Br. J. Pharmac. (1977), **61**, 189–197

LIGAND BINDING TO MUSCARINIC RECEPTORS IN INTACT LONGITUDINAL MUSCLE STRIPS FROM GUINEA-PIG INTESTINE

DAWN WARD & J.M. YOUNG

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge, CB2 2QD

1 The binding of ligands to muscarinic receptors in intact longitudinal muscle strips from guinea-pig small intestine has been determined by measuring the inhibition of the irreversible binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM).

2 The IC_{50} values for inhibition of [^{3}H]-PrBCM binding by a given ligand were generally higher in intact strips than those reported for broken-cell preparations. This effect is probably due, at least in part, to the presence of an access-limitation factor in the kinetics of the irreversible binding of [^{3}H]-PrBCM to the intact tissue.

3 The mean Hill coefficients for antagonist binding approached unity, but those for strong agonists were significantly less than unity. There was, with the possible exceptions of hexyltrimethylammonium and oxotremorine, reasonably good agreement with the Hill coefficients reported for brain homogenates.

Introduction

Following the classic study of Paton & Rang (1965) on the binding of [^{3}H]-atropine by longitudinal muscle strips from guinea-pig intestine, a number of groups have demonstrated muscarinic receptor binding by tritium-labelled reversible or irreversible receptor ligands (see Birdsall & Hulme, 1976 for a review). Some of these studies, like the original, have used pharmacologically intact tissues, generally in conjunction with the use of an irreversible ligand (Rang, 1967; Cuthbert & Young, 1973; Fewtrell & Rang, 1973; Burgen, Hiley & Young, 1974a), but there are a number of experimental difficulties in this approach and most groups have preferred to work with tissue homogenates where equilibrium measurements with reversible ligands can be conveniently made and where statistical errors are generally small. The values of the binding affinities for antagonists obtained by the use of tissue homogenates are in general in excellent agreement with those determined from inhibition of the contractile response (Birdsall & Hulme, 1976). However, the point of particular interest that has emerged from ligand binding studies is that the binding of muscarinic agonists does not follow a single simple mass-action curve (Burgen & Hiley, 1974; Young, 1974; Birdsall, Burgen, Hiley & Hulme, 1976; Hulme, Burgen & Birdsall, 1976; Birdsall & Hulme, 1976). The binding of agonists is not readily correlated with the contractile response and in these circumstances measurements on a tissue which is demonstrably pharmacologically intact become important, in order to establish that the process of homogenization has not in some way modified agonist binding. So far as comparison has been made, largely limited to the binding of carbachol, the agreement has been good, but a more extensive examination of muscarinic ligand binding to an intact tissue, which we describe here, was clearly desirable.

Methods

[^{3}H]-Propylbenzilylcholine mustard

[^{3}H]-Propylbenzilylcholine mustard ([^{3}H]-PrBCM), specific activity 1.0 Ci/mmol, was prepared and allowed to cyclise to the aziridinium ion before use as described previously (Burgen *et al.*, 1974a).

Binding measurements

The procedure used here has been described in detail elsewhere (Burgen *et al.*, 1974a). Briefly, longitudinal muscle strips (6–13 per incubation, each usually weighing 3–8 mg) from guinea-pig small intestine were suspended in 400 or 800 ml Krebs–Henseleit solution at 30°C gassed with 5% CO_2 in O_2 and incubated for 1 h before addition of [^{3}H]-PrBCM

(final concentration 2.2 nM). Unless specified otherwise, antagonists whose binding was to be studied were added 30 min, and agonists 1 min, before the [^{3}H]-PrBCM. Incubation with [^{3}H]-PrBCM (10 min) was terminated by transferring the strips briefly to 200 ml fresh Krebs and then to a further 200 ml Krebs at 30°C. Washing was continued for 75 min with two changes of the solution and the strips were then blotted dry, weighed, dissolved in Soluene (Packard) and the tritium determined by liquid scintillation counting.

Analysis of curves of inhibition of [^{3}H]-PrBCM binding

The curves of % of uninhibited binding of [^{3}H]-PrBCM *versus* concentration of inhibitor (e.g. Figure 1) can be considered to be made up of two components, an inhibitor-insensitive component presumed to represent non-receptor binding and a second component reflecting the receptor occupancy of the inhibitor. The time course of the receptor-specific binding of [^{3}H]-PrBCM approximates, over the first 40 min, to a single exponential function (Burgen *et al.*, 1974a), consistent with the reversible drug-receptor complex initially formed being converted into a covalently-bound complex in a step with a rate constant much greater than that for dissociation of the reversible complex (Gill & Rang, 1966). In the simplest case, assuming that the drug-receptor interaction is rate-determining, the total occupancy, p, which is equivalent in these circumstances to the irreversible binding measured, at any time t is given (Gill & Rang, 1966) by:

$$ln\,(1-p) = -k_1 \,.\, \mathrm{M} \,.\, t$$

where k_1 is the rate constant for formation of the reversible complex and M is the concentration of the aziridinium ion derivative of [^{3}H]-PrBCM. In the presence of a reversible inhibitor with affinity K_a and at a concentration A, the occupancy, p_1, becomes

$$ln\,(1-p_1) = \frac{-k_1 \,.\, \mathrm{M} \,.\, t}{1+A \,.\, K_a}$$

Hence

$$\frac{ln\,(1-p_1)}{ln\,(1-p)} = \frac{1}{1+A \,.\, K_a}$$

Expanding

$$\frac{-p_1-p_1^2/2 \cdot \cdots}{-p-p^2/2 \cdot \cdots \cdot} = \frac{1}{1+A \,.\, K_a}$$

Hence when p is small, so that $p \gg p^2/2$, then

$$\frac{p_1}{p} = \frac{1}{1+A \,.\, K_a}$$

(and the fractional inhibition, $1-p_1/p$, is equal to the occupancy of the antagonist). The proportion of the maximum specific binding achieved by [^{3}H]-PrBCM after 10 min incubation, equivalent on this analysis to p, is 0.16 and $p^2/2 = 0.013$, so that the condition that $p \gg p^2/2$ is apparently reasonably well satisfied.

