed in the normal animal. This may be difficult, as we have noted, because of the poor selectivity of antagonists and the fact that more than one receptor species may be expressed in a given tissue. Furthermore, affinity constants determined from binding studies are sensitive to incubation conditions (see later). Therefore there is a spread of reported affinity constants found in binding and functional studies. Nevertheless, by taking recent literature values and averaging the log mean values, it is possible to generate a table of "best estimates" of the affinity constants for the M_1, M_2, and M_3 receptors (Table 3). For most antagonists and receptor subtypes, the s.e.m. values of the log affinity constants are less than 0.1. In general, there is an excellent agreement between data obtained in binding and functional studies. The main discrepancy (factors of 2–5) seems to be associated with the estimates of the affinities of AF-DX 116 and methoctramine at M_3 receptors. This has been commented upon previously (125). Part of the problem arises because the binding studies have been carried out on exocrine glandular tissue and functional studies on smooth muscle contraction. Functional studies on glandular receptors using a range of selective antagonists have seldom been performed. The data of Kunysz et al (126) using the submaxillary gland do suggest that the discrepancies between antagonist-affinity constants for M_3 receptors, measured by binding and functional studies *within* a tissue, are retained, and there is thus no a priori reason to assume that the predominant muscarinic receptors in exocrine glands are different from those mediating contraction in smooth muscle, especially as Northern blots show the presence of m3 mRNA in both tissues (30). The complementary approach, that is the performance of binding studies on smooth muscle preparations, is com-

Table 3 Comparison of affinity constants[1] (log values) of putative M_1, M_2, and M_3 receptors determined on mammalian tissues in binding[2] and functional studies

	M_1		M_2		M_3	
	Binding	Function	Binding	Function	Binding	Function
(−)-NMS	9.7(5)	-	9.2(6)	9.3*[3](1)	9.6(6)	9.6*(1)
(−)-Atropine	9.5(9)	9.6*(2)	9.1(11)	9.3(11)	9.4(7)	9.4(9)
Pirenzepine	7.9(13)	8.1(4)	6.3(15)	6.5(6)	6.8(14)	6.8(4)
4-DAMP	8.9(10)	9.1*(1)	8.0(11)	7.9(5)	9.0(8)	9.0(4)
(±)-HS-/	7.9(4)	7.9*(1)	6.9(7)	6.5(5)	8.2(4)	8.1(4)
(±)-AF-DX 116	6.4(12)	6.9*(2)	7.1(13)	7.2(6)	5.9(12)	6.4(4)
Methoctramine	7.1*(4)	- ·	7.9(3)	7.8(7)	6.5(5)	6.2(7)
Himbacine	7.0*(2)	8.0*(1)	8.1*(3)	8.2(6)	6.9*(2)	7.3(5)

[1] Values have been collated from a (noncomprehensive) search of the recent literature and are expressed as log mean values; the number of independent reports is shown in parenthesis. In general, the standard deviation associated with the parameters is 0.1–0.25, with the s.e.m. being less than 0.1.

[2] Binding studies on M_1, M_2 and M_3 receptors have generally been carried out on forebrain (inhibition of ^{3}H-pirenzepine binding), myocardium and salivary (or lacrimal) glands respectively. Functional studies have generally been carried out on the vas deferens or superior cervical ganglia (M_1), heart (M_2) or longitudinal smooth muscle of the ileum (M_3). The values for (±)-atropine have been increased by 0.3 to reflect the potency of the more active (−)-enantiomer. Binding data, measured at low ionic strength, have been excluded from the analysis.

[3]* Indicates mean values for which there are few estimates and/or larger variances.

plicated by the predominance of what appears to be M_2 receptor binding sites over M_3 binding sites (see e.g. 127 for a discussion on the topic). The function, if any, of these M_2 receptors, whose existence is also suggested by Northern blots in smooth muscle (30), is unknown.

