*Br. J. Pharmacol.* (1988), **94**, 1270–1274

# Dicyclomine discriminates between $M_1$- and $M_2$-muscarinic receptors in the guinea-pig ileum

H. Kilbinger & A. Stein

Pharmakologisches Institut der Universität, Obere Zahlbacher Str. 67, D-6500 Mainz, F.R.G.

1 The affinity of the antagonist dicyclomine for subtypes of muscarinic receptors has been assessed in the myenteric plexus-longitudinal muscle preparation of the guinea-pig.

2 Dicyclomine had a high affinity ($pA_2$ 9.13) for the neuronal $M_1$-receptor whose activation by pilocarpine causes an increase in acetylcholine release. Dicyclomine had a low affinity for both the prejunctional $M_2$-receptor ($pA_2$ 7.61) mediating inhibition of the electrically-evoked acetylcholine release and the postjunctional $M_2$-receptor ($pA_2$ 7.21).

3 It is concluded that dicyclomine distinguishes between $M_1$- and $M_2$-muscarinic receptors in functional experiments.

## Introduction

Muscarinic receptors have been subclassified into $M_1$- and $M_2$-receptors according to their affinities for the antagonist pirenzepine (see reviews by Birdsall & Hulme, 1985; Eglen & Whiting, 1986). Both subtypes have been detected in the guinea-pig ileum. The muscular receptor mediating contraction of the longitudinal muscle as well as the prejunctional autoreceptor mediating inhibition of the depolarization-evoked acetylcholine release are of the $M_2$-subtype (Kilbinger *et al.*, 1984). $M_1$-receptors are present on the cell bodies of enteric neurones, and mediate a slow membrane depolarization (North *et al.*, 1985) and an increase in acetylcholine release (Kilbinger, 1984; Kilbinger & Nafziger, 1985).

Recent radioligand binding studies have shown that not only pirenzepine but also the antagonist dicyclomine is able to distinguish between $M_1$-sites in the central nervous system and the $M_2$-sites in the heart (Kenny *et al.*, 1985; Nilvebrant & Sparf, 1986; Giachetti *et al.*, 1986; Doods *et al.*, 1987). However, affinity constants ($pA_2$ values) for dicyclomine acting on $M_1$-receptors have so far not been obtained in functional studies.

In the present investigation we have determined the $pA_2$ values of dicyclomine on $M_1$- and $M_2$-receptors in the guinea-pig ileum. The increase by pilocarpine of the release of acetylcholine was taken as a parameter for $M_1$-receptor activation. Inhibition by either pilocarpine or oxotremorine of the electrically-evoked acetylcholine release, and increase in longitudinal muscle tension by oxotremorine were considered as parameters for $M_2$-receptor activation. The affinity of dicyclomine was determined by constructing complete concentration-response curves for the effect of the agonists in the absence and presence of various concentrations of dicyclomine. A preliminary account of this work was given at a recent symposium (Kilbinger *et al.*, 1988).

## Methods

### *Release experiments*

Longitudinal muscle myenteric plexus preparations of the proximal guinea-pig ileum were suspended isometrically in a 4 ml organ bath which contained Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, D-glucose 5.6 and choline chloride 0.001) of 37°C bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. After incubation for 30 min with [$^3$H]-choline (1.25 $\mu$Ci ml$^{-1}$) during which the tissue was stimulated electrically (0.1 Hz; 1 ms) the strips were superfused with the medium at a rate of 2 ml min$^{-1}$. Naloxone (0.1 $\mu$M) was added to the superfusate 30 min after the end of the labelling period and was present until the end of the experiment. For reasons discussed previously (Kilbinger, 1984) the facilitatory effect of pilocarpine on release of acetylcholine is more marked in the presence of naloxone. After a 60 min washout the superfusate was collected in 3 min fractions and tritium was determined by liquid scintillation spectrometry. At the end of the experi-

ment the tissue was minced with scissors and placed overnight in 3 ml of 0.4 M $HClO_4$. Radioactivity was determined in 100 $\mu$l of the supernatant. The outflow of tritium was expressed as a percentage of the tritium content of the tissue at the start of the respective collection period.