The limitation of this simple approach is that it makes no allowance for diffusional delays. If there is an access-limitation factor then it is unrealistic to assume that the time-course of the irreversible binding of [^{3}H]-PrBCM can be approximated by a single exponential function with a rate constant of $1.4 \times 10^5 \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ (Burgen *et al.*, 1974a). Reaction with [^{3}H]-PrBCM presumably occurs first with receptors on the outer surfaces of the superficial layer of cells and then subsequently as the drug diffuses inwards through the channels between cells, with receptors on cells in deeper layers — assuming that the distribution of receptors throughout the tissue is not grossly non-uniform. It seems likely that the rate constant for binding to the superficial receptors will be of the same order as that for homogenates, i.e. around $2.5 \times 10^6 \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ (Burgen, Hiley & Young, 1974b) and that the same rate constant will describe the binding to the inner receptors, except that there the concentration of [^{3}H]-PrBCM will only slowly approach that in free solution. In this situation measuring [^{3}H]-PrBCM bound at a time short compared with that required to achieve saturation does not achieve a low occupancy of a homogeneous population but selects a group of receptors, largely on superficial cells, with which rapid reaction occurs. On the simple model the effect of a 10 min incubation period with a rate constant of $2.5 \times 10^6 \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ instead of $1.4 \times 10^5 \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ would be to shift the % of uninhibited uptake *versus* [inhibitor] curve along the concentration axis by a factor of about 4, with a small increase in the Hill coefficient, 1.01 → 1.12. In practice a somewhat smaller shift might be expected since for some receptors where the concentration of [^{3}H]-PrBCM is only just starting to rise the condition that $p \gg p^2/2$ will be much better satisfied. However, this analysis does suggest that even though using the simple approach may give poor estimates of binding affinities, it still provides a useful model against which the experimental curves can be tested to see whether they are consistent with a single mass-action equilibrium between inhibitor and receptor. To do this, inhibitor binding was assumed to follow a Hill equation, $A^n \,.\, K_a/(A^n \,.\, K_a + 1)$, and the best-fit values of n, the Hill coefficient, determined. The actual equation fitted, using the Patternsearch procedure of Hooke & Jeeves (1961; Colquhoun, 1971), was

$$\%\ \text{of uninhibited binding} = \frac{100-NS}{A^n \,.\, K_a + 1} + NS$$

with n, K_a and NS (non-specific, i.e. non-receptor, binding) as unknown. Each point was weighted according to the reciprocal of the approximate variance associated with it. The Patternsearch method

tended to work well where $n \sim 1$ but located subminima much more frequently when n was small. The best fit values were taken to be those that gave the lowest residual sum of squares after repeated trials with different initial parameter estimates. An alternative non-linear fitting procedure using a modified Marquardt approach as implemented in the Harwell Library routine VBOIA on the Cambridge IBM 370/165 gave similar values, where it succeeded, but failed to find a solution in some cases. The difficulties with both techniques probably arise from the fact that if $n \neq 1$ then almost certainly the binding curve of the inhibitor will not have an invariant value of n over its whole length and the numerical value of n obtained serves only as a useful index of the deviation of the binding curve from a single mass-action equilibrium. The use of the Hill equation has advantages, since the slope of the curve is the parameter of particular interest (Reiche & Zinke, 1974), but at the same time the limitations of the equation as a model to fit the data at low n may introduce problems of convergence and evaluation of error estimates (Reich, Winkler & Zinke, 1974). Consequently the mean Hill coefficients were also determined by linear regression analysis of conventional unweighted Hill plots of log {(100−% of uninhibited binding)/(% of uninhibited binding−*NS*)} *versus* log [*A*], ignoring points within 5% of the 100% or non-specific (taken as 13%) limits.

For analysis of agonist inhibition curves as binding to two independent sites, the equation fitted, using the Harwell VBOIA routine, was:

$$\%\ \text{of uninhibited binding} = 100 - \frac{N1 \,.\, A}{A + K_1} - \frac{N2 \,.\, A}{A + K_2}$$

where A is the concentration of the inhibitor, K_1 and K_2 its dissociation constants for the two sites, and $N1$ and $N2$ the percentage of the total binding of [^{3}H]-PrBCM, after a 10 min incubation with the mustard, associated with each site. [^{3}H]-PrBCM is presumed not to distinguish between the two sites.

Organ bath experiments

Longitudinal muscle strips were suspended in 10 ml of Krebs–Henseleit solution, gassed with 95% O_2 and 5% CO_2, at 30°C in a conventional organ bath. Contractions were recorded isotonically. Agonists were in contact with the tissue for 15–30 s and doses were added at 3 min intervals.

