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# Experimental testing of Mackay's model for functional antagonism in the isolated costo-uterus of the rat

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**1** Several key predictions of a recently developed model for functional antagonism (Mackay, 1981) were experimentally tested using the rat isolated costo-uterine preparation.

**2** In the presence of the functional antagonist fenoterol (Fen), the functional affinity constants ($K_A^F$) for carbachol and oxotremorine (Oxo) were respectively 9.9 and 3.4 fold greater than their corresponding affinity constants ($K_A$). According to Mackay's model for functional antagonism, the higher $K_A^F/K_A$ ratio for carbachol indicates that this cholinoceptor agonist has a greater efficacy than Oxo. This was confirmed by using conventional pharmacological methods.

**3** As predicted from the model of functional antagonism, the plot of $K_A^F/K_A - 1$ against the fraction of cholinoceptors not irreversibly blocked by phenoxybenzamine (Pbz) was linear for both carbachol and Oxo and the lines of best fit crossed the axes at a point not significantly different from the origin.

**4** The value of 4.6 for the relative efficacy of carbachol to Oxo estimated from functional antagonism studies was comparable to the value of 5.6 calculated using the method of irreversible antagonism proposed by Furchgott (1966).

## Introduction

Many models have been developed to explain the marked changes in shape and position of agonist concentration-state curves observed to occur in the presence of functional antagonists (Ariens *et al.*, 1964; Van den Brink, 1973a,b; Ohashi, 1976; Mackay, 1981; Amidon & Buckner, 1982). Mackay (1981) applied a null method to the model of functional antagonism proposed by Van den Brink (1973a,b), to derive null equations which related together the concentrations of agonist required to induce equivalent states in a cell or tissue in the presence of different concentrations of the functional antagonist. The general form of the null equation is:

$$[A]_2/[A]_1 = \alpha + \beta[A]_2 + \gamma/[A]_1 \qquad (1)$$

$[A]_1$ and $[A]_2$ are the concentrations of agonist A which induce equivalent states in the presence of the two concentrations of functional antagonist. $\alpha$, $\beta$ and $\gamma$ are adjustable constants for each pair of agonist concentration-state curves and estimates of these constants are obtained by solving the general form of the null equation. Mackay proposed the existence of five different types of functional antagonism (Type I, II (general), IIA, IIB and III) which could be differentiated according to the values obtained for $\alpha$, $\beta$ and $\gamma$ and their relationship to each other.

Type I functional antagonism was shown by Mackay (1981) to lead to patterns of curves which were very similar to those predicted from the model proposed by Van den Brink (1973a,b); a model which satisfactorily described many experimentally observed functional interactions.

For Type I functional antagonism the parameters $(\alpha - 1)$, $\beta$ and $\gamma$ are significantly different from zero and are related such that,

$$(\alpha - 1)^2 = 4 \cdot \beta \cdot \gamma \qquad (2)$$

The estimated values of $\alpha$, $\beta$ and $\gamma$ can be used to provide an estimate for the quantity $K_A^F$ (the functional affinity constant) since for Type I functional antagonism,

$$K_A^F = (\alpha - 1)/2\gamma = 2\beta/(\alpha - 1) \qquad (3a,b)$$

However the functional affinity constant ($K_A^F$) and the affinity constant ($K_A$) are not equivalent for Type I functional antagonism but are related through the equation,

$$K_A{}^F = K_A\ (a/b \cdot f_A \cdot R_t + 1) \qquad (4)$$

where a and b are chain constants relating the initial and final stimuli and $f_A$ and $R_t$ are respectively the intrinsic efficacy of agonist A and the total concentration of receptors on which agonist A acts.

Mackay's model for functional antagonism has been quantitatively tested (Emmerson & Mackay, 1981) by examining its ability to describe cholinoceptor agonist concentration-state data obtained in the presence and absence of (−)-isoprenaline in guinea-pig isolated atrial preparations. Concentration-state data from the pair of cholinoceptor agonist curves was substituted into the general form of the null equation and multiple linear regression analysis used to estimate $\alpha$, $\beta$ and $\gamma$. On the basis that the general form of the null equation satisfactorily described the experimental curves and that the estimated values of $(\alpha - 1)$, $\beta$ and $\gamma$ were generally significant, Emmerson & Mackay (1981) suggested that the functional antagonism was Type I. However, they found that the estimated values of $\alpha$, $\beta$ and $\gamma$ did not satisfy the requirement for Type I functional antagonism that $(\alpha - 1)^2 = 4 \cdot \beta \cdot \gamma$.

