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Pharmacology of muscarinic acetylcholine receptor subtypes (m1–m5): high throughput assays in mammalian cells

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Abstract

Based on the ability of many receptors to amplify NIH 3T3 cells, we developed a high throughput assay of cloned receptor pharmacology. In this assay, receptors are transiently co-expressed with the marker enzyme β-galactosidase. Receptors that induce cellular proliferation select and amplify the cells that also express the marker, thus the ability of ligands to alter receptor activity are reported as changes in enzyme activity. We used this assay to evaluate the pharmacology of agonist and antagonist ligands for five cloned human muscarinic receptor subtypes (m1–m5). When cells were transfected with subtypes that prefer the G-protein G_q (m1, m3, m5) robust increases in enzyme activity were observed. The subtypes that prefer G_i (m2 and m4) only induced β-galactosidase when co-expressed with a chimera between the G-proteins G_q and G_i ($G_{q\text{-}i5}$). Overall, the rank-order of potency and intrinsic activity of most of the tested ligands were in remarkably good agreement with earlier results using cloned cell lines and isolated tissues. These data demonstrate that a high throughput colorimetric assay performed in 96-well plates can be used to evaluate subtle differences the pharmacology of ligands for cloned muscarinic receptor subtypes.

Keywords: Muscarinic receptor; G-protein; High-throughput assay; Molecular biology

1. Introduction

Based on pharmacological criteria, at least three muscarinic receptor subtypes have been defined (M_1–M_3) (Nathanson, 1987; Brown, 1989). Molecular cloning procedures have demonstrated the existence of five distinct genetic subtypes (m1-m5) (Kubo et al., 1986; Peralta et al., 1987; Bonner et al., 1987, 1988) that are expressed in distinct parts of the brain and body (Brann et al., 1993; Levey, 1993). With the broad therapeutic uses of muscarinic agents, and their equally broad side effect profiles, the assignment of individual subtypes to physiological responses has become of considerable interest, and the identification of subtype selective ligands has become a major target of many drug discovery efforts. Among their many physiological functions and locations, M_1 (m1) is prominent in brain, M_2 (m2) receptors control cardiac function, and M_3 (m3) controls salivation and intestinal motility (Nathanson, 1987; Brown, 1989; Brann et al., 1993; Levey, 1993). Using transfected cell lines, we and many others have examined the radioligand binding properties of the cloned receptors, and the abilities of agonist ligands to alter second messenger and electrophysiological responses (Jones et al., 1992; Caulfield, 1993). When antagonist ligands are considered, the radioligand binding data from the cloned receptors are in good agreement with similar data from tissues where the identity of the receptor subtypes are known (Buckley et al., 1989; Dörje et al., 1991; Wess et al., 1991). Agonist pharmacology is confounded by the fact that agonist affinities, potencies and intrinsic activities can vary dramatically, depending on the levels of receptors, the cell type assay, and the coupling efficiency of the receptor with the response being measured (Kenakin and Morgan, 1989; Sokolovsky, 1989). Because agonist binding is strongly influenced by assay conditions and G-protein coupling states it is difficult to quantitatively relate

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agonist binding to the pharmacology of physiological responses. To measure the intrinsic activity of agonists a functional response is needed. In these functional assays in transfected cell lines, many ligands have been shown to have modest differences in potency and intrinsic activity at the receptor subtypes (Novotny and Brann, 1989; Lazareno et al., 1993; Schwarz et al., 1993; Wang and El-Fakahany, 1993; Richards and Van Giersbergen, 1995).

Overall, we have found that the use of second messenger responses to evaluate receptor pharmacology in transfected cells is limited by several factors. Receptor levels and coupling properties of receptors change dramatically with time in culture. This factor and the fact that second messenger assays require large numbers of cells, dictate that the preparation and quality control of the recombinant cells is a major limitation of these cloned receptor assays. Also, since most second messenger assays require many complex steps and radioisotopes, they are very expensive and labor intensive to perform. In some cases, e.g., inhibition of adenylyl cyclase, these assays also have a limited dynamic range. Perhaps due to these difficulties no comprehensive study of reference agonist and antagonist ligands at all of the cloned muscarinic receptor subtypes has been reported.

