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THE EFFECTS OF pH ON THE AFFINITY OF PIRENZEPINE FOR MUSCARINIC RECEPTORS IN THE GUINEA-PIG ILEUM AND RAT FUNDUS STRIP

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1 Dose-ratios obtained with pirenzepine on the guinea-pig ileum at 30°C are indistinguishable from those obtained at 37°C.

2 In 0.1 M NaCl at 37°C the pK_a of pirenzepine for the loss of its last ionizable proton is 8.2. The ionization of pirenzepine is therefore markedly affected by changes in pH in the physiological range.

3 In experiments with pirenzepine on guinea-pig ileum and rat fundus made over a range of pH, the dose-ratio increases with the proportion of the protonated form present. As expected, the slope of the graph of dose-ratio against proportion protonated depends on the concentration of antagonist. The changes in pH produce only small effects on dose-ratios obtained with pirenzepine monomethiodide. These effects of pH can account for some of the differences between estimates of the affinity of pirenzepine.

4 The logarithm of the affinity constant of the protonated form of pirenzepine for the receptors in guinea-pig ileum is estimated to be 6.93, compared with 6.94 for the receptors in rat fundus. However, for the non-protonated form the values appear to be below 5 for the ileum compared with about 6.4 for the rat fundus.

Introduction

There has been considerable interest in pirenzepine ever since the report that it distinguishes between different subclasses of muscarinic receptors (Hammer, Berrie, Birdsall, Burgen & Hulme, 1980). Its affinity was measured by the inhibition of radioligand binding to 'isolated' receptors obtained from cell membrane fragments and in experiments of this type with dog fundus, Hammer (1980) found that the affinity of pirenzepine for receptors in the mucosa was about 6.7 times its affinity for receptors in the smooth muscle. It had still higher affinity for receptors in calf sympathetic ganglia. In experiments in which dose-ratios were measured with functional receptors, Brown, Forward & Marsh (1980) found that its affinity for muscarinic receptors in rat isolated ganglia was 23 times that for muscarinic receptors in rat isolated ileum. For muscarinic receptors in guinea-pig ileum and atria, however, there was little difference in affinity (Barlow, Caulfield, Kitchen, Roberts & Stubley, 1981), though there were some discrepancies between the results obtained in different laboratories.

This paper is concerned with the extent to which differences in experimental conditions might account for differences in the affinity of pirenzepine. The temperature was not exactly the same in the experiments on guinea-pig ileum and atria so measurements have now been made on guinea-pig ileum at 30° and 37°C. It was also likely that the pH of the Locke solution used in one set of experiments on atria was more alkaline than in the other set, made with Krebs solution. The lower affinity of pirenzepine observed in the first set might be attributable to lower ionization of the pirenzepine. Eberlein, Schmidt & Mielenz (1982) reported pK_a values of 2.05 and 8.05 for pirenzepine in water at room temperature: we have measured the pK_a at 37°C in 0.1M NaCl and obtained similar values. Accordingly changes in pH in the region 7.5 to 8.5 will greatly influence the proportion of pirenzepine which is in the monoprotonated form (Figure 1) and should therefore affect the dose-ratio produced.

Methods

Experiments were done with the guinea-pig isolated ileum and the rat fundus preparations in which the dose-ratios produced by pirenzepine were measured over a range of pH as in previous work with the ileum with hyoscine and hyoscine-*N*-oxide (Barlow & Winter, 1981). In this work, however, experiments were carried out with more than one concentration of antagonist in order to observe the effect of concent-

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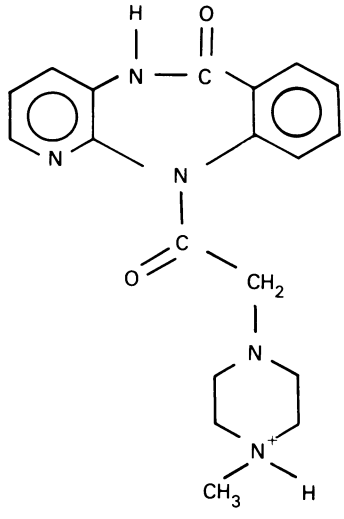

Figure 1 The monoprotonated form of pirenzepine.

ration, B, on the slope of the graph of dose-ratio, DR, against the proportion of pirenzepine protonated. If the affinity constant of the protonated form is K_i and that of the non-protonated form is K_u, and a fraction X is protonated, the observed dose-ratio,

$$\begin{aligned} DR &= 1 + XBK_i + 1 + (1-X)BK_u - 1 \\ &= 1 + BK_u + XB(K_i - K_u) \end{aligned}$$

so the graph of dose-ratio against X should be a straight line the slope of which will depend on the concentration of antagonist, B, as well as on the difference between the affinity constants of the protonated and non-protonated forms. Alternatively the equation can be written

$$\frac{DR-1}{B} = K_u + X(K_i - K_u)$$

so the graph of apparent affinity constant against X should also be a straight line along which all of the points should lie, regardless of the concentration of antagonist. From these graphs an attempt has been made to assess the separate affinities of the monoprotonated and nonprotonated forms for the receptors.

