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REVIEW

The muscarinic M_5 receptor: a silent or emerging subtype?

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Abbreviations: AF-DX 116, 11-({2-[diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6**H**-pyrido (2,3-b)(1,4) benzodiazepine-6-one; AQ-RA 741, 11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)5,11-dihydro-6**H**-pyrido(2,3-b)(1,4) benzodiazepine-6-one; CHO cells, chinese hamster ovary cells; DAG, (diacylglycerol); 4-DAMP, 4-diphenyl acetoxy-*N*-methyl piperidine methiodide; GRKs, G-protein coupled receptor kinases; Ins 1,4,5P_3, inositol 1,4,5-trisphosphate, mitogen activated protein kinase (MAP kinase); MT-3, *mamba* snake toxin 3; NOS, nitric oxide synthase; PD 102807, 3,6a,11,14-tetrahydro-9-methoxy-2-methyl-12**H**-isoquino[1,2-b] pyrrolo [3,2-f][1,3]benzoxazine-1-carboxylic acid ethyl ester; *p*-f-HHSiD, para-fluoro-hexahydro-silafenidol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC-β, phosphoinositide C-β; PLC-β, PTX (pertussis toxin); SCH 57790, 4-cyclohexyl-(-[4-[4-methoxyphenyl]-(**S**)-sulfinyl]-phenyl]-1-piperazineacetonitrile; TM, transmembrane; UH-AH 37, 6-chloro-5,10-dihydro-5-[1-methyl-4-piperidinyl)acetyl]-11**H**-dibenzo[b,e][1,4]diazepine-11-one hydrochloride

Introduction

The muscarinic acetylcholine receptors belong to the super-family of seven TM domain receptors that interact with G-proteins to initiate intracellular responses. Evidence from molecular cloning indicates that there are separate intronless human genes that encode five muscarinic receptor glycoproteins. Muscarinic receptor sequences have significant homologies with other members of this large super-family and the genes are very similar across mammalian species (Caulfield 1993; Felder 1995).

Despite over a decade following their molecular identification, the therapeutic exploitation of this crucial family of receptors remains disappointing. This results from a relative inability to pharmacologically distinguish between the subtypes, markedly hindering their investigation in native mammalian tissues. In particular, this has hampered investigation of the last-identified subtype, the muscarinic M_5 receptor. The inability to clearly distinguish it from the M_3 receptor has, moreover, led to confusion of its physiological role. Finally, determination of the precise distribution of M_5 receptors within tissues is complicated by inadequate selectivity of radioligands as well as the low sensitivity/selectivity of polyclonal antisera in immunocytochemical studies (Caulfield 1993; Reever *et al*., 1997). Despite these problems, this receptor has recently been assigned an upper case M_5 nomenclature (Caulfield & Birdsall, 1998) presumably reflecting recognition of its presence and function in native tissues despite the current incomplete characterization. In this respect the identification of a human A2058 melanoma cell that endogenously expresses the M_5 receptor (Kohn *et al*., 1996) should facilitate its investigation in endogenous tissues, although extensive use of these cells have not been reported to date. Consequently, the majority of the current information on the functional properties and regulation of coupling of this subtype still arises from their expression in model cells following cDNA transfection.

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The purpose of this short review is to critically evaluate current data on the muscarinic receptor M_5 subtype from several standpoints. Hopefully, this critique will stimulate further studies on the M_5 receptor that may raise it from a 'relatively ephemeral' or 'fact or fiction' status, described in recent reviews (Reever *et al*., 1997; Caulfield & Birdsall 1998).

Identification and structural features

The muscarinic M_5 receptor was the last of the muscarinic receptor family to be cloned in the human and is mapped to chromosome 15q26 (Bonner *et al*., 1988; Liao *et al*., 1989). The receptor sequence conforms to a predicted seven transmembrane glycoprotein consisting of 531 residues in the human (GeneBank accession number PO8912) and 532 in the mouse (PO8911; 89% homologous to human). Structurally, the M_5 receptor is the next largest muscarinic receptor to the M_3 subtype with both these subtypes possessing a large third intracellular loop. Differences in this cytoplasmic loop account for the sequence diversity between muscarinic receptor subtypes and also between muscarinic receptors from different species. However, of the five muscarinic receptors, the M_5 subtype demonstrates the least homology in this region when comparisons are made between human and rat sequences. Wess and colleagues (Wess *et al*., 1992; Pittel & Wess, 1994; Wess, 1997) have explored the nature of ligand binding and G-protein coupling by using chimeras of muscarinic M_2 and M_5 receptors. Most M_2/M_5 constructs are inactive but the presence of the M_2 sequence in TMVII and M_5 in TMI agonist activation of G-protein coupling is restored. Pittel & Wess (1994) argued that these data supported the bacteriorhodopsin model in which the seven transmembrane helices are arranged in a ring, such that TMI is adjacent to TMVII. A series of M_2/M_5 chimeras in which regions of the M_5 receptors have been systemically replaced by homologous regions of the M_2 receptor, indicated the higher affinity of the antagonist UH-AH 37 for the M_2 over the M_5 receptor was dependent upon a short stretch of 31 residues in TMVI as well as a short region

of the third intracellular loop. This however contrasts to the antagonist AQ-RA 741 which also preferentially binds to the M_2 receptor suggesting that different receptor epitopes may be involved in conferring different ligand specificities.

