

# International Union of Pharmacology. XVII. Classification of Muscarinic Acetylcholine Receptors<sup>a</sup>

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|  |     |
|--|-----|
| I. Introduction  | 279 |
| II. Nomenclature   | 280 |
| III. Molecular definition of subtypes                        | 280 |
| IV. Pharmacological definition of subtypes                   | 282 |
| A. Antagonists   | 282 |
| B. Allosteric agents   | 283 |
| C. Definition of individual receptor subtypes                | 283 |
| 1. M <sub>1</sub> receptors                                  | 285 |
| 2. M <sub>2</sub> receptors                                  | 285 |
| 3. M <sub>3</sub> receptors                                  | 285 |
| 4. M <sub>4</sub> receptors                                  | 285 |
| 5. M <sub>5</sub> receptors                                  | 286 |
| 6. Functional systems whose classification is open to debate | 286 |
| D. Agonists  | 286 |
| V. Transduction mechanisms and functional responses          | 287 |
| VI. Conclusions  | 288 |
| VII. Acknowledgments   | 288 |
| VIII. References   | 288 |

## I. Introduction

Actions of acetylcholine in the periphery are the result of activation of either the ionotropic nicotinic receptor or the metabotropic muscarinic receptor. In the mamma-

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lian central nervous system (CNS)<sup>c</sup>, both nicotinic and muscarinic receptor subtypes are present on neurons, although there is as yet very limited evidence for a physiological role for nicotinic receptors in synaptic function in the mammalian brain (Role and Berg, 1996). In the periphery, among other effects, muscarinic receptors mediate smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the CNS, there is evidence that muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation, and memory. Interest in the classification of muscarinic receptors involved in functions at different locations has been heightened by the potential therapeutic application of selective agents in areas such as Alzheimer's disease, Parkinson's disease, asthma, analgesia, and disorders of intestinal motility and cardiac and urinary bladder function.

Historically, the first indications of the existence of muscarinic receptor subtypes were the cardioselective actions of gallamine (Riker and Wescoe, 1951) and the

<sup>c</sup> Abbreviations: cAMP, cyclic adenosine monophosphate; CNS, central nervous system; LY297802, (+)-3-(S)-3-[4-butylthio-12,5-thiadiazol-3-yl]-1-azabicyclo [2,2,2]octane; McN-A-343, (4-Hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbonyl chloride; mRNA, messenger ribonucleic acid.

sympathetic ganglionic stimulant behavior of (4-hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbamate chloride (McN-A-343) (Roszkowski, 1961). Subsequently, Barlow *et al.* (1976) demonstrated significant differences in the pharmacological properties of ileal and atrial muscarinic receptors. The introduction of pirenzepine, a drug used in the treatment of peptic ulcer disease, had a major role in the appreciation of the existence of muscarinic receptor subtypes. Its selectivity in binding (Hammer *et al.*, 1980) and functional studies (Brown *et al.*, 1980; Hammer and Giachetti, 1982) provided an explanation of its *in vivo* selectivity. It seemed as if there were at least three subclasses of muscarinic receptors (Birdsall and Hulme, 1983).

Knowledge of the potential functions and roles of muscarinic receptors and their subtypes was advanced significantly by the cloning of five mammalian genes encoding muscarinic receptors. Their expression in cell lines has resulted in the generation of much information on potential coupling mechanisms, the production of selective antibodies, and the ability to localize sites of expression of messenger ribonucleic acids (mRNAs) encoding the receptors. Notwithstanding this progress, the definition of the receptor subtype(s) involved in a particular functional response still is accomplished best by the use of selective pharmacological tools. Although it is true that the presence of receptor gene-specific mRNA, or receptor-specific immunoreactivity can provide evidence supporting the pharmacological demonstration of a functional receptor subtype, it is equally true that firm pharmacological evidence for the involvement of a particular subtype stands alone: lack of supporting molecular data is not sufficient justification for rejecting the pharmacological evidence.

This review describes the naming and classification of muscarinic receptors in line with the guidelines of NC-IUPHAR. The main features of muscarinic receptor structure, pharmacology, and function that provide the basis for the classification are summarized. Because this review does not set out to be a comprehensive review of the literature, readers seeking more detail should refer to the many relevant reviews in the field (table 1).

## II. Nomenclature

The previous nomenclature was recommended by the Fourth Symposium on Subtypes of Muscarinic Receptors and the NC-IUPHAR Subcommittee on Muscarinic Acetylcholine Receptors (Levine and Birdsall, 1989). This nomenclature used a lower case 'm' followed by its number to describe a subtype when the muscarinic receptor gene or gene product was known unambiguously (for example, by transfection into a nonexpressing cell line). On the other hand, when the properties of the receptor were defined by its pharmacology and the molecular species contributing to these properties was not known unambiguously the receptor was denoted by 'M' and a subscript number (this being the nomenclature used before the cloning of the receptor genes). The aim was that, with the discovery of antagonists of greater selectivity, the dual molecular and pharmacological descriptors of muscarinic receptor subtypes would merge into a single definition.

Based on existing knowledge, summarized in this review, it is now recommended that M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>, M<sub>4</sub>, and M<sub>5</sub> be used to describe both the pharmacological subtypes (as defined previously) and the molecular subtypes (defined previously as m1–m5, respectively).

A pharmacological characterization of endogenous M<sub>5</sub> receptors in whole-tissue functional studies is still lacking. However, under the revised guidelines of the NC-IUPHAR Committee on Receptor Nomenclature and Drug Classification (Vanhoutte *et al.*, 1998), it is viewed by the above-mentioned committee and the muscarinic receptor subcommittee that the evidence presented in this review is sufficient to define the M<sub>5</sub> receptor.

