Pharmacological characterization of acetylcholine-stimulated [³⁵S]-GTPγS binding mediated by human muscarinic m1–m4 receptors: antagonist studies

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1 We have used dose-ratio analysis to estimate functionally the affinity constants (pK_b) and Schild slope factors of a range of selective or atypical antagonists at human muscarinic m1–m4 receptors.

2 The functional response was the stimulation by acetylcholine of [³⁵S]-GTPγS binding to membranes from Chinese hamster ovary (CHO) cells stably expressing individual receptor subtypes.

3 A novel experimental design and analysis was used which allowed the estimation of affinity and Schild slope factor from a single antagonist inhibition curve, and the results were compared with other methods of analysis, both theoretically valid and invalid.

4 In general, the affinity estimates were very similar to previously reported values obtained in binding studies with animal tissues and cloned human receptors and the Schild slope factors were close to unity.

5 These results demonstrate the validity of the assay and provide no evidence for species differences in antagonist affinity for muscarinic receptor subtypes.

6 The results confirm both the utility of himbacine in distinguishing between m1 and m4 receptors and a previously reported modest m4-selectivity for tropicamide and secoverine.

7 The cholinesterase inhibitor, tacrine (THA), had a potency profile similar to that of gallamine but with less selectivity. Its affinity could not be determined since it had Schild slope factors of about 2 at all subtypes.

8 *o*-Methoxy-sila-hexocyclium had only a modest selectivity for the m1 subtype.

Keywords: Muscarinic receptor subtype; cloned human receptors; guanine nucleotide exchange; GTPγS binding; competitive antagonist; allosteric effect; null method; affinity estimation; tacrine

Introduction

Muscarinic cholinergic receptors belong to the superfamily of G-protein coupled receptors. Five subtypes have been identified by molecular biological techniques and are referred to as m1, m2, m3, m4 and m5 receptors (Hulme *et al.*, 1990). Before this family was discovered by cloning techniques, three subtypes were identified pharmacologically and referred to as M₁, M₂ and M₃ receptors. These are now known to correspond to the m1, m2 and m3 receptors. The pharmacological M₄ receptor, corresponding to the m4 gene product, has recently been characterized in animal tissues in binding (Lazareno *et al.*, 1990; Waelbroeck *et al.*, 1990) and functional (Caulfield & Brown, 1991; Bernheim *et al.*, 1992) studies. The cloned m5 receptor has been characterized by binding studies (Buckley *et al.*, 1989; Dörje *et al.*, 1991b) but there have been no studies of the native m5 receptor.

The muscarinic receptor subtypes preferentially couple to different effector systems. The clearest distinction is that m1, m3 and m5 receptors preferentially couple to phospholipase-C (PLC)-mediated phosphoinositide hydrolysis (PI), while m2 and m4 receptors preferentially couple to inhibition of adenylyl cyclase. A single type of receptor can, however, mediate many independent responses: for example, m2 receptors can inhibit cyclase, open K⁺ channels and stimulate phosphoinositide hydrolysis (Pfaffinger *et al.*, 1985; Ashkenazi *et al.*, 1987; Nathanson, 1987), suggesting that a single type of receptor may activate more than one type of G-protein.

Although a receptor may mediate more than one independent response, this does not prove that the receptors act via different G-proteins. Katz *et al.* (1992) have recently demon-

strated that an m2 receptor can stimulate PLC-β2, presumably by causing dissociation of G-protein α and βγ subunits and thus increasing the free concentration of the βγ subunit which is the stimulus for this isoenzyme of PLC. These authors suggest that this mechanism may explain the stimulation of PI breakdown which is sometimes seen in cells expressing high levels of m2 or m4 receptors (e.g. Ashkenazi *et al.*, 1987; Tietje *et al.*, 1990), since both the cyclase and PI responses are sensitive to pertussis toxin (PTX). PTX causes the ADP ribosylation of certain G-proteins (Gi₁, Gi₂, Gi₃ and Go), and this prevents the G-protein from being activated by the receptor. The fact that two responses are sensitive to PTX does not prove that they are mediated by the same G-protein, but if only one response is PTX-sensitive it is likely that different G-proteins are involved. This is the case in m4-transfected CHO cells where PTX blocks the inhibitory effect on cyclic AMP levels but not the stimulatory effect seen with high agonist concentrations (Jones *et al.*, 1991). Conversely, if a response is only partially sensitive to PTX this implies that different G-proteins are involved, and this is the case with phosphoinositide hydrolysis stimulated by transfected m1 receptors in Chinese hamster ovary (CHO) cells (Ashkenazi *et al.*, 1989).

