Br. J. Pharmac. (1985), **86**, 601–607

Pharmacological analysis of muscarinic receptors coupled to oxyntic cell secretion in the mouse stomach

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1 In the light of recent attempts to subclassify muscarinic receptors, agonist-antagonist interactions at muscarinic receptors have been re-examined using improved techniques, on the mouse, isolated, lumen-perfused stomach gastric acid assay.

2 Using 5-methylfurmethide as the muscarinic agonist, the pK_B estimated for atropine was significantly lower on the stomach assay (7.78) than on the guinea-pig trachea (8.93). However pK_B values for N-methylatropine, the quaternary ammonium derivative of atropine, at concentrations producing dose-ratios above 20 on the stomach assay ($pK_B = 9.67$), and over the full concentration range studied on the trachea ($pK_B = 9.69$) were not significantly different.

3 The deviation from simple competitive behaviour at low dose-ratios with N-methylatropine in the stomach assay is consistent with the effects of a saturable uptake mechanism for quaternary ammonium compounds.

4 The pK_B values for pirenzepine on the stomach (6.67) and the trachea (6.87) were not significantly different suggesting that pirenzepine behaves more like N-methylatropine in terms of expressed affinity.

5 We conclude that the oxyntic cell muscarinic receptors are homogeneous with those in the guinea-pig trachea. An initial exploration suggests that there is a relationship between the lipophilicity (log P) of the antagonists and the degree of apparent underestimation of antagonist affinity in the stomach assay. This supports the hypothesis that the underestimation of antagonist affinity is due to the loss of antagonist into the gastric secretion from the receptor compartment. Apparently, relatively selective inhibition of acid secretion, compared to atropine, could be explained without the need to postulate heterogeneity of muscarinic receptor populations.

Introduction

Although Angus & Black (1979) found that atropine behaved as a simple competitive antagonist of bethanechol and carbachol at muscarinic receptors coupled to acid secretion in the isolated, lumen-perfused, stomach preparation of the mouse, the calculated pK_B values were significantly lower than the pK_B values found using other muscarinic receptor systems such as the guinea-pig ileum and trachea. These differences in pK_B values were attributed to a reduction of the steady state concentration of the antagonist in the receptor compartment compared with the applied concentration, due to the loss of the antagonist into the gastric secretion, rather than to the more orthodox and less conservative possibility of heterogeneity of muscarinic receptors. However, the existence of muscarinic receptor heterogeneity has recently received considerable and increasing support (Hirschowitz *et al.*, 1984) and this, in addition to the emergence of pirenzepine, proposed as a selective muscarinic antagonist by Hammer *et al.* (1980), have led us to re-examine the properties of muscarinic receptors on oxyntic cells.

Originally Angus & Black (1979) used a single exposure, 2 + 2 bioassay design to estimate pK_B values. This method gave values of pK_B with 95% fiducial limits such that differences in pK_B values of less than approximately 0.5 log units could not be reliably discriminated. Our recent development of the mouse stomach assay (Black & Shankley, 1985a) now permits the determination of pK_B values with greater precision (95% fiducial limits approximately 0.2 log units) and provides a more reliable instrument for either probing the homogeneity of muscarinic receptor populations or detecting non-equilibrium or saturating conditions.

In addition to improvements in the precision of the analytical method, we have tried to exploit the poten-

tially selective properties of N-methylatropine and pirenzepine. N-methylatropine, the quaternary ammonium derivative of atropine, was chosen as a ligand whose physico-chemical properties were expected to prevent loss into the gastric secretion. Pirenzepine was chosen as a ligand whose radioligand binding data suggested that it had a significantly higher affinity for so-called M_1-muscarinic receptors (Giachetti *et al.*, 1982). Finally, in the mouse stomach the existence of muscarinic receptors on histamine-secreting cells as well as oxyntic cells was predicated by the discovery (Angus & Black, 1982) that acid secretion evoked by nerve stimulation was equally sensitive to blockade by both atropine and metiamide. Therefore, in most experiments, the potential complication of histamine release has been eliminated by concurrent histamine H_2-receptor blockade. Tiotidine was chosen as the H_2-receptor antagonist because of its high potency and selectivity (Black *et al.*, 1985b). Similarly, 5-methylfurmethide (5mef) was chosen as the muscarinic agonist because its high potency and selectivity allowed a wide range of antagonist concentrations to be explored (Black & Shankley, 1985a). For comparative purposes we have also obtained pK_B values for the muscarinic antagonists on the guinea-pig trachea assay.