THE EFFECTS OF INCUBATION CONDITIONS ON THE BINDING OF ANTAGONISTS Before considering the binding properties of clonal muscarinic receptors (obtained by transfection, translation of cRNA in oocytes, or by clonal selection), it is necessary to emphasize that the binding of antagonists to muscarinic receptors is sensitive to incubation conditions. The effects are often not consistent between antagonists or between subtypes, although early studies had suggested that lowering the ionic strength (128) or incubation temperature (129) increased antagonist affinities. In unpublished studies of M_1, M_2, and M_3 receptors, we have found that lowering the incubation temperature from 30°C to 4°C in an 20 mM HEPES buffer containing 100mM NaCl produced relatively small changes in affinity constants of a number of antagonists except that a 3–10 fold increase was seen for (−)-atropine and NMS (M_1, M_2, M_3 receptors) and gallamine (M_1 receptors only) and a fivefold *decrease* in affinity for AF-DX 116 (M_1 receptors only) was observed.

Lowering the ionic strength at 4°C and changing to a medium where receptor-G protein coupling is enhanced (100mM NaCl → 1mM Mg^{2+})

produces small increases in antagonist-affinity constants (1- to 2.5-fold) except for a fivefold decrease in AF-DX 116 binding (M_2 only) and a tenfold increase in gallamine binding to the receptor (M_1, M_2, M_3).

Some indications of the cumulative effects of changing ionic strength and temperature is that the M_3/M_2 selectivity of hexahydrosiladifenidol is amplified from 30 (30°C, 100 mM NaCl) to 250 (4°C, 1mM Mg^{2+}).

A more drastic effect on the receptor environment may be produced by solubilization in detergent. Detailed data are available for muscarinic receptors (M_1–M_3) solubilized in digitonin and CHAPSO (130–132). These studies, performed at 4°C (1mM Mg^{2+}), have shown that the receptors are solubilized in each detergent in characteristic states which interconvert on exchanging or removing the detergent. The differences in binding affinity of a selective antagonist for the receptor subtypes may be amplified or attenuated by solubilization. For example, the binding of pirenzepine and hexahydrosiladifenidol to M_2 receptors is increased 25–50-fold on solubilization in digitonin, whereas their affinity for M_3 receptors decreases 20-fold and their affinity for M_1 receptors is almost unchanged. As with membrane studies, the binding properties of soluble receptors vary with temperature, and conflicting conclusions have been drawn regarding the effects of solubilization on the binding properties (for a discussion, see 127).

It is therefore clear that the antagonist-binding properties of muscarinic receptors are quite sensitive to the environment of the receptor. It remains to be determined whether this is due to the complexities associated with the different contributions of the ionizable groups on the receptor which regulate binding.

THE ANTAGONIST BINDING PROPERTIES OF CLONED MUSCARINIC RECEPTOR SUBTYPES The most complete set of data are those of Buckley et al (28) (Table 4). These pertain to clonal cell lines obtained by stably transfecting CHO-K1 cells with the m1–m5 genes. The binding assays were carried out in a 5mM Mg^{2+}/25 mM phosphate buffer, pH 7.4, 22°C and are thus not directly comparable to those in Table 2. Nevertheless, pirenzepine has its highest affinity for m1 receptors, AF-DX 116 and methoctramine for m2 receptors, and hexahydrosiladifenidol for m3 receptors. These data, together with the data of Fukuda et al (61), Peralta et al (21) (data from their Figure 2, not their Table 1), Akiba et al (27) and Mei et al (133) are in accord with the theory that m1, m2, and m3 gene products have the binding properties of M_1, M_2, and M_3 receptors, respectively.

Some additional binding data on m4 and m5 receptors (20, 21, 27) exists, but the only conclusion at present is that these two receptors appear to have a somewhat different pharmacological profile from M_1-M_3 receptors, but no antagonist examined to date is selective for these subtypes.

Somewhat surprisingly, in view of the fact that only one subtype is

Table 4 Binding of antagonists[1] to muscarinic receptors obtained by transfecting CHO cells with the m1–m5 genes[2]

	m1	m2	m3	m4	m5
(−)NMS	10.2	9.4	10.3	10.3	10.0
(−)-Atr	10.0	9.1	10.1	10.5[3]	10.0
Pirenzepine	7.8	6.0	6.7	7.1[3]	6.2
(±) HS	7.4	6.6	8.0	7.4[3]	7.2
(±) AF-DX 116	5.9	6.7	6.1	6.6[3]	5.6
Methoctramine	7.8	8.4	6.9	7.4[3]	7.2