In a series of studies, Brann and colleagues also attempted to identify key residues associated with agonist activation of M_5 receptors. Initially using random saturation mutagenesis they identified the amino acids 439, A440, A441 towards the C-terminal end of the third intracellular loop of the M_5 muscarinic receptor critical for G-protein coupling (Burstein *et al*., 1995). In a more recent paper, this group (Burstein *et al*., 1998a) constructed a further series of point mutants at each of these residues and characterized their functional phenotypes in order to find structure function relationship for G-protein coupling to the M_5 receptor. Their evidence suggests that residue 439 participates in G-protein activation through an ionic mechanism and that A440 fulfils more of a structural role, perhaps forming part of the G-protein coupling pocket. Further, A441 apparently contributes to receptor affinity for G-proteins. Collectively, these data suggest that the third intracellular loop of the M_5 receptor forms a G-protein coupling pocket comprised of a positively charged lip and a hydrophobic core.

Brann's group (Spalding *et al*., 1998) also investigated a potential switch between active and inactive conformations of the M_5 muscarinic receptor. There is much evidence from several G-protein coupled receptors to suggest that G-protein receptors exhibit constitutive activity (i.e. activation in the absence of agonist) and that agonists stabilize active whereas antagonists stabilise inactive conformations (Kenakin 1996; 1997). In a search for residues that participate in receptor function, several regions of the M_5 receptor were randomly mutated and tested for their functional properties. Mutations spanning the face of TMVI were found to induce high levels of constitutive activity of the receptor. The same face of TMVI contained several residues crucial to receptor activation by agonists and one residue was identified as a contact site for both agonists and antagonists. These results suggest that within TMVI of the M_5 receptor is a switch that defines the activation state of the receptor and the ligand interactions with TMVI stabilizing the receptor in either active or inactive conformations. In a further study (Burstein *et al*., 1998b) this group completed a systematic search of the intracellular loops in an attempt to identify further domains that govern G-protein coupling. A feature of the second intracellular loop was an ordered cluster of residues where substitutions also cause constitutive activation of the M_5 receptor. A second group of residues in the second intracellular loop have been identified where mutations compromise receptor/G-protein coupling. The residues of each group appear to alternate and are spaced three to four positions apart, perhaps suggesting an α-helical structure where the groups form opposing faces of the helix. The authors suggest that the constitutively activating face normally constrain the receptor in the off state while the other face couples to G-proteins with the receptor being in the on state.

Signal transduction

It is generally accepted that muscarinic M_1, M_3 and M_5 receptors couple preferentially *via* the pertussis toxin insensitive $Gq/_{11}$ protein to phosphoinositide C-β (PLC-β) (Caulfield, 1993). Agonist activation of these subtypes therefore accelerates the rate of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis leading to the formation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) and these products act as second messengers by mobilizing Ca^{2+} from intracellular stores and activating protein kinase(s) C (PCK) respectively (Berridge, 1997). Bonner *et al*. (1988) were the first group to observe that recombinant muscarinic M_5 receptors expressed in CHO-cells coupled to this signalling pathway. Confirmation of effective coupling of M_5 receptors to phosphoinositide hydrolysis-linked signalling has been reported in CHO cells (Jones *et al*., 1991; Wang & El Fakahany, 1993; Richards & van Giersbergan, 1995; Watson *et al*., 1999) as well as murine L-cells (Liao *et al*., 1990), A9L cells (Richards & van Giersbergan, 1995) and insect SF-9 cells (Kukkonen *et al*., 1996).

The assumption underlying these observations i.e. that the M_5 receptor activates a phospholipase C-β *via* $Gq/_{11}$ proteins, is based upon the fact that responses are insensitive to pertussis toxin (Liao *et al*., 1990). Direct evidence (as reported for the M_1 receptor; Berstein *et al*., 1992) for this pathway of M_5 receptor signalling remains to be established, although efficient coupling of this subtype to $Gq/_{11}$ in CHO cells using [^{35}S]-GTPγS binding and immunoprecipitation protocols (Smith, Eglen & Nahorski, unpublished, see below) or an antibody capture technique (De Lapp *et al*., 1999) has recently been observed. In contrast, Gusovosky *et al*. (1993) reported that M_5 receptors expressed in CHO cells stimulated tyrosine phosphorylation of PLC-γ and that tyrosine kinase inhibitors suppressed agonist activation of phosphoinositide hydrolysis. It is unlikely, however, that this response is a subtype-specific effect of M_5 receptors, as both M_1 and M_3 receptor activation of IP_3 generation in CHO cells is suppressed by the tyrosine kinase inhibitor, genistein (Umemori *et al*., 1997). Palmier *et al*. (1999) have recently reported very similar observations in rat myometrium, recently shown to contract by activation of the M_3 receptor (Choppin *et al*., 1999a).