Even though the different muscarinic receptor subtypes generate different second messenger responses, they have in common the effect of activating G-proteins by stimulating guanine nucleotide exchange. The details of the mechanism of G-protein activation are still being revealed (Cerione, 1991; Bourne & Stryer, 1992), but it seems that the major consequence of the interaction of agonist-occupied receptor and G-protein is that a molecule of GDP dissociates from the α subunit of G-protein and is replaced by a molecule of

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GTP. This activates the α subunit, which may dissociate from the receptor and from the $\beta\gamma$ subunit. The α -GTP subunit interacts with and modulates its effector until the GTP is hydrolysed to GDP by a GTPase intrinsic to the α subunit. The receptor does not affect this intrinsic GTPase, but it has recently been shown that the effector may itself act on the α -GTP subunit to increase the rate of GTP hydrolysis and hence inactivation of the G-protein (Berstein *et al.*, 1992). The α -GDP subunit remains inactive until the GDP dissociates and is replaced by GTP, the process which is stimulated by the receptor.

There are many ways of measuring guanine nucleotide exchange. One method uses [35 S]-GTP γ S. This GTP analogue is relatively resistant to hydrolysis and dissociates slowly from the G-protein, so it accumulates in membranes or vesicles containing G-proteins. Activation of muscarinic receptors increases the binding of [35 S]-GTP γ S in reconstituted systems (Kurose *et al.*, 1986; Florio & Sternweis, 1989) and in membranes from heart (Hilf *et al.*, 1989; 1992; Shiozaki & Haga, 1992; Chidiac & Wells, 1992) and smooth muscle (Liebmann *et al.*, 1992). We have measured the stimulation by acetylcholine (ACh) of [35 S]-GTP γ S binding to membranes from CHO cells stably transfected with single human muscarinic m1–m4 receptors (Farries, unpublished observations), and here we have characterized the response pharmacologically with a number of selective or atypical antagonists.

One aim of this study was to assess the validity of the assay as a pharmacological response by comparing the antagonist affinity estimates of a range of known selective antagonists, measured using this functional response at human receptors, with affinity estimates measured in binding studies with human, rat or rabbit receptors. Some atypical antagonists were also studied.

A second aim was to assess a novel experimental design and method of analysis described in the accompanying paper (Lazareno & Birdsall, 1993). This is an 'inhibition curve' design which uses a single agonist titration and a titration of antagonist in the presence of a fixed concentration of agonist. The data from both titrations are fitted simultaneously to the Schild equation (Waud, 1975; 1976) using non-linear regression analysis. We compare this method of analysis with other methods of analysis, both theoretically valid and invalid, which do not require non-linear regression analysis. The large data set (112 experiments) of this study provides an opportunity to compare the distribution of errors incurred by the (invalid) use of the Cheng-Prusoff (1973) correction with the distribution predicted by a theoretical model, and also to compare two slightly different (valid) procedures which are based on the null method of dose-ratio analysis.

Some of the results described in this paper have been presented in preliminary form (Lazareno *et al.*, 1993).

Methods

Cell culture and membrane preparation

CHO cells stably expressing cDNA encoding human muscarinic receptors m1–m4 (Buckley *et al.*, 1989) were generously provided by Dr N.J. Buckley (N.I.M.R., London). These were grown in MEM- α medium (GIBCO) containing 10% (v/v) new born calf serum and harvested by scraping the cells off the surface in a hypoosmotic medium (20 mM HEPES + 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40,000 g, 15 min), were washed once in 20 mM HEPES + 0.1 mM EDTA pH 7.4, and were stored at –70°C in the same buffer. The receptor densities (pmol receptor mg $^{-1}$ membrane protein) were 4, 1, 5 and 3 for m1–m4 receptors respectively.