We report a novel assay of cloned receptor pharmacology that overcomes the limitations that are inherent to the previously published functional assays using transfected cells. When transfected into NIH 3T3 cells, many genes are able to stimulate cellular proliferation and induce macroscopic colonies of cells called foci (Jainchill et al., 1969). This is the traditional assay of cellular transformation that has been used to identify many oncogenes. Many receptors only induce cellular proliferation and focus formation in NIH 3T3 cells when stimulated with agonist ligands (Julius et al., 1989; Allen et al., 1991; Gutkind et al., 1991). In the case of muscarinic receptor subtypes, only those subtypes that efficiently stimulate phosphatidyl inositol metabolism (m1, m3, m5) induce foci, and the dose-responses of agonists for focus induction and stimulation of phosphatidyl inositol metabolism are identical (Gutkind et al., 1991). Unfortunately, induction of foci is not a convenient assay of receptor pharmacology. The response is labor intensive and difficult to quantitate (colonies are stained and counted) and requires at least 10 days of ligand treatment to be observed. This requires several media and drug applications. Also, relatively large culture vessels are required for reliable measures. Our assay involves the co-expression of a convenient marker enzyme with the receptor of interest. Ligands stimulate proliferation of the cells that express the receptor (and thus the marker) and thus ligand induced effects are read by assay of the marker. In this assay receptors select and amplify cells containing the marker, and we therefore refer to the assay as *R*eceptor *S*election and *A*mplification *T*echnology (R-SAT). We report application of this assay to evaluation of the agonist and antagonist pharmacology of the m1–m5 muscarinic receptor subtypes. Assays were performed in a 96-well plate format using a standard colorimetric assay of β-galactosidase.

2. Materials and methods

2.1. Cell culture

NIH 3T3 cells (ATCC No. CRL 1658) were cultured at 37°C in a humidified atmosphere (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 4500 mg/l glucose, 4 nM L-glutamine, 50 U/ml penicillin G, 50 U/ml streptomycin (A.B.I. Columbia, MD) and 10% calf serum (Gibco, Grand Island, NY).

2.2. Transfection procedure and ligand addition

Cells were plated 1 day before transfection using 10^6 cells in 10 ml of medium per 10 cm plate. Cells were transfected by calcium precipitation as described by Wigler et al. (1977), using 5 μg of the human m1–m5 receptors (Bonner et al., 1987, 1988) in the pcD expression vector (Okayama and Berg, 1983), 5 μg pSV-β-galactosidase (Promega, Madison, WI) and 20 μg of salmon sperm DNA (Sigma, St. Louis, MO). Cells transfected with m2 or m4 were further co-transfected with 5 μg $G_{q\text{-}i5}$ (Conklin et al., 1993) in the pcDNAI vector (Invitrogen, San Diego, CA). 1 day after transfection medium was changed and after 2 days cells were trypsinized and aliquoted into the wells of a 96-well plate (100 μl/well). One 10 cm plate yields 96 wells. Ligands were combined with the cells to a final volume of 200 μl/well.

2.3. β-Galactosidase assay

After 4 (m1, m3, m5) or 5 (m2, m4) days of culture in the presence of ligands, the level of β-galactosidase was measured essentially as described by Lim and Chae (1989). The medium was removed from the wells and the cells were rinsed with PBS (136.9 mM NaCl, 2.68 mM KCl, 8.09 mM Na_2HPO_4 and 1.47 mM KH_2PO_4; pH 7.4). 200 μl of PBS with 3.5 mM ONGP (*o*-nitrophenyl-β-D-galactopyranoside) and 0.5% nonidet P-40 (Sigma, St. Louis, MO) was added to each well and the 96-well plate was incubated at room temperature. After 4–20 h the plates were read at 405 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices). Better signal-to-noise relationships were observed for the longer incubation times.

2.4. Data analysis

Data from both agonist stimulation and antagonist inhibition were fitted to the three parameter equation (Rodbard and Hutt, 1974):

$$R = D + \frac{(A - D)}{1 + (X/c)}$$

R = response, X = concentration of ligand. For agonists A = minimum response, D = maximum response and $c = EC_{50}$ (EC_{50} = concentration of agonist producing 50% of its maximal response). For antagonists D = minimum response, A = maximum response and $c = IC_{50}$ (IC_{50} = concentration of antagonist causing 50% inhibition of agonist response).

pA_2 analysis was performed as described by Arunlakshana and Schild (1959). Under conditions where agonist and antagonist concentration-response relationships obey mass action, the relationships described by Cheng and Prusoff (1973) can be applied to estimation of antagonist affinity constants (K_b) in functional assays (Craig, 1993):

$$K_b = \frac{IC_{50}}{(1 + [A]/EC_{50})}$$

where [A] is the agonist concentration. Best-fit parameters were obtained by non-linear least-squares curve-fitting using the program KaleidaGraph (Abelbeck Software) on a Macintosh Quadra 700 and were used to generate the curves shown in Figs. 3–5.

2.5. Ligands

Ligands were obtained from the following sources; arecoline hydrobromide, 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine) methiodide, methoctramine tetrahydrochloride, (±)-muscarine chloride, oxotremorine sesquifumarate, p-F-HHSiD ((±)-*p*-fluoro-hexahydrosila-difenidol hydrochloride) (Research Biochemicals, Natick, MA); atropine sulfate, carbachol, McN A-343 (4-[[*N*-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl-trimethylammonium chloride), pilocarpine hydrochloride (Sigma, St. Louis, MO); pirenzepine dihydrochloride (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT).