Methods

Acid secretion

Gastric acid secretion was measured in the isolated, lumen-perfused, stomach preparation of the mouse as described previously (Black & Shankley, 1985a). Briefly, stomach preparations were established with the pH-electrode system arranged to provide a 12 cmH_2O pressure to distend the stomach. Six preparations were used simultaneously and after an initial 60 min stabilization period those not producing a stable basal acid secretion (approximately 5%) were rejected. All drugs were added directly to the organ bath (serosal side) and, following a further 60 min equilibration period in the absence or presence of antagonist, a single cumulative agonist-concentration effect curve was obtained.

Guinea-pig trachea assay

Tissues were prepared to tracheal strips essentially as described by Emmerson & Mackay (1979). Briefly, tracheal strips were suspended with a constant load of 0.5 g in organ baths containing 20 ml Krebs solution (with 2.8 μM indomethacin to minimize interference from cyclo-oxygenase products) at 37°C, gassed with 95% O_2 and 5% CO_2. Responses were measured isotonically using rotary transducers. Six preparations were used simultaneously and after an initial 45 min stabilization period the tissues were incubated for a further 60 min in the absence or presence of antagonist before a single cumulative concentration-effect curve was obtained.

Experimental design

Antagonist treatments were allocated on a block design such that, as far as possible, all organ baths received each treatment during the course of an experiment.

Analysis

Acid secretion and trachea responses resulting from the addition of the muscarinic agonist 5-methylfurmethide (5mef) were recorded as the change in pH (Δ pH) of the lumen perfusate and changes in tissue length respectively, measured from the baseline immediately prior to starting the cumulative concentration-effect curve. The concentration-effect curve data from individual preparations were fitted to a logistic function which provided estimates of the mid-point location parameter (log [A_{50}]), maximal asymptote (a) and mid-point slope (n), as described previously (Black & Shankley, 1985a). For display purposes the individual computed parameter estimates for each treatment group were expressed as means and a single

Table 1 The effect of tiotidine on logistic curve parameters obtained from the fitting of 5-methylfurmethide concentration-effect data

Tiotidine (μM)	n	*log [A_{50}]* (M)	Mid-point slope (n)	Maximal asymptote (ΔpH) (a)
0	6	-7.44 ± 0.08	2.09 ± 0.07	0.55 ± 0.03
0.3	6	-7.19 ± 0.06*	1.88 ± 0.29	0.58 ± 0.02
1.5	4	-7.22 ± 0.06	2.89 ± 0.34*	0.47 ± 0.06
8.5	5	-7.11 ± 0.04*	2.74 ± 0.26	0.54 ± 0.06
100.0	4	-7.10 ± 0.08*	3.83 ± 0.33*	0.61 ± 0.05

*Significantly different from control value, $P < 0.05$.

logistic curve generated and superimposed upon the experimental data.

Agonist-antagonist interactions were analysed as described previously (Black *et al.*, 1985a).

Drugs

Drugs were freshly prepared in distilled water. The total volume added to the 40 ml organ bath did not exceed 400 µl. Drugs and their sources were as follows: N–methylatropine (Sigma), atropine (Sigma), 5-methylfurmethide (Wellcome Research Laboratories). Tiotidine and pirenzepine were generous gifts from Imperial Chemical Industries Ltd and A.B. Häsle Ltd, respectively.