[35 S]-GTP γ S binding assay

Membranes were suspended in a buffer containing 20 mM HEPES, 100 mM NaCl and 10 mM MgCl $_2$ pH 7.4 at a protein concentration of 25–50 μ g ml $^{-1}$. Polystyrene tubes (5 ml) containing 1 ml of membrane suspension were incubated with GDP (0.1 μ M for m1 and m3 receptors, 1 μ M for m2 and m4 receptors), ACh and antagonist at 30°C for 20 min and then transferred to ice for 15 min. [35 S]-GTP γ S was added to a final concentration of 100 pM and the samples incubated for 30 min at 30°C. The preincubation ensured that agonist and antagonist were at equilibrium throughout the labelling period. It was found necessary to pre-equilibrate the membranes with both agonist and antagonist before initiating the [35 S]-GTP γ S binding, since preincubation with pirenzepine alone led to concentration-dependent decreases in the agonist $E_{\max}$ at m1–m4 receptor subtypes (data not shown). Preincubation with ACh did not reduce either the potency of ACh or its $E_{\max}$ (data not shown). The samples were filtered over glass fibre filters (Whatman GF/B) using a Brandel cell harvester and washed with 5 ml water. The filter discs were extracted overnight in 3 ml scintillant and counted by liquid scintillation spectrometry at an efficiency of about 97%. There was no detectable cholinesterase activity in CHO cells membranes, so there was no need to include a cholinesterase inhibitor in these assays.

Materials

[35 S]-GTP γ S was purchased from Du Pont; AQ-RA 741 (11-([4-[4-(diethylamino)-butyl]-1-piperidinyl]acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one) was a gift from Dr Karl Thomae GmbH; *o*-methoxy-sila-hexocyclium (OMSH) was a gift from Dr G. Lambrecht (Frankfurt), secoverine was a gift from Dr J. Barnes (Glaxo); Himbacine was a gift from Prof. W. Taylor (Sydney); hexahydrosiladiphenidol (HHSiD) and methoctramine were from Research Biochemicals Inc. (Semat, U.K.); acetylcholine (ACh), pirenzepine (PRZ), tacrine (THA), tropicamide and GDP were from Sigma (U.K.). Benzyl-4-DAPine (benzyl-4-diphenyl acetoxy piperidine) was a gift from Dr R.B. Barlow; its results will be presented in detail elsewhere, but they have been included in the population of data used to compare methods of analysis (Figure 3).

Experimental design and analysis

Each assay contained a titration of ACh alone, and titrations of 4 or 5 antagonists in the presence of a fixed concentration of ACh. Assays were conducted in duplicate, with each set of replicates filtered together. In separate assays the effect of the antagonist was studied in the absence of ACh.

For each set of replicates the data were expressed as a % of basal activity. This transformation sometimes reduced the coefficient of variation of the data, and never significantly increased it. The data were analysed in four ways, which have been described in more detail by Lazareno & Birdsall (1993). The first method is the best, but requires a non-linear curve fitting programme which can handle user-defined equations with two independent variables. The other methods do not require non-linear regression. The second method is invalid, but the third and fourth methods are theoretically valid and are equivalent to using the Gaddum equation (Gaddum, 1957) to analyse agonist titrations in the absence and presence of a fixed concentration of antagonist.

(1) For a single antagonist, the combined ACh and antagonist titration data were fitted to the Schild equation (Waud, 1975; 1976)

$$Effect = (E_{\max} - basal) / (1 + \{ \frac{[EC_{50}^0] \cdot (\frac{[B]^f}{K_b} + 1)}{[A]} \}^b) + basal$$

Figure 1 Stimulation of [35 S]-GTP γ S binding to CHO cell membranes containing Hm1-Hm4 muscarinic receptors by acetylcholine (ACh) and inhibition of the effect of a fixed ACh concentration by tropicamide. The data are expressed as a % of basal activity, and each point represents the mean of duplicate observations. (○) ACh alone; (●) tropicamide in the presence of a submaximal concentration of ACh; solid lines show the fit of the combined data to the Schild equation with Schild slope constrained to unity; (▽) effect of tropicamide on basal activity; the dotted line shows the linear regression; these data were obtained in a separate experiment.