Results

Binding of muscarinic antagonists

The effect of methylatropinium on the amount of [^{3}H]-PrBCM bound during a 10 min incubation period with

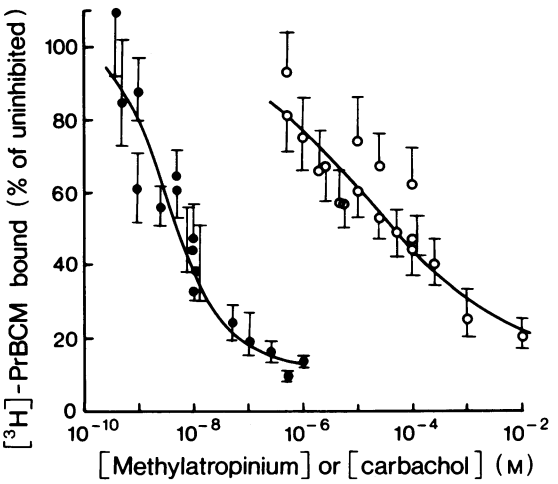

Figure 1 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding to muscle strips by methylatropinium and by carbachol. The experimental conditions and the method used to determine the weighted best-fit curves are described under Methods. The data in this Figure were derived from measurements on some 500 muscle strips. Error bars represent the 69% confidence limits. (●) Methylatropinium; (○) carbachol.

longitudinal muscle strips is shown in Figure 1. The Hill coefficient for the apparent binding curve for methylatropinium, 0.99 (Table 2), is consistent with a simple mass-action equilibrium, but the apparent binding affinity, $2.4 \times 10^8\ \text{M}^{-1}$, is lower than that determined from inhibition of the contractile response, $2.1 \times 10^9\ \text{M}^{-1}$ (Paton & Rang, 1965). A similar discrepancy, mirrored in the difference between the IC_{50} values in the intact strips and those reported for homogenates (Birdsall & Hulme, 1976; Yamamura & Snyder, 1974) exists for all the antagonists tested (Table 1) and is in the direction predicted if there is a diffusional factor in the kinetics of binding of [^{3}H]-PrBCM (cf Methods section). The extent of the shift for atropine is very similar to that apparent in the inhibition of [^{3}H]-BCM binding to muscle strips (Fewtrell & Rang, 1973). Whether the shifts can be wholly explained by diffusional effects is uncertain, but at least in the case of benzhexol, where the difference is particularly large, it seems likely that other factors are involved. If additional factors do exist then it seems equally likely that they involve some property of the intact muscle, since the binding affinities of antagonists to homogenates of mammalian cerebral cortex, measured using the same preparation of [^{3}H]-PrBCM (Burgen *et al.*, 1974b), agreed well with the values from organ bath studies.

The conclusion drawn from simple consideration of the effect of a diffusional barrier that the inhibition

Table 1 IC_{50} values for inhibition of receptor-specific [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by muscarinic ligands: comparison with broken cell preparations

		IC_{50}(M)		
			Homogenates	
	Non-specific binding (%)§	*Intact muscle*	*Cerebral cortex*†	*Longit. muscle*‡
Atropine§§	–	6×10^{-9}††	5.9×10^{-10}*	$3–4 \times 10^{-9}$
Benzhexol	11	1.2×10^{-7}††	7.1×10^{-9}	–
Methylatropinium	12	4.2×10^{-9}	3.5×10^{-10}*	$1–2 \times 10^{-10}$
Propylbenzilylcholine	9	4.0×10^{-8}	1.0×10^{-8}	–
Acetylcholine	11	8.9×10^{-6}	3.3×10^{-6}	–
Carbachol	15	2.0×10^{-5}	1.5×10^{-5}	$2–3 \times 10^{-5}$
(+)-Methacholine	(14)¶	1.3×10^{-5}	2.0×10^{-5}	$2–3 \times 10^{-6}$
Methylfurmethide	16	1.5×10^{-5}	2.2×10^{-6}	–
Oxotremorine	13	6.6×10^{-7}	4.6×10^{-7}	$5–8 \times 10^{-7}$
Pilocarpine	21	3.6×10^{-5}	6.0×10^{-6}	$7–9 \times 10^{-7}$
C_6TMA	14	1.7×10^{-5}	6.3×10^{-6}	–
	12	2.5×10^{-5}**		
C_{12}TMA	18	5.8×10^{-6}	–	–
	16	2.9×10^{-6}**		

Antagonists were preincubated with the muscle for 30 min, and agonists for 1 min, before [^{3}H]-PrBCM addition.

§ From weighted non-linear fit (see Methods); † Data taken from Birdsall & Hulme (1976); ‡ Data taken from Yamamura & Snyder (1974); §§ IC_{50} value for intact muscle taken from Burgen *et al.* (1974a). †† For comparison with the values for [^{3}H]-PrBCM, IC_{50} values for inhibition of [^{3}H]-BCM binding to intact strips, estimated from the data of Fewtrell & Rang (1973) were: atropine, 5×10^{-9} M; benzhexol, 3×10^{-8} M. * Value for (–)-isomer. ¶ Lower limit of the curve ill-defined (cf. text). Best-fit curve (Figure 3) obtained by setting non-specific binding of [^{3}H]-PrBCM to 14%. ** 30 min preincubation.

Abbreviations: C_6TMA, hexyltrimethylammonium; C_{12}TMA, dodecyltrimethylammonium.