3. Results

3.1. Assay setup

Based on protocols that we have previously used to evaluate induction of foci (Gutkind et al., 1991), we performed a series of experiments seeking to identify conditions where muscarinic agonists would induce β-galactosidase in receptor transfected NIH 3T3 cells. Concentrations of DNA were systematically titrated, as were ratios of receptor to marker DNA, and time in culture with ligands. In these initial studies, the largest responses were observed when 5 μg of receptor and 5 μg of marker cDNA per 10 cm dish was used. Using these conditions treatment of m5 transfected cells with carbachol led to a marked increase in β-galactosidase that plateaued by 5 days in culture (Fig. 1). Similar responses were observed for m1 and m3. m2 transfected cells did not respond to treatment with carbachol. When m2 receptors were co-transfected with $G_{q\text{-}i5}$, a G-protein chimera that has previously been shown to allow G_i selective receptors to stimulate G_q responses (Conklin et al., 1993), m2 induced β-galactosidase albeit with lower efficiency than the m5 receptor. The m4 receptor was similarly dependent on co-expression with $G_{q\text{-}i5}$. These data indicate that as for focus assays, only the muscarinic receptors that prefer the G-protein G_q are able to induce detectable β-galactosidase responses. A direct role of G_q in the β-galactosidase response is further supported by the ability of the G-protein chimera to allow responses to be mediated by m2 and m4. Further, we have recently observed that over expression of G_q shifts the concentration-response relationships of carbachol at m3 re-

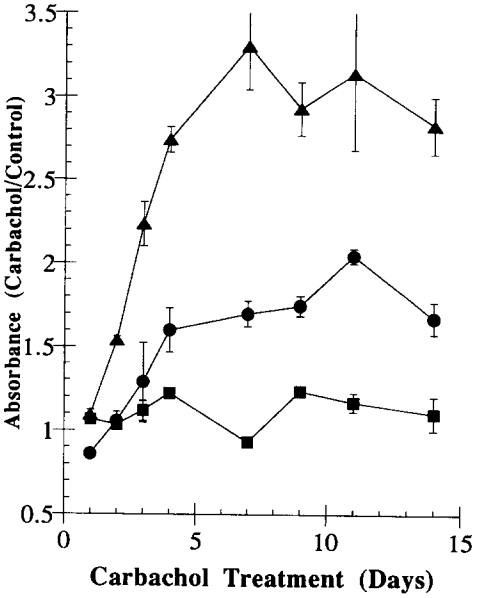

Fig. 1. Time-courses of β-galactosidase amplification by NIH 3T3 cells transfected with 5 μg pcD-m2+5 μg pcD-neo (■), 5 μg pcD-m2+5 μg pCMV-Gq-i5 (●) or 5 μg pcD-m5 (▲). All transfections also included 5 μg pCDNA-b-galactosidase. 2 days after transfection, the cells were split into 96-well plates and incubated with 10 μM (for m2) or 1 μM (for m5) carbachol for the indicated period of time. Controls were performed for each condition in the absence of carbachol. Data are plotted as the ratio of the absorbance at 420 nm determined in carbachol divided by the no carbachol control. Points are means from a representative experiment. Similar results were observed in three experiments.

Table 1
EC_{50} and maximum responses of agonists at muscarinic receptors

Agonist	m1	m2	m3	m4	m5
Arecoline	3.2 ± 0.7	0.025 ± 0.001	0.34 ± 0.11	0.13 ± 0.05	0.60 ± 0.05
	(86 ± 3)	(105 ± 0)	(66 ± 9)	(72 ± 3)	(77 ± 2)
Carbachol	6.5 ± 0.6	0.10 ± 0.04	1.4 ± 0.7	0.27 ± 0.07	0.11 ± 0.05
	(100)	(100)	(100)	(100)	(100)
McN A-343	1.1 ± 0.2	1.5 ± 0.6	2.2 ± 0.0	0.12 ± 0.02	1.0 ± 0.3
	(43 ± 2)	(108 ± 7)	(38 ± 2)	(84 ± 3)	(57 ± 4)
Muscarine	2.4 ± 0.8	0.060 ± 0.020	0.56 ± 0.25	0.32 ± 0.15	0.39 ± 0.18
	(84 ± 4)	(76 ± 1)	(84 ± 6)	(69 ± 2)	(86 ± 0)
Oxotremorine	0.39 ± 0.13	0.019 ± 0.010	0.21 ± 0.06	0.033 ± 0.014	0.055 ± 0.001
	(75 ± 10)	(100 ± 5)	(66 ± 5)	(102 ± 3)	(74 ± 2)
Pilocarpine	274 ± 30	25 ± 1	35 ± 3	60 ± 16	27 ± 10
	(79 ± 5)	(107 ± 4)	(54 ± 7)	(71 ± 8)	(71 ± 4)

NIH 3T3 cells were transfected with the indicated muscarinic acetylcholine receptor and assayed using R-SAT. EC_{50} is expressed in μM and maximal responses are presented (in parentheses) as a percentage of carbachol responses. Data represent the means (±S.E.M.) of 2–4 experiments. At least nine doses of each ligand were used.

ceptors to the left, and induces atropine and pirenzepine sensitive constitutive receptor activity (Burstein et al., 1995a).