Several modifications to the approach used by Emmerson & Mackay (1981) to test the model experimentally would provide a better indication of the applicability of the model. The major modifications adopted in the present study were as follows. (i) The use of the more statistically sound method of non-linear least squares regression analysis for the estimation of $\alpha$, $\beta$ and $\gamma$. (ii) The application of constrained curve-fitting methods to three (rather than a pair of) cholinoceptor agonist concentration-state curves obtained in the presence of different concentrations of the functional antagonist within individual preparations. The imposition of these constraints upon such a model may be necessary in order to obtain reasonable parameter estimates from the model (De Lean *et al.*, 1978).

In addition, in this study equation 4 was experimentally tested after rearranging it to give

$$(K_A{}^F/K_A) - 1 = (a/b) \cdot f_A \cdot R_t \qquad (5)$$

Since $R_t$ can be reduced in relation to the concentration of an irreversible antagonist such as phenoxybenzamine (Pbz), it was possible through the successive use of irreversible and functional antagonists on a series of preparations, to construct plots of $K_A{}^F/K_A - 1$ against $R_t$ for the cholinoceptor agonists, carbachol and oxotremorine (Oxo). According to Mackay's model for functional antagonism (Equation 5), the plots should be linear, have a slope of $(a/b) \cdot f_A$ and pass through the origin. In addition, the ratio of the slopes of the plots obtained for carbachol and Oxo $((a/b) \cdot f_{carbachol}/(a/b) \cdot f_{Oxo})$ is proposed to be a measure of the relative efficacies of the two agonists. This estimate of the relative efficacy obtained using functional antagonism was compared to estimates obtained by using conventional pharmacological methods (Furchgott, 1966) to test further Mackay's model for functional antagonism.

## *Methods*

### (1) *Rat isolated costo-uterine preparation*

Female Wistar rats (200–300 g) were injected intraperitoneally with 200 μg diethylstilboestrol (in ethanol, 0.1 ml) 24 h before killing them in order to induce a state of oestrus. This was confirmed by histological examination of vaginal smears (Ham & Cormack, 1979). The rats were killed by a blow to the head and exsanguinated. A body of tissue comprising the uterine horn, ovary, oviduct, kidney, costo-uterine smooth muscle band and attached fat and mesentery was removed from both the right and left sides of the animal and placed in a Petri dish containing precarbogenated low-$Ca^{2+}$ Krebs-bicarbonate solution of the following composition (mM: NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, $KH_2PO_4$ 1.03, $MgSO_4$ 0.57, $CaCl_2$ 0.54 and glucose, 11.1. Low $Ca^{2+}$ solutions were used to reduce the incidence of spontaneous activity. With the use of a dissecting microscope, the band of costo-uterine smooth muscle was identified, tied at both ends, isolated from surrounding tissue and transferred to an organ bath containing 20 ml of the physiological solution at 30°C, bubbled with carbogen (95% $O_2$/5% $CO_2$ gas mixture). The preparations were equilibrated for an hour under an applied tension of 100 mg. Changes in tension were detected by a FT-03C isometric force-displacement transducer and recorded on a Rikadenki chart recorder via a preamplifier. Carbachol was added in a cumulative manner until further drug addition produced no additional response. This cumulative concentration-state curve (CCSC) to carbachol was used to 'prime' the tissue and was not used in any calculations.

### (2) *Experimental protocol*

One of the three general experimental regimens was completed in each preparation.

(i) A control carbachol CCSC was completed ($C_1$). The preparation was incubated with Pbz (0,0.4,0.65 or 0.8 μM) for 20 min, washed repeatedly over 15 min to remove unbound Pbz, and exposed to cumulatively added carbachol ($C_2$). A third carbachol CCSC ($C_3$) was completed in the presence of the functional antagonist, fenoterol (Fen; 0.15–1.0 nM) which was incubated with the tissue for 5 min before the addition

of carbachol. The final CCSC to carbachol ($C_4$) was completed in the presence of a higher concentration of Fen (0.3–5.0 nM). Between each successive carbachol CCSC the preparation was repeatedly washed and rested for 40 min. In this series of experiments $K_A$ and $K_A^F$ vlaues for carbachol were estimated using Fen as the functional antagonist in preparations in which the population of cholinoceptors had been reduced with Pbz. The fraction of receptors not irreversibly bound with Pbz (q) was estimated by using carbachol as the agonist.

(ii) In these preparations $K_A$ and $K_A^F$ values for Oxo were estimated by applying the successive use or irreversible and functional antagonists as described in (i). The fraction of receptors not irreversibly bound to Pbz (q) was estimated using Oxo as the agonist.

(iii) In the final set of experiments the experimental procedure outlined in (i) was used; however, the value of q was estimated using Oxo as the agonist. The $K_A^F$ value for carbachol was then estimated using Fen as the functional antagonist.