Table 2 Mean Hill coefficients for muscarinic ligand binding: comparison with values from homogenates

	Mean Hill coefficients		
	Intact strips		
	*Non-linear fit**	*Hill plot**	*Homogenates with ref.***
Atropine	–	0.84 ± 0.06 (7)‡	~1.0 a
Benzhexol	1.00 (8)¶	0.88 ± 0.14 (6)	~1.0 a
Methylatropinium	0.99 (16)	0.96 ± 0.09 (13)	~1.0 a
Propylbenzilylcholine	0.90 (5)	0.93 ± 0.13 (4)	1.0 b
Acetylcholine	0.62 (10)	0.54 ± 0.06 (9)§	0.42, 0.50, 0.63, a,b,c
Carbachol	0.36 (18)	0.31 ± 0.05 (16)	0.33 a
(+)-Methacholine	0.46 (11)†	0.36 ± 0.06 (9)	0.52†† a
Methylfurmethide	0.58 (10)	0.53 ± 0.06 (10)	0.48 a
Oxotremorine	0.65 (17)¶	0.44 ± 0.06 (14)	0.88, 1.0 a,b
Pilocarpine	0.82 (8)	0.63 ± 0.12 (8)	0.95, 1.0 a,b
C_6TMA	0.55 (14)	0.53 ± 0.08 (13)	1.0 a
	0.72 (9)‡‡	0.82 ± 0.10 (8)	
C_{12}TMA	0.80 (9)	0.63 ± 0.10 (9)	
	0.90 (7)‡‡	1.09 ± 0.18 (6)	

Errors are ± s.e. with number of points in parentheses.

* Details of the weighted non-linear fit and unweighted Hill plot are given under Methods; ** References: a, Birdsall & Hulme (1976); b, Birdsall *et al.* (1976); c, Hulme *et al.* (1976). ‡ Calculated from Burgen *et al.* (1974a). § Robinson, Taylor & Young (1975). † Non-specific binding of [^{3}H]-PrBCM set to 14% (cf text and legend to Table 1). The fitted curve is shown in Figure 3. †† Racemate. ¶ Curve fitted to the filled points in Figure 2. If all points are fitted the value rises to 0.73 (23). ‡‡ 30 min preincubation.

Abbreviations: C_6TMA, hexyltrimethylammonium; C_{12}TMA, dodecyltrimethylammonium.

curves for ligands would probably be shifted but without a marked effect on the mean Hill coefficient is apparently borne out, since for the antagonists the values approached unity and gave no indication of being other than single mass action binding curves, in good agreement with the data for homogenates (Birdsall & Hulme, 1976; Birdsall *et al.*, 1976) (Table 2).

Binding of muscarinic agonists

The proportion of [^{3}H]-PrBCM binding insensitive to inhibition by the muscarinic agonists investigated was not markedly different from that for the antagonists (Table 1). However, in contrast to the antagonists, the binding of muscarinic agonists does not represent a mass action equilibrium with a single site. The inhibition curve for carbachol (Figure 1) has a mean Hill coefficient of 0.36, although as noted in the methods section the actual value serves only as an index of the deviation from a simple drug-receptor equilibrium. Even with the scatter of points there is some indication of a plateau in the carbachol inhibition curve (Figure 1), which would be anticipated whether the low value of the Hill coefficient was due to binding to two discrete sites with differing affinities or to some form of negative cooperativity. In both cases the value of Hill coefficient would vary over the length of the curve.

There is some indication that the amount of scatter observed on agonist binding curves made up of points derived from different groups of guinea-pigs at different times may not be due solely to experimental error. The inhibition of [^{3}H]-PrBCM binding by oxotremorine measured on guinea-pigs from three different batches from two suppliers is shown in Figure 2 (closed symbols). However, the values obtained from another batch of animals purchased and used in the period February–March 197 deviated systematically (open symbols, Figure 2) from the general trend and were not a property of the particular solution of oxotremorine used. A subsequent batch of animals gave points that were consistent with the original curve and consequently it has not been possible to investigate this phenomenon in more detail. Whether the effect is related to the seasonal variation that has been reported for the contractile response of guinea-pig ileum segments to acetylcholine (Weinstock & Shoham, 1974) is unknown, although we have not observed any consistent seasonal variation in the sensitivity of the contractile response to carbachol.

In view of the variable effect of oxotremorine, the best-fit value for the Hill coefficient, 0.65, neglecting the open points in Figure 2, must be treated with some reserve. An alternative fitting procedure (see Methods) gave a similar best-fit value, 0.63, but inspection of the experimental data and fitted curve (Figure 2) indicates

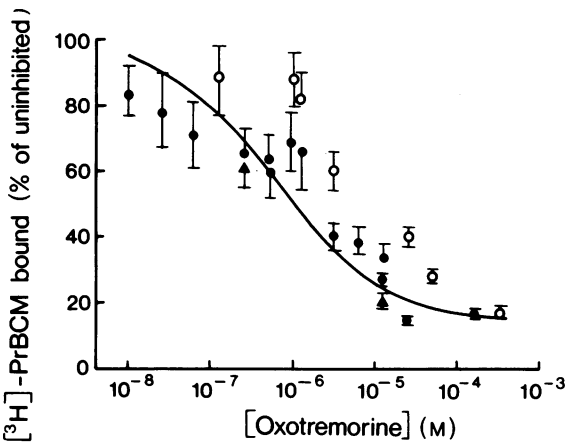

Figure 2 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by oxotremorine. The experimental conditions are described under Methods. Points obtained from one particular batch of guinea-pigs (○) differed systematically from those made on three others (●). The curve is fitted to the closed symbols (●) only. Error bars represent the 69% confidence limits. (▲) 30 min preincubation with oxotremorine.

the limitation of analysing the inhibition curves by a simple Hill equation when the Hill coefficient is not near unity. If all the points obtained (both closed and open symbols, Figure 2) are used, the best-fit value of the Hill coefficient rises to 0.73.