Foci are not observed until at least 10 days of carbachol treatment (Gutkind et al., 1991), while all of the β-galactosidase responses plateaued by 5 days (Fig. 1). Since it is not necessary to change media during the first 5 days of culture, this rapid time-course is an important technical advantage relative to focus assays.

For all of the following experiments, we chose 4 days of ligand treatment for the more robust m1, m3 and m5 receptors, and 5 days in culture for the m2 and m4 receptors. In no cases were media or ligands changed during the course of the experiments. We assayed the β-galactosidase using ONGP as a substrate essentially as reported (Lim and Chae, 1989). At room temperature levels of signal increased in proportion to the incubation time with substrate, being linear for at least

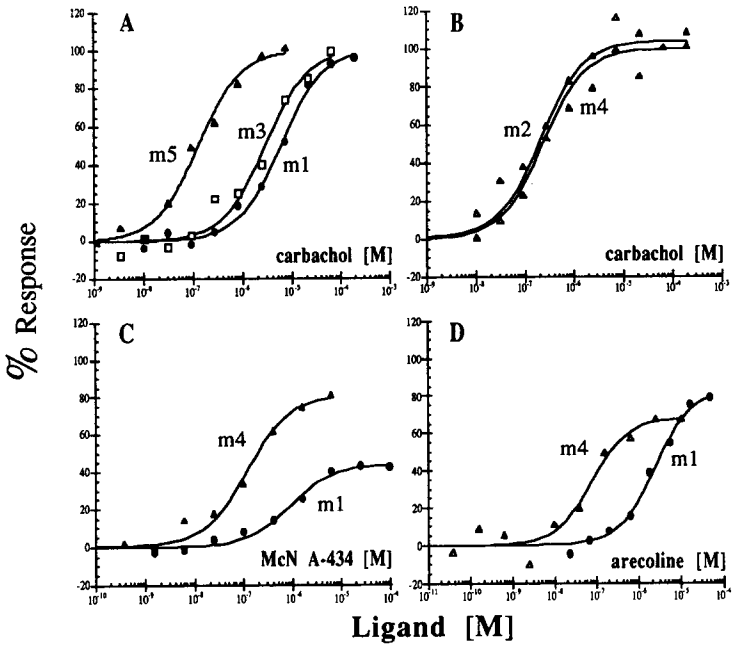

Fig. 2. Agonist concentration-response relationships of β-galactosidase amplification responses of cells transfected with m1 (●), m2 (▲), m3 (□), m4 (△) and m5 (▲) receptors. The m2 and m4 were co-transfected with $G_{q\text{-}i5}$. 2 days after transfection, the cells were split into 96-well plates and incubated for four (m1, m3, m5) or 5 (m2, m4) days with the indicated ligands. The agonists McN A-343 and arecoline are presented as percent of maximal carbachol response. Each point is the mean of a quadruplicate determination. Curves are mass-action fits of the data.

10 h. We also observed that there was no significant change in pharmacological parameters for at least 20 h (data not shown).

In theory, any one of a diversity of marker enzymes could be used for R-SAT assays. We have tested luciferase and acid phosphatase as alternative markers to β-galactosidase. These markers actually gave more robust responses to ligand treatment than the β-galactosidase assays (data not shown). For the reported pharmacological assays, we chose to use β-galactosidase as a marker because the assay was the easiest and least expensive to perform, and the magnitude of response was sufficient to accurately titrate concentration-response relationships.

3.2. Agonist pharmacology

As shown in Table 1 and Fig. 2, the agonist carbachol induced responses in cells transfected with each of the five muscarinic receptor subtypes. Each of these

Table 2
Rank orders of EC_{50} values and maximum responses of agonists at muscarinic receptor subtypes