[1] Data are expressed as log (affinity constant)
[2] Adapted from Buckley et al. (28)
[3] Apparently heterogeneous binding curves: data are expressed as -log IC_{50} values corrected for receptor occupancy by the tritiated radioligand.

expressed, several authors (21, 28, 107, 108) have reported that inhibition curves suggestive of binding heterogeneity were found for some antagonists and some subtypes. When the data were analyzed according to a two-site model, no consistency was found in the percentage of high- and low-affinity sites calculated for different antagonists binding to one subtype. Nor did the different groups agree as to which antagonist inhibition curves were complex. Therefore, either an uncontrolled factor is responsible for generating binding complexity, or an artifact is associated with the performance and/or the analysis of the binding data.

The outstanding feature of the binding properties of the five subtypes is that *no* antagonist exhibits greater than fivefold selectivity for one subtype over all other subtypes. The differences in structures of the binding sites for antagonists must be very subtle, as might be expected from the very strong conservation of relevant amino acid residues suggested by the model presented earlier.

Aside from cell lines transfected with known muscarinic genes, other immortalized cell lines exhibit a limited repertoire of expression of the muscarinic genes. As a consequence of their origin, these lines may be suitable for detailed functional studies because they could contain a more appropriate complement of G-proteins and effector mechanisms than, for example, fibroblasts. Some cell lines appear to express relatively pure populations of M_1, M_3 and m4 receptor species, and others express two receptor subtypes that mediate the same (PC12) or different (NIE-115) biochemical responses (114).

The Agonist-Binding Properties of Muscarinic Receptor Subtypes

As alluded to earlier in this review, the agonist-binding properties of muscarinic receptors are extremely complex (see 127 for a detailed discus-

sion). The agonist-receptor complex seems to exist in several states. The differences in agonist affinity for these states seems related to agonist efficacy. Those states with the highest affinity for an agonist are considered to be agonist-receptor-G-protein complexes and indeed molecularly distinct complexes can be solubilized from heart and cortical membranes (85, 132). Formation of these complexes are favored by the presence of divalent cations (e.g. Mg^{2+}) and disfavored by guanine nucleotides (GDP, GTP, or modified derivatives). However, the binding data cannot in general be modelled by a simple two-component (receptor and G-protein) system (Wong et al, 134); but see Ehlert 135). There must be either more than one receptor species (e.g. palmitoylated vs nonpalmitoylated), more than one G-protein interacting with the receptor, or additional components (e.g. arrestin-like molecules). It also seems that purified receptor preparations, where no other protein is present, can exhibit agonist binding heterogeneity. At present, we do not have the necessary control of the system to be able to analyze quantitatively the events associated with agonist binding and receptor activation.

Even in studies of expressed m1 species the estimate of the log-affinity constant of carbachol for the lowest-affinity (putative uncoupled) state of the receptor vary from 3.2 to 4.9 (mean 3.8 ± 0.6 s.d. n = 5). The equivalent range for m3 receptors is 3.0–4.2 (n = 3). However, in a comparative study, Bujo et al (136) state that carbachol binds 10 times more tightly to the m3 than to the m1 receptor. In functional and binding studies on NIE 115 cells, McKinney & Richelson (137) have also concluded that M_4 receptors have about a 20-fold higher affinity for carbachol than do M_1 receptors. The functional data of Fisher & Snider (138) suggests that carbachol binds 3–5-fold more weakly to M_1 receptors than (probably) to M_3 receptors in the striatum and on SK-N-SH cells. As yet binding studies offer no clear guidance to selective interactions of agonists with the receptor subtypes.

The Allosteric Site

Considerable evidence now exists in the literature for the presence of allosteric interactions between muscarinic drugs and other agents acting on muscarinic receptors (139), although this area of research is not free of controversy (127). The basic finding, exemplified by studies on the neuromuscular blocker gallamine, has demonstrated in binding studies the existence of a ternary complex between the receptor-gallamine and muscarinic drugs (140). An allosteric effect of gallamine has also been demonstrated in studies of the whole heart (141) and of muscarinic inhibition of myocardial adenylate cyclase (142).