## III. Molecular Definition of Subtypes

Cloning of complementary deoxyribonucleic acids for muscarinic receptor genes was spearheaded by the work of Numa and colleagues, who cloned the M<sub>1</sub> and M<sub>2</sub> genes (Kubo *et al.*, 1986a,b), and was extended by the discovery of the M<sub>3</sub>, M<sub>4</sub>, and M<sub>5</sub> genes (Bonner *et al.*, 1987, 1988; Peralta *et al.*, 1987). These five genes encode muscarinic receptor proteins (actually glycoproteins)

TABLE 1  
Recent reviews on muscarinic receptors

| Coverage   | Authors                                |
|--|--|
| Pharmacology, structure, coupling, localization                        | Hulme <i>et al.</i> (1990)             |
| Pharmacology, structure, coupling, localization                        | Caulfield (1993)                       |
| Pharmacology, classification techniques                                | Caulfield (1997)                       |
| Muscarinic receptors in smooth muscle                                  | Eglen <i>et al.</i> (1996)             |
| Roles for M <sub>2</sub> and M <sub>3</sub> receptors in smooth muscle | Eglen <i>et al.</i> (1994)             |
| Species differences  | Hall <i>et al.</i> (1993)              |
| Mutational analysis of ligand binding and G-protein-binding residues   | Wess <i>et al.</i> (1995); Wess (1996) |
| Muscarinic receptor toxins   | Jerusalinsky and Harvey (1994)         |
| Signal transduction  | Felder (1995)                          |
| Ion channel coupling   | Brown <i>et al.</i> (1995)             |
| Presynaptic muscarinic receptors                                       | Grimm <i>et al.</i> (1994b)            |
|  | Fuder and Muscholl (1995)              |
| Newer agonists and antagonists   | Eglen and Watson (1996)                |
|  | Grimm <i>et al.</i> (1994b)            |
| Muscarinic receptor subtypes symposium                                 | Levine and Birdsall (1997)             |

which have the structural features of the seven transmembrane helix G-protein-coupled receptor family. Muscarinic receptor sequences have significant homologies with other members of this receptor superfamily (Hulme *et al.*, 1990). The vertebrate receptor genes cloned so far are intronless within the coding regions and are notably similar across mammalian species (Hall *et al.*, 1993; Eglen *et al.*, 1996; table 2). The chromosomal localization of the human M<sub>1</sub>–M<sub>5</sub> genes are reported to be 11q12–13, 7q35–36, 1q43–44, 11p12–11.2, and 15q26, respectively (Bonner *et al.*, 1991).

In addition to being transfected into a variety of cells of mammalian/amphibian origin, either transiently (e.g., COS cells, *xenopus* oocytes) or stably (e.g., Chinese hamster ovary, A9L, Y<sub>1</sub> cells), the receptors have also been expressed in insect (Sf9) cells (Vasudevan *et al.*, 1995; Hepler *et al.*, 1996), Dictyostelium (Voith and Dingermann, 1995), yeast (Payette *et al.*, 1990; Huang *et al.*, 1992), and *Escherichia coli* (Curtis and Hulme, 1997).

The apparent similarities between muscarinic receptor subtypes across species, at least at the amino acid level, are potentially misleading. Precedents from other types of receptors clearly show that a minor sequence difference between receptors in different species can have a major impact on their pharmacological profiles. For example, a single amino acid difference between the human and rat 5-hydroxytryptamine<sub>1B</sub> receptor is manifest as a major difference in ligand affinities (reviewed by Kenakin, 1996).

In muscarinic receptors the existing evidence is that the pharmacology is *not* significantly different between mammalian receptor homologs (insofar as it has been studied; see Hall *et al.*, 1993, for example). However, the possibility cannot be excluded that novel ligands may make different interactions with regions of the receptor where there are sequence differences between species. Thus, there is a very good argument for using cloned human receptor genes expressed in cell lines to derive information about potential human therapeutic agents, rather

than attempting to use a receptor system from a nonhuman species that provides the 'best' pharmacological match to the human profile.

In common with most members of the subgroup of G-protein-coupled receptor family whose ligand-recognition site binds small molecules, the major features of muscarinic receptor structure are:

- The ligand recognition site is within the outer half of the membrane-embedded part of the protein.
- The transmembrane segments are probably  $\alpha$ -helices, three oriented approximately perpendicular to the membrane, four at a more acute angle (Baldwin *et al.*, 1997).
- There are two conserved cysteine residues that form a disulfide bond between the first and third extracellular loops (Kurtenbach *et al.*, 1990; Savarese *et al.*, 1992).
- There is a conserved triplet of amino acids (Asp Arg Tyr) at the cytoplasmic interface of TMIII with the second intracellular loop, which is important for both the expression and function of the receptor (Zhu *et al.*, 1994; Jones *et al.*, 1995; Lu *et al.*, 1997).
- The carboxy-terminus is on the intracellular side of the membrane because antibodies to the C-terminus sequences recognize cell-surface receptors only when cells are permeabilized (e.g., Lu *et al.*, 1997). Palmitoylation of a cysteine residue at the carboxy-terminus of M<sub>2</sub> receptors (C457) occurs in cells, but it is not an absolute requirement for the interaction with G-proteins even though function is enhanced (Hayashi and Haga, 1997).
- There are one or more glycosylation sites on the N-terminus, but glycosylation apparently is not crucial for receptor expression and function, at least for the M<sub>2</sub> receptor (Van Koppen and Nathanson, 1990). This study provides evidence for the extracellular location of the N-terminus.

TABLE 2  
Muscarinic receptor genes

| Species                     | SwissProt accession code<br>Number of amino acids (% identity with human sequence) |                                  |                     |                     |                     |
|-----------------------------|--|----------------------------------|---------------------|---------------------|---------------------|
|                             | M <sub>1</sub>   | M <sub>2</sub>                   | M <sub>3</sub>      | M <sub>4</sub>      | M <sub>5</sub>      |
| Human                       | P11229<br>460  | P08172<br>466                    | P20309<br>590       | P08713<br>479       | P08912<br>532       |
| Pig                         | P04761<br>460 (99%)  | P06199<br>466 (97%)              | P11483<br>590 (96%) |                     |                     |
| Cow                         |  |                                  | P41984<br>590 (95%) |                     |                     |
| Rat                         | P08482<br>458 (99%)  | P10980<br>466 (95%)              | P08483<br>589 (92%) | P08485<br>478 (95%) | P08911<br>531 (89%) |
| Mouse                       | P12657<br>460 (98%)  |                                  |                     | P30544<br>479 (95%) |                     |
| <i>Xenopus</i> <sup>a</sup> |  |                                  |                     | P30544<br>484 (78%) |                     |
| Chicken <sup>a</sup>        |  | P30372<br>466 (92%) <sup>b</sup> | P49578<br>639 (87%) | P17200<br>490 (76%) |                     |

<sup>a</sup> The nonmammalian *Xenopus* and chicken receptors have been included for comparison. A *Drosophila* muscarinic receptor (P13695, 722aa) also has been cloned (Shapiro *et al.*, 1989; Onai *et al.*, 1989), but its sequence does not fit into the current classification.

<sup>b</sup> Unusual pharmacology, with approximately ten times higher pirenzepine affinity than mammalian M<sub>2</sub> receptors (Tietje and Nathanson, 1991). From Hulme *et al.* (1990); Hall *et al.* (1993); Herrera *et al.* (1994); Eglen *et al.* (1996), and the SwissProt database.