Receptor	Method	(Ref.)	Rank order of EC_{50}					
m1	R-SAT		oxo >	mcn ≈	mus ≈	are ≈	car >	pil
m1	PI	(b)	oxo >			are ≈	car >	pil
m1	PI	(c)	oxo >	mcn ≈	mus ≈	are ≈	car ≈	pil
m1	GTPase	(a)	oxo >	mcn ≈	pil >	are >	car	
M_1	RVD	(d)	oxo >	mcn ≈		are ≈	car	
m2	R-SAT		oxo ≈	are >	car ≈	mus >	mcn >	pil
m2	cAMP	(e)	oxo >	are ≈	car ≈	mus ≈	mcn ≈	pil
m2	GTPase	(a)	oxo >	car >	are ≈		pil >	mcn
M_2	GPA	(d)	oxo >	are ≈	car >		mcn	
m3	R-SAT		oxo ≈	are ≈	musc ≈	car ≈	mcn >	pil
m3	GTPase	(a)	oxo >	are ≈		car ≈		pil
M_3	GPI	(d)	oxo >	are ≈		car >	mcn	
m3	PI	(c)	oxo ≈	are ≈	musc ≈	car >	mcn	pil
m4	R-SAT		oxo >	mcn ≈	are ≈	car ≈	mus >	pil
m4	GTPase	(a)	oxo ≈	mcn >	are ≈	car ≈		pil
m4	cAMP	(c)	oxo >	mcn ≈	are ≈	car ≈	mus >	pil
m5	R-SAT		oxo ≈	car >	mus ≈	are ≈	mcn >	pil
m5	AAR	(f)	oxo >	car ≈	mus ≈	are ≈	mcn ≈	pil
m5	PI	(c)	oxo ≈	car ≈	mus >	are >	mcn >	pil
			Rank order of maximum response					
m1	R-SAT		car >	are ≈	musc ≈	pil ≈	oxo >	mcn
m1	PI	(b)	car >	are >		pil ≈	oxo	
m1	PI	(c)	car ≈	are ≈		pil ≈	oxo >	mcn
m1	GTPase	(a)	car >	are ≈		oxo >	pil ≈	mcn
M_1	RVD	(d)	car ≈	are ≈			oxo ≈	mcn
m2	R-SAT		car ≈	oxo ≈	are ≈	pil ≈	mcn >	mus
m2	cAMP	(e)	car ≈	oxo ≈	are ≈	mus >	pil >	mcn
m2	GTPase	(a)	car ≈	oxo ≈	are >	pil >	mcn	
M_2	GPA	(d)	car ≈	oxo ≈	are >		mcn	
m3	R-SAT		car >	mus >	are ≈	oxo >	pilo >	mcn
m3	GTPase	(a)	car >		are ≈	oxo >	pilo >	mcn
M_3	GPI	(d)	car ≈		are ≈	oxo >		mcn
m3	PI	(c)	car ≈	mus >	are ≈	oxo >	pilo ≈	mcn
m4	R-SAT		car ≈	oxo >	mcn >	are ≈	pil ≈	mus
m4	GTPase	(a)	car ≈	oxo ≈	are >	mcn >	pil	
m4	cAMP	(c)	car ≈	oxo ≈	mcn ≈	are ≈	pil ≈	mus
m5	R-SAT		car >	mus >	are ≈	oxo ≈	pil >	mcn
m5	AAR	(f)	car ≈	mus >	are ≈	oxo >	pil ≈	mcn
m5	PI	(c)	car ≈	mus >	are ≈	oxo >	mcn >	pil

> indicates differences greater than 3-fold for EC_{50} values and 10% for maximum responses. Abbreviations: PI (phosphatidyl inositol), arecoline (are), carbachol (CCh), pilocarpine (pil), rabbit vas deferens (RVD), guinea pig atrium (GPA), guinea pig ileum (GPI), arachidonic acid release (AAR), m1–m5 molecularly defined subtype, M_1–M_3 pharmacologically defined subtype. (a) Lazareno et al. (1993). (b) Schwarz et al. (1993). (c) Richards and Van Giersbergen (1995). (d) Eltze et al. (1993). (e) McKinney et al., 1991. (f) Bymaster et al. (1995).

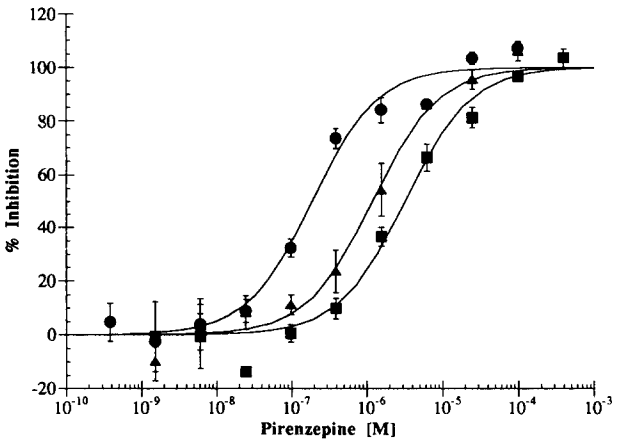

Fig. 3. Pirenzepine concentration-response relationships of β-galactosidase amplification responses. NIH 3T3 cells were transfected with m1 (●), m3 (■) and m5 (▲) receptors. 2 days after transfection, the cells were split into 96-well plates and incubated with ligands for 4 days. Pirenzepine concentration-response curves were obtained in the presence of 50 μM (m1), 10 μM (m3) or 1 μM (m5) carbachol. Each point is the mean (±S.E.M.) of triplicate determinations. Curves are mass-action fits of the data.

responses was dose-dependent and obeyed mass-action relationships. The illustrated computer generated lines are derived from an equation where the slope of a concentration-response curve is unity in a Hill plot. The rank-order of potency was m2 ≥ m4 = m5 > m3 > m1. A similar rank order of potency has been observed in second messenger and GTPase assays (Novotny and Brann, 1989; Lazareno et al., 1993; Wang and El-Fakahany, 1993; Richards and Van Giersbergen, 1995). Concentration-responses to the known partial agonists McN A-343 and arecoline are also shown in Fig. 2. Both were more potent at m4 than at m1 receptors, also consistent with earlier work using second messenger and GTPase assays (Novotny and Brann, 1989; Lazareno et al., 1993; Wang and El-Fakahany, 1993; Richards and Van Giersbergen, 1995).

