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A quantitative study of the effects of some muscarinic antagonists on the guinea-pig olfactory cortex slice

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1 Muscarinic depression of the electrically-evoked surface-negative field potential (N-wave) was measured in guinea-pig olfactory cortex slices maintained *in vitro*.

2 The effects of three muscarinic receptor antagonists, pirenzepine, atropine and gallamine on this muscarinic response were analysed in detail.

3 Pirenzepine was a potent competitive antagonist of carbachol (CCh)-evoked responses. Schild plot analysis yielded a pA_2 value of 7.9 (Schild slope constrained to unity). A similar analysis for atropine versus CCh responses gave a pA_2 of 8.9.

4 Combination experiments using pirenzepine and atropine produced dose-ratio shifts close to those expected for two antagonists competing for a similar receptor site.

5 Gallamine was only a weak antagonist of responses to CCh.

6 Oxotremorine behaved as a competitive antagonist at this muscarinic receptor ($pA_2 = 6.1$).

7 It is concluded that the presynaptic muscarinic receptor mediating depression of the N-wave in the olfactory cortex slice is of the M_1-subtype.

Introduction

Pirenzepine is currently the most useful agent available for distinguishing muscarinic receptor subtypes (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). Although its selectivity for M_1- over M_2-receptors is only twenty fold, it is a competitive antagonist and thus its affinity can be measured by pharmacological techniques in intact tissue, using the analysis of Arunlakshana & Schild (1959). It has thus been possible to confirm that the selectivity seen in radioligand binding studies (Hammer *et al.* 1980; Hammer & Giachetti, 1982) can also be observed in intact tissue. A twenty fold difference in the pA_2 value was determined for pirenzepine on the rat superior cervical ganglion compared with the ileum (Brown *et al.*, 1980b). In lower brain areas (pons) (where M_2-receptors are predominantly found; Mash & Potter, 1986) it has been shown that pirenzepine has a low pA_2 value ($\sim$6.2–6.6; Egan & North, 1985; 1986) as predicted from binding studies, but no detailed measurements have been made from cortical regions, which have a high density of M_1-binding sites showing high affinity for pirenzepine (Hammer *et al.*, 1980; Watson *et al.*, 1983). In the previous paper (Williams & Constanti, 1988) we described the properties of a muscarinic 'response' that can be measured in the guinea-pig olfactory cortex slice; namely, the muscarinic depression of the evoked surface-negative field potential (N-wave). In the present study, we have investigated quantitatively the actions of pirenzepine on this preparation, in an attempt to determine the receptor subtype responsible for generating this muscarinic response. We have also attempted to clarify the actions of oxotremorine, which appeared to antagonize the muscarinic responses (Williams & Constanti, 1988). Some of the data in this study have been reported in a preliminary form (Constanti & Williams, 1985; Williams & Constanti, 1986).

Methods

The methods used for preparation and recording from guinea-pig olfactory cortex slices are described in detail in the accompanying paper (Williams & Constanti, 1988). Schild plots (Arunlakshana &

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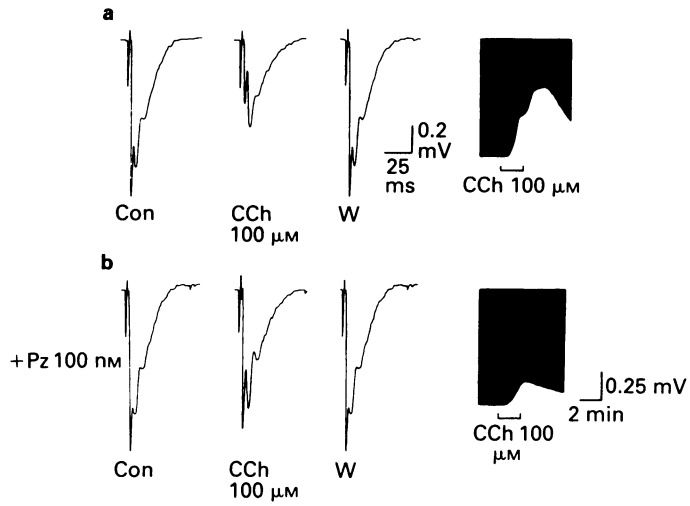