Other reports that the muscarinic M_5 receptor shows subtype selective signal transduction have also proven inconclusive. Wang *et al*. (1993; 1994; 1996) demonstrated that M_5 receptors efficiently couple to neuronal nitric oxide synthase (NOS) in CHO cells and Wotta *et al*. (1998) demonstrated that agonist activation of CHO M_5 cells leads to accelerated phosphoinositide hydrolysis and MAP kinase activation. However, there is no evidence that activation of NOS or MAPK is selective for M_5 receptors and these responses are almost certainly secondary to changes in either intracellular Ca^{2+} and/or protein kinase C activation in response to phospholipase C mediated PIP_2 hydrolysis. On the other hand, the identification and characterization of an endogenous M_5 receptor in the A-2058 human melanoma cell line (Kohn *et al*., 1996) could provide evidence of unusual transmembrane signalling. Activation of M_5 receptors expressed in these cells resulted in no evidence of phospholipase C-β or γ activation but both a robust Ca^{2+} mobilization from intracellular stores ($InsP_3$ independent?) and the resulting Ca^{2+} influx was accompanied by marked phospholipase A_2 activation and inhibition of forskolin-stimulated cyclic AMP accumulation. Alternatively these data may reflect the very low expression of M_5 receptors in A-2058 cells resulting in undetectable phosphoinositide hydrolysis with subsequent amplified Ca^{2+} mobilization and PLA_2 responses. Further studies on this endogenous M_5 receptor are clearly required.

However, there is now good evidence that the M_5 receptor differs from M_1 and M_3 receptors in its ability to couple to Gsα in order to activate adenylate cyclase. Jones *et al*. (1991) initially observed that matched expression of M_5 and M_3 receptors in CHO cells produces a robust phosphoinositide hydrolysis but M_3 receptors were 10 fold more active at stimulating cyclic AMP accumulation. Moreover, Liao *et al*.

(1990) failed to observe any M_5 mediated adenylate cyclase activity in transfected L-cells. Our own studies have revealed, using matched expression levels in CHO cells, that there are major differences in the coupling of M_1 and M_3 receptors to Gs-α and the activation of adenylate cyclase (Akam *et al*., 1998; Burford *et al*., 1995; Burford & Nahorski, 1996, see also Schwartz *et al*., 1993; Gurwitz *et al*., 1994; Heldmann, 1996). From these studies it would seem reasonable to propose a $M_1 > M_3 < M_5$ ranking for Gs coupling and adenylate cyclase activation at least when expressed in a common CHO cell signalling background.

Overall, these observations assume that muscarinic receptor subtypes are promiscuous in their coupling to G-proteins. The likelihood of multiple interactions of a single receptor to various G-proteins is obviously increased with high concentrations of the reactants. It is not only the receptor cell surface density but also variations in the amount and nature of G-protein available to interact with the receptor and the affinity of the receptor for those G-proteins that are critical. Kenakin (1997) has discussed the theoretical outcome of receptor G-protein promiscuity while others emphasise the stoichiometry of receptors and G-proteins, focusing on access between these proteins within microdomains of intact cells (Neubig 1994; 1998). The question therefore of the physiological significance of muscarinic receptor promiscuity is still hotly debated. However, it is the opinion of the current authors that there is now persuasive evidence from studies of relatively low levels of recombinant muscarinic M_1, M_3, and M_5 receptors in model cells that reveal interactions with Gs and Gi subtypes as well as $Gq/_{11}$ proteins.

Offermans *et al*. (1994) using subtype specific immunoprecipitation of G-protein-α subunits photo-labelled with (α-^{32}P) GTP azidoanilide, provided the first direct evidence that M_1 and M_3 receptor subtypes can interact with members of both the $Gq/_{11}$ and Gi classes of G-proteins in CHO cells. More recently, we have developed an alternative approach in which [^{35}S]-GTPγS labelled Gα subunits is immunoprecipitated by specific antisera (Akam *et al*., 1998 and submitted for publication). Using this approach in CHO cells matched for M_1 and M_3 receptor expression, major differences were seen in the ability of agonists to activate $Gq/_{11}$ and the various species of Gi-α subunits. Extension of these studies to M_5 receptors (unpublished observations) suggest that following methacholine stimulation of different muscarinic subtypes, the relative activation of $Gq/_{11}$ was $M_1 > M_5 > M_3$ subtype. In short, there are both subtype and agonist specific-profiles of Gα subclass activation. These data strongly suggest that the muscarinic receptor subtypes are promiscuous in their interactions with different G-proteins and that there are subtype-selective effects on the coupling to different species.