Table 2 illustrates a comparison of the pharmacologies of agonists determined by R-SAT with other assays of muscarinic receptor function. There is a strong correlation in the rank order of potencies (EC_{50} values) and intrinisic activities (maximum responses) of most ligands as assayed by R-SAT and the other systems. At all of the receptor subtypes, oxotremorine was the most potent and pilocarpine was the least potent. With the exception of the m2 receptor, there is also strong correlation in the rank order of maximum responses. For all of the recombinant receptor assays there is a much smaller range of maximum responses for the m2 and m4 receptors than the m1, m3, and m5. Given that different G-proteins are used to mediate the responses of the two groups of receptors (e.g., presumably endogenous G_q versus $G_{q\text{-}i5}$ for R-SAT and G_q versus G_i for the others), it is likely that the assays for the two groups of receptors may be influenced by different levels of G-protein and different receptor/G-protein coupling efficiencies. Our inability to discriminate the maximum responses of m2 receptors to the known partial agonists McN A-343 and pilocarpine from those of the full agonist carbachol could imply that we have a particularly high degree of functional spareness (Kenakin and Morgan, 1989) in the m2 R-SAT assays. This could be due to the necessity of using recombinant G-protein. On the other hand, like the GTPase assay, our m1 and m3 R-SAT assays showed much better discrimination of partial agonism than the assays based on peripheral tissues.

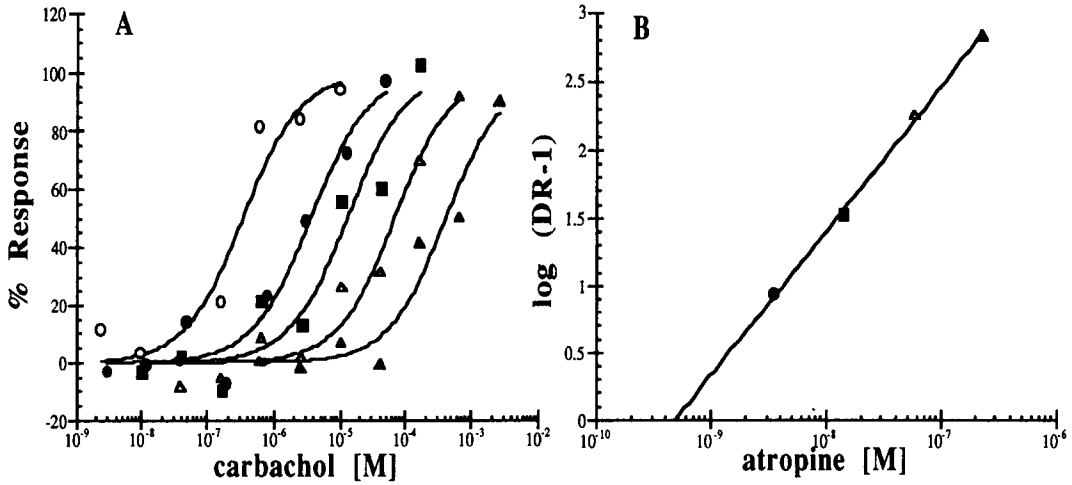

Fig. 4. Schild analysis of atropine at m5 receptors. Data are the means of duplicate determinations. (A) Concentration-responses for carbachol were evaluated in the presence of increasing concentrations of atropine (○, 0 nM; ●, 4 nM; ■, 14 nM; △, 57 nM; ▲, 228 nM). (B) Linear regression of the concentration-ratio shifts plotted versus the atropine concentrations gave a pA_2 of 9.3 with a slope of 1.07. The correlation coefficient was > 0.999.