In whole-cell studies, serine and threonine residues in the large postulated third intracellular loop of muscarinic receptors are phosphorylated by endogenous protein kinases (Pals-Rylaarsdam and Hosey, 1997), which indicates that the large i3 loop of the  $M_2$  receptor is indeed intracellular.

The projected three-dimensional structure of the receptors is expected to have more in common with that of rhodopsin, a G-protein-coupled receptor (Baldwin, 1993), than that of bacteriorhodopsin, a proton pump and another seven-transmembrane helix protein whose medium-resolution, three-dimensional structure is known (Henderson *et al.*, 1990). The latter structure has been assumed to be an appropriate model for G-protein-coupled receptors in some studies.

As with the cationic amine receptors, all muscarinic receptors have an Asp residue in the distal N-terminal part of the third transmembrane domain which is thought to interact with the polar headgroup of amine ligands, including acetylcholine. This residue is alkylated specifically by the agonist, acetylcholine mustard, and the antagonist, propylbenzilylcholine mustard (Curtis *et al.*, 1989; Spalding *et al.*, 1994). Uncharged muscarinic antagonists also will bind to muscarinic receptors but with lower affinity (e.g., Hou *et al.*, 1996). Residues important for the binding of these ligands have not been defined. Sites involved in binding different receptor-selective antagonists are probably quite diverse, depending on the antagonist (Wess *et al.*, 1990, 1992; Matsui *et al.*, 1995). It presently is difficult to identify amino acids that interact directly with the antagonists and to distinguish such residues from those which, when mutated, affect antagonist binding by indirectly changing the conformation of the binding site (or sites). Blüml *et al.* (1994) suggested that a conserved Asn residue in the sixth transmembrane segment is very important for the binding of certain subclasses of antagonists, notably atropine-like analogs.

An important molecular distinction between the different muscarinic receptor subtypes is the sequence divergence in the postulated third internal (i3) loops between the  $M_1/M_3/M_5$  sequences compared with the  $M_2/M_4$  sequences (Hulme *et al.*, 1990; Wess, 1996; Wess *et al.*, 1997) that probably determines the quite specific coupling preferences of these two groups (Wess, 1993). More recently, further mutational studies have shown that coupling specificity of the  $M_3$  receptor is determined by a small set of amino acids in the TMIII/i2 loop interface and in the membrane-proximal portions of the i3 loop (Blin *et al.*, 1995). Similar studies with the  $M_2$  receptor have identified a four amino acid sequence (Val Thr Ile Leu) located at the interface of the i3 loop and TM VI which couples this receptor to its target G-proteins ( $G\alpha_{O1}$ ) (Wess *et al.*, 1997); this sequence is also present in other receptors with high coupling preference for these G-protein  $\alpha$  subunits (Liu *et al.*, 1995).

Muscarinic receptor-G-protein interactions and activation can occur in the absence of agonists. This can be demonstrated in binding studies carried out at low ionic strengths in the presence of  $Mg^{2+}$  (Hulme *et al.*, 1981), as the existence of constitutive activity observed in functional studies by overexpression of the receptor (Vogel *et al.*, 1995) or G-protein (Burstein *et al.*, 1995), and also in a patch-clamp study in atrial cells (Soejima and Noma, 1984). Muscarinic antagonists regulate 'basal' activity [elevate cyclic adenosine monophosphate (cAMP) levels, inhibit inositol 1,4,5-trisphosphate production, or close atrial  $K^+$  channels, depending on subtype], but presently there is no evidence of differential effects of antagonists on their maximal effects. The existence of significant constitutive activity in vivo is not known, nor is there any therapeutic use of muscarinic antagonists acting as 'inverse agonists.'

Another feature of muscarinic receptors is the presence of a specific binding site which, when occupied by ligands, can modify the binding and behavior of ligands binding to the acetylcholine recognition site. This allosteric site has been characterized by equilibrium and kinetic binding studies (Stockton *et al.*, 1983; Ellis *et al.*, 1991; Proska and Tucek, 1995; Lazareno and Birdsall, 1995) as well as in functional studies (Ehlert, 1988; Lazareno and Birdsall, 1995). Studies of chimeric and mutant receptors (Ellis *et al.*, 1993; Leppik *et al.*, 1994; Matsui *et al.*, 1995) have begun to identify amino acids and receptor domains that may constitute the binding site.

#### IV. Pharmacological Definition of Subtypes

Pharmacological characterization of muscarinic receptor subtypes has long been dogged by a complete lack of agonists with any selectivity, and a lack of antagonists with very high selectivity for any single receptor subtype. Additionally, cells frequently coexpress more than one subtype, further adding to the difficulty of assigning a functional response to a single receptor subtype.

##### A. Antagonists

The definition of antagonist affinities for the five muscarinic receptors has been aided greatly by the use of radioligand binding techniques, with ligands such as [ $^3H$ ]pirenzepine and [ $^3H$ ]N-methylscopolamine, in combination with membrane preparations from cells transfected with the gene for a particular receptor, and thereby expressing a single receptor subtype. Affinity constants obtained from these experiments have been remarkably comparable with apparent affinity constants determined in functional experiments using Arunlakshana-Schild analysis (reviewed by Caulfield, 1993; table 3) or any of the acceptable variants (Lazareno and Birdsall, 1993a,b). Nevertheless, serious errors can be made in estimates of antagonist affinity constants by inappropriate design of radioligand binding experiments (Hulme and Birdsall, 1992). It also is possible to perturb grossly the antagonist structure-binding relationships by carrying out binding assays under condi-

TABLE 3

Antagonist affinity constants (log affinity constants or  $pK_B$  values) for mammalian muscarinic receptors<sup>a</sup>

| Antagonist        | Receptor subtype |                |                |                |                |
|-------------------|------------------|----------------|----------------|----------------|----------------|
|                   | M <sub>1</sub>   | M <sub>2</sub> | M <sub>3</sub> | M <sub>4</sub> | M <sub>5</sub> |
| Atropine          | 9.0–9.7          | 9.0–9.3        | 8.9–9.8        | 9.1–9.6        | 8.9–9.7        |
| Pirenzepine       | 7.8–8.5          | 6.3–6.7        | 6.7–7.1        | 7.1–8.1        | 6.2–7.1        |
| Methoctramine     | 7.1–7.8          | 7.8–8.3        | 6.3–6.9        | 7.4–8.1        | 6.9–7.2        |
| 4-DAMP            | 8.6–9.2          | 7.8–8.4        | 8.9–9.3        | 8.4–9.4        | 8.9–9.0        |
| Himbacine         | 7.0–7.2          | 8.0–8.3        | 6.9–7.4        | 8.0–8.8        | 6.1–6.3        |
| AF-DX 384         | 7.3–7.5          | 8.2–9.0        | 7.2–7.8        | 8.0–8.7        | 6.3            |
| Tripitramine      | 8.4–8.8          | 9.4–9.6        | 7.1–7.4        | 7.8–8.2        | 7.3–7.5        |
| Darifenacin       | 7.5–7.8          | 7.0–7.4        | 8.4–8.9        | 7.7–8.0        | 8.0–8.1        |
| Guanylpirenzepine | 7.7              | 5.5            | 6.5            | 6.5            | 6.8            |
| PD 102807         | 5.3              | 5.7            | 6.2            | 7.3            | 5.2            |
| MT3 <sup>b</sup>  | 7.1              | <6             | <6             | 8.7            | <6             |
| MT7 <sup>b</sup>  | 9.8              | <6             | <6             | <6             | <6             |