### (3) *Estimation of agonist* $K_A$ *values and q*

Concentration-state data from $C_1$ (absence of Pbz) and $C_2$ (presence of Pbz) were substituted into equations 6 and 7 respectively, which describe rectangular hyperbola and allow for the presence of a threshold effect (McPherson *et al.*, 1983).

$$S_1 = \frac{(S_{m1} + S_t)\cdot[A]_1}{[A]_1 + K_1} - S_t \qquad (6)$$

$$S_2 = \frac{(S_{m2} + S_t)\cdot[A]_2}{[A]_2 + K_2} - S_t \qquad (7)$$

Equations 6 and 7 were solved simultaneously using a nonlinear least squares regression computer programme and estimates obtained for $S_{m1}$ and $S_{m2}$ (the maximum state levels induced by agonist A in the absence and presence of Pbz respectively), $K_1$ and $K_2$ (the concentrations of agonist A producing 50% of $(S_{m1} + S_t)$ and $(S_{m2} + S_t)$ respectively) and $S_t$ (the threshold state). Estimates for $K_A$ and q were obtained from the linear double reciprocal plots of the concentrations of agonist (calculated from the equations of the concentration-state curves) required to produce equivalent states ($S_1 = S_2$) in the presence ($1/[A]_2$) and absence ($1/[A]_1$) of Pbz. According to Furchgott (1966),

$$q = 1/\text{slope} \qquad (8)$$

and $$K_a = (\text{slope} - 1)/\text{ordinate intercept} \qquad (9)$$

### (4) *Calculation of relative efficacy*

*(i) Irreversible antagonism (method of Furchgott, 1966)* The efficacy of carbachol relative to Oxo was calculated as the antilogarithm of the distance between the log (fractional receptor occupancy) versus State curves for the two agonists. The fractional receptor occupancy (y) for any agonist concentration [A], was calculated from the equation,

$$y = [A]/([A] + K_a) \qquad (10)$$

The value of $K_a$ used was the geometric mean of those $K_a$ values estimated for the agonist. The concentrations of agonist ([A]) substituted into equation 10 were the geometric means of the concentrations required to produce 20, 40, 50, 60 and 80% of the maximum agonist contraction produced in $C_1$.

*(ii) Functional antagonism (method derived from the model proposed by Mackay, 1981)* The relative efficacies of carbachol and Oxo were estimated from the ratio of the slopes of the respective plots of $K_A^F/K_A - 1$ versus $q\cdot R_t$. The slopes of the plots were estimated using linear least squares regression analysis.

### (5) *Estimation of* $K_A^F$ *values*

The three agonist CCSCs, $C_2$ (no functional antagonist), $C_3$ and $C_4$ (in the presence of different concentrations of Fen) were analysed to estimate $K_A^F$ values. The CCSCs were constructed by drawing smooth curves by eye through the plots of state against the log molar concentration of the agonist. Five pairs of agonist concentrations ($A_2$, $A_3$) which produced equivalent state levels were read from $C_2$ and $C_3$ at state levels spread evenly through the state range common to both curves. A similar method was used to obtain five pairs of agonist concentrations ($A_2$, $A_4$) which corresponded to five equivalent state levels common to $C_2$ and $C_4$. A computer-based nonlinear least squares regression programme used ($A_2$, $A_3$) and ($A_2$, $A_4$) coordinate pairs to solve respectively equations 11 and 12 which describe Type I functional antagonism,

$$A_3 = \frac{\alpha_1\cdot A_2 + (\alpha_1 - 1)^2/4\beta_1}{1 - \beta_1\cdot A_2} \qquad (11)$$

$$A_4 = \frac{\alpha_2\cdot A_2 + (\alpha_2 - 1)^2/4\beta_2}{1 - \beta_2\cdot A_2} \qquad (12)$$

The subscripts of $\alpha_1$ and $\beta_1$ indicate that these constants were relating data from $C_3$ with that of $C_2$. Similarly $\alpha_2$ and $\beta_2$ describe the relationship between

values of $A_2$ and $A_4$ obtained from $C_2$ and $C_4$ respectively. The estimates of $\alpha_1$, $\beta_1$, $\alpha_2$ and $\beta_2$ were used to calculate estimates for $K_{A_1}^F$ and $K_{A_2}^F$ where

$$K_{A_1}^F = 2\beta_1/(\alpha_1 - 1) \text{ and } K_{A_2}^F = 2\beta_2/(\alpha_2 - 1) \quad (13a,b)$$

The values of $K_{A_1}^F$ or $K_{A_2}^F$ describe how one agonist CCSC ($C_3$ or $C_4$) is related to another ($C_2$). Since the value of the functional affinity constant is independent of the concentration of functional antagonist concentration it would be predicted that within a single preparation a single $K_A^F$ value should describe the relationship between all three agonist CCSCs. This constraint was imposed by insisting that the calculated values of $K_{A_1}^F$ and $K_{A_2}^F$ were equal. Hence equations 11 and 12 are no longer independent since from equation 13a,b.