For electrical stimulation, square wave pulses (1 ms) were applied at a frequency of 1 Hz (3 min). The voltage drop between the electrodes was 11 V $cm^{-1}$. Stimulation periods started 9 min (S1) and 60 min (S2) after the end of the washout period. Pilocarpine or oxotremorine were added 30 min before S2. The outflow of tritium evoked by electrical stimulation or by pilocarpine was obtained from the difference between the total tritium outflow in the samples during and after stimulation, and the calculated spontaneous outflow; the spontaneous outflow was assumed to decline linearly from the 3 min period before to that 15 min (electrical) or 24 min (pilocarpine) after the onset of stimulation. The effects of oxotremorine or pilocarpine on the electrically-evoked outflow of tritium are expressed as the ratio of the outflow caused by S2 over that caused by S1. $EC_{50}$ values for oxotremorine and pilocarpine were calculated from the average concentration-response curves obtained from a number of determinations in individual experiments; dose-ratios were calculated at 50% of the maximal effect.

*Contraction of smooth muscle*

In separate experiments longitudinal muscle strips were suspended isometrically in Tyrode solution. Three cumulative concentration-response curves to oxotremorine were obtained at 45 min intervals. The potencies for oxotremorine did not differ significantly between the first curve ($-\log EC_{50}$: $7.53 \pm 0.09$, $n = 8$), the second ($7.42 \pm 0.06$) and third ($7.31 \pm 0.08$) curve. Dicyclomine was added in two cumulatively increasing concentrations immediately after the first concentration-response curve had been obtained. The curves in the presence of dicyclomine were not corrected for the small and insignificant decrease in potency of oxotremorine observed in control experiments. Responses of the longitudinal muscle to oxotremorine were expressed as a percentage of the maximal response in the first curve. $EC_{50}$ values were obtained from individual concentration-response curves, and dose-ratios were calculated for each experiment at the level of the $EC_{50}$.

*Statistics*

Results are given as means $\pm$ s.e.mean. The significance of differences was calculated by $t$ test. The $pA_2$ values were calculated from Schild plots by linear regression analysis (Arunlakshana & Schild, 1959).

*Drugs*

The following drugs were used: [methyl-$^3$H]-choline (NEN, Dreieich, F.R.G.); dicyclomine hydrochloride (Merrell Dow, Egham, U.K.); oxotremorine sesquifumarate (Aldrich- Europe, Nettetal, F.R.G.); pilocarpine hydrochloride (Merck, Darmstadt, F.R.G.).

## Results

Figure 1 shows the time course of the release experiments. Pilocarpine caused a transient increase in spontaneous outflow, and a decrease in the electrically-evoked outflow of tritium. Previous experiments have shown that the increase in tritium outflow evoked by electrical stimulation or by pilocarpine is due to the release of [$^3$H]-acetylcholine (Kilbinger, 1984; Kilbinger & Nafziger, 1985). For interaction experiments, dicyclomine was present in the superfusate from 30 min before S1 onwards. In the presence of 1 $\mu$M dicyclomine the outflow during S1 ($3.46 \pm 0.14\%$; $n = 12$) was slightly but significantly ($P < 0.01$) larger than in control experiments without dicyclomine ($2.39 \pm 0.09\%$; $n = 39$). Smaller concentrations of dicyclomine did not cause a statistically significant increase in the evoked outflow during S1. In the presence of 1–100 nM dicyclomine the concentration-response curve for the facilitatory effect of pilocarpine on tritium outflow was shifted in parallel to the right (Figure 2a). The curves generated in the presence of 0.1 and 1 $\mu$M dicyclomine did not reach the same maximal response as in the presence of the lower dicyclomine concentrations; however, the effects of higher concentrations of pilocarpine (> 100 $\mu$M) were not tested. From the effects of 1, 10 and 100 nM dicyclomine a $pA_2$ value of 9.25 was calculated (Figure 4). The inhibition by pilocarpine of the electrically-evoked tritium outflow was antagonized only by higher concentrations of dicyclomine (Figure 2b). From the shift of the pilocarpine curve at the level of the $EC_{50}$ (0.56 $\mu$M) in the presence of 0.1 and 1 $\mu$M dicyclomine, two individual $pA_2$ values of 7.6 and 7.8 were calculated according to $pA_2 = pA_x + \log(\text{dose ratio} - 1)$ (Schild, 1947).