where $[A]$ and $[B]$ are the agonist and antagonist concentrations respectively, EC_{50}^0 is the EC_{50} of the agonist alone, b is the logistic slope factor of the agonist effect, K_b is the antagonist dissociation constant and s is the Schild slope factor. Lazareno & Birdsall (1993) have discussed the derivation and utility of this equation and have validated its use in both the traditional Schild-plot type of design and in the inhibition-curve design used here. The non-linear regression analysis was performed with SigmaPlot (Jandel). The data were analysed twice. Initially, the Schild slope factor was unconstrained, and the estimates of this parameter are those reported here. The data were reanalysed with the Schild slope factor constrained to be 1, and the pK_b estimates from these analyses are reported here. When terms such as 'affinity' or ' pK_b ' are used they refer to the estimates from this second, constrained, analysis. The results of the two analyses were also used to assess whether the Schild slope factor was significantly different from unity, using the 'extra sum of squares' F-test (Munson & Rodbard, 1980). A pK_b value can only be considered as a true estimate of affinity if the corresponding Schild slope factor is not different from 1.

(2) The data from each assay (titrations of ACh alone and with a fixed concentration in the presence of titrations of 4 or 5 antagonists separately) were simultaneously fitted to logistic functions with the programme Allfit (De Lean *et al.*, 1978, gift of Dr P. Munson, NIH). The 'basal' parameter was constrained to be shared between all the curves (this was always statistically valid), and the 'maximum' parameter was constrained to be shared among the antagonist curves, while the 'maximum' parameter of the ACh curve was unconstrained. The EC_{50} and IC_{50} estimates were used to calculate the K_{b-CP} using a version of the Cheng-Prusoff equation (Cheng & Prusoff, 1973)

$$K_{b-CP} = IC_{50}/(A/EC_{50} + 1)$$

where A is the fixed agonist concentration. It is theoretically invalid to use this equation to estimate antagonist affinities from functional inhibition curve data, and Lazareno & Birdsall (1993) have derived some theoretical predictions about the size and distribution of the errors incurred by the use of this form of analysis.

(3) The logistic parameter estimates for the ACh titration obtained in the preceding analysis were used with the IC_{50} estimates to calculate the K_{b-P} estimate (Pösch *et al.*, 1992)

$$EC_{50}^* = (A^b/((A/EC_{50})^b + 2))^{(1/b)}$$

$$K_{b-P} = IC_{50}/(A/EC_{50}^* - 1)$$

where b is the slope factor of the ACh curve and EC_{50}^* is the ACh concentration causing 50% of the effect caused by the fixed concentration. This form of analysis is valid (Pösch *et al.*, 1992; Lazareno & Birdsall (1993).

(4) The data from each assay (titrations of 4 or 5 antagonists and of ACh alone up to the fixed agonist concentration) were simultaneously fitted to logistic functions with the programme Allfit. Both the 'basal' and 'maximum' parameters were constrained to be shared between all the curves. This analysis yielded EC_{50}^* and IC_{50}^* estimates which were used to calculate the K_{b-LR} (Lazareno & Roberts, 1987)

$$K_{b-LR} = IC_{50}/(A/EC_{50}^* - 1)$$

These EC_{50}^* and IC_{50}^* estimates were usually slightly different from the EC_{50} and IC_{50} values used to calculate the K_{b-P} . This form of analysis is valid and is equivalent to defining the effect of the fixed agonist concentration as E_{max} and estimating EC_{50}^* and IC_{50}^* with Hill (logit-log) analysis (Lazareno & Roberts, 1987).

Results

ACh caused a dose-dependent increase in [35 S]-GTP γ S binding (Figure 1, Table 1). The rank order of ACh potency

Table 1 Logistic parameters of acetylcholine (ACh) stimulation of [3 S]-GTP γ S binding to CHO cell membranes containing single subtypes of human m1–m4 muscarinic receptors

Subtype	N	pEC ₅₀ (log M)	Slope	E _{max} (% basal)
m1	8	5.91 $\pm$ 0.11	0.56 $\pm$ 0.02	232 $\pm$ 5
m2	9	7.40 $\pm$ 0.06	0.69 $\pm$ 0.02	217 $\pm$ 13
m3	9	5.34 $\pm$ 0.08	0.81 $\pm$ 0.03	189 $\pm$ 11
m4	7	6.79 $\pm$ 0.09	0.67 $\pm$ 0.02	336 $\pm$ 18

Values are mean $\pm$ s.e.mean.

between the subtypes was m2 > m4 > m1 > m3. This differed from the rank order of receptor density, which was m3 > m1 > m4 > m2, and maximal effect, which was m4 > m1 > m2 > m3. The concentration-effect curves of ACh had logistic slope factors less than 1 with a rank order m3 > m4 = m2 > m1. It is difficult to interpret differences between EC₅₀ or E_{max} values because both parameters are modified by GDP in a subtype-dependent manner (Farries, unpublished; Lazareno *et al.*, 1993).