Results

The effect of H_2-receptor blockade on 5-methylfurmethide concentration-effect curves

Tiotidine significantly displaced and increased the mid-point slope of the fitted 5mef concentration-effect curves (Table 1) at concentrations which produced selective H_2-receptor blockade (Black *et al.*, 1985b). This result points to the possibility of the presence of muscarinic receptors on histamine secreting cells in the mouse stomach. Therefore, to eliminate the potential complication of the release of endogenous histamine, all subsequent 5mef-muscarinic antagonist interactions were studied in the presence of tiotidine 10^{-4}M.

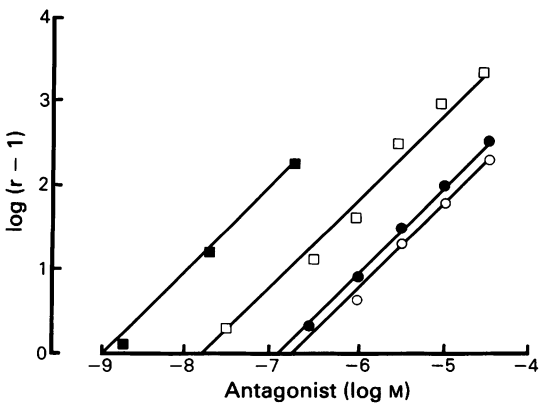

Figure 1 Schild plots for 5-methylfurmethide (5mef)/atropine (■) and 5mef/pirenzepine (●) assays on the guinea-pig trachea (closed symbols) and mouse stomach (open symbols). Dose-ratios (r) were calculated from the $[A_{50}]$ values estimated for the 5mef concentration-effect curves. Estimates of slope parameter and pK_B were obtained by model fitting and are presented in Table 2.

Atropine

Atropine produced a significant concentration-dependent parallel displacement of 5mef concentration-effect curves with no significant change in the maximal asymptote. Dose-ratio analysis indicated a Schild plot slope not significantly different from unity (Figure 1). The estimate of pK_B (7.78 ± 0.07) was significantly lower than that obtained from identical analysis on the guinea-pig trachea assay ($pK_B = 8.93 \pm 0.16$). The value obtained on the stomach assay compares with values of 7.65 estimated by Angus & Black (1979) and 7.78 by Szelenyi (1982) with bethanechol using the 2 + 2 bioassay design on the same preparation.

N-methylatropine

N-methylatropine also produced a significant concentration-dependent parallel displacement of the 5mef concentration-effect curve with no significant change in the maximal asymptote (Figure 2a). Dose-ratio analysis revealed that the corresponding Schild plot (Figure 2b) deviates significantly from unit slope at low concentrations of N-methylatropine (3–10 nM). However, analysis of the data obtained in the presence of concentrations of N-methylatropine corresponding to dose-ratios between 20 and 1,500, produced a Schild slope not significantly different from unity with estimated $pK_B = 9.67 \pm 0.11$. This value is not significantly different from that obtained on the guinea-pig trachea assay ($pK_B = 9.69 \pm 0.09$) where competitive behaviour was obtained over the full concentration range with N-methylatropine.

The downward concave nature of the Schild plot in Figure 2b could be accounted for by a simple model describing the saturable uptake of antagonist from the receptor compartment. The line drawn through these data is a simulation of this model which was based on the model developed by Furchgott (1972) describing the effects of a saturable uptake process for agonists on Schild plots. A detailed description of this model will be the subject of a subsequent paper. In brief it is assumed that the deviation from unit slope observed with low concentrations of antagonist is due to the loss of N-methylatropine from the receptor compartment via a saturable removal process. In the simulation, the model parameters were fixed such that concentrations of N-methylatropine above 10^{-8}M saturate the removal process and the receptor compartment concentration effectively attains equilibrium with the organ bath serosal solution.

