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ANALYSIS OF ANOMALOUS pK_B VALUES FOR METIAMIDE AND ATROPINE IN THE ISOLATED STOMACH OF THE MOUSE

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1 In the isolated, lumen-perfused, stomach preparation of the mouse, metiamide was found by kinetic analysis to behave like a simple competitive antagonist of histamine-stimulated acid secretion. However, the pK_B estimate of 5.08 was significantly lower than that found in guinea-pig atrium (6.0) or rat uterus (6.1) suggesting that H_2-receptors might not be homogeneous.

2 A similar analysis showed that atropine also behaved like a simple competitive antagonist of bethanechol-stimulated acid secretion and the estimated pK_B (7.65) was significantly lower than the standard estimate of this parameter in guinea-pig ileum (9.0). Either the muscarinic cholinoceptors in mouse stomach were also anomalous or the preparation was introducing a systematic error. Lumen perfusion might distort this type of kinetic analysis by allowing steady-state conditions but not true equilibrium to develop at the receptor compartment due to loss of antagonist into the gastric secretion. Drug interactions at receptors in the muscle layers of the stomach would be expected to be much less sensitive to this error.

3 When the atropine–bethanechol interaction was measured on the contraction of the isolated, lumen-perfused, stomach of the mouse the necessary conditions for simple competition were not met even though the sensitivity to atropine was obviously increased. The criteria for the expected simple competition were being obscured by events at low antagonist concentrations. Alterations in agonist or antagonist concentrations could be more or less eliminated so that physiological antagonism, perhaps by release of 5-hydroxytryptamine, was considered. This was supported, to some extent, by finding that, when stomachs from animals pretreated with reserpine were used, the kinetic analysis was normalized and gave a pK_B of 8.99. Apparently, the muscarinic receptors in mouse stomach are homogeneous with those in other tissues.

4 Therefore, we conclude that our results no more point to heterogeneity among histamine receptors than they point to differences in muscarinic cholinoceptors because this type of kinetic analysis can be readily distorted by special features of the measuring system.

Introduction

Metiamide and related compounds behave as simple competitive antagonists of histamine on acid secretion in the isolated, lumen-perfused stomach preparation of the mouse and pK_B values (negative logarithms of the estimated dissociation constants) can be calculated (Angus, Black & Stone, 1978). However, the estimated pK_B values for burimamide, metiamide and cimetidine were all significantly lower (by as much as 1 unit) than the pK_B values reported for these compounds in other H_2-receptor systems, such as guinea-pig heart and rat uterus. Further, with dimaprit (the selective H_2-receptor agonist) in place of histamine, similar low pK_B values were obtained. We have considered two possible explanations. Either there is a subset of histamine receptors involved in gastric acid secretion different from those subserving tachycardia and uterine relaxation or there is some undefined process interfering with the acid secretion assay. Evidence from the biochemical assay of histamine-stimulated adenylate cyclase in cells from dog gastric mucosa suggests that the H_2-receptors on parietal cells are homogeneous with those receptors in atria and uterus because the pK_B values of metiamide and burimamide were similar in all three assays (Scholes, Cooper, Jones, Major, Walters & Wilde, 1976). Therefore, before reaching any conclusions about the heterogeneity of H_2-receptors, the reliability of the stomach assay needed to be examined.

Cholinoceptor agonists of the muscarinic type also stimulate acid secretion and atropine would be expected to be a simple competitive antagonist with a pK_B value of around 9 (Arunlakshana & Schild,

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1959). We have used these agents to examine the possibility that assays based on acid secretion may systematically give low antagonist potencies.

Methods

Acid secretion

Young adult mice (25 to 28 g) were killed and the stomachs rapidly cannulated at both the pyloric sphincter and fundus. After tying the oesophagus the stomachs were transferred to baths holding 40 ml of buffered solution gassed with 95% O_2 and 5% CO_2 (Angus & Black, 1978). Each stomach lumen was continuously perfused at 1 ml/min with an unbuffered oxygenated solution (Angus & Black, 1978) at 37°C and the perfusate passed over a flow-type glass pH electrode raised 18 cm above the stomach to keep it distended. All drugs were added to the bath; atropine was allowed to equilibrate for 30 min before adding the agonists bethanechol or carbachol. A 2 + 2 bioassay design of 24 stomachs was employed to estimate dose-ratios and confidence limits (Colquhoun, 1971). To avoid problems of desensitization, serial correlation and time-dependence, each stomach was used for only one agonist response. Two concentrations of atropine, 10^{-7} M and 10^{-6} M, were used.