Some confidence that the agonist inhibition curves do represent some aspect of muscarinic cholinergic action is provided by the difference in potency between the (+)- and (–)-isomers of β-methacholine (Figure 3). The high concentration and consequently large amount of the (–)-isomer required for the highest levels of inhibition of [^{3}H]-PrBCM binding precluded the determination of a reliable value of the Hill coefficient so that it is uncertain whether the two curves are 'parallel'. Even with the (+)-isomer the lack of a sufficient number of points at high inhibition led to a best-fit curve with only 2% non-specific binding, which in comparison with other values in Table 1 is unlikely. The potency ratio between the two isomers for production of the contractile response, measured in 3 independent experiments (2×2 dose assay) was 470 ± 30. This is rather larger than the reported value of 240 (Beckett, Harper, Clitherow & Lesser, 1961), but could reflect a more complete resolution of the isomers in our sample (prepared by Dr K.S. Scott). Both potency ratios were measured in the absence of a cholinesterase inhibitor. (+)-Methacholine is a substrate for acetylcholinesterase but, at least at higher concentrations of the isomer, the presence of physostigmine 10^{-6} M made no apparent difference to the level of inhibition of [^{3}H]-PrBCM binding

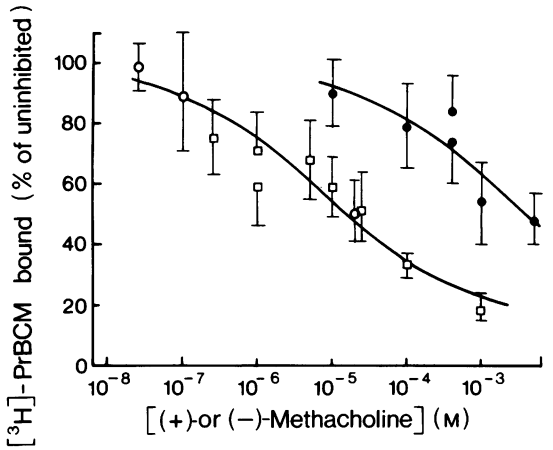

Figure 3 Comparison of (+)- and (−)-methacholine as inhibitors of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding to muscle strips. Error bars represent the 69% confidence limits. (□) (+)-Methacholine, no cholinesterase inhibitor present; (○) (+)-methacholine, with physostigmine 10^{-6} M present in both uninhibited and methacholine incubation vessels; (●) (−)-methacholine, no cholinesterase inhibitor present. The curve through the (−)-methacholine points has been drawn by inspection, that through the points for the (+)-isomer is calculated, but see footnote † to Table 2.

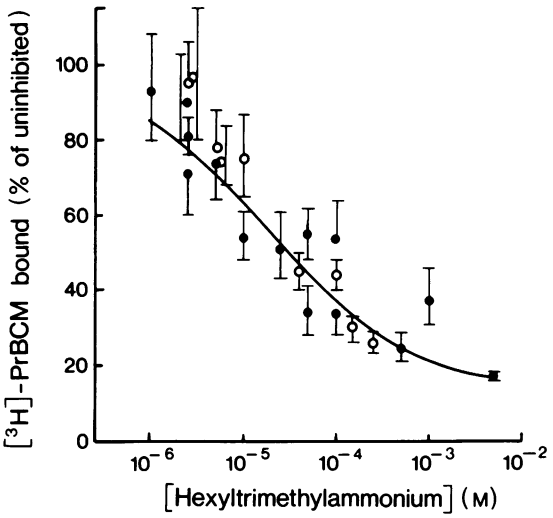

Figure 4 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding to muscle strips by hexyltrimethylammonium. The hexyltrimethylammonium was preincubated with the strips for either 1 min (●) or for 30 min (○) before the addition of [^{3}H]-PrBCM. The curve drawn has been fitted to the 1 min (●) points. Error bars represent the 69% confidence limits.

observed. The potency ratio for binding, measured at the 50% inhibition level was approximately 200, but the accuracy of this figure is clearly limited by the uncertainty of the slope and position of the curve for the (−)-isomer (Figure 3).

The IC_{50} values for the inhibition of [^{3}H]-PrBCM binding to the intact muscle by muscarinic agonists were shifted to higher concentrations than those reported for homogenates (Table 1), just as for the antagonists. Only carbachol was a clear exception to this rule. However, the point of prime interest is that the Hill coefficients determined from the agonist inhibition curves are, with the reservations about oxotremorine noted above and hexyltrimethylammonium, discussed below, in reasonable agreement (Table 2) with those reported for binding to homogenates of cerebral cortex (Birdsall & Hulme, 1976; Birdsall *et al.*, 1976; Hulme *et al.*, 1976), the tissue for which the most extensive data are available. Making comparisons between two different tissues raises problems, but the evidence currently available suggests a close similarity between the biochemical properties of muscarinic receptors in cerebral cortex and in smooth muscle (see review by Birdsall & Hulme, 1976). The measure of agreement of the mean Hill coefficients for the agonists binding to the intact muscle and to cortical homogenates provides strong evidence that the low values are not diffusional or homogenization artifacts.