Pirenzepine

Pirenzepine produced a significant concentration-dependent parallel displacement of the 5mef concentration-effect curves, with no change in maximal asymp-

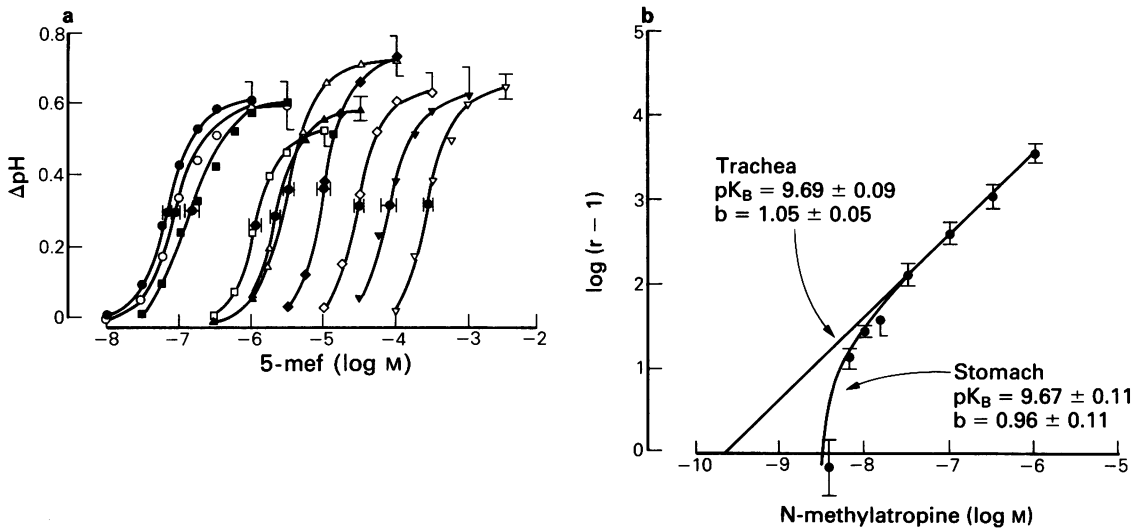

Figure 2 N-methylatropine pK_B determination on the mouse stomach and guinea-pig tracheal assays. (a) 5-methylfurmethide (5mef) concentration-effect curves obtained in the absence (●) and presence of N-methylatropine, (○) 3×10^{-9}, (■) 4×10^{-9}, (□) 6×10^{-9}, (▲) 10^{-8}, (△) 1.5×10^{-8}, (◆) 3×10^{-8}, (◇) 10^{-7} and (▽) 10^{-6} M, on the mouse stomach assay with H_2-receptor blockade (10^{-4}M tiotidine). The curves drawn through the mean experimental data points (n = 5/6) were obtained from a logistic curve fitting procedure. (b) Schild plots for 5mef/N-methylatropine assays on the mouse stomach and guinea-pig trachea. For clarity the experimental data from the trachea assay have been omitted. The pK_B and slope parameter estimates on the mouse stomach assay were obtained by model fitting of the data obtained in the presence of 15, 30, 100, 300 and 1000 nM N-methyl-atropine. The curve drawn through the stomach data was simulated from a model describing the effect of a saturable uptake process for a competitive antagonist (see text for details).

tote, in both the mouse stomach and the guinea-pig trachea assays. Dose-ratio analysis (Figure 1) produced pK_B values of 6.87 ± 0.09 and 6.67 ± 0.09 in the trachea and stomach, respectively.

The estimates of pK_B values obtained on the mouse stomach and guinea-pig trachea assays for the three muscarinic receptor antagonists are summarized in Table 2. The difference in pK_B values on each assay has been assigned the quantity, ΔpK_B. Thus the larger the ΔpK_B value the greater the apparent underestimation of antagonist activity in the mouse stomach assay.