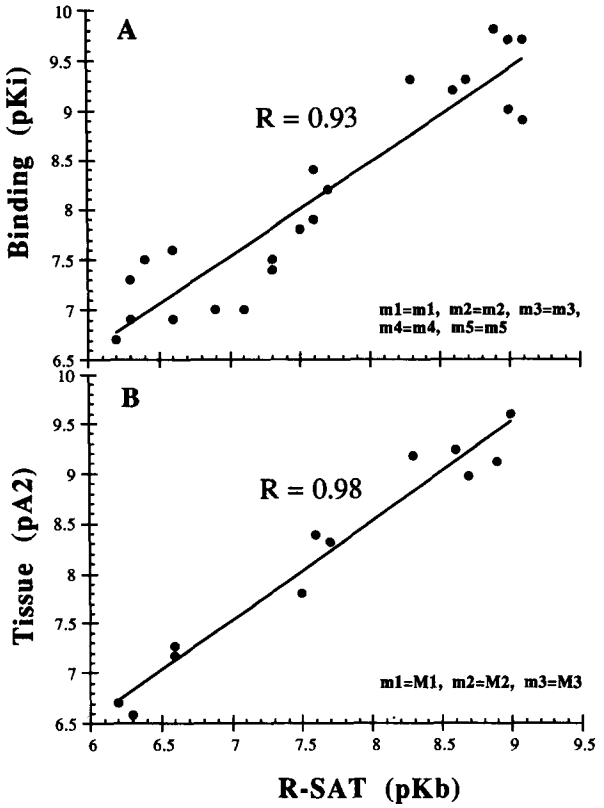

Fig. 5. Correlation of antagonist affinities determined by R-SAT versus traditional assays. (A) pK_d values determined using transfected cell-lines (Buckley et al., 1989; Dörje et al., 1991; Wess et al., 1991). (B) pA_2 values from tissues (Eltze et al., 1993). M_1 and M_2 were measured in rabbit vas deferens and M_3 was measured in guinea-pig ileum (Eltze et al., 1993). Antagonists used were atropine, pirenzepine, 4-DAMP, p-F-HHSiD and methoctramine.

3.3. Antagonist pharmacology

As shown in Fig. 3, the antagonist pirenzepine inhibited carbachol responses with concentration-response relationships that obeyed mass-action. When the illustrated data were fit to an equation where the Hill value could vary from unity, Hill values of 0.99 ± 0.17, 0.94 ± 0.21 and 0.99 ± 0.12 were observed for m1, m3 and m5 respectively. Since both agonist (Fig. 2) and antagonist (Fig. 3) concentration-responses obey these mass-action relationships, one would predict that affinity constants (K_b) can be calculated from IC_{50} values (Craig, 1993). To confirm the validity of these relationships for calculating pK_b values from inhibition curves, we examined carbachol concentration-response relationships in the presence of increasing concentrations of atropine (Fig. 4A). From the resulting Arunlakshana-Schild plot (Fig. 4B) a pA_2 of 9.3 was calculated (slope 1.07). These data comply with competitive antagonism, and are in excellent agreement with the calculated pK_b value (Table 2). pK_b values for several muscarinic antagonists are shown in Table 3. The 'M_1 selective' antagonist pirenzepine had highest affinity for m1 followed by m4 > m5 > m3 > m2. Atropine and 4-DAMP both showed lower affinity for the m2 receptor than for the four other receptors, whereas the 'M_2-selective' methoctramine showed preference for the m2 receptor. The 'M_3-selective' p-F-HHSiD showed highest affinity for m3 and m4. A comparison of the antagonist affinities obtained using R-SAT versus binding assays is presented in Fig. 5A. A similar comparison with data from functional studies using isolated peripheral tissues is presented in Fig. 5B. As illustrated, there is an extremely high correlation in the rank-order of potencies of antagonists with both recombinant cell lines and tissue preparations. The K_I values for R-SAT were 3–4-fold lower than similar values obtained from binding assays, but nearly identical to values from functional assays. The lower absolute affinities of the muscarinic antagonists in functional assays are consistent with the known effects on antagonist affinities of low ionic strength of the buffers used in most binding assays (Pedder et al., 1991).

4. Discussion

Overall, the pharmacology of most ligands at recombinant muscarinic receptors are remarkably similar when evaluated using either R-SAT or a wide variety of other functional assays. Both agonists and antago-

Table 3
pK_b values of antagonists at muscarinic receptor subtypes

Antagonist	m1	m2	m3	m4	m5
Atropine	9.0 ± 0.1	8.3 ± 0.3	8.9 ± 0.2	9.1 ± 0.0	9.1 ± 0.0
Pirenzepine	7.7 ± 0.0	6.2 ± 0.0	6.6 ± 0.2	7.3 ± 0.2	6.9 ± 0.0
4-DAMP	8.6 ± 0.0	7.6 ± 0.2	8.7 ± 0.3	9.1 ± 0.1	9.0 ± 0.3
p-F-HHSiD	6.6 ± 0.2	6.3 ± 0.1 [a]	7.5 ± 0.1	7.3 ± 0.1	7.1 ± 0.2
Methoctramine	6.3 ± 0.1	7.6 ± 0.1 [a]	< 6.0 [a]	6.4 ± 0.1	< 6.0 [a]

NIH 3T3 cells were transfected with a muscarinic acetylcholine receptor and ß-galactosidase DNA. Cells with m2 and m4 were further co-transfected with G_{q-i5}. After 2 days the cells were split into 96-well plates and ligands were added. Data were derived after 4 (m1, m3, m5) or 5 (m2, m4) days of incubation with drugs. Data represent the mean of 2–4 experiments (±S.E.M.). K_b values were calculated from the IC_{50} (Craig, 1993). [a] Estimation from partial curves, cytotoxicity was observed at the highest doses.

nists have identical rank orders of potency at m1–m5 receptors when measured using R-SAT and other assays, and at least in the case of the m1, m3 and m5 receptors, R-SAT assays were able to discriminate subtle differences in the efficacies of partial agonists.