$$2\beta_1/(\alpha_1 - 1) = 2\beta_2/(\alpha_2 - 1) \quad (14)$$

The parameter $\beta_1$ was substituted by the term $[\beta_2 \cdot (\alpha_1 - 1)]/(\alpha_2 - 1)$ in equation 11 such that for this constrained model for Type I functional antagonism, the null equations were:

$$A_3 = \frac{\alpha_1 A_2 + [(\alpha_1 - 1)(\alpha_2 - 1)]/4\beta_2}{1 - [\beta_2(\alpha_1 - 1)]/(\alpha_2 - 1) \cdot A_2} \quad (15)$$

$$A_4 = \frac{\alpha_2 A_2 + (\alpha_2 - 1)^2/4\beta_2}{1 - \beta_2 \cdot A_2} \quad (16)$$

Estimates for $\alpha_1$, $\alpha_2$ and $\beta_2$ were obtained by solving equations 15 and 16 simultaneously using a computer-assisted nonlinear least squares regression programme. The equation $K_A^F = 2\beta_2/(\alpha_2 - 1)$ was then used to estimate $K_A^F$.

### (6) *Discrimination of the experimental data*

In order to experimentally test equation 4 it was necessary to obtain estimates of $K_A^F$, $K_A$ and q. Each data point in the plot of $K_A^F/K_A - 1$ against $q \cdot R_t$ was obtained from the analysis of at least four agonist CCSCs completed successively in a single preparation. Hence it was essential that changes in shape and position of agonist CCSCs induced by the irreversible and functional antagonists were conducive to the accurate determination of $K_A^F$, $K_A$ and q values.

In order to determine accurately $K_A^F$ values from estimates of $\alpha$ and $\beta$ (equation 13), the agonist CCSCs completed in the presence of the functional antagonist ($C_3$,$C_4$) should be significantly displaced to the right of the control agonist CCSC ($C_2$). If the degree of functional antagonism induced by the set concentration of Fen in $C_3$ or $C_4$ is not of sufficient magnitude then the estimated values of $(\alpha_1 - 1)$ and $(\alpha_2 - 1)$ will approach zero and may produce erroneously high estimates of $K_A^F$. To ensure that $C_2$, $C_3$ and $C_4$ were sufficiently shifted and accurate estimates of $K_A^F$ produced, it was arbitrarily decided that the estimates of $K_A^F$ would be accepted only when $\alpha_1 > 1.4$ and $\alpha_2 > 1.9$. In the set of experiments described in 2(i),$\alpha$ values in the range 1.4–1.6 and 1.9–2.3 were associated with carbachol CCSCs positioned respectively $2.5 \pm 0.1$ ($n = 19$) and $5.2 \pm 0.3$ ($n = 10$) fold to the right of the corresponding control CCSC.

### *Drugs*

The drugs used were: carbamylcholine chloride (carbachol), oxotremorine sesquifumarate (Sigma), fenoterol hydrobromide (Boehringer-Ingelheim), phenoxybenzamine hydrochloride (Smith, Kline and French).

Phenoxybenzamine was dissolved in ethanol. All other drugs were freshly prepared in ascorbic acid (100 μM) Krebs-bicarbonate solution. All drugs were kept on ice for the duration of the experiment.

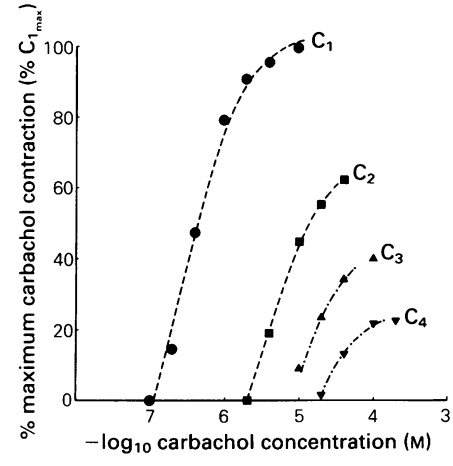


**Figure 1** Cumulative $\log_{10}$ concentration-state data for carbachol determined before ($C_1$) and following irreversible ($C_2$) and functional antagonism ($C_3$ and $C_4$) in a single rat isolated costo-uterine preparation. Broken lines (------) are the computed lines of best fit through the experimental concentration-state data predicted from solving equations 6 and 7 simultaneously. The calculated $K_a$ value was 7.9 μM and the fraction of cholinoceptors not irreversibly bound to phenoxybenzamine (q) was estimated to be 0.083. The second set of broken lines (-·-·-·-·-·-) are the computed lines of best fit through the experimental concentration-state data predicted from solving equations 15 and 16 simultaneously, which describe the constrained model for Type I functional antagonism. The estimated $K_A^F$ value was $1.92 \times 10^5 \text{ M}^{-1}$.