Stomach wall contraction

Lumen perfused. Stomachs were prepared as outlined for measuring acid secretion. A stainless steel hook was firmly tied to the surface of the stomach at the base of the oesophagus and the isometric tension was recorded by a Grass transducer (FTO3C) with an initial resting tension of 1 g. Cumulative concentration–response curves to bethanechol were constructed on each stomach (using a 2 min cycle and common dose-ratio of 3) before and 30 min after contact with atropine, 10^{-8} M to 10^{-6} M. Only one concentration of atropine was used with each stomach.
Stomachs not perfused Whole stomachs were suspended perpendicularly in the organ bath for measurement of isometric or isotonic tension. Small cuts were made in the stomach wall to allow both stomach surfaces to be oxygenated by the bathing medium. Cumulative dose–response curves to bethanechol were constructed. Three concentrations of atropine 10^{-8} M, 10^{-7} M, 10^{-6} M were used.

Statistics

In the acid secretion assays, responses were calculated as log peak H^+ secretion rate (nmol/min) to produce reasonable experimental variance. Dose-ratios and 95% confidence limits were calculated from each assay of 24 stomachs for one concentration of atropine. Each assay met the criteria for parallelism and significant linear regression of agonist concentration-response (Colquhoun, 1971). From the dose-ratios (r) obtained for 10^{-7} M and 10^{-6} M atropine, a Schild plot was constructed and the line extrapolated to log $(r - 1) = 0$ to estimate pK_B.

Concentration-response curves for muscle contraction were analysed by first using an iterative logistic curve-fitting programme to determine ED_{50} values, maximum tension and slope of the logistic curve at the ED_{50} value (Parker & Waud, 1971). Dose-ratios (r) were then estimated and Schild plots constructed where values of log $(r - 1)$ were plotted against log B (antagonist concentration). Least squares linear regression analysis of the Schild plot was used to estimate pK_B values and the slope of the regression line with confidence limits. In each regression analysis there were 3 or 4 points at each atropine concentration of 10^{-8} M, 10^{-7} M and 10^{-6} M. Comparison of slopes and intercepts of regression lines were performed by analysis of covariance (Snedecor & Cochran, 1967).

Drugs

Atropine sulphate (Sigma), methyl atropine bromide (MacFarlan Smith), bethanechol (carbamyl β-methylcholine chloride, Sigma), carbachol (Koch-Light), neostigmine (prostigmine, Roche), phentolamine mesylate (Rogitine, Ciba), reserpine (Sigma) and tetrodotoxin (Sigma) were used. Reserpine was dissolved as the acetate. The 5-methyl furmethide was a generous gift from Professor W.D.M. Paton.

Results

Acid secretion

Atropine caused parallel displacement of the two-point dose-response lines for both bethanechol and carbachol. The two-point bethanechol assays gave a Schild plot with a regression slope of 1.08 providing a pK_B estimate of 7.65 (Figure 1 and Table 1). Similar results, a pK_B of 7.52 and slope 0.94, were found with carbachol. Although these results are compatible with simple competition between atropine and these cholinoceptor agonists, the pK_B values are more than 1 log unit lower than the reference value of 9.0 for atropine found by Arunlakshana & Schild (1959) on guinea-pig ileum.