That different agonists may direct or 'traffic' single receptors to different transducing G-proteins is of much interest to pharmacologists since it suggests that not only is the concentration and intrinsic efficacy of an agonist important but that the *nature* of the agonist determines the extent and also the 'quality' of an overall cellular response by influencing different G-protein mediated signalling cascades. There are several examples of 'receptor channelling' (Kenakin 1996; Gudermann *et al*., 1996) but as yet not convincing data with muscarinic receptors. However, studies on muscarinic receptor subtypes do indicate that partial agonists, such as pilocarpine or McN-A-343, display distinctly selective activation of M_1 over M_3 receptors in cells in which these subtypes are expressed at relatively similar densities (Heldman *et al*., 1996). Richards and van Giersbergan (1995) also compared the relative efficacies of partial agonists at M_1, M_3 and M_5 receptors expressed in CHO cells. Of interest was the observation that while M_5 receptors were less efficiently coupled to phospholipase C than M_1 and M_3 receptors, some partial agonists (arecoline and oxotremorine) were more efficacious at activating M_5 receptors. This suggests that this receptor may utilize different G-proteins and/or different phospholipase C(s) to stimulate phosphoinositide hydrolysis. Furthermore, differences in the relative potency of particular agonists at M_1, M_3 and M_5 receptors (Wang & El Fakahany 1993; Jones *et al*., 1991; Richards & Van Giersbergan, 1995) may not only relate to receptor density but also the assay conditions of phosphoinositide hydrolysis.

Overall, there is emerging evidence that muscarinic receptor subtypes (like many other seven TM receptors) adopt multiple active states to promote selective G-protein coupling in response to different agonists. Although it will be important to establish if such models occur widely in intact cells, it provides the potential to design receptor active agents to selectively activate or inhibit an effector cascade by trapping or inhibiting particular conformations of the receptor subtype. This approach may reveal new information on the selectivity of M_5 receptor signalling and provide clues to its physiological role.

Receptor regulation

There is now substantial evidence that the activation of G protein coupled receptors is attenuated by receptor phosphorylation, uncoupling from G-proteins and by internalization. These modifications appear to modulate various aspects of signal transduction such as desensitization, resensitization and switching of signalling cascades (Lefkowitz 1998). Muscarinic receptors are no exception to this, although details of the regulation of PLC coupled subtypes lags behind those that act *via* Gs and Gi to influence the activity of adenylate cyclase. Thus, while well established that M_1 and M_3 receptors undergo agonist mediated phosphorylation and that this accompanies the rapid desensitization of initial steps of signalling, the relative roles of the kinases involved (G-protein coupled receptor kinases GRKs, PKC, casein kinase 1α) in intact cells remains to be established (Tobin, 1997). To our knowledge only one study has addressed the question of whether M_5 receptors show acute regulation. Tsuga *et al*. (1998) examined the sequestration of M_5 muscarinic receptors expressed in COS-7 cells. Cell surface M_5 muscarinic receptors were reduced by 20–25% following 30 min incubation with a maximal concentration of carbachol. Receptor internalization was increased substantially (to 60%) in cells that were also overexpressing GRK-2 but not influenced by expression of a dominant negative GRK-2. These data suggest that both GRK-dependent and independent components may be involved in the sequestration of the M_5 subtype and further studies are awaited.

In addition to desensitization/resensitization, phosphorylation-dependent 'switching' of receptor specificity for G-proteins provides a further dimension for signal diversity *via* G-protein coupled receptors (Lefkowitz, 1998). Coupled to the evidence discussed above that muscarinic receptors may adopt multiple active states that promotes selective G-protein coupling in response to different agonists, additional modulation by receptor phosphorylation could provide a further level of complexity.

Pharmacology

The pharmacology of the M_5 receptor was first defined by the use of recombinant cell systems, enabling a series of antagonist

affinities to be generated at a singular receptor subtype (Bonner *et al*., 1988; Jones *et al*., 1991; Dorje *et al*., 1991). Although cloned over ten years ago, and in marked contrast to the other four muscarinic receptor subtypes, there remains a paucity of functional pharmacological studies undertaken against a cellular or tissue response exclusively mediated by M_5 receptor activation. Consequently direct comparison with other potential endogenously expressed M_5 receptors cannot presently be made. In lieu of an endogenous correlate of the muscarinic M_5 receptor, one must turn to recombinant systems to characterize the pharmacology. Antagonist affinity data from our group (Watson *et al*., 1999; Loury *et al*., 1999; Table 1) confirms previous reports on the receptor affinity profile. Historically, the M_5 receptor exhibits a low affinity for AF-DX 116, AQ-RA 741 and an intermediate affinity for methoctramine and pirenzepine; no compound has been reported that exhibits a preferential high affinity for the receptor. It can also be seen that, apart from nonselective antagonists such as tolterodine or atropine, all compounds exhibit a low affinity at the receptor, including novel ligands such as PD 102807, and MT-3 (Table 2). Comparison of the values estimated in Tris-EDTA and Tris-Krebs' buffer reveal few effects on their affinity at this receptor and thus their selectivity (see also Caulfield & Birdsall, 1998).