## Results

Of the 33 preparations used in the study outlined in Methods, section 2 (i), 20 were exposed to Pbz before using the functional antagonist Fen (Figure 1). The mean $EC_{50}$ (estimated from $C_1$) and $K_a$ values for carbachol were 0.44 μM (95% confidence limits, 0.41–0.48 μM, $n = 33$) and 11.5 μM (9.3–14.3 μM, $n = 20$) respectively. The mean $K_A^F$ value for carbachol determined in the 13 preparations not exposed to Pbz was $8.6 \times 10^5\ M^{-1}$, a value 9.9 fold greater than the affinity constant ($1/K_a$). The $K_A^F$ values estimated for carbachol in the 33 preparations were used to construct a plot of $K_A^F/K_A - 1$ against $q \cdot R_t$ (Figure 2). The data points in the plot were located predominantly in two regions; one corresponding to q values of less than 0.25 and the other for q values equal to one. In those preparations exposed to Pbz ($q < 1.0$, $n = 20$) the line of best fit through the plot of $K_A^F/K_A - 1$ against $q \cdot R_t$ had a slope of 9.45 ± 2.25 and passed through the axes at a point not significantly different from the origin (Table 1). No significant change in the character of the plot was observed when $K_A^F/K_A - 1$ values estimated in the 13 preparations not exposed to Pbz were included in the plot (Table 1). The line of best fit through these 33 points had a slope of 9.56 ± 1.68 and passed through the axes at a point not significantly different from the origin. A feature of the plot (Figure 2) was the considerable interpreparation variability in the estimates of $K_A^F$ for carbachol in the 13 preparations not exposed to Pbz. Although in 11 of the preparations the range of $K_A^F$ values estimated for carbachol was 3.9 to 12.1, in the other two preparations the $K_A^F$ values for carbachol were much higher, 18.5 and 22.0. The higher carbachol $K_A^F$ values obtained in the two preparations may be due to them being significantly more sensitive to carbachol than the other 11 preparations because in this sample of 13

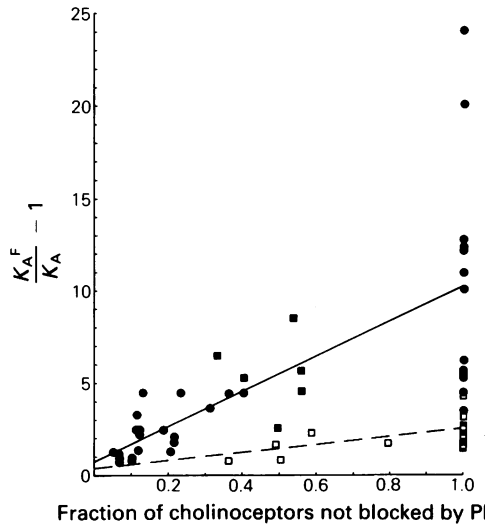


**Figure 2** Experimentally determined relationship between the fraction of cholinoceptors not blocked by phenoxybenzamine (Pbz) (q) and the estimated values of $K_A^F/K_A - 1$ for carbachol (solid symbols) and oxotrimorine (Oxo; open symbols) in 53 rat isolated costo-uterine preparations. In 33 preparations (●), q was determined using carbachol as the agonist (2(i) in Methods) and on the remaining 20 preparations (■, □) Oxo was the agonist used to estimate q (2(ii) and 2(iii) in Methods). The $K_A^F$ values for carbachol and Oxo were esimated by using fenoterol as the functional antagonist in all preparations. Linear least squares regression analyses were used to determine the line of best fit through the respective plots of $K_A^F/K_A - 1$ against $q \cdot R_t$ for carbachol (————, $n = 39$) and Oxo (- - - - -, $n = 14$).

**Table 1** Characteristics of the plots for carbachol and oxotremorine (Oxo) of $K_A^F/K_A - 1$ against the fraction of cholinoceptors not irreversibly blocked by phenoxybenzamine

| *Agonist used to functionally antagonize fenoterol* | *Agonist used to estimate q (q value)* | *Number of preparations used* | *Slope (± s.e.mean) of the line of best fit* | *y-intercept (± s.e.mean) of the line of best fit* | *Correlation coefficient* |
|---|---|---|---|---|---|
| Carbachol | Carbachol ($q < 1.0$) | 20 | 9.45 ± 2.25 | 0.77 ± 0.99 | 0.70** |
| | Carbachol ($q \leqq 1.0$) | 33 | 9.56 ± 1.68 | 0.76 ± 4.00 | 0.72** |
| | Carbachol or Oxo ($q \leqq 1.0$) | 39 | 9.46 ± 1.57 | 0.82 ± 3.80 | 0.70** |
| Oxo | Oxo ($q \leqq 1.0$) | 14 | 2.05 ± 0.93 | 0.35 ± 0.81 | 0.54* |

*$P < 0.05$, **$P < 0.001$, significant correlation.