In most of the pharmacological studies of cloned receptors that have been reported, well characterized stable cell lines have been used. R-SAT is based on the ability of ligands to select and amplify cells directly after transfection. Given that levels of receptors strongly influence agonist pharmacology, the former strategy would appear to have advantages relative to R-SAT. In practice, we have found that the pharmacological properties of 'stable' cell lines are not as consistent as one would suspect. Because functional spareness is often observed when one uses cells with high receptor levels, one attempts to prepare cell lines with relatively low receptor levels. This exacerbates the problem that the cells tend to lose receptors with time in culture, often losing all responsiveness to ligands. It should also be considered that in the process of antibiotic selection, one is actually creating entirely new subclones of the original cell line. These new lines can have many differences (other than receptor levels) that influence functional responses to ligands. The varying properties of 'stable' cell lines make quality control of these materials of critical importance. In practice as for most traditional pharmacological assays, test ligands must be directly compared with reference compounds.

It is difficult to precisely define the levels of receptors that are expressed in the NIH 3T3 cells during R-SAT. Receptor levels almost certainly change with time during the 4–5 days of drug treatment. Initially, levels are likely to be higher due to high levels of transient expression, while at the end of the time of ligand treatment, only stably integrated receptors are likely to be expressed. Levels at the start of an R-SAT experiment are difficult to measure because less than 1% of the cells are transfected (data not shown). Muscarinic receptor induced foci to express 100–200 fmol receptor/mg protein, providing a rough measure of receptor levels at the end of the drug treatment period. Because antibiotic selection is avoided in R-SAT, several receptors can be compared in an extremely similar cellular context (assays are performed within a few cell divisions of transfection). When one is comparing the pharmacological properties of a large number of ligands, cells for R-SAT can be prepared in large quantities and frozen as aliquots, samples of which can be tested using reference compounds. As for other functional assays that require extensive incubation times, desensitization is likely to contribute to agonist potency. Thus for R-SAT assays, as for other functional assays, comparison with reference compounds is essential.

R-SAT assays involve very few steps, no expensive reagents, and are performed with standard colorimetric assays in 96-well plates. These properties make R-SAT assays amenable to high throughput applications such as mass screening of extremely large compound libraries. Using R-SAT assays, one person can manually perform more than 5000 assays per day. Several other high throughput assays have been reported. One particularly facile assay is based on the ability of a wide diversity of receptors to alter pigmentation of frog melanophores (Potenza et al., 1992; Graminski et al., 1993). Unfortunately, the pharmacological comparability of frog and mammalian systems has yet to be demonstrated. Another system is based on the ability of recombinant receptors to elicit the release of hexosaminidase from transfected rat basophilic leukemia (RBL) cells (Jones et al., 1991). Hexosaminidase is readily measured in colorimetric assays in 96-well plate formats. The extreme difficulty of transfecting these cells has limited use of this assay. By co-expression of G-proteins, there has been recent success in engineering yeast cells to respond activation of mammalian receptors (King et al., 1990). Considering the marked differences in the properties of mammalian and yeast membranes, one would be concerned about the validity of this approach for evaluation of mammalian pharmacology. The assays that are most similar to R-SAT involve the use of marker genes that are under the transcriptional control of receptors in mammalian cells (Montmayeur and Borelli, 1991; Himmler et al., 1993; Weyer et al., 1993). While these assays have been successfully applied to mass screening of ligands, they appear to have very limited ability to discriminate subtle differences in ligand efficacy. These assays have recently been applied to the assay of responses to m2 receptors (Goldman and Nathanson, 1994).

Another important feature of R-SAT is that extremely small amounts of receptor DNA are required to yield functional responses. We have exploited this property of R-SAT to develop efficient screens for mutant receptors. R-SAT assays have been used to screen libraries of randomly mutated receptors to study structure/function relationships of receptor/G-protein coupling (Burstein et al., 1995b), and to identify a constitutively active version of m5 receptors (Spalding et al., 1995). Using these approaches it is possible for one person to functionally screen ~ 200 mutant receptors per day.

In conclusion, we have developed a high throughput assay which overcomes many of the limitations of previous systems. This assay is performed using living mammalian cells and discriminates between full and partial agonists. Using this assay we report the first comprehensive study of the agonist and antagonist pharmacology of reference muscarinic ligands at all five of the muscarinic receptor subtypes. In addition to the muscarinic receptor subtypes described in this re-

port, we have used R-SAT to measure the pharmacological properties of many other receptor subtypes, successfully including adrenergic, dopamine, endothelin, glutamate, neurokinin, opiate, prostanoid, serotonin, GM-CSF, insulin, and trk receptor families.

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