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COMMENTARY

PHARMACOLOGY AND SECOND MESSENGER INTERACTIONS OF CLONED MUSCARINIC RECEPTORS

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The first publications on genetically defined muscarinic receptor subtypes, which appeared in October and December, 1986 [1, 2], laid to rest any remaining doubts about the existence of distinct subtypes of muscarinic receptors and opened the way to obtaining cell lines expressing pure populations of receptors. In the succeeding months other groups presented evidence for additional genes encoding muscarinic receptors—there are currently five genetically defined subtypes—which have now been cloned and expressed in multiple types of cells. According to the nomenclature for muscarinic receptor subtypes proposed at the Fourth International Symposium on Subtypes of Muscarinic Receptors [3], M1–M4 designates the pharmacologically defined receptors and m1–m5 the cloned receptors. The proteins encoded by m1 genes appear to correspond to M1 receptors in neuronal tissues, m2 gene products to M2 receptors found, for examble, in heart or cerebellum, and m3 to M3 receptors in smooth muscles and glandular tissues. Cloned m4 receptors were available before the corresponding tissue receptors were demonstrated. Recently, *in situ* hybridization studies [4, 5] and binding studies [6] have provided evidence for M4 receptors in specific regions of rat brain. Although mRNA for m5 receptors has been located in discreet regions of rat CNS [5, 7], there are as yet no reports of pharmacological studies of this receptor in tissues so that cloned m5 receptors are currently the only means to study this receptor subtype. The distribution of the five muscarinic receptor subtypes in human tissues remains to be defined.

In this commentary, observations obtained with cells transfected with genes for a type of muscarinic receptor will be compared, where possible, with results obtained in tissues. In addition, some of the problems which may arise using cell lines transfected with genes for a receptor subtype are discussed.

Muscarinic receptor subtypes and second messengers

Muscarinic receptors are part of a large family of receptors that are linked through guanine-nucleotide binding proteins (G proteins) to effectors that translate activation of a plasma membrane receptor into an intracellular response. In tissues, complexes between muscarinic receptors and G proteins enhance PI† hydrolysis, inhibit adenylyl cyclase and modulate ion channel activity [8]. During the last three years, such receptor–effector interactions have also been established in various cell lines transfected with genetic material encoding one of the subtypes of muscarinic receptors. In general, m1, m3 and m5 cloned receptors were found to be linked through uncharacterized G protein(s) to phosphoinositidase(s) (phospholipase C), while m2 and m4 receptors were negatively linked, through G_i proteins, to adenylyl cyclase.

The interactions between these effectors and a given subtype of cloned muscarinic receptor appear not to be exclusive. Activation of m2 and m4 cloned receptors leads to an inhibition of cyclic AMP formation but in some cell systems these cloned receptors could also produce, at higher agonist concentrations, a small stimulation of IP formation (Table 1). Some but not all cloned m1, m3 and m5 receptors (Table 1) enhanced the formation of both IP and cyclic AMP at similar agonist concentrations.

The first reports of multiple effector activation by a single receptor subtype considered that either changes in one effector were secondary to changes in a primary effector [9–11] or that a given receptor could interact with different G proteins [9, 12]. Experimental evidence for both possibilities has been reported. Enhanced accumulation of cyclic AMP by m1 receptors cloned in A9L‡ cells may be due to calmodulin activation of adenylyl cyclase subsequent to an elevation of intracellular levels of calcium by m1 receptor activation of PI turnover [10]. The ability of muscarinic receptor subtypes to interact with multiple G proteins was illustrated by the observations that m1 and m3 receptors expressed in CHO cells could stimulate PI hydrolysis through both pertussis toxin-sensitive and -insensitive pathways [13].

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† Abbreviations: AF-DX 116, [11[[2-(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; cyclic AMP, adenosine cyclic 3′,5′-monophosphate; IP, inositol phosphate; NMPB, *N*-methyl-4-piperidylbenzilate; NMS, *N*-methylscopolamine; PI, phosphatidylinositol; PZ, pirenzepine; and QNB, quinuclidinylbenzilate.

‡ Cell lines used to express cloned muscarinic receptor subtypes:

A9L, L, and B82, murine fibroblast cells; CHO, Chinese hamster ovary cells; COS-7, monkey kidney cells; HEK, human embryonic kidney cells; NG108-15, mouse neuroblastoma × rat glioma hybrid cells; RAT-1, rat cells; origin not described; Y1, murine adrenal carcinoma cells; and oocytes from *Xenopus laevis*.