**Table 2** At various state (% $C_{max}$) levels, fractional receptor occupancy (y) values have been estimated for carbachol and oxotremorine (Oxo) through application of Equation 10

| *Carbachol* | | | *Oxotremorine* | | |
|---|---|---|---|---|---|
| *State* (% $C_{max}$) | *Concentration of carbachol* (μM) | *Fractional receptor occupancy* | *State* (% $C_{max}$) | *Concentration of* Oxo (μM) | *Fractional receptor occupancy* |
| 20 | 0.236 (0.196–0.281 | 0.0201 | 14.7 | 0.112 (0.102–0.123) | 0.087 |
| 40 | 0.347 (0.319–0.378 | 0.0293 | 29.3 | 0.166 (0.152–0.181) | 0.124 |
| 50 | 0.439 (0.405–0.478) | 0.0368 | 36.7 | 0.216 (0.199–0.236) | 0.156 |
| 60 | 0.553 0.512–0.599) | 0.0459 | 44.0 | 0.302 (0.277–0.330) | 0.205 |
| 80 | 1.103 (0.94–1.095) | 0.0810 | 58.6 | 0.72 (0.64–0.81) | 0.381 |

The mean agonist concentrations ([A]) for carbachol and Oxo were determined in 33 and 14 preparations respectively. The mean $K_a$ values estimated for carbachol and Oxo were 11.5 μM ($n = 20$) and 1.17 μM ($n = 5$) respectively. Numbers in parentheses represent the 95% confidence limits of the meaned values.

preparations, a significant positive linear relationship ($P < 0.001$) existed between the corresponding estimates of $K_A^F$ and $pD_2$ for carbachol. This positive relationship probably reflects the quantitative dependence of both $K_A^F$ and $pD_2$ values for carbachol on preparation-dependent parameters such as receptor number and the characteristics of the chain of stimuli. Another feature of the plot of $K_A^F/K_A - 1$ against $q \cdot R_t$ for carbachol was that in seventeen of the twenty preparations exposed to Pbz the estimated q value was less than 0.25 (Figure 2). This was not the consequence of using too high a concentration of Pbz but due to the existence of spare cholinoceptors. In general, 75% of the receptors through which carbachol acted had to be irreversibly blocked before the maximum contractile response to carbachol was significantly reduced. At maximum concentrations, Oxo induced 73.3 ± 3.4% ($n = 7$) of the maximum tension induced with carbachol in the rat isolated costo-uterine preparation. It was postulated that because Oxo was a partial agonist it would need to occupy fully the receptor population in order to elicit its maximum contraction, and any reduction in the concentration of cholinoceptors would reduce the maximum level of Oxo-induced contracture. Hence the upper limit of calculable q values obtained following Pbz exposure would be expected to be greater for Oxo than the value of 0.25 obtained using carbachol. When Oxo was used as the agonist in six preparation exposed to Pbz (section (iii) in the Methods) the range of calculable q values was 0.33 to 0.56 (Figure 2). The $K_A^F$ values for carbachol were estimated in these preparations by using Fen as the functional antagonist and the values obtained added to the plot of $K_A^F/K_A - 1$ against $q \cdot R_t$ for carbachol (Figure 2). For this purpose it was assumed that Oxo and carbachol induced contractile responses through activation of the same population of receptors and hence the estimates of q were independent of the agonist used. In addition it was assumed that in these six preparations the $K_a$ for carbachol was 11.5 μM. The use of Oxo for the estimation of q values effectively increased the range over which $K_A^F/K_A - 1$ values were estimated for carbachol (Figure 2). The line of best fit through the plot of $K_A^F/K_A - 1$ against $q \cdot R_t$

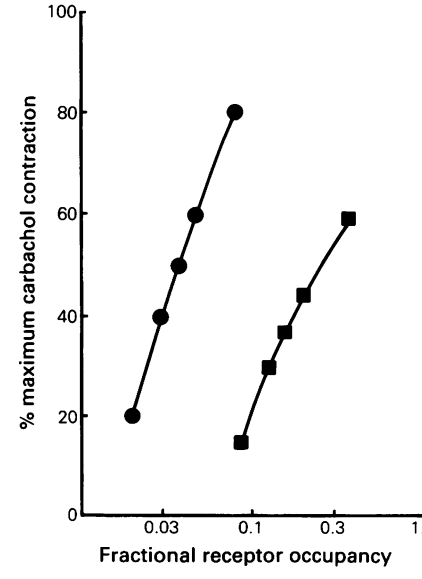


**Figure 3** Data presented in Table 1 expressed as a plot of State (% maximum carbachol contraction) against $\log_{10}$ (fractional receptor occupancy) for carbachol (●) and oxotremorine (■).

had a slope of 9.46 ± 1.57 and intercepted the axes at a point not significantly different from the origin (Table 1).