Figure 1 Effects of pirenzepine (Pz) on the carbachol (CCh)-evoked depression of the surface-negative field potential (N-wave) recorded in a guinea-pig olfactory cortex slice. (a) Control responses to 100 μM CCh. The centre field potential records show the peak N-wave depression measured after a 2 min bath-application of CCh, and on the far right is the corresponding slow-speed chart record of peak N-wave amplitude (individual downward deflections cannot be distinguished). (b) Pirenzepine (100 nM), bath-applied for 1 h, had no effect on the field potential alone, but markedly depressed the response to 100 μM CCh. Wash periods following CCh applications were 45 min.

Schild, 1959) were analysed by a weighted (1/variance) linear regression programme (courtesy of Mr S. Marsh, The School of Pharmacy) using an HP 9820A calculator. The derivation of such weighting is described in detail in Tallarida *et al.*, (1979). Slope and intercept estimates are expressed with $\pm$ standard error (s.e.). All drugs (BDH or Sigma Ltd.) were bath-applied in Krebs solution at 23–25°C. Agonists were applied for 2 min periods and antagonists were preincubated for at least 45 min before agonist testing. Pirenzepine was obtained from Karl Thomae GmbH, FRG.

Results

Action of pirenzepine

In the experiment shown in Figure 1, control responses were obtained to a 100 μM dose of carbachol (CCh) that produced a ~50% reduction of the field potential after a 2 min application (see Williams & Constanti, 1988). When consistent responses had been obtained to this dose (Figure 1a), the slice was perfused with 100 nM pirenzepine for 1 h and a 100 μM CCh dose reapplied (Figure 1b). Clearly, in the presence of pirenzepine, there was a marked reduction in the action of CCh. Note also, that pirenzepine itself had no action on the field potential amplitude. This depression of the CCh response could be overcome by increasing the agonist dose (not illustrated). The effect of pirenzepine was not, however, readily reversible even after 6 h of washout. In one set of experiments, the effects of different pirenzepine incubation periods was studied. In these experiments, pirenzepine was perfused for a set time and then a test dose of CCh applied, followed after a period of 45 min with a second test dose. If the time between the start of pirenzepine application and CCh test dose was less than 30 min, the first CCh test dose gave a larger response than subsequent doses. If pirenzepine was preapplied for 1 h however, all test CCh doses gave the same response. In all the experiments described here using pirenzepine (and also atropine and gallamine), a preincubation of 1 h was used before agonist testing.

To determine the pA_2 value for an antagonist by the Arunlakshana-Schild technique the agonist concentration required to elicit a set response under control conditions is compared to that concentration required in the presence of the antagonist (i.e. the dose-ratio). Conventionally, this is estimated by measuring the parallel shift in the agonist dose-response curve in the presence and absence of antagonist. Unfortunately, the long duration and prolonged washout time of the muscarinic response precluded the measurement of full dose-response curves in the presence and absence of antagonist. Instead, in any one experiment, two doses of agonist were chosen to