Table 1. Receptor–effector interactions of cloned muscarinic receptors

↓ *cAMP*, ↑ *IP*	↑ *IP*, ↑ *cAMP*
Hm2 and Hm4 in HEK [9]	Hm1 and Hm3 in A9*
Pm2 in CHO [12]	Hm1 and Hm3 in CHO [9]
Rm1 in RAT-1 [14]	Hm5 in CHO [63]
	Rm3 in RAT-1 [71]
	Mm1 in Y1 [72]
↓ *cAMP, no change IP*	↑ *IP, no change cAMP*
Pm2 and Rm4 in NG108-15 [66]	Rm5 in L cells [11]
Pm2 in Y1 [72]	

Genes for human (H), rat (R), porcine (P), or mouse (M) muscarinic receptor subtypes m1–m5 were transfected into various cell lines (abbreviations are given above) and receptor modulation of the formation of inositol phosphates (IP) or cAMP was determined. References are indicated by numbers in brackets.

* Unpublished data of Brann *et al.* cited in Ref. 63.

An additional factor to be considered concerns the densities of muscarinic receptors expressed in the different cell lines, which varied from 29 fmol/mg protein ([^{3}H]NMS binding to m1 receptors expressed in RAT-1 cells [14]) to 2.5×10^6 [^{3}H]QNB binding sites/cell on m2 receptors expressed in CHO cells [12] which corresponds roughly to 5000 fmol/mg protein (calculated from data in Ref. 9 and assuming that membrane protein yield per cell is similar for the two cell lines). For comparison, densities of [^{3}H]QNB binding sites in mammalian tissues range from 30 to 3000 fmol/mg protein [15]. In some cell lines with a given complement of G proteins, receptors expressed at higher densities may interact with certain of these G proteins but may fail to form such complexes at lower densities. In muscarinic receptor cloning experiments the number of receptors produced by the various expression systems is an experimental variable but the types and relative amounts of G proteins in the different cell lines remain, for the most part, uncharacterized.

Although the above examples suggest that one cloned receptor may activate more than one G protein, the physiological significance of "promiscuous" muscarinic receptors interacting with multiple types of G proteins remains to be established. One possibility may be the modulation of the force of atrial contractions. In some species, low concentrations of muscarinic agonists decrease (negative inotropism) whereas higher concentrations augment (positive inotropism) the force of atrial contractions (see references in [16]). In reserpinized guinea pigs and rats the negative (but not the positive) inotropic response was abolished by pretreatment of the animals with pertussis toxin whereas the positive inotropic response was attenuated by chronic treatment with lithium [16] suggesting that the two responses are coupled to different effectors. Muscarinic binding sites in membranes prepared from atria appear to be of only one subtype [17–19]. Blot hybridization analysis using probes for m1–m4 mRNA found only m2 mRNA in this tissue [20]. Recent publications report pharmacological evidence that is consistent with both the positive and negative inotropic responses induced by carbachol in rat left atria as being mediated by one receptor subtype [21, 22].

Pharmacology of muscarinic receptor subtypes

Muscarinic receptor agonists. Before the advent of "selective" muscarinic antagonists, a common observation was that the displacement from tissue membranes of a radioactive muscarinic receptor ligand by antagonists followed the law of Mass Action, i.e. binding to a homogeneous population of sites, whereas displacement by muscarinic agonists did not. Heterogeneity of agonist binding sites has been found in virtually every tissue or cell type examined. Some parameters of muscarinic agonist binding, such as the absolute values of agonist affinities and the reduction of agonist affinity induced by GTP and its analogs, are tissue dependent whereas the ratios of agonist affinity states in a given tissue are agonist dependent [23–26]. Despite a great deal of progress in our understanding of the functions of muscarinic receptors, the relationship between the agonist affinity states of the receptor and the receptor–effector complexes that produce a functional response is not yet clear.

With the demonstration, first with antagonists and then with molecular biology techniques, that muscarinic receptor subtypes exist, the possible explanations for agonist binding heterogeneity in a given tissue were expanded to include not only different agonist affinity states of a receptor but also the displacement from different receptor subtypes, or a combination of both. Since most tissues are endowed with multiple subtypes of muscarinic receptors, the best means to study these factors would be in cells which have only one type of muscarinic receptor.