In the final series of experiments (section (ii) in the Methods) estimates of q, $K_a$ and $K_A^F$ were obtained in fourteen preparations using Oxo as the agonist. Although Oxo was a partial agonist the mean $EC_{50}$ value for Oxo of 0.216 μM made it twice as potent as carbachol. The greater potency of Oxo was a consequence of it having a ten fold higher affinity constant than carbachol. The mean $K_a$ value for Oxo was 1.17 μM (95% confidence limits, 0.4–3.4 μM, $n = 5$). The mean $K_A^F$ value estimated for Oxo ($q = 1.0, n = 9$) of $2.89 \times 10^6$ $M^{-1}$ was 3.4 fold greater than the mean $K_A$ ($1/K_a$) value. For Oxo, the line of best fit through the plot of $K_A^F/K_A - 1$ against $q \cdot R_t$ had a slope of 2.05 ± 0.93 and passed through the respective axes at a point not significantly different from the origin (Figure 2, Table 1). The ratio of the slopes (carbachol/Oxo) estimated from the plots was 4.6 (= 9.46/2.05). This estimate of the relative efficacy of carbachol and Oxo obtained from using functional antagonism was comparable to the value of 5.6 calculated using irreversible antagonism. The mean concentrations of agonist ([A]) and the agonist dissociation constants ($K_a$) substituted into equation 10 to calculate fractional receptor occupancy (y) are presented in Table 2. The calculated y values are plotted against the corresponding state levels in Figure 3. At the 36.7% $C_{max}$ state level the antilogarithm of the distance between the respective carbachol and Oxo plots of $\log_{10}$ y against % $C_{max}$ was 5.6.

## Discussion

Of central importance to the model of Type 1 functional antagonism is equation 4 which describes the relationship between the functional affinity constant ($K_A^F$) and the affinity constant ($K_A$) for any agonist (Mackay, 1981). The ratio of $K_A^F/K_A$ was proposed to be equal to the term $(a/b) \cdot f_A \cdot R_t + 1$. It was predicted that for a constant value of (a/b), $K_A^F$ will approach the value of $K_A$ as the concentration of receptors approaches zero. In addition, the ratio $K_A^F/K_A$ should be closer to unity for agonists with lower efficacies. A third quantitative prediction of the model was that for any pair of agonists which acted through the receptor population $R_t$, the ratio of the slopes of the lines of best fit through the respective plots of $K_A^F/K_A - 1$ against $q \cdot R_t$ would provide an estimate of their relative efficacies. Each of these three quantitative predictions of the model for functional antagonism was experimentally tested in the present study, which used the rat isolated costo-uterine preparation to investigate the changes in agonist CCSCs induced by functional and irreversible antagonists.

When Pbz was used to reduce the number of functional cholinoceptors, the ratio of $K_A^F/K_A$ decreased for both carbachol and Oxo (Figure 2). Hence the estimate of $K_A^F$ did indeed approach the value of $K_A$ as the concentration of receptors was reduced. Furthermore the results (Figure 2) showed that in preparations not exposed to Pbz, the ratio of $K_A^F/K_A$ estimated for carbachol and Oxo were 9.9 and 3.4 respectively. According to Mackay's model the higher $K_A^F/K_A$ ratio observed for carbachol is indicative of this agonist having a greater efficacy than Oxo. At least two independent lines of evidence support this supposition. Firstly, since the maximum contraction induced by carbachol was greater than that induced by Oxo, carbachol must have the greater efficacy. Secondly, the efficacy of carbachol was calculated to be 5.6 times that of Oxo when determined using the null method of irreversible antagonism proposed by Furchgott (1966). The third quantitative prediction of equation 4 was that for two agonists the ratios of the slopes of the lines of best fit through the plots of $K_A^F/K_A - 1$ against $q \cdot R_t$ will provide a measure of their relative efficacies. Indeed the value of 4.6 obtained for the ratio of the slopes of the carbachol and Oxo plots, respectively, was similar to the value of 5.6 for the relative efficacy obtained using the null method of irreversible antagonism.