<sup>a</sup> Data are from a variety of mammalian species, including human: Hulme *et al.*, 1990; Caulfield, 1993; Maggio *et al.*, 1994; Eglén *et al.*, 1996; Nunn *et al.*, 1996; Adem and Karlsson, 1997; Caulfield, 1997; Levine and Birdsall, 1997; Jolkkonen *et al.*, 1994.

<sup>b</sup> Toxins from Eastern Green Mamba venom.

tions (particularly ionic strength, temperature, and solubilization in detergents) that differ substantially from those of the functional assays (Pedder *et al.*, 1991). Similarly, the estimation of apparent affinity constants for antagonists in functional experiments can be flawed unless care is taken in experimental design (Caulfield, 1997).

Table 3 summarizes the (log) affinity constants (and apparent affinity constants in functional studies) of atropine, some selective antagonists, and the two most selective muscarinic toxins for the different muscarinic receptor subtypes. The chemical structures of the selective nonpeptide antagonists are given in figure 1. The data in table 3 include information from binding studies and functional studies (e.g., Lazareno and Birdsall, 1993a,b) on cloned receptors, and on natively expressed receptors (where the subtype involved has been defined satisfactorily; see Section IV.C.1–4).

The ranges of values represent the differences in values determined by different laboratories, and the inference is that when a value for a given antagonist lies outside the range for a given receptor, then that receptor is not detected (in a binding study) or does not mediate the response measured (in a functional study). Less well-characterized, but nevertheless potentially useful molecules are also included in table 3 (e.g., guanylpirenzepine, darifenacin, and PD102807). Ranges of values for these antagonists are not given and caution should be taken in interpreting data obtained with these compounds until more information is available from further studies on cloned receptors and from a wider range of functional studies. These antagonists are of special interest because they have been reported to have a considerably higher affinity for one subtype over all other subtypes.

Also included in table 3 are two muscarinic snake toxins, MT3 and MT7. These toxins are two of several components of the venom of the green (*Dendroaspis angusticeps*) and black mamba (*Dendroaspis polylepis*) which have a high affinity for muscarinic receptors. These two toxins show great promise in that their apparently high

subtype selectivity should be extremely useful in muscarinic receptor classification. However, whether they and the other muscarinic toxins have additional actions on nonmuscarinic systems has yet to be determined.

It is evident from table 3 that convincing evidence for the involvement of a particular receptor necessitates the use of more than one antagonist. It must be emphasized that, because of the lack of very high subtype selectivity of any single antagonist (except perhaps for the M<sub>1</sub> receptor selectivity of MT7 toxin), it should not be acceptable to state that a particular receptor is involved when the experimental design involves procedures such as “block” of an agonist response by a fixed high concentration of antagonist, determination of IC<sub>50</sub> values, “potency rank orders” of antagonists, or any other design that does not involve determination of affinity constants (or apparent affinity constants). Similarly, descriptions of compounds such as pirenzepine as “M<sub>1</sub> receptor-selective” should be resisted. Most importantly, it is not advisable to use a “majority verdict” approach to receptor classification. Thus, if even one antagonist has a  $pK_B$  that is significantly different from its  $pK_B$  at one defined (preferably cloned) receptor, say M<sub>1</sub>, it is not acceptable to ignore that discrepancy and to classify the receptor as M<sub>1</sub> without an experimental explanation for the discrepancy.

### B. Allosteric Agents

Experiments with the allosteric compound gallamine (which acts as a selective allosteric antagonist at M<sub>2</sub> receptors) gave important early indications of muscarinic receptor heterogeneity (Riker and Wescoe, 1951; Clark and Mitchelson, 1976; Stockton *et al.*, 1983). The effects of allosteric ligands can be detected in studies on cloned and expressed human muscarinic receptors, both in radioligand binding studies (e.g., Ellis *et al.*, 1991; Lazareno and Birdsall, 1995), and in functional studies (Lazareno and Birdsall, 1995). It is expected that the use of selective ligands acting at this site will be useful in muscarinic receptor classification, although the interpretation of their effects is complex (Lazareno and Birdsall, 1995).

Several allosteric ligands have been described (see e.g., Birdsall *et al.*, 1987; Lee and El-Fakahany, 1991). Most ligands exhibit negative cooperativity with agonists and antagonists. Alcuronium and strychnine, however, are allosteric agents that are positively cooperative with the antagonist, N-methylscopolamine, at one or more muscarinic receptor subtypes (Tucek *et al.*, 1990; Lazareno and Birdsall, 1995; Proska and Tucek, 1995), and brucine and certain analogs exhibit positive cooperativity with acetylcholine at specific receptor subtypes in both binding and functional studies (Birdsall *et al.*, 1997; Jakubik *et al.*, 1997; Lazareno *et al.*, 1998)

### C. Definition of Individual Receptor Subtypes

The affinity constants for the partially selective antagonists given in table 3 represent the basis for assigning a response or a binding site to a particular muscarinic recep-