A few reports have been published on the binding affinities of agonists as displacers of specifically bound radiolabeled ligands from muscarinic receptors expressed in different cell lines, and these are summarized in Table 2. These values may be compared with pharmacologically determined agonist affinities at muscarinic receptors in tissues [27]. In most but not all cell lines, carbachol and

Table 2. Binding constants of muscarinic agonists at cloned muscarinic receptors

	HEK[a]	CHO[b]	A9L[c]	RAT-1[d]	B82[e]	B82[f]	Y1[g]
Carbachol							
m1							
pK_H	5.1			7.0		5.7–5.1	5.4
pK_L	3.3			3.9		4.5–4.3	3.9
%H	30%			25%		40–60%	26%
pIC_{50}			6.3		5.3		
n_H			0.4		1.0	0.7	
GTP-shift			4X		8X		None
%H + GTP			ND	0%			
m2							
pK_H	7.0	5.8					
pK_L	3.9	3.8					
%H	28%	30%					
%H + GTP		9%					
m3							
pK_H	5.1			4.1			
pK_L	3.3			3.0			
%H	6%			56%			
n_H				0.8			
%H + GTP				30%			
m4							
pK_H							
pK_L	3.7						
%H	0%						
Oxotremorine							
m1							
pK_H	—			7.8			
pK_L	5.4			5.1			
%H	0%			23%			
pIC_{50}			6.1				
n_H			0.9				
m2							
pK_H	8.1	6.7					
pK_L	5.3	5.3					
%H	20%	24%					
%H + GTP		10%					
m3							
pK_H	—			5.1			
pK_L	5.4			4.3			
%H	0%			46%			
n_H				0.7			
m4							
pK_H	7.7						
pK_L	5.4						
%H	5%						

The negative logs of dissociation constants were calculated from binding data obtained by the displacement of specifically bound radiolabeled ligands by muscarinic agonists from cloned muscarinic receptors expressed in various cell lines. The source of the genes, the buffer and the radioligand used in the binding assays are included with the references. pK_H and pK_L indicate high and low affinity values; "GTP shift", the change in IC_{50} values in the presence of GTP; and %H, the percent of the total binding sites that display high affinity for the agonist. ND = not indicated.

References:

a. 10 mM Na_2HPO_4, 1 mM EDTA; [^{3}H](−)-quinuclidinyl benzilate ([^{3}H]QNB); human m1–m4 [69].
b. Buffer as for HEK; [^{3}H]QNB; pig m2 [12].
c. 10 mM HEPES, 5 mM $MgCl_2$; [^{3}H]QNB; rat m1 [64].
d. Phosphate-buffered saline; [^{3}H]*N*-methyl-4-piperidylbenzilate; rat m1 and m3 [14, 71].
e. Krebs-phosphate buffer; affinity constants calculated from IC_{50} values; [^{3}H]QNB; rat m1 [67].
f. Intact cells, culture medium; [^{3}H]MeQNB; rat m1; clones with different receptor densities/cell [30].
g. 50 mM sodium potassium phosphate buffer; [^{3}H]QNB; mouse m1 [72].

oxotremorine displaced specifically bound radioligands in a multiphasic manner. These observations support the hypothesis [28, 29] that the multiple agonist affinity states found in tissues are due to the recognition by an agonist of different configurations of a particular muscarinic receptor subtype instead of or in addition to binding to multiple subclasses of receptors. The reports that agonist displacement curves at a given receptor subtype were multiphasic in some cells lines but monophasic in others indicate that the proportions of affinity states may depend on factors extraneous to the receptor *per se*.

One such factor determining the ratio of agonist affinity states may be receptor density. Mei *et al.* [30] observed in B82 cells that as the m1 receptor density increased, the proportion of high affinity agonist binding states decreased. This probably reflects, in part, the decreasing proportion of receptor–G protein complexes as a percent of the total receptor density. (In these experiments, it was assumed that the types and levels of G proteins were similar in the different clones since the same cell line was used to express m1 receptors at different densities.) As expected, the *affinities* of carbachol for the low and high agonist affinity states remained constant throughout the range of receptor densities and changing proportions of these states. Similar studies using full and partial agonists will provide a means of testing the hypothesis that the *efficacy* of an agonist at a given muscarinic receptor subtype depends on the *ratio* of its affinities for the high and low agonist affinity states [31–33].

In rat forebrain, the presence of GTP or Gpp(NH)p has little effect on the displacement of specifically bound ligands by carbachol [24, 25, 34, 35], and this was also observed with cloned m1 receptors in most cell types. GTP or Gpp(NH)p induces significant changes in agonist binding curves at M2 muscarinic receptors in heart [23, 25, 35] or cerebellum and brainstem [24, 35], and this was observed with m2 receptors cloned in CHO cells [12].