In several respects, the profile of antagonist affinities at the M_5 subtype resembles that determined at the muscarinic M_3 receptor (Table 1), a finding highlighted by several workers in the area (Buckley *et al*., 1989; Jones *et al*., 1991; Dorje *et al*., 1991). Importantly, therefore, several ligands, including AQ-RA 741, himbacine, and darifenacin are preferential for the M_3 over the M_5 receptor (Buckley *et al*., 1989; Jones *et al*., 1991; Wallis & Napier 1999, Loury *et al*., 1999; Watson *et al*., 1999). Recently, our group has also shown that older compounds, including oxybutynin, racemic secoverine and (S) secoverine, possess similar selectivity (Choppin *et al*., 1999b). Inclusion of all these compounds in classifying a putative endogenous M_3 receptor is thus important in defining the subtype. Given the similarity in M_3 and M_5 binding profiles, it would be interesting to re-examine (in terms of the displacement isotherms of such M_3/M_5 selective antagonists) tissues previously thought to express exclusively M_3 receptors, and those now believed to co-express M_5 receptors. Noteworthy in this respect is the salivary gland, a tissue that appears to express both M_3 and M_5 receptors (Flynn *et al*., 1997). Prior to the cloning of the muscarinic receptor family, membranes of this tissue were extensively used to assess the affinity of ligands at the nominal M_3 receptor (e.g. Hammer *et al*., 1980; Nilvebrant & Sparf, 1988).

Currently, the majority of studies of antagonists at the M_5 receptor have employed radioligand binding techniques to determine affinity (Table 2). Very few functional studies have been reported in which the antagonist affinity has been operationally determined. Brauner-Osborne & Brann (1996) reported an assay using activation of the proliferative marker β-galactosidase and observed good agreement with antagonist affinity estimates derived from radioligand binding studies at the M_5 receptor. Watson *et al*. (1999) have reported an antagonist affinity profile using null methods against agonist mediated phosphoinositide hydrolysis in CHO M_5 cells. Ligand affinities obtained by these techniques also agreed with those determined in radioligand binding studies (Table 2), providing confidence that the binding profiles are relevant. In another model, Burstein *et al*. (1997) utilized a constitutively active recombinant M_5 receptor system, in which $Gq\alpha$ is overexpressed. As expected, all antagonists studied acted as inverse agonists and maximal responses of agonists of low efficacy were enhanced. This system could also prove useful in the detection of novel antagonists for the receptor. Finally, recently DeLapp *et al*. (1999) used [^{35}S]-GTPγS binding, allied to an antibody capture technique, to assess agonist potency at M_5 receptors in CHO cells. Antagonist affinities at M_5 receptors by this technique were not reported, although it clearly has the potential for use in this kind of analysis. However, the absence of a robust functional assay for the M_5 receptor in an endogenous setting remains a serious deficit in the area, particularly when assessing the potential selectivity of muscarinic agonists. No such agonists to our knowledge have been identified to date.

Evidently, judicious use of several antagonists, including those with M_3/M_5 selectivity, must be used to pharmacologically define the nature of the receptor mediating a cellular response. For these reasons the operational identification of the M_5 receptor when expressed in an endogenous tissue,

Table 1 Antagonist affinity estimates (pKi) derived from radioligand binding studies at muscarinic M_1, M_2, M_3, M_4, and M_5 human recombinant receptors

Compound	*M_1*	*M_2*	*M_3*	*M_4*	*M_5*
4-DAMP	9.4 (9.2)	8.4 (8.1)	9.1 (9.2)	8.9 (8.5)	9.3 (8.9)
AQ-RA 741	7.6 (7.8)	8.9 (8.6)	7.5 (7.4)	8.0 (8.2)	6.0 (5.8)
Atropine	9.0 (9.0)	8.8 (8.7)	9.3 (9.2)	8.9 (8.9)	9.2 (8.9)
Darifenacin	7.8 (7.8)	7.0 (7.0)	8.8 (8.8)	7.7 (7.7)	8.0 (8.0)
Himbacine	6.8 (6.7)	7.7 (8.0)	6.9 (6.9)	7.5 (7.8)	6.1 (6.1)
MT-3	6.7 (6.5)	5.9 (<5.0)	6.0 (<5.0)	8.1 (8.2)	6.0 (5.9)
Methoctramine	7.5 (6.7)	8.7 (7.7)	7.0 (6.1)	7.6 (7.0)	7.0 (6.3)
Tripitramine	8.9 (8.5)	9.9 (9.4)	7.8 (7.1)	8.5 (8.0)	7.9 (7.3)
Oxybutynin	8.2 (8.5)	7.5 (7.8)	8.3 (8.7)	8.1 (8.2)	7.7 (7.6)
p-F-HHSiD	7.4 (7.3)	6.7 (6.6)	7.7 (7.7)	7.2 (7.2)	6.9 (6.7)
Pirenzepine	8.2 (8.0)	6.5 (6.3)	6.9 (6.8)	7.4 (7.1)	7.2 (6.9)
PD 102807	5.6 (5.5)	5.4 (5.9)	6.1 (6.7)	7.7 (7.5)	5.3 (5.5)
(S) Secoverine	8.0 (8.5)	7.9 (9.0)	7.7 (8.4)	7.7 (8.6)	6.5 (7.0)
Tolterodine	8.4 (8.5)	8.1 (8.4)	8.2 (8.5)	7.9 (8.1)	8.4 (8.6)
Zamifenacin	7.7 (7.6)	7.7 (8.2)	8.2 (7.9)	7.0 (6.9)	7.6 (7.3)

Values are mean pKis estimated in a Tris-EDTA buffer. Those values in parenthesis are estimated in a Tris Krebs' buffer (see Loury *et al*., 1999; Watson *et al*., 1999, for additional details).