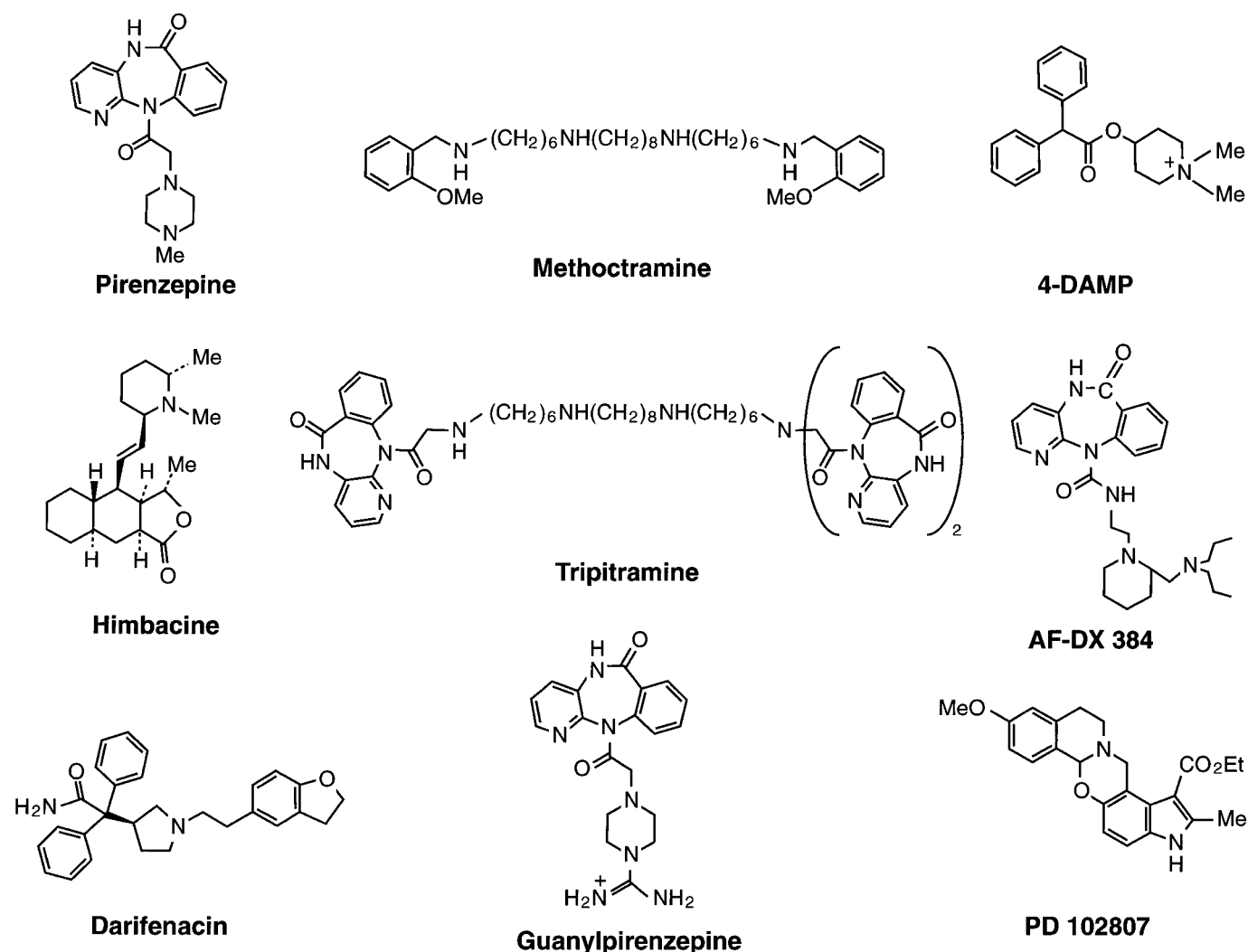


FIG. 1. Muscarinic antagonists.

tor subtype. Definition of the selectivity of a novel muscarinic antagonist, or of the actions of a putative selective agonist, can be accomplished best using recombinant muscarinic receptors expressed in cell lines (Buckley *et al.*, 1989; Lazareno and Birdsall, 1993a; Dörje *et al.*, 1991b; Maggio *et al.*, 1994). If the agent is intended to have therapeutic utility, it would seem logical to do these tests on cloned and expressed human receptors. Nevertheless, there have been many studies of new muscarinic agonists and antagonists on native receptors, often mediating functional responses, possibly because there is a view that a profile obtained with native functionally coupled receptors is "better" than similar data obtained either in binding or functional studies with cloned receptors. This might be true if there were reason to believe that native receptors behaved significantly differently from cloned receptors (e.g., because of posttranslational modifications). However, there is currently no evidence for such a difference (Caulfield, 1993). What is clear is that the behavior of agonists in particular (including their binding properties) will be determined by levels of expression of signal transduction

proteins, including receptors, receptor kinases, G-proteins, RGS proteins, enzymes generating second-messengers, and other effectors, such as ion channels. For example, increasing the expression of *Gaq* increased the potency of agonists and induced constitutive activity (Burstein *et al.*, 1995, 1997), which is what would be expected on theoretical grounds (Kenakin, 1996). A further complication that is likely to arise with high expression levels of receptors or other components of the signal transduction pathway is coupling to multiple effectors, especially with highly efficacious agonists (Kenakin, 1996). This has been observed in functional studies in membranes (Lazareno *et al.*, 1993) and in whole-cell studies (see e.g., Ashkenazi *et al.*, 1987; Gurwitz *et al.*, 1994).

A true resolution of these problems will only be made when there is a full definition of the levels of each constituent of the signal transduction pathway involved in a given response, together with an understanding of how agonists work at the molecular level. Although there has been considerable progress in this regard, we are nevertheless a long way from this ideal state.

Notwithstanding this, many reports still use nonhuman functional systems to define the actions of agonists and antagonists at muscarinic receptor subtypes, often with a therapeutic end in mind. For this reason, we will outline briefly some model functional responses, which can be recorded by fairly simple techniques and which have been defined satisfactorily as involving a particular muscarinic receptor subtype.

### 1. $M_1$ receptors.

a. RAT SUPERIOR CERVICAL GANGLION. Muscarinic agonist depolarization of rat isolated superior cervical ganglion, recorded extracellularly, is mediated by  $M_1$  receptors (Brown *et al.*, 1980). This is probably the result of inhibition of opening of the voltage-gated M-type  $K^+$  channels in these neurons (Marrion *et al.*, 1989; Bernheim *et al.*, 1992), although  $M_1$  receptors can modulate other conductances which could contribute to the depolarizing response (e.g., the protein kinase C-dependent  $Cl^-$  current described by Marsh *et al.*, 1995). The pharmacology of this system has not been investigated using the more recently discovered antagonists. However, the ablation of M-current inhibition in sympathetic ganglion neurons of the  $M_1$ -knockout mouse argues convincingly for the linkage (Hamilton *et al.*, 1997.)

b. CANINE SAPHENOUS VEIN. Contraction of this preparation probably is mediated by  $M_1$  receptors, because the apparent  $pK_B$  values of a range of partially selective antagonists is entirely consistent with an  $M_1$  receptor profile (O'Rourke and Vanhoutte, 1987; Sagrada *et al.*, 1994; Watson *et al.*, 1995). It should be noted that there is a low receptor reserve associated with the contraction. Most agonists, notably those of low intrinsic efficacy (including McN-A-343!), act as antagonists.