The guinea-pig isolated ileum

The ileum was set up as described by Edinburgh Staff (1974) with the responses recorded isotonically and a load of about 0.5 g. Carbachol was used as agonist, added by machine once every 90 s and allowed to act for 30 s, as in previous work (Barlow & Burston, 1979). Hexamethonium (0.28 mM) was present in all experiments. Alternate small and large control responses were obtained, usually with 0.1 and 0.2 μM carbachol, and when these were regular the ileum was exposed to a solution of the antagonist and the concentration of carbachol was increased to try to obtain responses which roughly matched the controls. When these were regular, usually after 10–20 min with pirenzepine, the size of the responses could be used to obtain an estimate of the exact dose-ratio (Edinburgh Staff, 1974).

To study the effects of temperature experiments were done at 30° and at 37°C in aerated Tyrode solution. The effects of pH were studied at 37°C: some experiments were done using modified Tyrode solutions as described by Barlow & Winter (1981). These all contained (mM): NaCl 137, KCl 2.7, $MgSO_4$ 1.0, $CaCl_2$ 1.8, glucose 5 and hexamethonium 0.28. The more acidic solution also contained NaH_2PO_4 0.84 and $NaHCO_3$ 5.9 mM; the more alkaline solution contained glycine 50 mM and sodium hydroxide 5 mM instead of phosphate and bicarbonate. Both solutions were aerated with 95% O_2 and 5% CO_2. It was found that similar changes in pH could also be obtained simply by using normal Tyrode solution (Edinburgh Staff, 1974) and changing the aerating gas from 95% O_2 plus 5% CO_2 to pure O_2 and many experiments were done in this way. In all experiments the pH was measured after the solution had been in contact with the tissue.

The rat fundus strip (Vane, 1957)

The rat fundus strip was set up as described by Edinburgh Staff (1974) with the responses recorded isotonically but with an increased load (about 2 g). Carbachol was agonist, added by machine (operated from a PET computer) once every 5 min and allowed to act for 2 min. In these conditions it was not necessary to increase the load to stretch the preparation after a contraction. Experiments were carried out at 37°C with the same modified Tyrode solution, aerated with 95% O_2 and 5% CO_2, as was used with the ileum and the pH was measured after the solution had been on contact with the tissue. Because the log dose-response curve was flatter with this tissue than with the ileum, four concentrations of agonist were tested, usually 0.12, 0.18, 0.28 and 0.42 μM, with the order such that the responses were alternately small and large. When these were regular the preparation was exposed to the antagonist and the concentration of carbachol was increased, as in the experiments on the ileum. The exact dose-ratio was estimated by fitting the responses, Y, by least-squares to the logistic expression

$$Y = M \frac{A^p}{A^p + K^p}$$

assuming that the slope of the dose-response curve is unaltered by the antagonist (i.e. p is common to both

Table 1 Effect of temperature on dose-ratio of pirenzepine

	2 μM	10 μM	20 μM	50 μM	100 μM
37°C	10.2 ±0.5 (8)	45.1 ±1.8 (8)	87.1 ±5.1 (2)	223 ±11.5 (4)	429 ±31 (2)
30°C	11.7 ±0.4 (2)	44.4 ±5.8 (2)			458 ±43 (2)

Mean dose-ratios (±s.e.) are shown for the concentration of pirenzepine and temperature indicated.
A least-squares fit of log (dose-ratio − 1) against log (antagonist concentration) gives: slope = 0.990; X-intercept (log K) = 6.665, r = 0.996 at 37°C and slope = 0.963, X-intercept (log K) = 6.742, r = 6.742, r = 0.997 at 30°C.

lines and the dose-ratio is given by the ratio of the values of K; Waud & Parker, 1971; Barlow 1975).

Measurement of pK_a

This was determined electrometrically with a Metrohm Model E 500 pH meter, as in previous work (Armstrong & Barlow, 1976: Barlow & Burston, 1979). The calculations incorporate activity coefficients based on the ionic strength and should estimate the thermodynamic pK_a. The temperature was 37 ± 0.1°C and the experiments were carried out in 0.1 M NaCl.