Table 2 Antagonist affinity estimates (pKi) at human recombinant muscarinic M_5 receptors in CHO-K1 cells

Antagonist	*Buckley* et al. (1991)	*Dorje* et al. (1991)	*Eglen & Hegde* (1998)	*Watson* et al (1999)
Atropine	9.7		9.1	8.7
Pirenzepine	6.2	7.1	6.9	6.4
Tripitramine			7.9	
Methoctramine	7.2	6.9	6.4	6.3
AQ-RA 741		6.1		6.1
Himbacine		6.3	6.1	6.3
MT-3			<6.0	
SCH 57790			6.5[1]	
4-DAMP		9.0	8.9	8.6
p-F-HHSiD		7.0	6.9	6.6
PD 102807			5.3	
Darifenacin			8.1	7.7
Oxybuytnin			7.6	6.6
(R,S) Secoverine			6.2	

Values are pKis determined in competition radioligand binding studies, with the exception of the data from Watson *et al*. (1999) in which the numbers were operationally derived from studies using incorporation of hydrolysis of inositol phospholipids as the cellular response. [1]from Lachowicz *et al*. (1999).

particularly when co-expressed with the M_3 receptor remains difficult. A good example of the problem is the precise nature of the muscarinic receptors mediating contraction of rabbit isolated iris-ciliary muscle. Bognar *et al*. (1989; 1992) reported an antagonist profile at receptors mediating contraction of this tissue inconsistent with M_1, M_2, M_3 and M_4 receptor activation, notably with affinities determined for 4-DAMP and racemic secoverine. One explanation was the participation of M_5 receptors in the contraction. However subsequent work in our laboratory has not confirmed these discrepant values. Furthermore, use of an extensive series of antagonists suggests that the pharmacological profile is similar to that of the M_3 receptor mediating contraction of rabbit bladder (Choppin *et al*., 1998).

Nonetheless the iris-ciliary muscle from other species remains of interest in terms of a functional role of the M_5 receptor. McIntyre & Quinn (1995) demonstrated that zamifenacin, a forerunner of darifenacin, was highly selective for muscarinic receptors mediating contractions of canine ileum over canine isolated iris suggesting either differences in M_3 receptors or that contractions of this iris were mediated by more than one receptor, thereby contrasting with the ileum. Recent work from our group (Choppin *et al*., 1999c) supports these differences in the antagonist affinity profile at muscarinic receptors mediating contraction of dog isolated iris muscle is inconsistent with activation of muscarinic M_3 receptors, possibly revealing involvement of M_5 receptors. This is most evident with the antagonists AQ-RA 741 and darifenacin (Choppin *et al*., 1999c) in which the antagonism by these compounds clearly differs from that seen at archetypal M_3 receptors in dog urinary bladder. Similar observations have secretly been made in human isolated iris muscle (Choppin *et al*., 2000). The fact remains, however, that in the absence of ligands preferential for the M_5 receptor, definition of its pharmacology in endogenous tissues is defined only by exclusion criteria, resulting in imprecise definition.

Distribution

The distribution of muscarinic receptor subtypes in mammalian tissues have been investigated using both immunological (receptor proteins) and mRNA approaches. These studies, although valuable, have proved contradictory and in some cases confusing (see Caulfield 1993). Studies with complementary nucleic acid sequences to hybridize with part of the muscarinic receptor mRNA either in Northern blots from tissues or with *in situ* hybridization have resulted in a potential extension of knowledge on the localization of muscarinic receptor subtypes in mammalian tissues. However, it must always be emphasized that such studies may give a misleading picture of receptor localization when the site of production of mRNA is remote from the site of expression of the receptor protein. Likewise, although the use of receptor specific antibodies produced initially encouraging and interesting results, it is still too early to make firm judgement on receptor localization. The likely specificity and cross reactivity of the antibodies is not always thoroughly established and there is a clear need for further work with different subtype specific antibodies certainly at higher resolution and with quantitative information about their immunoprecipitating efficiency. To illustrate these confusions with the M_5 receptor, Levey *et al*. (1991) found no receptor immunoprecipitation with an M_5 receptor antibody throughout the brain despite the presence of M_5 receptor message in hippocampus, substantia nigra, thalamus and hypothalamus. However, Yasuda *et al*. (1993) has found low levels of M_5 receptor immunoreactivity in striatum, hippocampus midbrain pons medulla and cerebellum. Early studies reported RNA encoding muscarinic receptors in the substantia nigra compacta is apparently exclusively of the M_5 type through more recent work using RT–PCR (Wei *et al*., 1994) revealed M_5 and M_3 mRNA quite uniformly expressed in brain.