The concentration-response curve for the effect of pilocarpine on the electrically-evoked outflow of tritium was rather flat with a maximal inhibition of only 35%. Therefore, the effect of dicyclomine was also studied against oxotremorine which caused a steep curve and a maximal inhibition of the evoked outflow of 77% (Figure 3a). Oxotremorine did not affect the spontaneous outflow of tritium as has been reported previously (Kilbinger, 1984). In the presence

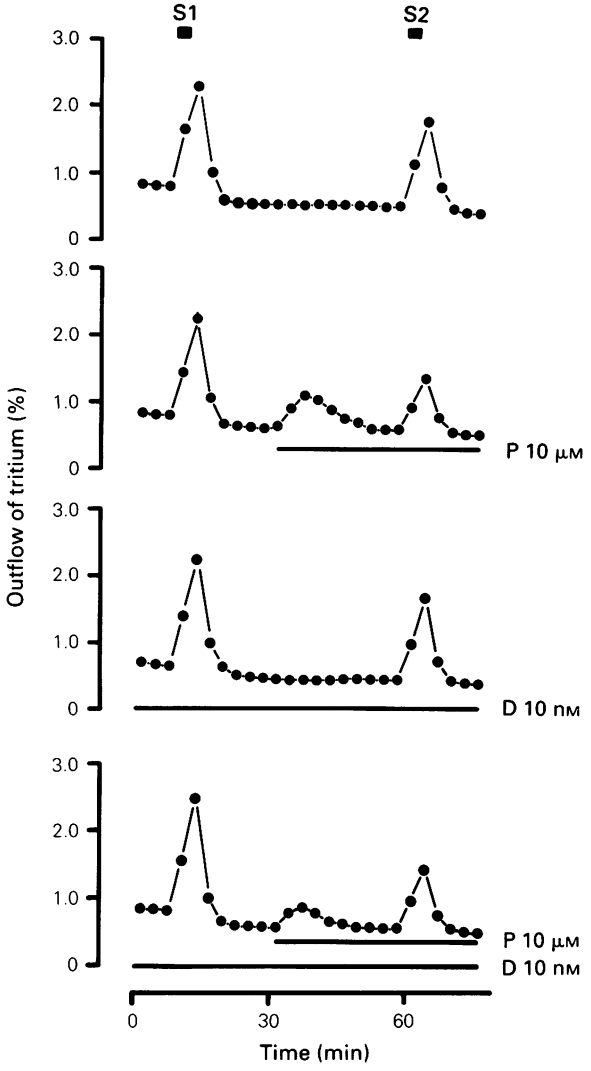


**Figure 1** Effects of pilocarpine 10 $\mu$M in the absence and presence of dicyclomine 10 nM on outflow of tritium from strips preincubated with [$^3$H]-choline. Outflow is shown as percentage of the tritium present in the strips at the start of each fraction. Electrical stimulation (1 Hz, 3 min) is indicated at the top of the figure (S1, S2). The abscissa scale starts at the end of the washout period. Horizontal bars indicate superfusion with pilocarpine (P; $n = 12$), dicyclomine (D, added 30 min before S1; $n = 5$), and P in the presence of D ($n = 5$). Upper panel, control experiments ($n = 9$). Mean values are shown ± s.e.means.

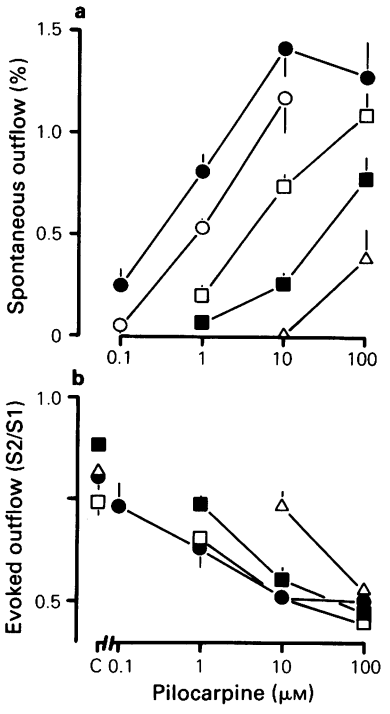


**Figure 2** Concentration-response curves for the effects of pilocarpine on spontaneous outflow (a) and on electrically-evoked outflow (b) of tritium in the absence (●) and presence of the following concentrations of dicyclomine ($\mu$M): 0.001 (○), 0.01 (□), 0.1 (■), 1 (△). The pilocarpine-induced increase in outflow is expressed as a percentage of the amount of tritium in the strips at the start of superfusion with pilocarpine. The inhibition by pilocarpine of the electrically-evoked outflow is expressed by the ratio of the outflows in the presence (S2) and absence (S1) of pilocarpine. C, control experiments. Means of 4–12 experiments with s.e. shown by vertical lines.