Discussion

When investigating agonist-antagonist interactions it

Table 2 Estimates of pK_B for muscarinic receptor antagonists on assays of acid secretion in the mouse, isolated, lumen-perfused stomach and muscle contraction in the guinea-pig trachea

	$pK_B \pm s.e.$			
Angatonist	*Guinea-pig trachea: contraction* (A)	*Mouse stomach: acid secretion* (B)	$\Delta pK_B \pm s.e.$ (A − B)	*log* P_{OCT/H_2O}
Atropine	8.93 ± 0.16	7.78 ± 0.11*	+ 1.15 ± 0.19	1.83
N-methylatropine	9.69 ± 0.13†	9.67 ± 0.11	+ 0.02 ± 0.17	− 0.40
Pirenzepine	6.87 ± 0.09	6.67 ± 0.09	+ 0.20 ± 0.13	0.20

*Significantly different from pK_B value obtained on the guinea-pig trachea assay.
†Estimated from dose-ratios above 20 (see Figure 2b).
log P_{OCT/H_2O} values estimated with aqueous buffers 2 pH units above the pK_A values of the compounds.

is desirable to maximize the possibility of exposing non-competitive behaviour and to increase the confidence in the conclusion that the observed results do not disagree with the expectations for competitive behaviour. Previous investigations (Angus & Black, 1979) with muscarinic antagonists on the mouse, lumen-perfused stomach assay were limited to the study of dose-ratios less than 100 due to the relatively low potency of the muscarinic agonists used. 5-Methylfurmethide is approximately 30 fold more potent than bethanechol in stimulating gastric acid secretion in the mouse stomach assay and allowed for the determination of dose-ratios as high as 1,500 with the muscarinic antagonists. If dose-ratios between 10 and 1,500 had not been determined with N-methylatropine it is conceivable that the apparent deviation from competitive behaviour at low dose-ratios (Figure 2b) would have precluded an estimate of the pK_B for this antagonist.

The ability to obtain fully-defined cumulative concentration-effect curves provided a further indication, in addition to the observed dose-ratio, that tiotidine 10^{-4}M pretreatment significantly altered the 5mef concentration-effect curve (Table 1). The possible significance of the steepening of the 5mef concentration-effect curve in the presence of H_2-receptor blockade is discussed in a subsequent paper (Black & Shankley, 1985b). The observation of the tiotidine-sensitive component is indicative of an effect of 5mef to release histamine (see Introduction). Failure to detect this component could possibly have confounded the subsequent interpretation and analysis of the interactions between 5mef and the muscarinic receptor antagonists on the oxyntic cell receptor. Similarly, the full definition of cumulative concentration-effect curves allows for a more rigorous examination of competitive interactions between agonists and antagonists. The modified assay allows tests to be made for detecting changes in maximal asymptotes and mid-point slope parameters obtained by curve fitting of the experimental data obtained in the presence and absence of antagonist.

The pK_B value for atropine on the mouse stomach assay in this study (7.78) is comparable to the pK_B values of 7.65, obtained by Angus & Black (1979), and 7.78, found by Szelenyi (1982) using the 2 + 2 bioassay design. This confirms the underestimation of the affinity of atropine in this assay. If the underestimation of the pK_B value for atropine is due to the loss of antagonist into the gastric secretion rather than an expression of binding to a subclass of muscarininc receptors then it would be anticipated that the underestimation should also occur in other experimental preparations where the gastric mucosa is intact and the acid secreted is compartmentalized from the tissue bathing fluid. Using the rat isolated gastric mucosa, Main & Pearce (1981) also obtained a low pK_B for atropine (8.40) against methacholine-stimulated gastric acid secretion, although this value is about 0.6 log unit higher than that found in the mouse preparation. However, in the same study the H_2-receptor antagonist metiamide had a pK_B value of 6.49 against histamine-stimulated acid secretion, a value about 0.5 log unit higher than that found in other tissues of any species. Apparently the difference in pK_B value between atropine and metiamide is roughly the same in rat and mouse preparations although the measured values are different. However, comparison of the pA_2 values found by Main & Pearce (1981) and pK_B values obtained in this assay are not strictly valid due to the low Schild plot slopes obtained in the former studies. In general, if low Schild plot slopes are obtained, but still not significantly different from unity, the refitting of the experimental data with the Schild plot constrained to unity results in estimates of pK_B lower than the pA_2 value obtained from the unconstrained regression. It is therefore conceivable that the data from the study of Main & Pearce (1981) are consistent with the results of Angus & Black (1979) and Angus *et al.* (1980) who demonstrated the underestimation of pK_B values of three histamine H_2-receptor antagonists as well as for atropine in the mouse stomach preparation. If the underestimation is a result of loss from the oxyntic cell receptor compartment to the acid secretion the degree of underestimation would be anticipated to be independent of the receptor selectivity of the compound but to be dependent on the ability of the compound to pass through oxyntic cell membranes.