Flynn *et al*. (1997) have exploited the kinetic and equilibrium binding of several muscarinic antagonists to identify, by exclusion, the distribution of muscarinic M_5 receptors in rat brain and some peripheral tissues, including salivary glands. Using this technique Reever *et al*. (1997) demonstrated a labelling pattern partially overlapping the M_3 receptor, although this was not seen in all CNS areas. In general, highest densities were seen in the outermost layer of the cerebral cortex and the caudate putamen. Distinct labelling was also seen in the substantia nigra/ventral tegmetum area, with dense staining in the substantia nigra pars compacta. This localization is consistent with the *in situ* hybridization data, although the identification of cortical sites, possibly microglia, was not anticipated from the earlier immunoprecipitation experiments. The Flynn group (Reever *et al*., 1997) thus proposed that the receptor modulates dopaminergic transmission *via* a location on nigro-striatal dopaminergic terminals. In contrast, lesions studies have indicated that only a fraction of M_5 receptors were associated with these terminals, and the majority of these were expressed on afferent on intrinsic striatal neurones (Wall *et al*., 1994). Muscarinic M_5 receptors are also expressed in murine P19 derived neurones from an embryonic carcinoma cell line (Parnas *et al*., 1998), from which it has been suggested that the expression could be primarily presynaptic. This suggestion accords with the neuronal location of the receptor and its putative role in regulating transmitter release, as discussed above.

There is little data regarding the expression and function of the M_5 receptor in peripheral tissues. Philipps *et al*. (1997) reported, by RT–PCR, the presence of muscarinic M_5 receptor mRNA in rat basilar, pulmonary, mesenteric and tail artery, although the functional significance of these data is unknown. Indeed, arterial endothelial cells express M_3 receptors through which nitric oxide-mediated relaxation occurs. In terms of vascular smooth, pharmacological data provides no evidence to support the presence of a contractile M_5 receptor (Eglen & Whiting, 1990). Consequently, the role of the M_5 receptor in the cardiovascular system remains to be established.

It has been known for some time that the iris-ciliary body muscle of the eye expresses M_3 receptors, through which it is presumed the tissue contracts (see Eglen *et al*., 1996 for review). Concordantly, limited radioligand binding studies in cultured human iris cells also suggests the presence of M_3 receptors (Woldemussie *et al*., 1993). Subsequent immunoprecipitation data from this group (Gil *et al*., 1997) confirm a predominant M_3 population (60–75%), but also indicates minor (5%) expression of the M_5 receptor in human iris-ciliary muscle. Antagonism of the muscarinic M_3 receptor is useful in urinary incontinence or chronic obstructive pulmonary disease (Eglen & Hegde, 1998). This finding of Gil *et al*. (1997) is of interest since it suggests that M_3/M_5 selective antagonists could have a reduced propensity for mydriasis, providing an advantage over current anticholinergic therapies (Eglen & Hegde, 1998). However, the study of Gil *et al*. (1997) has not been confirmed to-day and it is unknown if such receptors are functional. Interestingly, Zhang *et al*. (1999) report that, in primary cultures of human ciliary muscle cells (H7CM cells),

chronic exposure to muscarinic agonists down regulates M_3 receptor mRNA. No data were reported for the expression patterns of the M_5 receptor mRNA.

Flynn *et al.* (1997) have shown that, by exclusion radioligand binding criteria, M_5 receptors are present in rat salivary gland tissue, contrasting with an earlier report by Watson & Culp (1994) who demonstrated that the mucous acini from rat sublingual gland contain abundant amounts of only M_1 and M_3 receptors. However, most functional studies of salivary gland tissue have been conducted *in vivo* or have determined the inhibitory potency, rather than affinity, of muscarinic antagonists. Consequently, definition of the muscarinic receptor(s) mediating salivation has not been conducted over ideal conditions. Our group (Meloy *et al.*, 1998) has reported preliminary affinity data for several antagonists in a primary culture of rat submaxilliary gland cells (Table 3). These data were obtained by using microphysiometry and subsequent operational analysis to determine the nature of the subtype mediating the increase in the acidification of the perfusate (used as a readout for muscarinic receptor activation). The results reveal a series of antagonist affinities intermediate to those obtained in CHO M_3 or M_5 cells. It is presently unclear if these data result from activation of both subtypes, in which case it is anticipated that both would be functional, or if the culturing conditions influence the pharmacology of a single atypical muscarinic receptor.

Potential physiological roles

As is clear from the limited studies discussed above, the function of the M_5 receptor is speculative. Muscarinic M_5 receptors are selectively enriched in the substantia nigra and ventral tegmental areas of rat brain, suggesting that they may have a role in the modulation of dopaminergic transmission (Reever *et al.*, 1997). In dispersed cultures of foetal cells from ventral mesencephalon, muscarinic receptor activation potentiates NMDA stimulated dopamine release. This appears to be *via* a receptor coupled to inositol phospholipid hydrolysis, *via* a pertussis toxin insensitive G protein. Although extensive pharmacology was unreported, these findings are consistent with activation of an M_5 receptor (Allaoua *et al.*, 1993).

Classically, a functional role of a receptor in the CNS can be evaluated in terms of changes in expression during disease or development. Surprisingly, little work has been done in this area regarding the M_5 receptor, although one report (Flynn *et al.*, 1995) has assessed changes in the subtype during Alzheimer's disease. The subtype M_5 expression was unchanged in contrast to the marked reduction in M_2 immunoreactivity and upregulation of M_4 immunoreactivity. The ontogeny of the M_5 receptor has been reported by Wall *et al.* (1992) in which it was found that the expression levels of the receptor in rat brain were consistently low ($<1\%$ of the total receptor density) at all ages.