Bunce, Marsh & Parsons (1977) have also reported unexpectedly weak antagonism of atropine against acetylcholine on rat isolated stomach preparations and raised the question about species (rodent) specificity of the results. Fortunately, the mouse stomach

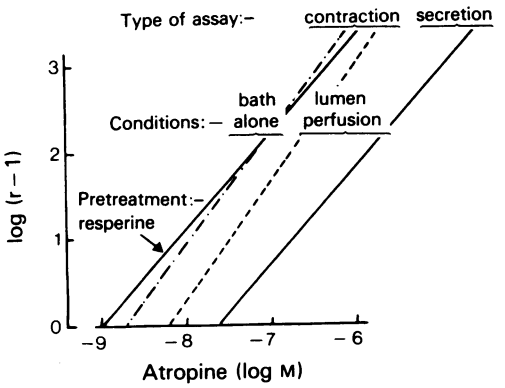

Figure 1 Schild plot regression lines for bethanechol/atropine interaction. Lines right to left: acid secretion assay, lumen perfused; whole stomach isometric wall contraction, lumen perfused; isometric wall contraction without lumen perfusion; isometric wall contraction without lumen perfusion in reserpine pretreated stomachs. pK_B values for each line from right to left 7.65, 8.22, 8.71 and 8.98.

preparation allowed us to measure a pK_B value for muscle contraction in the same preparation under identical conditions for direct comparisons with the assays based on acid secretion.

Wall contraction

Concentration–response curves to both bethanechol and carbachol were displaced to the right in parallel by atropine without significant depression of maxima so that dose-ratios and pK_B values could be calculated. The pK_B values for atropine estimated from linear regression in the Schild plots were 8.22 and 8.47 for bethanechol and carbachol respectively (Table 1). These values were almost 1 unit higher than the pK_B estimates for atropine obtained for acid secretion under similar conditions. However, the slopes in the Schild plots for muscle contraction were significantly greater than unity (1.34 and 1.26 for bethanechol and carbachol respectively, Table 1).

This departure from the necessary conditions for simple competitive antagonism means that the pK_B values for the muscle and secretion assays cannot be compared directly. This is a surprising result because the atropine/bethanechol interaction is usually considered to be a standard example of simple competition. However, the use of lumen perfusion for the contraction assay is an obvious difference between these and standard assays. Therefore the assays were repeated on whole mouse stomachs without lumen perfusion but with all other conditions the same.

In this assay the pK_B was 8.71 and the slope was 1.28 (Figure 1, Table 2). The analysis of covariance for comparison of regression lines (Snedecor & Coch-

Table 1 Dose-ratios and regression analysis of Schild plots for cholinoceptor agonists/atropine interactions on assays of acid secretion and stomach wall contraction in the lumen-perfused isolated mouse stomach. Analysis of histamine/metiamide interaction on acid secretion assay and atrial tachycardia in guinea-pig are given for comparison.

Antagonist	*Agonist*	*Perfused stomach-acid secretion*			*Perfused stomach-wall contraction*		
		Dose-ratios atropine			*Regression analysis*		
		10^{-7} M	10^{-6} M	pK_B[a]	Slope[a]	pK_B	Slope
Atropine:	Bethanechol	5.04[b] (2.3, 12.6)	62.7[b] (15.6, 535)	7.65	1.08	8.22[f] (7.8, 8.7)	1.34[f] (1.19, 1.48)
	Carbachol	4.17[b] (0.7, 13.5)	28.7[b] (8.2, 67.6)	7.52	0.94	8.47 (7.8, 9.2)	1.26 (1.00, 1.52)
	Acetylcholine	3.1[c]	26.5[c]	7.30	1.1	—	—
						Guinea-pig atria tachycardia	
Metiamide:	*Histamine*	—	—	5.08[d] (4.76, 5.40)	0.96[d] (0.74, 1.18)	6.03[e] (5.94–6.13)	0.98[e] (0.92–1.04)

Notes: [a]estimated from 2-point regression line; [b]estimated from 2 + 2 bioassay; [c]data from Thorpe & Durbin (1972) on frog mucosa; [d]data from Angus *et al.* (1978); [e]data from Parsons (1973); [f]in reserpine pretreated stomachs pK_B = 8.99 (8.57, 9.47) slope = 1.11 (0.96, 1.25).
Brackets enclose ± 95% confidence limits.

ran, 1967) gave an F value for comparison of slopes of 0.29 (not significant) and F value for comparison of intercept 37.25 ($P < 0.001$). Apparently, lumen perfusion had interfered with the assay but was not the source of the deviation from unity in the slope.

In case the stomach muscle presented special problems of *'access'* the experiments were repeated with the non-quarternary agonist methyl furmethide or the quarternary antagonist methyl atropine. The results from these assays were not significantly different from the atropine/bethanechol interaction (Table 2).