Neither functional nor irreversible antagonism provided a means for directly measuring relative agonist efficacy. Estimates of this parameter were derived from substituting the experimentally determined values of $K_A$, q, $K_A^F$ and [A] into the appropriate algebraic equations. Hence the precise determination of estimates for these parameters is fundamental to obtaining precise measures of the relative efficacy. Estimates of $K_A$ and q were obtained using the approach described by McPherson *et al.* (1983). The use of nonlinear least squares regression analysis to fit a rectangular hyperbola to agonist concentration-state data while simultaneously allowing for a threshold phenomenon provides a more precise method for determining $K_A$ and q values than the widely used method of transforming a hyperbolic function to a linear function by use of a double reciprocal plot (McPherson *et al.*, 1983). In addition, this rectangular hyperbolic function which allowed for a threshold phenomenon also provided a more satisfactory qualitative description of the changes in the character of CCSCs of the cholinoceptor agonists which occurred in the presence of Pbz (Figure 1). The estimates of the equilibrium dissociation constants ($K_a$) obtained for carbachol and Oxo are in agreement with the values published. The $K_a$ value of 1.17 μM for Oxo in the present study is similar to the $K_a$ values of 1.2 μM (Emmerson & Mackay, 1981), 1.09 μM (Ringdahl & Jenden, 1983), 0.68 μM (Ringdahl, 1984) and 0.5 μM (Takeyasu *et al.*, 1979) found in the guinea-pig

ileum. The $K_a$ value for carbachol of 11.5 μM calculated in the present study compared well with the value of 12 μM, found by Furchgott (1978) in the rabbit stomach fundus and the value of 16 μM estimated in guinea-pig ileum (Ringdahl, 1984).

In addition to $K_a$ values, the estimates of the relative efficacies obtained from the functional antagonism studies were dependent upon agonist $K_A^F$ values. The determination of a single $K_A^F$ value involved the simultaneous analysis of concentration-state data from three CCSCs by utilizing constrained curve-fitting methods and nonlinear least squares regression analysis. The validity of using a single $K_A^F$ value to describe the change in shape and position of an agonist CCSC in the presence of the functional antagonist is dependent on the estimates of $K_{A_1}^F$ and $K_{A_2}^F$ being similar for each preparation. In the present study small but statistically significant differences existed between the respective estimates of $K_{A_1}^F$ and $K_{A_2}^F$ for carbachol but not for Oxo. In preparations not exposed to Pbz ($q = 1$, $n = 13$) the mean ratio of $K_{A_1}^F/K_{A_2}^F$ for carbachol was 1.23, whereas in 20 preparations exposed to Pbz the mean ratio of $K_{A_1}^F/K_{A_2}^F$ was 0.85. Both of these mean ratios were significantly different from 1.0. The reason why this significant difference was observed between the estimates of $K_{A_1}^F/K_{A_2}^F$ for carbachol but not for Oxo is at present not clear. Nevertheless, the magnitude of the difference was small and neither $K_{A_1}^F$ nor $K_{A_2}^F$ was significantly different from the single $K_A^F$ value estimated using the constrained method. Hence it is probable that the single $K_A^F$ values derived for carbachol satisfactorily reflect the Type I functional antagonism of carbachol CCSCs by Fen.

Although in the present study several key quantitative and qualitative predictions of Mackay's model for functional antagonism have been confirmed, clearly it is necessary to complete further studies of this nature in order to obtain a greater insight into the ability of Mackay's model to describe accurately functional antagonism. Similar studies to those described here need to be completed with other cholinoceptor agonists such as methacholine, pilocarpine and bethanechol, which have different affinities and efficacies to each other (Furchgott, 1978; Takeyasu *et al.*, 1979), in order to investigate further the applicability of Mackay's model for determining the magnitude of such parameters from agonist concentration-state data.

In addition to satisfying several quantitative aspects, the model of functional antagonism proposed by Mackay (1981) also provided an adequate qualitative description of the Fen-induced functional antagonism of cholinoceptor agonist mediated contractions in the rat isolated costo-uterine preparation. However, in view of the report by Van den Brink (1973 a,b) that changes in the magnitude of tissue-dependent parameters such as the relative sizes of the agonist receptor reserves may invoke marked changes in the characteristics of agonist CCSCs constructed in the presence of the functional antagonists, there exists a need to establish experimentally whether the model has the versatility to describe functional antagonism in other smooth muscle preparations. Indeed, in tissues such as the guinea-pig isolated ileum or the calf isolated trachea, which have large cholinoceptor reserves, (Van den Brink *et al.*, 1977; Takeyasu *et al.*, 1979) the influence of functional antagonists on the character of cholinoceptor agonist CCSCs can be studied over a wide range of agonist concentrations.

In conclusion, the present study has comprehensively investigated some predictions of Mackay's model for functional antagonism. Data analysis methods such as nonlinear least squares regression and constrained curve fitting techniques have been used to solve the null equations derived for functional and irreversible antagonism and provide estimates of parameters including relative agonist efficacy. We showed that the relative efficacies of two cholinoceptor agonists carbachol and Oxo estimated using the model of functional antagonism proposed by Mackay (1981) were comparable to the estimates obtained using the method of irreversible antagonism originated by Furchgott (1966).

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