In the past, differences in the potency of a given agonist, determined in functional studies using different tissues, have been considered indicative of variable receptor reserves and/or receptor subtypes among the tissues. However, additional factors are now thought to play a role in determining agonist potencies. A theoretical study of the interactions between a receptor and multiple G proteins suggests that agonist potency will be dependent on the relative quantities of the different G proteins capable of interacting with the receptor and thus become a tissue-dependent as well as a receptor-dependent factor [36]. In addition, the discovery of isoforms of enzymes in effector systems, for example isozymes of phosphoinositidase [37], could possibly contribute to differences in agonist potency. This emphasizes the importance of comparing agonist potencies at the different muscarinic receptor subtypes which have been expressed at similar receptor densities in the same cell line.

Not unexpectedly, the maximum response obtained with cloned receptors was related to the receptor density. A 15-fold increase in the density of human m3 receptors expressed in HEK cells correlated with a 13-fold increase in the formation of IP [9]. A 15-fold increase in the density of human m1 receptors expressed in CHO cells led to a 9-fold increase in IP formation induced by carbachol but a further increase in receptor density did not further enhance the biological response [13], indicating, perhaps, a saturation of the available G_p proteins (which activate phosphoinositidases) with receptors. In seven clones of B82 cells transfected with rat m1 receptors, there was a significant correlation between the maximum formation of IP induced by carbachol and m1 receptor densities [30]. However, in these cells, a 20-fold increase in receptor density gave only an 8-fold increase in the maximum response. In CHO cells expressing porcine m2 receptors [12], the receptor density varied by 23-fold but the small increases in IP induced in these cells by carbachol differed maximally by 4-fold between cells with the highest and lowest receptor densities.

There are several possible explanations for a lack of a strong correlation between receptor density and biological response. The phenomenon of "spare receptors" is often cited when higher half-maximal concentrations of agonist are required for receptor occupancy than for eliciting a biological response. However, the relationship between total receptor concentration and maximum response is complex and a simple correlation between these two parameters is not sufficient to demonstrate spare receptors [30]. At higher receptor densities, saturation of G proteins could occur, flattening the receptor density–response curve or, as reported for cloned β-adrenergic receptors, the curve may become bell-shaped [38]. Receptor density may influence the kinetics of a response such that at low densities the response appears qualitatively different from that measured at high densities [39]. Differences in the binding affinity of an agonist compared to its potency could indicate the presence of non-functional receptors as was shown recently for mutated β-adrenergic receptors [40]. Loss of receptors with increasing passage number, which occurs with endogenous muscarinic receptors in cloned cell lines [41], may also occur with cloned receptors and underlines the importance of determining agonist binding affinities and potencies using cells of similar passage number.

Muscarinic receptor antagonists. The pK_D or pK_i values of four muscarinic receptor antagonists, atropine, pirenzepine, AF-DX 116 and hexahydro-sila-difenidol (HHSiD), obtained from binding studies using cells (intact or membrane preparations) transfected with genes encoding one receptor subtype, are presented in Table 3. Because affinity values or the number of antagonist binding states may depend, to some extent, on the ionic conditions of the binding assay [42] and the hydrophobicity of the radioligands used [43], these conditions are included with the references at the end of the table. The affinity values of pirenzepine, AF-DX 116 and HHSiD found with cloned muscarinic receptors can be compared with data obtained in binding assays or functional tests in tissues as summarized by Mitchelson *et al.* [44].

It was reassuring to find that affinity values and selectivity ratios observed in tissues with these antagonists were also found with most of the cloned

Table 3. Affinity values of muscarinic antagonists at cloned muscarinic receptors