### 2. $M_2$ receptors.

a. GUINEA-PIG HEART. Activation of muscarinic receptors in these preparations produces a reduction in force of contraction and (in nonpaced tissues) a decrease in the rate of beating. These effects are probably the consequence of inhibition of voltage-gated  $Ca^{2+}$  channels and activation of inwardly rectifying  $K^+$  channels, respectively. Extensive studies with many antagonists have defined this response as being mediated by the  $M_2$  receptor (reviewed by Caulfield, 1993).

$M_2$  receptors can mediate both negative and positive inotropic responses in the left atrium of the reserpinized rat, that latter effect being insensitive to pertussis toxin (Kenakin and Boselli, 1990).

It also has been suggested that an  $M_1$  muscarinic receptor stimulates phospholipase C, and increases  $Ca^{2+}$  currents in pertussis toxin-treated guinea-pig and rat ventricular myocytes (see Sharma *et al.*, 1997 for references). Supporting evidence for this contention was that subtype-specific antibodies detected  $M_1$  receptor protein in myocytes, and reverse transcriptase polymerase chain reaction detected significant  $m1$  mRNA (Gallo *et al.*, 1993; Sharma *et al.*, 1997). However, one caveat to the report of Gallo *et al.* (1993) is that the effect of

pirenzepine in antagonizing the muscarinic stimulation of phospholipase C extrapolated to an apparent  $pK_B$  value of approximately 9.5, which is not consistent with any known muscarinic receptor.

### 3. $M_3$ receptors.

a. GUINEA-PIG ILEUM. The muscarinic receptors mediating contraction of guinea-pig ileum (and indeed of many other smooth muscle preparations) are defined pharmacologically as  $M_3$  (reviewed by Eglen *et al.*, 1996). There is a large population of  $M_2$  receptors in many smooth muscles, and it seems likely that they are involved in antagonizing the relaxant effects of agents that elevate cAMP (Thomas *et al.*, 1993; Eglen *et al.*, 1994).  $M_2$  receptors in guinea-pig ileum also stimulate the opening of cation-selective channels that depolarize the muscle cells (Bolton and Zholos, 1997).

Several studies have indicated that the receptor mediating relaxation of vascular smooth muscle (via release of relaxing factors from endothelial cells) is  $M_3$  (reviewed by Eglen and Whiting, 1990; Caulfield, 1993; Van Zwieten and Doods, 1995) but there is also evidence, summarized in the reviews above, for differences in  $pK_B$  values for selective antagonists in blocking the relaxant responses in some blood vessels.

There also have been suggestions that smooth muscle  $M_3$  receptors from different tissues may be heterogeneous. Thus, compounds such as zamifenacin, darifenacin, and p-F-HHSiD have been reported to distinguish between muscarinic agonist responses in tissues such as trachea, ileum, and urinary bladder (reviewed by Eglen *et al.*, 1996). However, as pointed out by Eglen *et al.* (1996), it seems premature to speculate about subtypes of  $M_3$  receptor (or a new receptor subtype) in the absence of supporting molecular evidence, either in the form of new genes or posttranslational modifications which change antagonist affinities.

### 4. $M_4$ receptors.

a. RABBIT ANOCOCCYGEUS MUSCLE. In preparations in which the tone has been raised by histamine, muscarinic agonists relax the precontraction. This apparently is an exclusively presynaptic effect, involving the release of an inhibitory nonadrenergic noncholinergic neurotransmitter, probably nitric oxide (Gross *et al.*, 1997). Muscarinic antagonists inhibit the relaxation, the  $pK_B$  values indicating that  $M_4$  receptors mediate this response (Gross *et al.*, 1997). There is a low receptor reserve for this response because agonist potencies are low and several agonists of low intrinsic efficacy act as antagonists.

b. NG108-15 CELLS. The neuroblastoma-glioma hybrid cell line NG108-15 expresses  $M_4$  mRNA (Peralta *et al.*, 1987) and  $M_4$  receptors can be detected readily in radioligand binding assays (Lazareno *et al.*, 1990).

Inhibition of adenylyl cyclase activity by muscarinic agonists in rat corpus striatum probably is mediated by  $M_4$  receptors (Caulfield, 1993; Olanas *et al.*, 1996). However, a 3- to 10-fold discrepant value for the  $pK_B$  of methocarbamol has been reported (Onali and Olanas, 1995).

5.  $M_5$  receptors. Despite evidence for the presence of the  $M_5$  protein and its mRNA in the brain and periphery (Weiner *et al.*, 1990; Flynn *et al.*, 1997), it has not yet been possible to delineate a whole-tissue response whose location and pharmacology matches that predicted for the expressed gene product. There have been several studies of the function of the cloned  $M_5$  receptor, so this gene product does represent a functional receptor. However, it has been shown only recently that the  $pK_B$  values for several selective antagonists in blocking function in cells transfected with the  $M_5$  gene agree with the binding affinities measured in membranes from the same cell line (Watson *et al.*, 1998). Reeve *et al.* (1997) have summarized the current knowledge about this somewhat ephemeral receptor subtype.

There is evidence that the A2058 human melanoma cell line expresses only  $M_5$  receptors (Kohn *et al.*, 1996). This may provide a useful model for an endogenous  $M_5$  receptor in a human cell line, but it also should be noted that the coupling mechanisms in this cell line are somewhat unusual. Another potentially useful system is the eosinophilic leukemia cell line (EoL-1) where  $M_5$  (and  $M_3$ ) receptors can be induced on differentiation with interferon- $\gamma$  (Mita *et al.*, 1996).

The conclusion is that the characterization of the  $M_5$  receptor is still incomplete but, based on the revised NC-IUPHAR guidelines, there is now sufficient evidence to warrant a  $M_5$  (rather than  $m_5$ ) nomenclature.