of increasing concentrations of dicyclomine the curve to oxotremorine was shifted in parallel to the right with no significant attenuation of the maximal effect of oxotremorine. Dose ratios were obtained at the level of the mean $EC_{50}$ value of the oxotremorine control curve (30 nM), and a $pA_2$ value of 7.58 was determined from the Schild plot shown in Figure 4. This value was similar to the $pA_2$ values obtained for the antagonistic effect of dicyclomine against the inhibition of outflow by pilocarpine.

Dicyclomine was further studied at the muscarinic receptors of the longitudinal muscle. Figure 3b shows that concentrations between 0.1 and 3 $\mu$M shifted the curve for oxotremorine in parallel to the right without appreciably altering the maximal responses, whereas at 10 $\mu$M dicyclomine the maximum was considerably depressed. From the data for the dicyclomine concentrations up to and including 3 $\mu$M, a $pA_2$ value of 7.26 (7.01–7.67 confidence limits; $n = 25$) was determined (Figure 4).

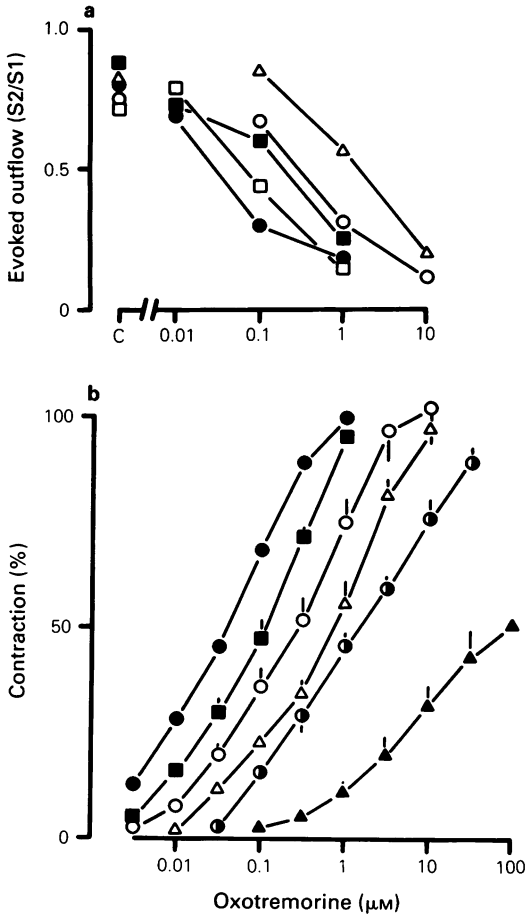


**Figure 3** Concentration-response curves for the effects of oxotremorine on electrically-evoked outflow of tritium (a) and on longitudinal muscle (b) in the absence (●) and presence of the following concentrations of dicyclomine (μM): 0.03 (□), 0.1 (■), 0.3 (○), 1 (△), 3 (◑), 10 (▲). For further details see legend to Figure 2. Means of 4–13 experiments with s.e.mean shown by vertical lines.