The antagonists caused a dose-dependent inhibition of [3 S]-GTP γ S binding stimulated by a fixed concentration of ACh (Figure 1, Table 2). They had little effect on basal activity (Figure 1 and data not shown). Often the higher concentrations seemed to inhibit basal activity by 5–10%, but the effect was too small and variable to quantify. We saw a similar lack of effect when a sodium-free Tris-HEPES-Mg assay buffer was used (data not shown).

The Schild slope factors were generally not different from 1 (Table 2). The single exception was THA which had slopes of about 2 at all subtypes, and the slope factors in each experiment with THA were significantly different from unity. In general, the logistic slope factors of the antagonist inhibition curves were always less than the corresponding Schild slope factor.

The antagonist affinity estimates were generally similar to previously reported values (Lazareno *et al.*, 1990; Michel *et*

Figure 2 Comparison of log affinity estimates of six antagonists at m1–m4 receptors measured in two studies: the current study using a functional response and cloned human receptors, and the binding study of Lazareno *et al.* (1990) using native rat and rabbit receptors. Each drug is represented by a different symbol and the four instances of each symbol correspond to the drug's affinity at each of the four receptor subtypes. The drugs are: himbacine (○); HHSiD (●); methoctramine (▽); tropicamide (▼); secoverine (□); and pirenzepine (■). The line has a slope of 1 and intercept of 0.

al., 1990; Dörje *et al.*, 1991b; see Mitchelson, 1988 and Caulfield, 1993 for reviews). Pirenzepine were most potent at m1 receptors, but also had high affinity at m4 receptors. Himbacine had log affinities at about 8 at m2 and m4 receptors and about 7 at m1 and m3 receptors; it was the best drug for distinguishing between m1 and m4 receptors. Gallamine, methoctramine and AQ-RA 741 had different

Table 2 Estimates of pK_b and Schild slope factors of antagonists inhibiting acetylcholine (ACh)-stimulated [3 S]-GTP γ S binding to CHO cell membranes containing single human m1–m4 muscarinic receptors

Drug/subtype	pK _b (– log M) and Schild slope factor			
	m1	m2	m3	m4
Pirenzepine	8.35 $\pm$ 0.03	6.65 $\pm$ 0.01	6.88 $\pm$ 0.12	7.65 $\pm$ 0.04
Gallamine	1.05 $\pm$ 0.06	1.02 $\pm$ 0.04	1.06 $\pm$ 0.17	1.07 $\pm$ 0.03
	5.35 $\pm$ 0.17*	6.24 $\pm$ 0.13	4.60 $\pm$ 0.16	5.42 $\pm$ 0.09
	0.75 $\pm$ 0.22*	1.07 $\pm$ 0.08	1.04 $\pm$ 0.18	0.90 $\pm$ 0.08
Himbacine	7.24 $\pm$ 0.06*	8.29 $\pm$ 0.05	7.04 $\pm$ 0.12	8.13 $\pm$ 0.11
	0.98 $\pm$ 0.07*	1.24 $\pm$ 0.12	1.23 $\pm$ 0.06	1.20 $\pm$ 0.01
Methoctramine	7.16 $\pm$ 0.04	8.16 $\pm$ 0.06	6.27 $\pm$ 0.06	7.13 $\pm$ 0.10
	0.98 $\pm$ 0.16	1.08 $\pm$ 1.12	1.10 $\pm$ 0.10	1.29 $\pm$ 0.16
AQ-RA 741	8.00 $\pm$ 0.22*	8.99 $\pm$ 0.06	7.19 $\pm$ 0.08	8.56 $\pm$ 0.07
	0.89 $\pm$ 0.04*	1.25 $\pm$ 0.09	1.09 $\pm$ 0.03	1.11 $\pm$ 0.02
HHSiD	8.30 $\pm$ 0.07	7.20 $\pm$ 0.08	8.10 $\pm$ 0.07	7.88 $\pm$ 0.09
	0.93 $\pm$ 0.08	1.14 $\pm$ 0.07	1.13 $\pm$ 0.05	1.17 $\pm$ 0.10
Tropicamide	7.27 $\pm$ 0.03*	7.34 $\pm$ 0.04	7.30 $\pm$ 0.05	7.82 $\pm$ 0.10
	0.98 $\pm$ 0.12*	1.27 $\pm$ 0.11	1.04 $\pm$ 0.01	1.09 $\pm$ 0.04
Secoverine	8.59 $\pm$ 0.18*	8.63 $\pm$ 0.14	8.12 $\pm$ 0.06	8.96 $\pm$ 0.16
	0.94 $\pm$ 0.08*	1.32 $\pm$ 0.16	1.15 $\pm$ 0.10	1.19 $\pm$ 0.15
<i>o</i> -Me-Si-HexC	7.44 $\pm$ 0.11	6.36 $\pm$ 0.10	6.68 $\pm$ 0.12	7.18 $\pm$ 0.08
	0.94 $\pm$ 0.06	1.52 $\pm$ 0.33	1.32 $\pm$ 0.05	1.12 $\pm$ 0.03
THA ¹	6.18 $\pm$ 0.09*	6.30 $\pm$ 0.13*	5.46 $\pm$ 0.11*	5.75 $\pm$ 0.11
	2.01 $\pm$ 0.08*	1.98 $\pm$ 1.20*	2.33 $\pm$ 0.48*	1.75 $\pm$ 0.08