Table 3 Antagonist binding affinity estimates (pK_B) to muscarinic receptor(s) present in rat submaxilliary gland using microphysiometry

Compound	*pK_B*	*pA_2*	*Schild slope*
4-DAMP	8.88±0.05	8.93	0.95
AQ-RA 741	6.63±0.02	6.65	0.98
Atropine	8.85±0.04	8.73	1.07
Himbacine	6.82±0.04	6.98	0.84
Methoctramine	5.90±0.09	5.73	1.26
Oxybutynin	7.94±0.06	7.78	1.14
Pirenzepine	6.85±0.04	6.96	0.86
(S) Secoverine	7.18±0.03	7.15	1.05
Tolterodine	8.51±0.04	8.45	1.06
Darifenacin	8.89±0.21*	10.74	0.47
p-F-HHSiD	7.33±0.03*	7.14	1.19
Zamifenacin	8.09±0.10*	7.68	1.40

*Unsurmountable, pseudo-pK_B. Data are taken from Meloy *et al.* (1998).

It is intriguing that M_5 receptors are expressed in some blood cells, immortalized cell lines of which could prove useful model systems. For example, M_5 receptors are expressed in specifically microglia/macrophage cells (Ferrari-DiLeo & Flynn, 1995). Treatment with interferon γ increased both the expression of M_5 mRNA in monocytic/macrophage cells differentiated from eosinophilic leukaemic EoL-1 cells (Mita *et al.*, 1996). Although muscarinic receptor stimulation induces chemotaxis in these cells, it is unclear if the M_5 receptor mediates the response. Again, extensive pharmacological analysis is needed to determine the nature of the muscarinic receptor subtype mediating the response.

The lack of selective M_5 receptor antagonists has led to the development of alternative approaches to elucidate the function of the endogenous M_5 receptor. Yeomans *et al.* (1999) have reported preliminary data concerning a transgenic mouse with a null mutation in the M_5 gene. The phenotype of the animal showed that the maintenance phase of salivary gland secretion, induced by pilocarpine, was impaired, and no other gross abnormalities apparent. This phenotype agrees with the presence of the M_5 receptor in rat salivary gland tissue (Flynn *et al.*, 1997) and potentially, the atypical antagonist affinities seen by Meloy *et al.* (1998). Oxybutynin, an antagonist extensively used in the treatment of urinary incontinence, is selective for the muscarinic M_3 over the M_5 receptor (Watson *et al.*, 1999; Table 1). One may expect, therefore, oxybutynin to have reduced effects on salivary gland secretion, when compared to nonselective antagonists such as atropine, while retaining equivalent potency on the urinary bladder (in which contractile activity is augmented by the muscarinic M_3 receptor; Hegde *et al.*, 1997). However, extensive clinical and preclinical experience with oxybutynin shows no 'bladder selective' actions (Yarker *et al.*, 1995). A similar argument may be advanced for darifenacin in clinical evaluation for urinary incontinence (Wallis & Napier, 1999) in view of its selectivity for the M_3 over the M_5 receptor. In this case, however, *in vivo* bladder selectivity, at least at low doses, is present (Wallis & Napier, 1999), although this has been disputed (Hegde *et al.*, 1997).

Overall, if the muscarinic M_5 receptors modulates salivary gland secretion, then identification of agents with reduced affinity for this receptor, but with high M_3 receptor affinity, represents a novel approach to therapeutics for diseases in which smooth muscle overactivity needs to be reduced, but salivary gland function preserved. A similar argument can be made assuming that the M_5 receptor plays a role in the control of pupilliary diameter (Gil *et al.*, 1997; Alabaster, 1997). In this case muscarinic M_3/M_5 selective antagonists may possess a lower propensity for mydriasis, particularly at doses at which inhibition of smooth muscle activity occurs. Preclinical data to support this suggestion is that zamifenacin, also an M_3 over M_5 selective antagonist, does not affect dog pupilliary diameter *in vivo* at doses that inhibit gastrointestinal motility (McRitchie *et al.*, 1993).

Conclusion

The muscarinic M_5 receptor remains the least studied of the five muscarinic receptors, even though it is over a decade since the identification of the receptor gene. Expression of the receptor in

recombinant systems illustrates the use of the receptor as model to study agonist channelling of responses; a process that may provide the basis for its role *in vivo*. Currently, it is clear that the receptor, due to its probable restricted CNS distribution, probably has a discrete role to play in dopaminergic transmission. In the periphery, the identification of its expression in salivary gland and iris-ciliary muscle suggests a broader role, but the data is sparse and requires extensive confirmation. Nonetheless, the distribution of the receptor in tissues commonly associated with side-effects of anticholinergic therapy has implications for novel drug design.

Therefore, it is clearly important to define the role of the receptor in both central and peripheral, nervous systems. The lack of good antisera and limited use of transgenic animals has undoubtedly impeded this progress. Most critically, a major deficit in this area is an absence of an antagonist preferential for the subtype, pivotal in characterizing the physiology of the receptor. Whether the M_5 receptor will emerge from the shadows rests critically on the development of its pharmacology.

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