Before concluding that there is an unidentified source of variation present in mouse stomach muscle the bethanechol/atropine interaction was studied on guinea-pig ileum using both isometric and isotonic measurements. As shown in Table 2 these assays gave pK_B values not different from one another or from the value reported by Arunlakshana & Schild (1959): moreover the slopes of the Schild regression were not significantly different from unity.

Mouse stomach muscle, therefore, seems to present a special problem. Nerve plexuses as a source of variation were probably eliminated by finding that pretreatment with tetrodotoxin, 10^{-7} M, which has been shown to block the effect of nerve stimulation in this preparation (Angus & Black, 1978), had no effect on the parameter values.

Involvement of cholinesterase was considered possible because no reports about bethanechol as a substrate for this enzyme could be found and because atropine inhibits butyrylcholinesterase with a K_i of 5×10^{-6} M (Roepke, 1937). However, pretreatment with neostigmine 7×10^{-8} M, a concentration greater than that found by Edge (1970) to produce maximum potentiation of acetylcholine on visceral muscle, did not alter the assay results.

Inspection of all these steep Schild plots showed that the dose-ratios obtained at the lowest atropine concentration used (10^{-8} M) were lower than would be predicted by linear extrapolation from the results obtained at 10^{-7} M or 10^{-6} M (Figure 2). This suggested the possibility that physiological antagonism, perhaps by released 5-hydroxytryptamine, might be occurring. Stomachs taken from mice pretreated with reserpine 2 mg/kg intraperitoneally 24 h beforehand gave a pK_B value of 8.99 with slope 1.11, a value not significantly different from unity. This assay was repeated using isotonic measurements and gave a pK_B of 8.98 and Schild slope of 0.96. This decrease in the slope of the dose-ratio regression line produced by reserpine was associated with an increase in the dose-ratios at 10^{-8} M atropine. Consequently the intercept at log $(r - 1) = 0$ was significantly increased ($F = 21.1$; d.f. = 1,3; $P < 0.05$). This value is now not different from that found by Arunlakshana & Schild (1959).

There was a possibility that pretreatment with reserpine, having normalized the assay of wall contraction, might also alter the pK_B estimate in the acid secretion assay. However, in the 2 + 2 acid secretion assay for atropine versus bethanechol the dose-ratio (±95% confidence limits) for atropine 10^{-7} M, was 2.5 (0.3, 16.3) and for atropine 10^{-6} M was 20.3 (3.7, 79.7). These dose-ratios were not significantly different from those found without reserpine pretreatment (Table 1).

Table 2 Schild plot analysis for contraction induced by cholinoceptor agonists of non-perfused mouse stomach and guinea-pig ileum

Antagonist	*Agonist*	*Non-perfused stomach Pretreatment*	pK_B (±95% *c.l.*)	*Slope* (±95% *c.l.*)
Atropine:	Bethanechol	—	8.71 (8.22, 9.26)	1.28 (1.10, 1.46)
	Bethanechol	Neostigmine	8.57 (8.19, 8.99)	1.21 (1.07, 1.35)
	Bethanechol	Tetrodotoxin	8.74 (8.45, 9.04)	1.18 (1.08, 1.28)
	Bethanechol	Phentolamine	8.65 (8.22, 9.16)	1.26 (1.07, 1.45)
	Methyl furmethide[a,b]	—	8.47 (8.27, 8.70)	1.58 (1.39, 1.77)
	Bethanechol[a]	Reserpine[c]	8.98 (8.36, 9.81)	*0.96 (0.76, 1.17)
	Bethanechol	Reserpine[c]	8.99 (8.54, 9.50)	*1.11 (0.96, 1.26)
Methyl atropine:	Bethanechol	—	8.81 (8.48, 9.18)	1.22 (1.10, 1.35)
		Guinea-pig ileum[d]		
Atropine:	Bethanechol[a]	—	9.01 (8.25, 10.01)	*1.06 (0.97, 1.15)
	Bethanechol	—	8.84 (8.61, 9.10)	*1.05 (0.98, 1.12)

Pretreatments included reserpine (2 mg/kg i.p. 24 h before experiment), neostigmine (7×10^{-8} M), tetrodotoxin (10^{-7} M) and phentolamine (3×10^{-6} M) added to the bath 30 min before agonists.
[a]Isotonic measurement; [b]only concentrations at 10^{-8} M and 10^{-7} M atropine; [c]see note f, Table 1, for lumen perfusion; *not significantly different from unity, $P > 0.05$; [d]Magnus's method (*Pflügers Arch. ges. Physiol.* (1904), **102**, 123).