The parameters were estimated by fitting data directly to the Schild model using non-linear regression analysis. pK_b values were estimated with Schild slope factor set to 1. Results are mean $\pm$ s.e.mean $n \geq 3$ except * $n = 2$. ¹pK_b values are not affinity constants since Schild slopes are steep.

Figure 3 Deviations of antagonist K_b estimates at Hm1–Hm4 receptors derived from different analyses of the same experimental data, expressed as a ratio of the corresponding K_b obtained from a direct fit to the Schild equation with Schild slope constrained to unity. The estimates (see methods) used (a) the Cheng-Prusoff equation (analysis 2), (b) the method of Pösch *et al.* (1992) (analysis 3), (c) the method of Lazareno & Roberts (1987) (analysis 4). In (a) (○) correspond to experiments in which the agonist titration had a logistic slope factor of <0.65 ; (●) experiments with agonist slopes ≥ 0.65 . The lines indicate the linear regression, and are included as a guide to the eye.

potencies but similar profiles; they were most potent at m2 receptors, about 50 fold weaker at m3 receptors, and with intermediate potency at m1 and m4; gallamine and methoctramine had similar affinities at m1 and m4, while AQ-RA 741 was more potent at m4 than at m1 receptors. HHSiD is known as an m3-selective drug, but it did not distinguish between m1, m3 and m4 receptors. In confirmation of a previous binding study (Lazareno *et al.*, 1990) tropicamide and secoverine showed small degrees of m4-selectivity (3 and 2 fold respectively).

OMSH had a similar selectivity pattern to pirenzepine ($m1 > m4 > m3 > m2$) but with only limited m1 selectivity.

THA had a pattern of selectivity ($m2 > m1 > m4 > m3$) similar to gallamine, though it was much less selective. Its rank order of potency was similar to that found in binding studies of M_1 – M_3 receptors (Kiefer-Day *et al.*, 1991). The values quoted in Table 2, however, may not correspond to affinity estimates since THA had Schild slopes greater than 1.

It is of interest to compare the results obtained in this study, using a functional response and cloned human receptors, with those obtained in a binding study which used rat and rabbit tissues (Lazareno *et al.*, 1990). Six drugs were common to both studies. There was a correlation of 0.93 between the affinity estimates of the six drugs at the four receptors in the two studies, the slope of the regression line was not different from 1 and the intercept was not different from 0 (Figure 2). The largest discrepancy (up to 5 fold) was with methoctramine at m1, m3 and m4 receptors, and this is explained by the lack of Mg^{2+} in the binding assays with these receptors.

The pK_b data shown in Table 2 were obtained by fitting the agonist and antagonist titrations directly to the Schild equation, as described in Methods. The fact that the Schild slope factors were generally about 1 (as expected for competitive antagonists) supports the validity of this form of analysis. Other ways of analysing the data, which may be more convenient, were described in Methods. The ratios of the K_b estimates obtained with these methods to those obtained from fitting to the Schild equation are shown in Figure 3.