Cloned receptor	Oocytes[a,f,g]	COS-7[b]	HEK[c]	CHO[d,e,n,o]	A9L[h,i]	RAT-1[j,k]	Y1[l]	B82[m]
Atropine								
m1	9.42[a] 9.36[f]		8.48	9.68[n]			9.49	9.54
m2	8.82[f]		7.78	8.39[e] 8.82[n]				
m3			8.96	9.82[n]				
m4			8.92	(2.1 nM)[n*]				
m5				9.68[n]	8.69[i]			
Pirenzepine								
m1	7.92[a] 8.00[f] 7.74[g]	8.07	6.30	7.82[d] 7.80[n] 8.20[o]	8.68[h]	7.39 (pK_H)[j] 5.35 (pK_L)	8.00	7.48
m2	6.30[f] 6.18[g]	6.30	4.90	6.03[d] 5.96[e] 6.04[n] 6.65[o]				
m3	6.74 (pig)[g] 6.89 (rat)[g]	7.72	5.92	6.74[n] 6.86[o]		7.12 (pK_H[k]) 5.21 (pK_L)		
m4	6.92[g]	7.79	5.57	(561 nM)[n*] 7.43[o]				
m5				(390 nM)[d*] (628 nM)[n*] 7.05[o]	6.82[i]			
AF-DX 116								
m1	5.74[f] 5.06[g]		7.3 (pK_H) 5.2 (pK_L)	5.89[d] 5.89[n]	5.62[h]	4.62[j]		5.85
m2	6.44[f] 6.14[g]		7.7 (pK_H) 6.1 (pK_L)	6.58[d] 6.59[n]				
m3	5.64 (pig)[g] 5.51 (rat)[g]		6.5 (pK_H) 6.0 (pK_L)	6.09[d] 6.07[n]		5.40[k]		
m4	5.64[g]		5.59	6.35[d] (1.8 μM)[n*]				
m5				5.55[d] 5.55[n]	5.70[i]			
Hexahydro-sila-difenidol								
m1	7.29[g]			7.36[n]				
m2	6.55[g]			6.60[n]				
m3	8.36 (pig)[g] 8.40 (rat)[g]			8.00[n]				
m4	7.70[g]			(298 nM)[n*]				
m5				7.20[n]				

The negative logs of dissociation constants were obtained in binding studies using muscarinic antagonists to displace specifically bound radiolabeled ligands from cloned muscarinic receptors expressed in different cell lines (abbreviations are given above). The source of the genes, the buffer and the radioligand used in binding assays are indicated. The abbreviations Na^+/PO_4 or K^+/PO_4 are used to indicate sodium phosphate or potassium phosphate buffers. Where multiple binding sites were reported, the high and low affinity values are indicated by pK_H and pK_L and the percent of high affinity sites (R_H) are included with the references.

References:

a. Porcine m1; 320 mM sucrose, 10 mM K^+/PO_4, 1 mM EDTA; [^{3}H]QNB [1].
b. Rat m1 and m3; human m2 and m4; 10 mM HEPES, 5 mM $MgCl_2$; [^{3}H]QNB.
Affinity constants calculated from IC_{50} values in Fig. 2 of Ref. 56.
c. Human genes; 10 mM Na^+/PO_4, 1 mM EDTA; [^{3}H]QNB. AF-DX 116: m1 R_H = 14%, m2 R_H = 41%, m3 R_H =20% [69].
d. Rat m1, human m2–m5; 25 mM Na^+/PO_4, 5 mM EDTA; [^{3}H]NMS [63].
e. Porcine m2; buffer as in "c"; [^{3}H]QNB [70].
f. Porcine m1 and m2; buffer as in "a"; [^{3}H]QNB [65].
g. Porcine m1–m3; rat m3 and m4; buffer as in "a"; [^{3}H]QNB [62].
h. Rat m1; buffer as in "b", [^{3}H]QNB. Affinity constants calculated from IC_{50} values [64].
i. Rat m5; phosphate-buffered saline (PBS); [^{3}H]NMS [11].
j. Rat m1; PBS; [^{3}H]NMPB; PZ: R_H = 70% [14].
k. Rat m3; PBS; [^{3}H]NMPB; PZ: R_H = 49% [71].
l. Mouse m1; 50 mM Na^+/PO_4; [^{3}H]QNB [72].
m. Mouse m1; 10 mM Na^+/PO_4, 1 mM $MgCl_2$; [^{3}H]QNB [68].
n. Rat m1, human m2–m5; 25 mM Na^+/PO_4, 5 mM $MgCl_2$; [^{3}H]NMS [50, 73].
o. Human m1–m5, 25 mM Na^+/PO_4, 5 mM $MgCl_2$; [^{3}H]NMS [52]. (* = IC_{50}; $n_H < 1$)

muscarinic receptors. Pirenzepine, an antagonist selective for M1 receptors in tissues [45], displaced specifically bound ligands from m1 receptors in the various cell lines with high affinity (pK_i of 7.4 or greater) with the exception of HEK cells (Table 3). Consistent with the M2 selectivity of AF-DX 116 in tissues [46], the highest pK_i value for this compound was found with m2 receptors expressed in the different cell lines (Table 3) but in these cells, as in tissues, the selectivity ratios among the different receptor subtypes were small. In tissues, HHSiD had similar affinities at M1 and M3 receptors whereas its affinity at M2 receptors was 20–30 times lower [47]. Similar affinity values and selectivity ratios among the different receptor subtypes were observed in CHO cells (Table 3).