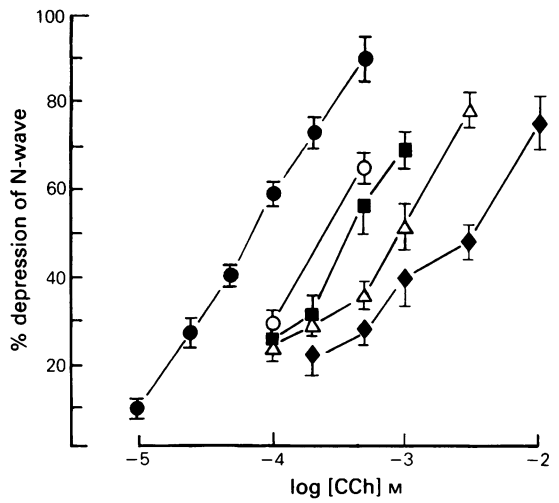

Figure 2 Dose-response curves for carbachol (CCh) showing percentage depression of peak N-wave amplitude (ordinate scale) versus log CCh concentration (abscissa scale) measured in control solution (●) and in the presence of increasing concentrations of pirenzepine (Pz): Pz 20 nM (○); 50 nM (■); 100 nM (△); 500 nM (◆). Note that pirenzepine shifted the CCh dose-response relation to the right in a competitive-type manner. Points represent means of pooled data from 27 experiments with s.e. mean shown by vertical lines.

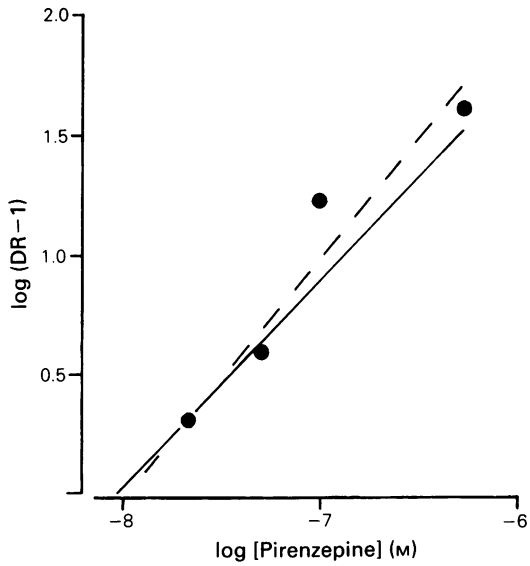

Figure 3 Schild plot for the antagonism of cabachol (CCh) responses by pirenzepine. Ordinate scale: log (DR − 1), where DR = dose ratio measured from the shift of CCh dose-response lines by antagonist. Abscissa scale: log molar concentration of pirenzepine. Points are means obtained from 5–10 individual experiments where dose-ratio shifts produced by a single pirenzepine concentration were determined. Continuous 'best fit' line was estimated by weighted (1/variance) linear regression allowing both slope and intercept to vary; broken line is least squares estimate constrained to unity slope (see Table 1).

represent the control dose-response relation (usually points at 20–35% and 60–80% field depression). After a few consistent responses to these doses, antagonist was applied for 1 h and a second dose-response line obtained. The dose-ratio was measured from the shift in the dose-response line. Pooled data from 27 such experiments with various concentrations of pirenzepine are shown in Figure 2. Clearly, pirenzepine produced a dose-dependent rightward shift in the CCh dose-response curve. The curves remained approximately parallel and there was no apparent depression of the maximum response, suggesting that pirenzepine was acting in a competitive manner.

To quantify the antagonism of pirenzepine, data from the experiments illustrated in Figure 2 were used to construct a Schild plot (Figure 3). For each pirenzepine dose, a mean dose-ratio was measured (5–10 experiments per concentration). Lines were determined by linear regression using a 1/variance weighting (Tallarida *et al.*, 1979). The best fit line had a slope of 0.90 ± 0.14, a value not significantly different from unity ($P < 0.05$), suggesting that pirenzepine was indeed acting as a competitive receptor antagonist. The pA_2 value was 8.11 ± 0.14 according to this line. Another, possibly more accurate, method of determining the pA_2 is to constrain the regression line to a slope of one and then measure the intercept on the abscissa when log (DR − 1) = 0 (Tallarida *et al.*, 1979). Using this analysis, a pA_2 value of 7.99 ± 0.12 was obtained. The pA_2 values estimated by either method were not significantly different from each other ($P < 0.05$).