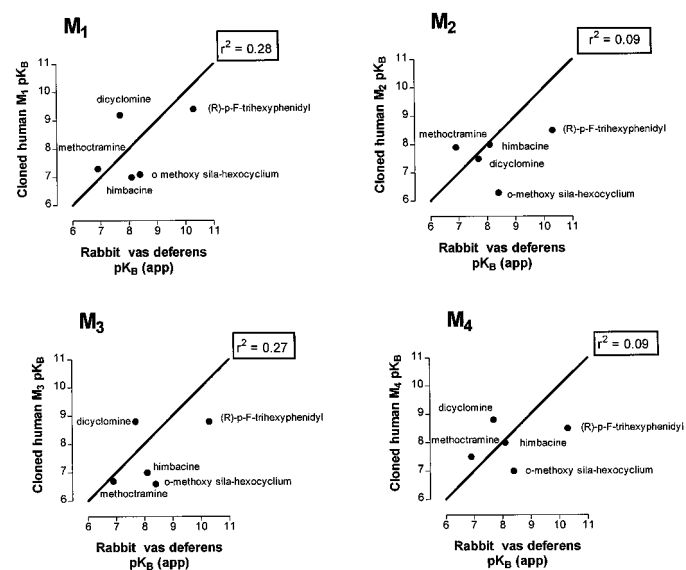


FIG. 2.  $pK_B$ (app) values for rabbit vas deferens functional assays versus  $pK_B$  values on cloned muscarinic receptors. The functional (apparent)  $pK_B$  values for six selective muscarinic antagonists obtained in Arukashana-Schild-type experiments on rabbit vas deferens (Eltze, 1988; Sagrada *et al.*, 1994; Grimm *et al.*, 1994a; Waelbroeck *et al.*, 1994) are compared with the log affinity values ( $pK_B$ ) from binding studies on cloned human receptors (Dörje *et al.*, 1991b; Lambrecht *et al.*, 1997). Where data are not available from work on cloned receptors, results are included from work on defined subtypes in nonhuman species (dicyclomine, Lazareno *et al.*, 1990; O-methoxy sila-hexocyclium, Waelbroeck *et al.*, 1994). Data have been included for those antagonists whose apparent  $pK_B$  values on the rabbit vas deferens preparation deviate by more than 0.5 log units from the  $pK_B$  values at either  $M_1$  or  $M_4$  receptors.

6. *Functional systems whose classification is open to debate.* a. RABBIT VAS DEFERENS. In rabbit vas deferens, it has been argued that the inhibition of field stimulation-evoked twitch responses by muscarinic agonists such as McN-A-343 is mediated by  $M_1$  receptors, because the effect is antagonized by pirenzepine with an apparent  $pK_B$  of 7.8 (Eltze, 1988, Grimm *et al.*, 1994a). However, the  $pK_B$  values obtained with some other antagonists are not consistent with this conclusion. Also, it previously has been suggested that the receptor mediating this functional response may be the  $M_4$  subtype, given the  $pK_B$  value for himbacine (Caulfield, 1993). However, for a wider range of antagonists, comparison between  $pK_B$  values obtained on this preparation with  $pK_B$  values at cloned human receptors (or other well-defined systems where cloned receptor data are not available) clearly indicates that neither of these hypotheses can be true (fig. 2). Thus, the rabbit vas deferens presynaptic muscarinic receptor subtype apparently is still not defined adequately. The possibility also remains that more than one muscarinic receptor subtype can couple to inhibit transmitter release in this preparation. A potentially further confounding factor is that most muscarinic agonists *potentiate* the field-stimulated twitch response by activating a receptor that has a  $M_2$ -like pharmacology (Eltze, 1988).

b. RAT DUODENUM. McN-A-343 produces relaxation of this preparation, thought to be caused by stimulation of nonadrenergic, noncholinergic neurons (Micheletti *et al.*, 1988), and it has been suggested, again on the basis of a pirenzepine  $pK_B$  in the region of 8.0, that the receptor involved is an  $M_1$  receptor. This is not so, because the  $pK_B$  values for 4-DAMP (Micheletti *et al.*, 1990a), guanylpirenzepine (Micheletti *et al.*, 1990b), and (S)-dimethindene (Pfaff *et al.*, 1995) in this assay differ from the  $M_1$  binding affinities by approximately 1, 0.5, and 0.7 log units, respectively.

Both the above-mentioned systems involve presynaptic muscarinic receptors. The rabbit ear artery preparation is another presynaptic system in which the subtype that inhibits noradrenaline release has an atypical pharmacology (Darroch *et al.*, 1992).

#### D. Agonists

There are no muscarinic agonists with a high selectivity for one particular subtype. Early studies of muscarinic receptors led to the suggestion that compounds such as McN-A-343 were selective for the  $M_1$  receptor, but this is not the case. In fact McN-A-343, if anything, may show a modest degree of  $M_4$  selectivity (Lazareno *et al.*, 1993; Richards and Van Giersbergen, 1995). Extensive studies with functional systems involving both native and cloned receptors have demonstrated that the potency of an agonist is not a function of the receptor subtype, but rather is a function of the tissue or cell under study (reviewed by Caulfield, 1993, 1997; Eglen *et al.*, 1996).

The concept of functional selectivity has been applied to the design of muscarinic agonists, which might, for



example, be used in the treatment of the cognitive deficit in Alzheimer's disease (e.g., Freedman *et al.*, 1993; Lambrrecht *et al.*, 1993; Ensinger *et al.*, 1993; Jaen *et al.*, 1995, Fisher *et al.*, 1996). A selective action in such a disease is difficult to predict, however. The approach depends on the agonists having their greatest potency at the receptors in the target tissue. The effective receptor reserves in tissues cannot be manipulated, and there is no guarantee that an advantageous receptor reserve in the target tissue, relative to other tissues, can be maintained during prolonged agonist treatment or during the pathological progression of the disease. The new agonist (+)-3-(S)-3-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo[2,2,2]octane (LY297802) is an antinociceptive agent at doses that do not produce parasympath mimetic effects. Its functional selectivity may be mediated via  $M_4$  receptors in the spinal cord (Shannon *et al.*, 1997).

Although muscarinic agonists with receptor selectivity are certainly worthwhile targets, it will not be possible to define that selectivity until there is better control and understanding of the transduction processes mediating responses [including the nature of agonist-induced conformational change(s) at the receptor], and definition of the stoichiometry and nature of the G-proteins and other components of the transduction pathway.

## V. Transduction Mechanisms and Functional Responses

It is well established that the "odd-numbered" muscarinic receptors ( $M_1$ ,  $M_3$ ,  $M_5$ ) typically couple via the  $\alpha$  subunits of the  $G_{q/11}$  family, whereas the "even-numbered" members ( $M_2$ ,  $M_4$ ) couple via  $G_i$  and  $G_o$   $\alpha$  subunits. This preferential coupling resides at the molecular level mainly in the postulated membrane-proximal regions of the i2 and i3 loops of the different receptors, which are notably different between the "odd" and "even" receptor groups, and similar within the two groups. The coupling selectivity at the G-protein level is reflected in the generally, but not exclusively, observed downstream second-messenger pathways activated by the two groups of muscarinic receptors; phospholipase  $C\beta$  is activated by the "odd" receptors, whereas adenylyl cyclase is inhibited by the "even" receptors (reviewed by Caulfield, 1993; Felder, 1995). Thus, responses to activation of the latter group of receptors usually can be blocked by pertussis toxin-catalyzed adenosine diphosphate ribosylation of  $G_{\alpha_i}$  and/or  $G_{\alpha_o}$ .