Binding of alkyltrimethylammonium salts

The general agreement between the Hill coefficients observed in homogenates and in the intact muscle is less clear for hexyltrimethylammonium (C_6TMA). There were, however, experimental difficulties with this compound, as also with nonyltrimethylammonium (C_9TMA), dodecyltrimethylammonium (C_{12}TMA) and pilocarpine, since at the concentrations necessary for higher degrees of inhibition a distinct detergent-like action was apparent. What effect this may have on the smooth muscle membrane or on the diffusional properties of the tissue is unknown. One consequence was that it was difficult to ascertain with any degree of confidence the level of inhibitor-insensitive binding and indeed with C_9TMA it was only feasible to determine half the inhibition curve.

Analysis of the curve for C_6TMA under normal conditions for agonists (Figure 4, closed symbols) is further complicated by the scatter of experimental values at concentrations of 5×10^{-5} M and above, although the weighing should to a large extent compensate for this. Even so the best-fit Hill coefficient, 0.55, was rather less than would have been expected in comparison with the value of 1.0 in cortical homogenates (Birdsall & Hulme, 1976). Increasing the period of incubation of the tissue with C_6TMA from 1 min, the period used for the agonists, to 30 min (Figure 4, open symbols), as employed for

the antagonists, did not lead to any statistically significant increase, as judged from unweighted Hill plots, in the Hill coefficient, although the best-fit value, 0.74, was numerically greater. Increasing the incubation for C_{12}TMA from 1 to 30 min also resulted in a numerical, but again non-significant, increase in the best-fit value (Table 2). Whether for C_6TMA there is a discrepancy from the homogenate value requires further investigation, but it is interesting to note that in muscle strips exposed to distilled water, a treatment which may influence the position of agonist binding curves without apparently having any marked effect on the slope, the Hill coefficient for C_6TMA with 1 min incubation is of the order 0.6–0.7 (Elliott, Tayler & Young, unpublished observations).

Analysis of agonist binding curves as double hyperbolae

The observations presented above, taken together with the published data from broken cell preparations, make it highly unlikely that the low values of the mean Hill coefficients for strong agonists are artifactual, but give little indication of the underlying mechanism. The simplest explanation (Burgen & Hiley, 1974) is that the agonist curves do not represent binding to a single site, but to two or more with differing affinity. Only for 4 of the agonists are the data for the binding to the intact muscle strips sufficiently extensive to warrant analysis of the curves as binding to two independent sites (Tables 3 and 4). The percentage of high and low affinity sites is reasonably similar for each agonist (Table 4). There is no simple correlation with the ED_{50} for the contractile response (Table 3). In any event such correlations need to be made with care since the ED_{50} for contraction may vary widely. The ED_{50} for carbachol was calculated from measurements made at irregular intervals over a period of some 3 years, the ED_{50} varying between 3×10^{-8} M and 2.5×10^{-7} M. The measurements for oxotremorine, (+)-methacholine and methylfurmethide were made during a limited period on muscle strips from guinea-pigs which were unusually sensitive to carbachol (ED_{50} of this group, 5 experiments, $5.2 \pm 0.5 \times 10^{-8}$ M).

Discussion

The degree of agreement of the values determined for the mean Hill coefficients for muscarinic ligand binding to 'intact' longitudinal muscle strips from guinea-pig small intestine with those derived from broken cell preparations leaves little doubt that the low values of the coefficients which characterize the binding of strong agonists is not an artifact of homogenization. At the same time the general shift in the IC_{50} values underlines the problems of kinetic analysis in the intact tissue. At first sight the

Table 3 Agonist inhibition curves analysed as binding to two independent sites: dissociation constants and comparison with ED_{50} for contraction

	Dissociation constants (M) K_1	K_2	*ED_{50} contraction (M)*
Carbachol	$7.2 \pm 3.6 \times 10^{-7}$	$2.9 \pm 1.6 \times 10^{-4}$ (18)	$1.1 \pm 0.2 \times 10^{-7}$ (14)
(+)-Methacholine	$1.8 \pm 1.0 \times 10^{-7}$	$5.3 \pm 1.4 \times 10^{-5}$ (13)	$3.3 \pm 0.7 \times 10^{-8}$ (3)
Methylfurmethide	$0.95 \pm 2.2 \times 10^{-6}$	$4.2 \pm 3.9 \times 10^{-4}$ (10)	$4.9 \pm 2.4 \times 10^{-8}$ (3)
Oxotremorine*	$0.66 \pm 1.3 \times 10^{-8}$	$2.4 \pm 0.96 \times 10^{-6}$ (17)	$2.6 \pm 0.5 \times 10^{-8}$ (3)

Values for K_1 and K_2, ± estimated standard deviations (number of points), were obtained using VBOIA (see Methods). Values for ED_{50} are means ± s.e. (number of determinations).
* Only the filled points in Figure 2 were fitted.