Combination experiments

A Schild plot was constructed for the 'classical' competitive muscarinic antagonist atropine (not shown), using the same protocol as was used for pirenzepine. Again, a linear Schild plot was obtained (slope 0.86 ± 0.28), and pA_2 values of 9.06 ± 0.24 using a 'best-fit' line or 8.87 ± 0.15 from the constrained plot were estimated (Table 1).

Although a unity slope from a Schild plot suggests a competitive type interaction, such a value could be obtained fortuitously. Competitive antagonists must, by definition, produce slopes of one, but non-competitive antagonists could yield any slope value,

Table 1 Summary of data obtained from Schild plots

Antagonist	pA_2 (± s.e.) Best fit	Constrained	Slope of 'best fit' Schild plot (± s.e.)	No. of expts
Pirenzepine	8.11 ± 0.14	7.99 ± 0.12	0.90 ± 0.14	27
Atropine	9.06 ± 0.24	8.87 ± 0.15	0.86 ± 0.28	5
Oxotremorine	6.30 ± 0.13	6.13 ± 0.04	0.87 ± 0.18	5

'Best fit' lines to Schild plots were determined by linear regression (as described in Methods section). pA_2 values (± standard error) were determined from the x-axis intercept, i.e. when $\log(DR - 1) = 0$.

including unity, partly depending on the 'receptor reserve' of the tissue (Barlow, 1980). Fortunately, receptor theory provides another check for competitiveness of antagonists. If two competitive antagonists are applied together, their combined dose-ratio should be equal to the sum of their individual dose-ratios minus one, while if they act at independent sites their combined dose-ratio is the product of the individual dose-ratios (see Barlow, 1980). From the data presented so far, both pirenzepine and atropine appeared to act as competitive antagonists at the cortical muscarinic receptor. To confirm this observation, some experiments were performed with these two antagonists acting in combination.

Two point dose-response lines were constructed under control conditions and then in the presence of 10^{-8} M atropine alone, or 10^{-7} M pirenzepine alone or both antagonists combined. Using experimental data for individual antagonist applications, the dose-ratios predicted for combination were calculated for the two models (single or independent site). The experimentally observed combined mean dose-ratio (32.6) was very close to that calculated for two antagonists acting at a single site (26.5) and clearly differed significantly from the value predicted by the independent site model, 165.5 (t test, $P < 0.05$). These data are summarised in Table 2.

Action of gallamine

The antimuscarinic properties of the skeletal neuromuscular blocking agent gallamine appear selective toward muscarinic receptors found in the myocardium (Clark & Mitchelson, 1976). It was therefore of interest to investigate the sensitivity of the cortical muscarinic receptor to gallamine.

Low doses of gallamine (1–10 μM) had no detectable effect on responses to CCh. At higher concentrations however, gallamine had a direct effect on the field potential producing up to a 25% enhancement of the population e.p.s.p., an effect that was well maintained during gallamine perfusion, but was readily reversed on washing. The mechanism of this enhancement was not investigated further, though a similar effect was observed when the K^+ channel blocker, tetraethylammonium, was applied (> 1 mM) (data not shown). At gallamine doses of 30 μM, a small reduction in responses to CCh was observed, and at 100 μM this effect was quite pronounced. A total of 7 experiments were conducted with gallamine; one at 1 μM, and two each at 10, 30 and 100 μM. One such experiment is shown in Figure 4(a,b). Gallamine markedly reduced the response to CCh; also note that gallamine itself increased the field potential amplitude by ~10%. The data from this experiment are summarised in the form of dose-response curves in Figure 4c.