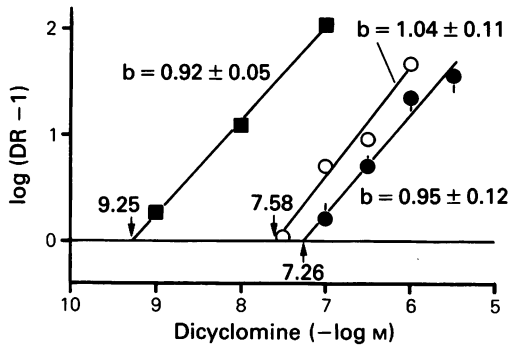


**Figure 4** Schild plot for the antagonism between dicyclomine and pilocarpine at release-enhancing $M_1$-receptors (■), and between dicyclomine and oxotremorine at prejunctional (○) and postjunctional (●) $M_2$-receptors in guinea-pig ileum. The points for the postjunctional effects of oxotremorine are the means of 6–7 experiments and the s.e.means are indicated by vertical lines. The points for the prejunctional effects of oxotremorine and for the effects of pilocarpine are obtained from the dose-ratios of the average concentration-response curves shown in Figure 2a and Figure 3a; b = mean ± s.d. of slopes of the respective regression lines. None of the slopes differed significantly from unity. Arrows indicate $pA_2$ values. When the $pA_2$ values for dicyclomine were calculated by constraining the slopes of the Schild plot to unity, the following (slightly different) values were obtained: 9.13 ± 0.06 ($M_1$-receptor), 7.61 ± 0.06 (prejunctional $M_2$-receptor), and 7.21 ± 0.07 (postjunctional $M_2$-receptor).

## Discussion

Muscarinic agonists such as pilocarpine, muscarine or methacholine cause an increase in acetylcholine release from guinea-pig myenteric plexus, which is calcium-dependent and prevented by tetrodotoxin. This enhancement has been detected irrespective of whether the release of endogenous acetylcholine was determined or the outflow of tritium from preparations that had been preincubated with [$^3$H]-choline (Kilbinger, 1978; 1984). Oxotremorine had no effect on the outflow of tritium from such preparations, and it has been suggested that oxotremorine exhibits only low potency at the release-enhancing muscarine receptors (Kilbinger & Nafziger, 1985). Pirenzepine has a high affinity for this receptor ($pA_2$ 8.5; Kilbinger & Nafziger, 1985) which by definition is thus of the $M_1$-subtype. The present paper shows that dicyclomine is also a selective antagonist on this myenteric $M_1$-receptor. Dicyclomine had an affinity 33–83 times higher for the muscarinic receptor mediating the increase in acetylcholine release than for the receptors mediating smooth muscle contraction or autoinhibition of the evoked acetylcholine release. To our knowledge, affinity constants of dicyclomine obtained in functional studies on $M_1$-receptors have not been reported so far. The present $pA_2$ value is in good agreement with the dissociation constant for dicyclomine (9.0, expressed as negative log value) found in radioligand binding experiments for the high affinity site in the rat cerebral cortex (Nilvebrant & Sparf, 1986). A lower affinity of dicyclomine to hippocampal $M_1$-sites was found by Doods *et al.* (1987) ($pK_I$ 8.0), but in these binding experiments the $pK_I$ value for pirenzepine on $M_1$-sites (7.43) was also one log

unit lower than the $pA_2$ values for pirenzepine determined in functional studies on myenteric neurones (Kilbinger & Nafziger, 1985; North *et al.*, 1985).

The inhibition by oxotremorine of the depolarization-evoked release of acetylcholine as well as the smooth muscle contraction are due to $M_2$-receptor activation (Kilbinger *et al.*, 1984). Dicyclomine in concentrations up to 3 $\mu$M demonstrated competitive antagonism against both effects of oxotremorine. The $pA_2$ values for pre- and postjunctional antagonistic effects of dicyclomine were similar, and agree well with $pA_2$ values found by others in functional studies on guinea-pig ileum (6.87; McGrath *et al.*, 1964), and on human and rabbit urinary bladder *in vitro* ($pA_2$ 7.1–7.4; Downie *et al.*, 1977). Moreover, our $pA_2$ values for dicyclomine on the $M_2$-receptors are also in good agreement with dissociation constants for the low affinity binding sites in cerebral cortex, heart or urinary bladder (7.3–7.6; neg. log of dissociation constants; Nilvebrant & Sparf, 1986). Dicyclomine itself did not enhance the evoked outflow during S1 except at a concentration of 1 $\mu$M which caused a 45% increase. This indicates that autoinhibition of acetylcholine release is of no great functional importance under the present experimental conditions, which are thus appropriate for the determination of prejunctional $pA_2$ values.

In conclusion, the present investigation shows that dicyclomine can discriminate in functional studies between $M_1$- and $M_2$-muscarinic receptors in the guinea-pig ileum.

This work was supported by the Deutsche Forschungsgemeinschaft. We thank Miss Doris Wolf for expert technical assistance, and Merrell Dow for the donation of dicyclomine. The paper contains part of the Dr. med. Thesis of A.S.

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*(Received December 21, 1987*
*Revised March 14, 1988*
*Accepted March 25, 1988)*