There were, however, some striking differences between cloned receptors and those in tissues as well as significant differences in antagonist affinities at a given receptor subtype expressed in different cell lines. Atropine, a putatively non-selective muscarinic antagonist, displayed differences in affinity of 4- to 5-fold in functional [48] and binding [49] studies in tissues. In contrast, the affinity constants for atropine observed in cells (except HEK) expressing m1–m5 receptors varied by 10- to 20-fold (Table 3). Pirenzepine was 60-fold more active at m3 receptors expressed in COS-7 cells than at m3 receptors in HEK cells. In Xenopus oocytes, there was a 13-fold difference in affinities for HHSiD at m3 compared to m1 receptors, whereas in CHO cells, the affinity values of HHSiD for the two receptors differed by 4-fold.

The possible contributions of binding conditions and expression systems to the differences in antagonist affinities at muscarinic receptor subtypes were eliminated in one study where rat m1 and human m2–m5 muscarinic receptors were expressed in the same cell line (CHO-K1) and similar binding conditions were used to determine muscarinic receptor antagonist affinities [50]. In general, the order of selectivities found with muscarinic antagonists at these receptors was similar to those found in tissues using functional tests but the magnitude of the selectivity ratios for some of the antagonists tended to be greater at the cloned receptors. For example, atropine displayed 10-fold selectivity in this study. The M1/M2 selectivity of pirenzepine, obtained by comparing its inhibitory activity at M1 receptors in rabbit vas deferens ($pA_2 = 7.6$ [51]) versus antagonism of atrial contractions through M2 receptors ($pA_2 = 6.6$–6.8 [17]), was maximally 10-fold, whereas the ratio of m1/m2 affinity values in CHO-K1 cells was 57.

Binding data obtained with all four antagonists displayed Hill coefficients of less than unity at one or more of the cloned receptors listed in Table 3. Some of these observations may be due to technical differences since a low Hill coefficient reported for pirenzepine at human m4 receptors expressed in CHO-K1 cells [50] was not found in a later study [52]. Possible interpretations of low Hill coefficients include negative cooperativity, multiple, non-interacting binding sites or multiple interconvertible affinity states [53]. Multiple interconvertible affinities are observed with agonists in binding to receptors in the presence of G proteins and GTP but this is usually not the case with antagonists. If cells without detectable muscarinic binding sites are transfected with the genetic material for one muscarinic receptor subtype, then the second possibility, multiple receptors, does not seem to be a likely explanation. The possibility of multiple muscarinic receptors arising from post-translational changes, as was reported recently for dopamine D_2 receptors [54, 55], seems unlikely because of the lack of introns in the genes for muscarinic receptors [56]. Negative cooperativity would be seen if the antagonist binds to a secondary, allosteric site such that the binding kinetics of the radioligand are perturbed. The observations that low Hill coefficients occur at a given receptor subtype expressed in some but not all cell lines indicate that the expression system may contribute to this parameter. That the muscarinic receptor subtypes are glycoproteins [57] suggests that perhaps differences in glycosylation of the cloned muscarinic receptors in different cell lines may contribute to differences in affinities. However, mutation of glycosylation sites of cloned m2 receptors [58] or enzymatic deglycosylation [59, 60] of endogenous muscarinic receptors had no effect on antagonist affinities or Hill coefficients. Another possible explanation for the low Hill coefficients resulting from ligand interactions with cloned muscarinic receptors could be that the cellular environments were sufficiently different among the various cell lines to influence the binding site recognition by these antagonists. In studies with purified muscarinic receptors from porcine heart and cerebral cortex, reconstituted into different lipid environments, the affinities for [^{3}H]QNB and pirenzepine, as well as the proportions of sites that displayed a high affinity for pirenzepine, were influenced significantly by the lipid composition [61].

In conclusion, pure populations of muscarinic receptors will provide the means of obtaining pharmacological profiles of each subtype of receptor. This information is necessary to maximize the possibility of synthesizing compounds that are selective for a given receptor subtype although there is no guarantee of success. The high degree of amino acid sequence homology among the subtypes of muscarinic receptors may be a stumbling block. The differences observed between receptors in tissues compared to cloned receptors or even among cloned receptors expressed in different cell lines mean that animal studies will, in the end, be the final arbitrator of pharmacological selectivity.

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