Antagonism by oxotremorine

Experiments described in the accompanying paper (Williams & Constanti, 1988) suggested that oxotremorine could act as a muscarinic antagonist at this receptor. To investigate this phenomenon further, some experiments were undertaken using an antagonist protocol identical to that used to study atropine and pirenzepine. In contrast to pirenzepine and atropine however, a preincubation time of only 15 min was required before 'full' antagonism was seen. Assuming that the action of oxotremorine was mediated through an interaction at the muscarinic receptor, the surmountability of its antagonism was tested (Figure 5). Control responses to 100 μM CCh were obtained and then repeated in the presence of 1 μM oxotremorine; oxotremorine clearly reduced the response to CCh, although it had no detectable effect when applied alone. This antagonism could be partially overcome by increasing the agonist dose, suggesting a competitive-type action.

To test further the mode of antagonism of oxotremorine, a series of doses were tested. In all cases, oxotremorine appeared to produce a parallel shift in the CCh dose-response lines. These data were transformed into a Schild plot (Figure 6). The best fit regression line had a slope of 0.87 ± 0.18 (not significantly different from unity). The abscissal intercept of this line when $\log(DR - 1) = 0$, yielded a pA_2 value of 6.30 ± 0.13, while the line constrained to unity slope gave a pA_2 of 6.13 ± 0.04 (Table 1).

Table 2 Summary of dose ratio (DR) data from antagonist combination experiments

Antagonist combination	*Mean dose-ratio (DR)* (± s.e.)	*No. of expts*
A *Pirenzepine + atropine*		
Atropine 10^{-8} M	8.9 ± 1.2 (DR_1)	6
Pirenzepine 10^{-7} M	18.6 ± 4.1 (DR_2)	10
Predicted single-site DR ($DR_1 + DR_2 - 1$)	26.5	
Predicted independent-site DR ($DR_1 \times DR_2$)	165.5	
Atropine 10^{-8} M + pirenzepine 10^{-7} M	32.6 ± 9.4	4
B *Atropine + oxotremorine*		
Atropine 10^{-8} M	8.9 ± 1.2	6
Oxotremorine 10^{-5} M	8.4 ± 1.3	4
Predicted single-site DR	16.3	
Predicted independent-site DR	73.9	
Atropine 10^{-8} M + oxotremorine 10^{-5} M	19.7 ± 5.1	4

Although Schild plot data suggested oxotremorine was acting competitively, further verification was attempted by combination studies with atropine as the second competitive antagonist. Once again, individual dose-ratios for the antagonists applied alone were measured and used to predict single site and independent site dose-ratios; these values were compared with experimentally observed values for two antagonists. The data are summarized in Table 2. It is clear that oxotremorine behaved more in accordance with the single site model i.e. it was a competitive muscarinic antagonist.

Discussion

The results of this study clearly indicate that pirenzepine is a potent competitive antagonist at the muscarinic receptor present in the guinea-pig olfactory cortex slice. The estimated pA_2 value for pirenzepine (~8) is in close agreement with other pharmacological studies where tissues containing mainly M_1-receptors have been investigated, including: rat superior cervical ganglion (~8.4, Brown *et al.*, 1980b) and myenteric neurones (~8, North *et al.*, 1985); also radioligand binding methods (Hammer *et al.*, 1980) and biochemical studies of phosphoinositide turnover (Lazareno *et al.*, 1985). It is important to note that the muscarinic receptors described in our study are most probably located presynaptically on terminals of the lateral olfactory tract (Williams *et al.*, 1985). Although several studies have suggested that presynaptic receptors in other areas of the nervous system are of the M_2-type, these appear to be located on cholinergic terminals (Choo *et al.*, 1985; North *et al.*, 1985). For non-cholinergic terminals, receptors appear to be of the M_1-type (Raiteri *et al.*, 1984; De Belleroche & Gardinier, 1985), and our data would support these findings. Autoradiography with ^{3}[H]-pirenzepine has revealed that approximately 75% of muscarinic binding sites in the olfactory cortex are of the M_1-subtype (Cortes & Palacios, 1986). The postsynaptic muscarinic receptors located on olfactory pyramidal neurones and coupled to potassium channels (slow calcium-dependent K^+ current and M-current) may be of the M_2-subtype (Constanti & Sim, 1987a, b) and therefore presumably contribute to the M_2-binding sites (some M_1-sites may also be postsynaptically located).