The pattern of deviations (-fold difference in K_b) obtained from using Cheng-Prusoff analysis is consistent with the theoretical predictions of Lazareno & Birdsall (1993). The antagonist affinity was underestimated, the error was less at high fixed agonist concentrations, increased as the fixed agonist concentration approached the agonist EC_{50} , and was more marked when the agonist curve had a low slope. The estimates obtained by the method of Pösch *et al.* (1992) were similar to those obtained from fitting to the Schild equation and were unrelated to subtype, fixed agonist concentration or the slope of the agonist curve. Overall, the mean and s.e.mean of the deviations obtained with this analysis was 1.04 ± 0.02 ($n = 112$). The method of Lazareno & Roberts (1987) tended to yield small overestimates of antagonist affinity; the mean and s.e.mean of the deviations was 0.86 ± 0.01 ($n = 112$). There was, however, a strong correlation between the size of the deviation and the ratio of fixed agonist concentration to EC_{50} and when this effect was removed by linear regression there was a much smaller residual variance in the deviations of the data derived by the method of Lazareno & Roberts (1987) than in the deviations of the data derived by the method of Pösch *et al.* (1992).

The correlation between the deviations of the Lazareno & Roberts (1987)-derived estimates and the ratio of fixed agonist concentration to EC_{50} is presumably caused by the small but consistent errors caused by forcing a truncated agonist titration into a logistic function. The increased variability of the deviations of the data obtained with the method of Pösch *et al.* (1992) compared with the deviations associated with the method of Lazareno & Roberts (1987) presumably reflect the fact that the parameter estimates were less accurate when the fitting procedure allowed the agonist E_{max} to float than when the E_{max} was constrained. If we

consider data where the fixed agonist concentration was at least 10 fold greater than the agonist EC_{50} , then these K_d estimates, as a ratio of the estimated value derived from the Schild equation, were 1.01 ± 0.03 and 0.92 ± 0.01 using the method of Pösch *et al.* (1992) and Lazareno & Roberts (1987) respectively.

Discussion

In this study we have characterized a range of selective or atypical antagonists at Hm1–Hm4 muscarinic receptors using as the functional response the stimulation by ACh of GTP γ S binding. In general, the antagonist affinity estimates obtained in this study with selective antagonists are in excellent agreement with previously reported values. Many of the drugs used in this study were also studied in binding studies using rat and rabbit tissues (Lazareno *et al.*, 1990) or cloned human receptors (Dörje *et al.*, 1991b) and the affinity estimates in the three studies were very similar (see also Mitchelson, 1988 and Caulfield, 1993). Our current results with secoverine and tropicamide confirm the earlier finding in binding studies (Lazareno *et al.*, 1990) that these antagonists have 2 to 3 fold overall selectivity for m4 receptors.

OMSH was reported to have similar affinities to pirenzepine at M_1 – M_3 receptors in functional studies, with a pA_2 of 8.3 at rat superior cervical ganglion and 8.4 at rabbit vas deferens (two putative functional M_1 preparations), but in binding studies at M_1 sites its log affinity constant was only 7 (Lambrecht *et al.*, 1988; Feifel *et al.*, 1990). Our results at m1–m3 receptors are almost identical to the binding results at M_1 and functional results at M_2 and M_3 receptors. OMSH had a similar selectivity pattern to pirenzepine (m1 > m4 > m3 > m2) but with only limited m1-selectivity.

These results are mainly confirmatory, but have two important implications. Firstly, they provide no evidence for mammalian species differences in the antagonist profile of muscarinic receptor subtypes. The potential for species differences in muscarinic subtype pharmacology must, however, always be considered. Secondly, the results demonstrate that both the functional GTP γ S binding assay with cloned human receptors and the radioligand binding assay with rat/rabbit tissues yield valid data for selective antagonists, despite the fact that most whole tissues contain more than one muscarinic receptor subtype (Dörje *et al.*, 1991a).

The logistic slope factors of the antagonist titrations were usually less than 1, but these slope factors cannot be interpreted in isolation from the slope factor of the agonist titration (Lazareno & Birdsall, 1993). By fitting the combined data of the antagonist and agonist titrations to the Schild equation it was possible to estimate the Schild slope of the antagonist. The slope factors were generally not different from unity, as expected for competitive antagonists. This result also supports the validity of this experimental design and form of analysis.