Table 4 Agonist inhibition curves analysed as binding to two independent sites: percentage of each site

	Site (% of total binding) *High affinity*	*Low affinity*	*Non-Spec.†*
Carbachol	41.7 ± 4.0 (50)	40.9 ± 4.6 (50)	17
(+)-Methacholine	34.4 ± 4.2 (41)	50.0 ± 4.2 (59)	16
Methylfurmethide	30.7 ± 21.0 (39)	48.1 ± 19.0 (61)	21
Oxotremorine*	27.6 ± 8.9 (32)	57.8 ± 8.7 (68)	15

Values ± estimated standard deviations were obtained from VBOIA (see Methods). The figures in parentheses are the values expressed as a percentage of the receptor-specific binding.
* Only the filled points in Figure 2 were fitted. † Non-receptor binding of [^{3}H]-PrBCM 100– (site 1 + site 2).

longitudinal muscle strip has several advantages. It separates from the gut as a thin sheet of largely smooth muscle tissue, some 6–8 cells thick, with Auerbach's plexus usually adhering to the inner surface. The contractile response to agonists is practically indistinguishable from that of whole intestinal segments, while at the same time it is sufficiently 'enriched' in smooth muscle cells to give a high-proportion of receptor-specific binding in the presence of low concentrations of [^{3}H]-PrBCM. It seems very probable however that diffusional problems remain.

The question of the presence or otherwise of a 'biophase' barrier in this tissue in the onset and offset of antagonist action has been discussed by earlier authors (Rang, 1966; Thron & Waud, 1968) and more recently Roberts & Stephenson (1976) have shown that the kinetics of antagonist action are not satisfactorily described by a simple interaction-limiting model. The sequential binding sites model of Paton & Rang (1965), which largely accounts for the anomalous kinetics of [^{3}H]-atropine uptake and release from longitudinal muscle strips, can be viewed as another expression of the diffusional problem, since it may be expected with some confidence that the process of diffusion in the inter-cellular channels will be strongly influenced by reversible interactions with numerous low affinity binding sites. We have also concluded from the increase in the rate of binding [^{3}H]-PrBCM in homogenates as compared with intact preparations that the presence of a diffusion barrier is very probable (Taylor, Cuthbert & Young, 1975) and the displacement of inhibition curves to higher inhibitor concentrations is in accord with this. However, the extent of the difference between IC_{50} values in homogenates and in intact muscle is variable and it is by no means certain that it can be accounted for entirely by the simple considerations applied here (cf. Methods section). For particular ligands other factors are clearly involved. The discrepancy for benzhexol is notably large while the IC_{50} for carbachol is in curiously good agreement with broken cell systems. Indeed, earlier measurements with carbachol (Young, 1974) indicated a leftward shift of the % of uninhibited uptake *versus* [carbachol] curve without any apparent change in slope as the period of incubation with [^{3}H]-PrBCM was reduced from 10 to 5 min, which might be anticipated on the simple theory developed under Methods, without the necessity of invoking ongoing desensitization. These particular cases require further examination and it would be of particular interest to know whether the changes in the ED_{50} values for the contractile response to carbachol are mirrored by corresponding changes in the position of the carbachol binding curve.

Although little doubt can remain that the binding of strong agonists cannot be described by a single mass-action equilibrium, the data presented here allow few conclusions to be drawn of the mechanism by which the flattened binding curves result. The two main possibilities open are that the curves represent binding to two or more independent sites or, alternatively, that the low Hill coefficients reflect the kinetic mechanism operating. The evidence currently available is consistent with the presence of two independent sites (Birdsall *et al.*, 1976; Hulme *et al.*, 1976). There is little indication of any negative cooperativity of muscarinic agonist binding and Birdsall *et al.* (1976) have demonstrated that occlusion of some 90% of muscarinic receptor sites in cortical homogenates by treatment with an irreversible antagonist (PrBCM) did not alter the mean Hill slope for carbachol binding to the remaining 10%. Similarly a suggestion that desensitization might be involved, on the basis of the effect of a large dose of carbachol of increasing the Hill coefficient of the binding curve for carbachol, measured immediately after a brief wash, in intact muscle strips (Young, 1974), now seems unlikely since no similar effect has been found in cortical homogenates (Birdsall & Hulme, 1976). The mechanism of the carbachol effect in intact muscle remains to be established.

Of reports in the literature of more than one site for muscarinic agonist action in the longitudinal muscle strip preparation (Burgen & Spero, 1968; Bolton, 1975; Kilbinger, 1975; Kilbinger & Wagner, 1975) that of Burgen & Spero (1968), who compared the effects of muscarinic agonists on causing contraction and, at higher concentrations, on promoting the efflux of K^+ and Rb^+, provides the most extensive data for comparison with agonist binding. However, there is no apparent correlation between the affinities of agonists for their two sites and the dissociation constants for binding, deduced from analysis of curves as double hyperbolae (Table 3), although presumably the Rb^+ efflux site will have been labelled by [^{3}H]-PrBCM since the affinity of antagonists for this site was the same as against contraction (Burgen & Spero, 1968). It is interesting to note, however, that the one antagonist which appeared to have different affinities against efflux and contraction was benzhexol and although the Hill coefficient for benzhexol binding is unity there is an unusually large difference between IC_{50} values in strips and in homogenates.

The weight of the evidence rests with two independent sites, but there is little indication what their function might be. It is, however, of particular interest that the curve for the stimulation of phosphatidylinositol turnover in longitudinal muscle strips (Jafferji & Michell, 1976) corresponds closely to the binding curve for carbachol. If phosphatidyl-inositol breakdown is intrinsic to the mechanisms of muscarinic receptor systems, as has been suggested (Michell, 1975), then both binding sites would seem to be involved with the production of a response.

We thank the Science Research Council for financial support (B/RG/5409).

References

BECKETT, A.H., HARPER, N.J., CLITHEROW, J.W. LESSER, E. (1961). Muscarinic receptors. *Nature, Lonc* **189**, 671–673.