The pK_B values obtained from analysis of the N-methylatropine/5mef interaction on the mouse stomach assay for concentrations of N-methylatropine above 10^{-8}M, and also on the trachea assay over the full concentration range of antagonist, indicated that the affinity of N-methylatropine is not underestimated in the stomach assay (Table 2). This result appears to support the hypothesis that the underestimation of antagonist affinity in this assay is a function of their ability to pass through biological membranes.

We have explored the theoretical effect of a saturable removal process to account for the deviation from competitive behaviour observed at low concentrations of N-methylatropine (Figure 2b). The suggestion of such a removal process operating in the mouse stomach assay is based on the reports of quaternary ammonium ion uptake processes which have been described in the gastrointestinal tract, kidney and liver (Ruifrok, 1981; 1982). Preliminary attempts have been made to explore this hypothesis with the ultimate aim of blocking the removal process so that competitive behaviour is achieved over the full concentration range of N-methylatropine. Taurocholic acid 5 mM has been found (Ruifrok, 1982) to decrease the uptake

of quaternary ammonium compounds, including N-methylatropine, into rat liver plasma membranes, but its use to block a similar process in the mouse stomach assay was precluded because this agent also inhibited 5mef control responses.

The pK_B value obtained for pirenzepine in the guinea-pig trachea assay (6.87 ± 0.09) agrees reasonably well with pA_2 values found by Szelenyi (1982) against bethanechol in guinea-pig ileum (6.60) and right atrium (6.45) and that found by Barlow & Chan (1982) in the guinea-pig ileum (6.67). Like Szelenyi (1982) we did not detect a significant difference between the pK_B obtained in the stomach assay (6.67 ± 0.09) and that found in the trachea assay (6.87 ± 0.09). Thus, pirenzepine does not show any selectivity for the oxyntic cell musarinic receptor in terms of its expressed affinity in the guinea-pig trachea and mouse stomach assays.

If we accept that the mouse stomach oxyntic cell muscarinic receptor is homogeneous with that found in the guinea-pig trachea assay it would, then, appear that pirenzepine behaves more like the quaternary ammonium ion N-methylatropine than atropine in terms of its loss from the receptor compartment in the stomach assay. The ability of a compound to penetrate biological membranes has often been correlated with log P values, that is their oil/water partition coefficients (Hansch & Leo, 1979). Therefore, we have explored the relationship between partition coefficients estimated between octanol and water and the ΔpK_B values obtained in this study for the muscarinic receptor antagonists (Table 2). Although there is not sufficient data to ascertain clearly the relationship between log P and ΔpK_B, these initial results suggest that increased lipophilicity of the compounds is paralleled by an increase in the apparent underestimation of affinity in the mouse stomach assay.

Our conclusion then is that the inconsistencies among the antagonist pK_B values for atropine, N-methylatropine and pirenzepine on tracheal muscle and acid secretion can be accounted for without the need to postulate heterogeneity of receptor populations. In particular, the ability of pirenzepine to display selective inhibition of acid secretion, *in vivo*, compared to atropine could simply be due to lack of loss through oxyntic cells and hence an effective blocking concentration would occur at much lower plasma levels.

This work was supported by The Wellcome Foundation Ltd. The authors wish to thank Dr Paul Leff (Department of Pharmacology, The Wellcome Foundation Ltd) for valuable scientific discussion and Mrs H.D. Williams for help in preparing the manuscript.

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(Received March 4, 1985.
Revised June 28, 1985.
Accepted July 8, 1985.)