Associated with the allosteric interaction of gallamine with muscarinic receptors is a change in the kinetics of the radioligand used to monitor the interaction (138, 140, 143). In general, a slowing down of the rate constants

has been observed. However, Ellis & Seidenberg (144) have observed that low concentrations of gallamine speed up the dissociation rate of [^{3}H]-quinuclidinylbenzilate. At equivalent concentrations, the off-rate of [^{3}H]-N-methylscopolamine is retarded. At much higher (about 10^3-fold) concentrations of gallamine a profound decrease occurs of the [^{3}H]-quinuclidinylbenzilate off rate which overcomes the acceleration observed at lower concentrations. This biphasic effect implies two sites of action of gallamine. The effects of high concentrations of gallamine (10^{-4}-10^{-3} M) have also been observed for a variety of ligands (145) including atropine (W. J. Kromer, N. J. M. Birdsall, E. C. Hulme, unpublished results). Therefore a kinetic effect seems to operate at low concentrations of gallamine (and other allosteric ligands) which is associated with formation of the ternary complex. Very high concentrations of many positively charged ligands will also slow down the kinetics in a seemingly less specific manner, maybe by interacting with the negatively charged sugar residues on the N-terminus of the receptor, providing a positively charged "haze" surrounding the receptor, and therefore making access to the binding site more difficult.

As discussed earlier, there are two acidic residues (pK 5.4, 6.8) on muscarinic receptors whose protonation strongly affects the binding of ligands and two aspartate residues (Asp^{99} and Asp^{105}, m1 sequence) may be labelled by the covalent antagonist [^{3}H]-propylbenzilylcholine mustard. The titratable acidic residues are close enough together to be bridged by methoctramine and a bivalent ligand (Birdsall et al, unpublished results). Furthermore, gallamine binds to the pK 6.8 residue on heart receptors and changes the pK of the residue to which [^{3}H]-methylscopolamine binds. The evidence points to the binding site of muscarinic receptors being quite complex, with the two conserved aspartate residues being the titratable groups. Some ligands appear to have the choice of which residue they bind. Two ligands may also bind within the same pocket, each ligand interacting with one aspartate residue, leading to allosterism. Alternatively, even if two ligands prefer to interact with different aspartate residues it may be impossible, because of steric constraints, to accommodate both within the binding site; such ligands will exhibit competitive behavior. These ideas can be tested by studies on receptors that have the aspartate residues altered by mutagenesis.

CONCLUSIONS

Future research on the mAChRs will address a number of important questions, among which we may anticipate the following:

(i) Do the muscarinic agonist and antagonist binding sites overlap? Precisely which amino acids in the mAChR sequences are responsible for pro-

ductive high-affinity agonist binding? Can we hope to determine or model the 3-dimensional structures of these receptors?

(ii) What precise molecular interactions underlie the different mechanisms of antagonist selectivity? Can they be exploited to synthesize ligands of greater selectivity?

(iii) Do the mAChR subtypes embody intrinsic differences in agonist affinities or kinetics? If so, are such differences functionally important? Can they be targeted pharmacologically?

(iv) Is discrimination between G-proteins exclusively encoded in the cytoplasmic domains of the mAChR subtypes, or are unknown cell-biological factors implicated in functional selectivity? Does G-protein selectivity extend beyond the PI/cyclase disjunction to encompass other coupling modalities?

(v) Are there mAChR-specific kinases? Are they different for PI than for cyclase-coupled subtypes? What is their role in promoting down-regulation? Does down-regulation feed back on mAChR gene expression? Which domains of the receptor are responsible for interacting with the cytoskeleton?

(vi) What are the physiological implications of the discrete distribution of mAChR subtypes? Is the potentially mitogenic role of the PI-coupled mAChRs implicated in synapse specification and development?

(vii) What is the distribution of mAChRs on the cell surface? How is their distribution affected by cell contact and diffusible factors? The production of receptor-specific antibodies will be necessary to answer these questions.

(viii) Are there additional mAChR subtypes? How is the differential expression of mAChR genes accomplished? What are the physiological and developmental consequences of interfering with these processes? Transgenic mouse models will be essential tools in addressing these questions.

(ix) Are these long-term effects of activation of muscarinic receptors? Can mAChR stimulation activate transcription of other genes? How does the cell membrane communicate information to the cell nucleus?

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