The differences in affinity for pirenzepine seen between M_1- and M_2-receptors cannot be explained by postulating different conformations of the same receptor since pirenzepine should bind to the receptor in its non-activated state. This interpretation is strongly confirmed by the elucidation of the primary sequence of the M_1-receptor (Kubo *et al.*, 1986) and the finding that there were no hybridizations between the complementary DNA for the M_1-receptor and RNA from the myocardium and medulla-pons.

Although the actions of gallamine (a 'cardioselective' M_2-receptor antagonist) were not investigated extensively, it was clear that it had a low potency against the muscarinic response. In the myocardium, Clark & Mitchelson (1976) showed that 10 μM gallamine produced a non-competitive shift of the acetylcholine dose-response curve to the right by about 10 fold, clearly a much more dramatic shift than observed in the present study. This further supports our hypothesis that the cortical receptor described here is of the M_1-subtype. The observed increase in the population e.p.s.p. seen in the presence of gallamine alone could not be accounted for by antagonism of endogenous acetylcholine action since neither atropine or pirenzepine ever had any effect *per se* on field potential amplitude, nor did (+)-tubocurarine (Williams *et al.*, 1985). A more likely explanation is that gallamine was reducing

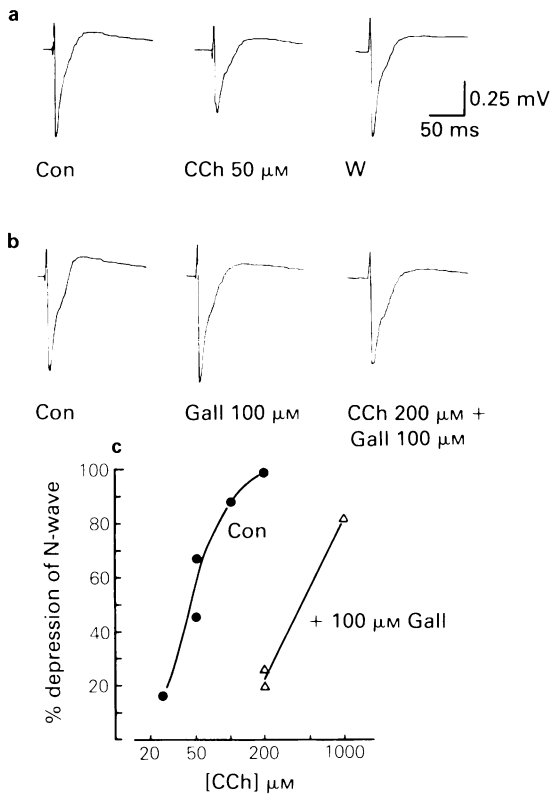

Figure 4 Effect of gallamine (Gall) on carbachol (CCh)-evoked depression of the N-wave. (a) Control response to 50 μM CCh (2 min) showing a clear depression of the field potential (wash period = 45 min). (b) Addition of 100 μM gallamine (60 min) produces a slight *increase* in the peak N-wave amplitude. In the presence of gallamine the responses to CCh are greatly diminished such that a 200 μM CCh dose now produces a smaller % depression of the field potential than produced by the control 50 μM dose. (c) The results of this experiment are summarised in the form of log dose-response curves showing % N-wave depression vs. CCh concentration (log scale). The CCh dose-response curve was shifted to the right.