Two drugs, gallamine and THA, are thought to act, at least in part, through an allosteric mechanism. An apparent allosteric action of gallamine has been detected in functional studies utilising full Schild analysis (Clarke & Mitchelson, 1976; Ehler, 1988; Kenakin & Boselli, 1989; Pösch *et al.*, 1992), but we saw no evidence for noncompetitive action. We might have seen signs of allosterism if higher fixed agonist concentrations had been used, but the single inhibition curve design is less sensitive than the full Schild analysis for detecting negative allosteric effects. THA has been found to inhibit the binding of antagonist radioligands with logistic slope factors of about 1.5–2 at M_1 , M_2 and M_3 sites (Potter *et al.*, 1989; Kiefer-Day *et al.*, 1991), and it also has steep slopes for the allosteric effect of inhibiting antagonist radioligand dissociation (Potter *et al.*, 1989). Interestingly Potter *et al.* (1989) reported that THA inhibited agonist radioligand binding with slopes of about 1 and did not affect agonist ligand

dissociation. Here we have found THA to inhibit ACh-stimulated GTP γ S binding with similar potency and subtype specificity as has been reported from binding studies, and with Schild slopes of about 2 at all subtypes. This is the first report that THA inhibits muscarinic receptor activation with a steep concentration-response function.

Hilf & Jakobs (1992) found that antagonists inhibit basal GTP γ S binding to cardiac membranes by up to 25% with potencies corresponding to their affinities at the m2 receptor. This phenomenon has been interpreted as evidence for 'negative intrinsic efficacy' of the antagonists at receptors pre-coupled to G-proteins (Hilf & Jakobs, 1992; Costa *et al.*, 1992). We saw little if any inhibition by antagonists of basal response using the standard assay conditions, but that was expected since the phenomenon is strongly inhibited by sodium ions (although the specificity of sodium, as opposed to a general monovalent cation effect, has not been explored). We also failed to see the inhibitory effect, however, when we used a sodium-free buffer. We have no explanation for this discrepancy.

This study demonstrates that accurate estimates of antagonist affinity and Schild slope can be obtained from functional assays with an inhibition-curve design as long as the data are analysed by a valid method. This design is often used with biochemical functional measures, but is more usually analysed with the Cheng-Prusoff equation (e.g. McKinney *et al.*, 1991; Ford *et al.*, 1992). As well as being theoretically invalid, this analysis will often underestimate (or overestimate) the antagonist affinity by up to 3 fold or more (Lazareno & Birdsall, 1993, this study). The best method of analysing such data is to fit them to the Schild equation, since this makes use of all the data without transformation and provides an estimate of Schild slope. If powerful curve-fitting facilities are unavailable, and the Schild slope can be assumed to be 1, then it is still possible to obtain accurate K_b estimates by logistic curve fitting or Hill analysis, or even measurement off a graph. The essence of this method is to estimate the concentration of agonist alone causing half the effect of the fixed concentration, i.e. the same effect as the fixed agonist concentration in the presence of the IC_{50} concentration of antagonist. We have assessed two slightly different methods of making this estimation. Both methods have strengths and weaknesses, but the errors associated with them are small and either method may be used with confidence, especially if the fixed agonist concentration is at least 10 fold greater than the EC_{50} .

The advantage of the single inhibition curve design in assessing antagonist affinity from functional experiments is that it requires less data than full Schild analysis containing a number of agonist (or antagonist) titrations in the presence of different fixed concentrations of antagonist (or agonist). The disadvantage, of course, is that it does not test the assumptions which underlie the analysis i.e. parallel displacement of agonist curves, and lack of effect on basal or maximal activity. In many cases the inhibition curve design will be quite adequate for the rapid assessment of antagonist affinity and Schild slope, and the effect of the antagonist on basal activity can be assessed in separate experiments if required. The full Schild design and analysis, however, preferably using non-linear regression, remains essential for the most thorough characterization of an antagonist.

In conclusion, the stimulation of [3 S]-GTP γ S binding by muscarinic agonists provides a quick and convenient assay which allows accurate estimation of antagonist affinities, and estimates of antagonist Schild slopes can be obtained from single inhibition curves.

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