Results

The dose-ratios obtained for pirenzepine on the guinea-pig ileum at 30°C are indistinguishable from those at 37°C and are consistent with competitive antagonism (Table 1).

The results of the electrometric titrations (Table 2) are very similar to those of Eberlein *et al.* (1982) and confirm that at physiological temperature, ionic strength and pH, pirenzepine is largely present as the singly protonated species shown in Figure 1 but there is a proportion which is not protonated at all. At pH 8.2 this will be 50%, so differences in the pH at which experiments are carried out could well account for some of the differences in dose-ratio observed. From the pK_a, taken as 8.20, and the pH of the fluid in contact with the tissue, the proportion of the protonated form was calculated and the dose-ratio was plotted against this (Figure 2). As expected there is a separate line for each concentration of antagonist.

The change in pH could itself alter the dose-ratio by affecting the receptors but the results obtained with hyoscine methobromide by Barlow & Winter (1981) indicate that for the guinea-pig ileum this effect is small: for the concentration of hyoscine methobromide used (50 nM), the graph of the dose-ratio against pH indicated a change from a dose-ratio of 355 at pH 7.6 to 350 at pH 8.6. Similar experiments with guinea-pig ileum and 10 μM pirenzepine monomethiodide (Figure 3) suggest a bigger effect, from 107 at pH 7.6 to 88 at pH 8.6. However, the value of Student's t test for $r = -0.31$ and 14 results is 1.12 so the probability that the slope is different from zero is only about 0.25. The slope is certainly very small compared with the changes shown in Figure 2.

Discussion

The results are affected by errors in the assessment of the pH as well as those usually associated with measuring a dose-ratio. As has has been noted previously (Barlow & Winter, 1981) it is remarkable how alkaline solutions in contact with ileum can become and there must be considerable uncertainty about the exact pH at the receptor. There is the further uncertainty about the exact effect of pH on the receptor

Table 2 pK_a values of pirenzepine by electrometric titration at 37°C in 0.1 M NaCl

	pK_1	pK_2
5.5 mM	3.18 ± 0.04 (9)	8.22 ± 0.02 (8)
9.8	2.94 ± 0.02 (14)	8.23 ± 0.01 (15)
15.4	2.70 ± 0.04 (3)	8.16 ± 0.01 (15)

The material used was the dihydrochloride. The initial concentration is shown and the mean (±s.e.) of the number of estimates of pK_a obtained in each titration.