Functional responses mediated by these major coupling pathways include the contraction of many smooth muscles and the stimulation of glandular secretion (by  $M_3$  receptors), and there is evidence that the  $M_2$  receptor-mediated inhibition of voltage-gated calcium channels in the heart is the result of adenylyl cyclase inhibition (see Méry *et al.*, 1997 for references). The ablation of M-current inhibition in sympathetic ganglion neurons in the  $M_1$ -knockout mouse provides direct evidence for this  $M_1$  receptor-mediated transduction mechanism in this

tissue (Hamilton *et al.*, 1997). Clearly, there are also many muscarinic responses that involve neither phospholipase  $C\beta$  nor adenylyl cyclase inhibition. Thus, the muscarinic activation of cardiac inward rectifier  $K^+$  channels (by  $M_2$  receptors) results from a direct action of  $G\beta\gamma$  subunits (released from the  $G_{\alpha_i\beta\gamma}$  heterotrimer) on the channels (see Wickman and Clapham, 1995). There are also several reports of muscarinic stimulation of adenylyl cyclase activity. This response is blocked by pertussis toxin pretreatment in membranes from the rat olfactory bulb and is thought to involve an indirect but synergistic interaction with  $G_s$  and a differential modulation of different isoforms of adenylyl cyclase (Onali and Olanas, 1995). In contrast Dittman *et al.* (1994) have provided evidence that  $M_4$  receptors can couple directly to  $G_s$  to activate adenylyl cyclase. Most unusually, it has been reported that  $M_5$  receptors in A2058 cells inhibit forskolin-stimulated cAMP production but do not stimulate inositol trisphosphate production (Kohn *et al.*, 1996). The cAMP response is not sensitive to pertussis toxin pretreatment of the cell but depends on calcium influx.

Table 4 summarizes information on the preferred coupling mechanisms and the functional roles of the different muscarinic receptor subtypes. It also indicates some of the tissues where muscarinic receptors have been detected which are relevant to mediation of the functional response. Autoradiographic localization studies of muscarinic receptors have used selective radiolabeled antagonists, e.g., [ $^3$ H]pirenzepine and [ $^3$ H]AF-DX 384, but their relatively low subtype selectivity only results in preferential localization of one or more subtypes. A more discriminative method, originally developed by Waelbroeck *et al.* (1990), exploits the combination of the different kinetics of binding of [ $^3$ H]N-methylscopolamine to the subtypes, together with the use of appropriate concentrations of combinations of selective antagonists, to allow a much more selective autoradiographic localization of  $M_1$  to  $M_5$  receptors (Flynn *et al.*, 1995). Subtype localization has been aided further by the development of subtype-selective antibodies, which have been useful in immunoprecipitation experiments and in immunocytochemical studies (e.g., Li *et al.*, 1991; Wall *et al.*, 1991a,b; Dörje *et al.*, 1991a; Levey, 1993; Yasuda *et al.*, 1993; Hersch and Levey, 1995; Rouse *et al.*, 1997). These latter studies have allowed a comparison of the proportions of neurons expressing muscarinic receptor proteins and their mRNAs, as determined by in situ hybridization (Hersch and Levey, 1995; Weiner *et al.*, 1990; Bernard *et al.*, 1992; Vilaro *et al.*, 1994). Immunocytochemical studies have been extended to the electron microscope level and have shown, for example, that the location of the  $M_2$  receptor protein is compatible with its acting as a presynaptic autoreceptor, a presynaptic heteroreceptor, and a postsynaptic receptor in the septohippocampal pathway (Rouse *et al.*, 1997).

Coexpression of different receptor subtypes can be an important issue in classification of muscarinic recep-

TABLE 4  
*Muscarinic receptors: G-protein coupling, transduction pathways, localization, and functional responses*

|                                   | Receptor subtype   |  |   |                                   |   |
|-----------------------------------|--|--|---|-----------------------------------|---|
|                                   | M <sub>1</sub>   | M <sub>2</sub>   | M <sub>3</sub>  | M <sub>4</sub>                    | M <sub>5</sub>                                    |
| Preferred G-protein               | q/11   | i/o  | q/11  | i/o                               | q/11  |
| Second messengers <sup>a</sup>    | PLC IP <sub>3</sub> /DAG<br>Ca <sup>2+</sup> /PKC            | AC (-)   | PLC IP <sub>3</sub> /DAG<br>Ca <sup>2+</sup> /PKC   | AC (-)                            | PLC IP <sub>3</sub> /DAG<br>Ca <sup>2+</sup> /PKC |
| Locations                         | Brain (cortex, hippocampus)<br>Glands<br>Sympathetic ganglia | Heart<br>Hindbrain<br>Smooth muscle  | Smooth muscle<br>Glands<br>Brain  | Basal forebrain<br>Striatum       | Substantia nigra                                  |
| Functional responses <sup>b</sup> | M-current inhibition   | K <sup>+</sup> channels<br><br>(βγ activates)<br>Inhibit Ca <sup>2+</sup> channels<br>Decrease heart rate and force<br>Decrease neurotransmitter release (presynaptic) | Smooth muscle contraction<br><br>Gland secretion<br>Decrease neurotransmitter release (presynaptic) | Inhibit Ca <sup>2+</sup> channels |   |

<sup>a</sup> DAG, diacylglycerol; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C.

<sup>b</sup> Main responses; several other responses have been suggested to be elicited by defined subtypes.

tor(s) mediating a functional response, especially where the response concerned (e.g., smooth muscle contraction) is the result of many potentially interacting steps in the transduction pathway. This is well illustrated by recent demonstrations of a function for M<sub>2</sub> receptors in regulation of gut smooth muscle tone under certain circumstances (Thomas *et al.*, 1993; Eglén *et al.*, 1994). Clearly, the involvement of a mixture of subtypes will result in a confusing pharmacological profile, which may account for many of the controversies in the literature.

## VI. Conclusions

Five muscarinic receptor genes have been characterized, and the understanding of their coupling characteristics is increasing, largely because of studies of cloned receptors expressed in cell lines. The use of selective antibodies has allowed the localization of muscarinic receptor subtype proteins. However, the paucity of highly selective antagonists, and the lack of any selective agonists has impeded the unambiguous identification of muscarinic receptor subtypes mediating many important responses. It is hoped that the discovery of compounds (and toxins) with greater receptor subtype selectivity will aid this process.

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