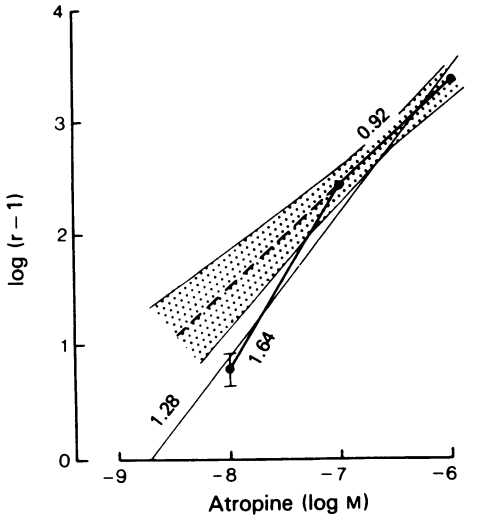

Figure 2 Schild plot for atropine/bethanechol interaction in the isometric wall contraction assay without lumen perfusion. The linear regression of the log (r − 1) values at atropine 10^{-7} M and 10^{-6} M had a slope of 0.92 and 95% confidence limits indicated by the shaded area. The mean ± s.e. value of log (r − 1) is indicated for atropine 10^{-8} M and lies below the 95% confidence band. The regression line of all the points had a slope of 1.28 (thin line) while the regression line of points at 10^{-8} M and 10^{-7} M atropine only was very steep with slope of 1.64.

Discussion

Metiamide behaved like a simple competitive antagonist of histamine in an assay of acid secretion by mouse isolated stomachs (Angus *et al.*, 1978). However, metiamide could not be classified as an H_2-receptor antagonist in that system because the pK_B values were significantly lower than that found with cardiac or uterine tissues. Like histamine, cholinoceptor agonists are also powerful stimulants of parietal cells. This paper shows that, as expected, atropine appears to be a simple competitive antagonist of bethanechol or carbachol. However, the potency of atropine was significantly less in this secretion assay than that found using contraction of mouse stomach (this paper), or that reported for contraction of guinea-pig ileum (Arunlakshana & Schild, 1959), cat isolated nictitating membrane (Langer & Trendelenburg, 1969) or atrial pacemaker frequency (Thron & Waud, 1968). These two systems involving acetylcholine and histamine receptors on parietal cells are apparently independent; atropine does not antagonize histamine, and metiamide does not antagonize acetylcholine (Tepperman, Schofield & Tepperman, 1975; Bunce *et al.* 1977). Therefore, the existence of this sytematic error, points to the possibility that the method of measurement itself is responsible.

An obvious difference between measurements of acid secretion and contraction is that the flow of specific ions and molecules across the parietal cell membrane, and their continuous removal by the perfusion process, means that for these substances their local concentration in the region of the basement membrane could be in a steady-state but not in equilibrium (Furchgott, 1972). Where the antagonist passes the parietal cell membrane and appears in the effluent then the concentration of antagonist in the region of the receptors will also be in a steady-state and presumably at a lower value than would be obtained if equilibrium has been reached.

However, isolated gastric mucosal cells (Scholes *et al.*, 1976; Soll, 1978) cell fragments (Dousa & Code, 1974) and isolated gastric glands (Berglindh, 1977) would be expected to come into equilibrium with their environment and, therefore, to be free from this discrepancy. Indeed Scholes *et al.* (1976) have reported pK_B values for metiamide of 6.46, a finding similar to those reported for guinea-pig atrium (6.04) and rat uterus (6.12) (Black, Duncan, Durant, Ganellin & Parsons, 1972).