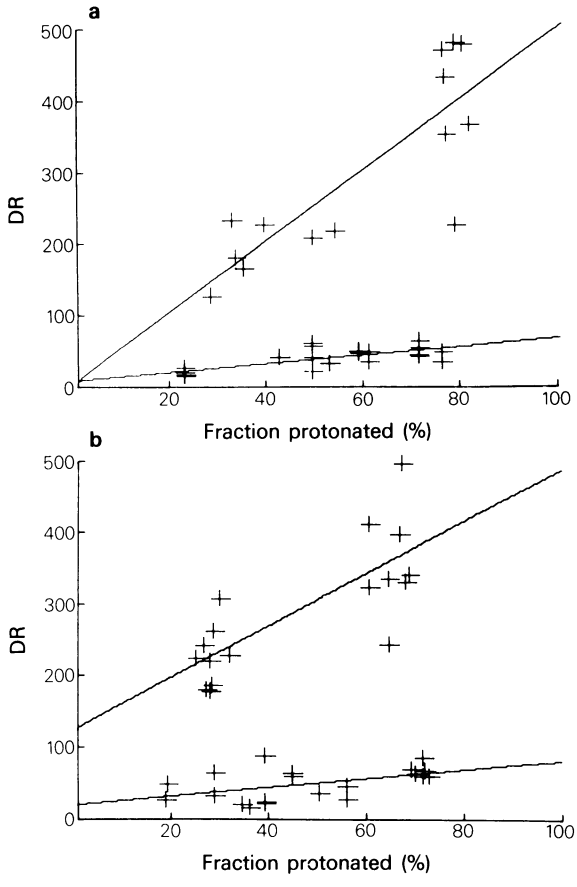

Figure 2 Values of dose-ratio (DR) at 37°C, plotted against the fraction protonated (X as %), calculated from the pH and the pK_a of pirenzepine (8.20). The upper section (a) shows results on the guinea-pig ileum: the lower section (b) shows results on the rat fundus. The flatter line in each is for 10 μM pirenzepine and the steeper line is for 50 μM pirenzepine. These lines were obtained by a least-squares fit of dose-ratio to percentage protonated and have the following equations: (a) Ileum, 10 μM pirenzepine, $DR = 0.610X + 6.8$: $r = 0.76$, 27 results: s.e. slope 0.104: s.e. constant 5.5: DR for X = 100%, 68; 50 μM pirenzepine, $DR = 4.999X + 6.6$: $r = 0.84$, 15 results: s.e. slope 0.883: s.e. constant 53: DR for X = 100%, 506. (b) Fundus, 10 μM pirenzepine, $DR = 0.625X + 19.4$: $r = 0.52$, 20 results: s.e. slope 0.244: s.e. constant 12: DR for X = 100%, 81; 50 μM pirenzepine, $DR = 3.638X + 125$: $r = 0.78$, 17 results: s.e. slope 0.766: s.e. constant 37: DR for X = 100%, 489.

itself. It is, nevertheless, clear that increasing the concentration of pirenzepine increases the slope of the graph of dose-ratio against proportion ionized. For the results on the rat fundus, multiplying the concentration by 5 appears to multiply the slope by

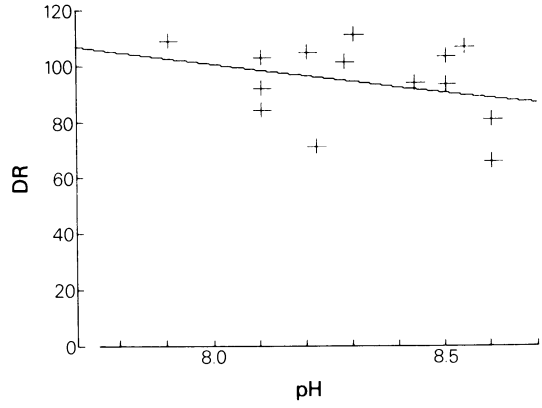

Figure 3 The effect of pH on dose-ratios produced by 10 μM pirenzepine monomethiodide. The line is the least-squares fit of dose-ratio (DR) to pH and has the equation: $DR = -19.4\,pH + 254$: $r = -0.307$, 14 results.

5.8: for those on the ileum this change in concentration appears to multiply the slope by 8.2 but with the variance attached to the estimates of the slopes it is doubtful whether this difference has any appreciable significance.

The estimates of the dose-ratios for the fully ionized form of pirenzepine are very similar for the two tissues, 68 and 81 for 10 μM solutions and 506 and 489 for 50 μM, corresponding to log K between 6.8 and 7.0. When estimates of affinity constant ($\times 10^6$) are plotted against percentage ionization the lines obtained by least-squares have the equations $K = 0.084X + 0.016$: $r = 0.76$ (42 results) for the guinea-pig ileum and $K = 0.061X + 2.52$: $r = 0.56$ (37 results) for rat fundus.

These give estimates of log K for the protonated form of 6.93 for the guinea-pig ileum and 6.94 for the rat fundus. From the considerable effect of pH on affinity it is clear that differences between the estimates of log K for guinea-pig atria of 6.71 and 6.20 reported by Barlow *et al.* (1981) could well have arisen simply from the differences in pH already mentioned. Literature values of log K for pirenzepine obtained at pH 7.6 will apply to solutions containing only 80% of the protonated form and are likely to be underestimated by 0.1 log units. Values of log K calculated from the results shown in Table 1, for instance, probably refer to about 60% protonation.

The results with the rat fundus suggest that the non-protonated form of pirenzepine has appreciable affinity with log K estimated to be 6.26 or 6.40 from the graphs of dose-ratio against ionization: from the graph of K against X the value is 6.40. However, with the guinea-pig ileum this form appears to have low affinity. From the graphs of dose-ratio against ionization, log K is estimated to be 5.76 or 5.05: from the

graph of K against X the value is only 4.20. Although these estimates must be very approximate they suggest that the contribution made by the positive charge to the affinity of pirenzepine may not be the same at the receptors in the two types of tissue.

For its size pirenzepine does not have very high affinity for muscarinic receptors in the guinea-pig ileum, atria and rat fundus; for instance, log K for propantheline, which is also tricyclic, is approx. 8.5 (Edinburgh Staff, 1974). Possibly the rigidity of pirenzepine, associated with the amide bond in the side-chain as well as its tricyclic nucleus, reduces binding to these receptors. The much higher affinity for other muscarinic receptors such as those in rat ganglia is more what might be expected for a molecule of this size.

This work establishes the extent to which differences in estimates of the affinity of pirenzepine for muscarinic receptors may be due to differences in the pH at which they were made. Estimates observed with receptors in tissues such as rat ganglia are clearly well outside this range.

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