BIRDSALL, N.J.M., BURGEN, A.S.V., HILEY, C.R. HULME, E.C. (1976). Binding of agonists ar antagonists to muscarinic receptors. *J. Supramole Struct.*, **4**, 367–371.

BIRDSALL, N.J.M. & HULME, E.C. (1976). Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem.*, **27**, 7–16.

BOLTON, T.B. (1975). Effects of stimulating the acetylcholine receptor on the current-voltage relationships of the smooth muscle membrane studied by voltage clamp of potential recorded by a microelectrode. *J. Physiol., Lond.*, **250**, 175–202.

BURGEN, A.S.V. & HILEY, C.R. (1974). Two populations of acetylcholine receptors in guinea-pig ileum. *Br. J. Pharmac.*, **51**, 127P.

BURGEN, A.S.V., HILEY, C.R. & YOUNG, J.M. (1974a). The binding of [^{3}H]-propylbenzilylcholine mustard by longitudinal muscle strips from guinea-pig small intestine. *Br. J. Pharmac.*, **50**, 145–151.

BURGEN, A.S.V., HILEY, C.R. & YOUNG, J.M. (1974b). The properties of muscarinic receptors in mammalian cerebral cortex. *Br. J. Pharmac.*, **51**, 279–285.

BURGEN, A.S.V. & SPERO, L. (1968). The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth-muscle of the guinea-pig intestine. *Br. J. Pharmac.*, **34**, 99–115.

COLQUHOUN, D. (1971). *Lectures on Biostatistics.* pp. 257–266. Oxford: Clarendon Press.

CUTHBERT, A.W. & YOUNG, J.M. (1973). The number of muscarinic receptors in chick amnion muscle. *Br. J. Pharmac.*, **51**, 279–285.

FEWTRELL, C.M.S. & RANG, H.P. (1973). The labelling of cholinergic receptors in smooth muscle. In *Drug Receptors,* ed. Rang, H.P., pp. 211–224. London: Macmillan.

GILL, E.W. & RANG, H.P. (1966). An alkylating derivative of benzilylcholine with specific and long lasting parasympatholytic activity. *Mol. Pharmac.*, **2**, 284–297.

HOOKE, R. & JEEVES, T.A. (1961). 'Direct search' solution of numerical and statistical problems. *J. Ass. comput. Mach.*, **8**, 212–229.

HULME, E.C., BURGEN, A.S.V. & BIRDSALL, N.J.M. (1976). Interactions of agonists and antagonists with the muscarinic receptor. *INSERM,* **50**, 49–70.

JAFFERJI, S.S. & MICHELL, R.H. (1976). Muscarinic cholinergic stimulation of phosphatidylinositol turnover in the longitudinal smooth muscle of guinea-pig ileum. *Biochem. J.*, **154**, 653–657.

KILBINGER, H. (1975). Modulation by oxotremorine and atropine of acetylcholine release evoked by electrical stimulation of guinea-pig ileum longitudinal muscle strips. *Naunyn-Schmiedebergs Arch. Pharmac.*, **287**, 28.

KILBINGER, H. & WAGNER, P. (1975). Inhibition by oxotremorine of acetylcholine resting release from guinea-pig ileum longitudinal muscle strips. *Naunyn-Schmiedebergs Arch. Pharmac.*, **287**, 47–60.

MICHELL, R.H. (1975). Inositol phospholipids and cell surface receptor function. *Biochim. biophys. Acta,* **415**, 81–147.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. Roy. Soc. B.*, **163**, 1–44.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. Roy. Soc. B.*, **164**, 488–510.

RANG, H.P. (1967). The uptake of atropine and related compounds by smooth muscle. *Ann. N.Y. Acad. Sci.*, **144**, 756–767.

REICH, J.G. & ZINKE, I. (1974). Analysis of kinetic and binding measurements. IV. Redundancy of model parameters. *Studia Biophys.*, **43**, 91–107.

REICH, J.G., WINKLER, J. & ZINKE, I. (1974). Analysis of kinetic and binding measurements. II. Reliability of parameter estimates. *Studia Biophys.*, **43**, 181–193.

ROBERTS, F. & STEPHENSON, R.P. (1976). The kinetics of competitive antagonists on guinea-pig ileum. *Br. J. Pharmac.*, **58**, 57–70.

TAYLOR, I.K., CUTHBERT, A.W. & YOUNG, J.M. (1975). Muscarinic receptors in rat intestinal muscle: comparison with the guinea-pig. *Eur. J. Pharmac.*, **31**, 319–326.

THRON, C.D. & WAUD, D.R. (1968). The rate of action of atropine. *J. Pharmac. exp. Ther.*, **160**, 91–105.

WEINSTOCK, M. & SHOHAM, S. (1974). Seasonal variation in sensitivity of guinea-pig tissues to agonists. *Nature, Lond.*, **251**, 427–428.

YAMAMURA, H.I. & SNYDER, S.H. (1974). Muscarinic cholinergic receptor binding in the longitudinal muscle of the guinea-pig ileum with ^{3}H-Quinuclidylbenzilate. *Mol. Pharmac.*, **10**, 861–867.

YOUNG, J.M. (1974). Desensitisation and agonist binding to cholinergic receptors in intestinal smooth muscle. *FEBS Letters,* **46**, 354–356.

(Received December 20, 1976. Revised March 29, 1977.)