Returning to the present study, if it is assumed that diffusion of antagonist across the parietal cell to the stomach lumen follows passive diffusion, then by analogy the rate of loss of antagonist will be directly proportional to the concentration at the basement membrane (Simon, 1977). Therefore, as the antagonist concentration is increased in the bath (serosal surface) a constant fraction of this concentration would be lost in the perfusate. The plot of log (r − 1) against log B would, therefore, be displaced to the right in parallel.

This model places the steepest part of the concentration gradient in the region of the parietal cell membranes. If this effect were significantly interfering with the acid secretion assay, then an assay based on muscle contraction would be expected to have a dose-ratio regression parallel and to the left. In fact, although the muscle assay was shifted to the left, it was significantly non-parallel to the acid secretion assay. Factors other than simple competitive antagonism alone were apparently influencing the dose-ratio regression so that no conclusions about the relevance of the model seemed safe until the significance of the steep slopes was clarified.

The criteria for defining simple competitive antagonism include, and attach much weight to, the slope of the dose-ratio regression. If we are to be impressed with the slope of the dose-ratio regression when it is not significantly different from unity, then we must be equally resolute about rejection of the simple competition hypothesis when the slope is significantly different from unity. The steep slopes of these regres-

sions for the atropine/bethanechol interaction on mouse isolated stomach muscle were, therefore, provocative because the conditions for a simple competitive interaction were expected to be met.

Furchgott (1972) has analysed model systems involving drug-receptor interactions combined with saturable removal processes for the agonist. He showed that when a substance giving simple competitive interaction at receptors also inhibits the removal process then non-linear, flat or steep dose-ratio regressions can occur depending upon the chosen parameter values. Analysis of the three-point dose-ratio regression involved in the atropine/bethanechol interaction in the contraction assays showed a significant degree of curvature (Table 2). As shown in Figure 2 the curvature appeared to be due to dose-ratios at the lowest atropine concentration which were significantly lower than predicted by the extrapolation of the regression from the two higher atropine concentrations. Although no saturable removal processes for bethanechol are recognised, nevertheless, on the one hand, there are no clear reports about bethanechol as a substrate of cholinesterase and, on the other hand, atropine is known to be an inhibitor of cholinesterase. However, the steep slope of the Schild plot was not altered by neostigmine so that drug interactions involving removal processes are unlikely.

From a formal point of view, physiological antagonism by agonist-induced release of another agonist could have the same effect on the slope of Schild plots. The possibility that bethanechol was interfering with the cholinoceptor reaction by stimulating nerve elements was probably ruled out by finding that tetrodotoxin did not alter the slope of the regression. However, we have rationalized the finding that pretreatment with reserpine normalized the Schild plot by proposing that bethanechol might be releasing 5-HT in the stomach wall and that reserpine depletes this 5-HT tissue store (Häkanson, Owman, Sjöberg & Sporrong, 1970). There is evidence that acetylcholine markedly increases 5-HT release in the dog and chicken intestine (Burks & Long, 1966; Gross & Sturkie, 1975). So far, we have no evidence that this is the explanation of its action here.

Whatever the reason for the normalizing effect of reserpine, the outcome is that we have grounds for believing that the locations of the Schild regressions for both the contraction and secretion assays are dominated by the simple competitive model and that the displacement of the two lines could be due to the steady-state effect.

Furthermore when muscle contraction assays with and without lumen perfusion were compared (an experimental procedure not available to investigation with the acid secretion assays) the Schild plots were also found to be significantly displaced in parallel. Presumably the deviation was due to the steady-state conditions imposed by lumen-perfusion and it seems reasonable to expect this. This factor would be even more marked if satisfactory contraction and secretion assays could be compared. Interestingly, Thorpe & Durbin (1972) also reported a low estimate of the pA_2 for atropine versus acetylcholine on the isolated gastric mucosal sheet preparation of the bull-frog. They concluded that the gastric cholinoceptors were intermediate in sensitivity for atropine between ileum 'muscarinic' receptors and 'nicotinic' receptors in frog rectus where the pA_2 was 5.2. Our results and interpretation suggests that acetylcholine (and histamine) receptors are quite homogeneous with muscarinic receptors (and H_2-receptors) found in other tissues but that local tissue factors can obscure the feature of simple competitive antagonism.

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