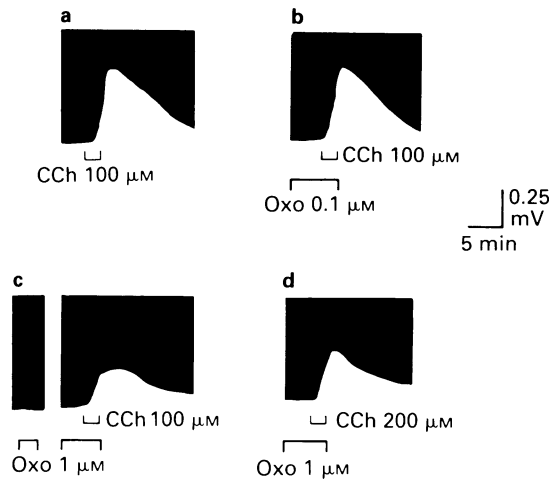

Figure 5 Effect of oxotremorine (Oxo) on carbachol (CCh)-evoked depression of N-wave. Slow chart speed records of peak N-wave amplitude show: (a) Control response to 100 μM CCh; (b) a 15 min preapplication of 0.1 μM oxotremorine does not reduce the sensitivity of the slice to CCh; (c) a 1 μM oxotremorine preapplication (15 min) however, markedly reduces the CCh response; (d) this antagonism is partially overcome by doubling the dose of CCh.

presynaptic potassium conductances, as reported by Smith & Schauf (1981).

Oxotremorine was found, by Schild analysis and combination studies, to act in a manner indistinguishable from a 'classical' competitive antagonist. This finding was somewhat curious since oxotremorine is known to be a potent agonist on other tissues e.g. ileum (Ringdahl & Jenden, 1983), locus coeruleus (Egan & North, 1985) and ganglia (Brown *et al.*, 1980a; North *et al.*, 1985). However, according to Stephenson (1956), an antagonist is an agonist with zero efficacy. Agonist efficacy can vary between

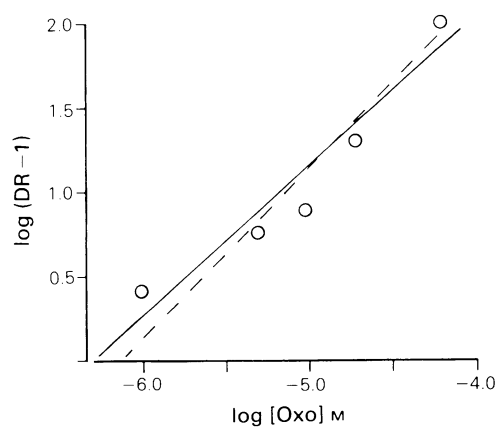

Figure 6 Schild plot for oxotremorine (Oxo) versus carbachol (CCh). Points represent dose-ratio shifts determined from 5 individual experiments where a single dose of oxotremorine was tested. The continuous line shows the 'best fit' and the broken line the constrained fit (slope = 1), both obtained by linear least squares regression. The corresponding pA_2 values ($\pm$ s.e.) were 6.30 $\pm$ 0.13 and 6.13 $\pm$ 0.04 respectively (Table 1).

tissues and could conceivably be zero at some sites. It is possible that oxotremorine was exerting some agonist action, but the magnitude of the response produced was below detection threshold in our study. Interestingly, the pA_2 measured for oxotremorine (~ 6) corresponded well with an estimated affinity constant in guinea-pig ileum, where it is a 'full' agonist ($-\log K_A = 6.17$, Ringdahl, 1984).

In conclusion, our experiments represent the first pharmacological measurement of a pA_2 for pirenzepine acting at a functional M_1-receptor in an 'intact' brain slice preparation. These data confirm previous radioligand binding studies suggesting there was a high affinity pirenzepine binding site in the cortex (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). We have shown, by use of Schild analysis and combination experiments, that pirenzepine acts as a purely competitive antagonist at this muscarinic receptor. It is unclear however, what physiological function these presynaptic muscarinic M_1-receptors subserve, and how the depression in synaptic transmission is mediated. Whatever the physiological significance of this response, the olfactory cortex slice provides a useful pharmacological model for studying central muscarinic receptors, and for testing new potentially selective M_1-receptor agonists and antagonists.

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