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Changes in muscarinic ligand binding to intestinal muscle strips produced by pre-exposure to hypotonic conditions

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The extent of the binding of [^{3}H]propylbenzilylcholine mustard (^{3}H-PrBCM) to muscarinic receptors in longitudinal muscle strips from guinea-pig small intestine is increased by nearly 50% when the strips are preexposed to distilled water before measurement of ^{3}H-PrBCM binding in Krebs-Henseleit solution. The apparent rate constant for ^{3}H-PrBCM-receptor complex formation is more than double that of intact strips. The curves for the inhibition of ^{3}H-PrBCM binding by methylatropinium bromide in normal and treated strips are superimposable, but, in contrast, distilled water pretreatment shifts the inhibition curve for carbachol to lower concentrations by a factor of 5–6. The inhibition curve for methylfurmethide is also shifted, by a factor of approximately 4, but the effect on the curve for hexyltrimethylammonium (C_6TMA) is slight. The relative inhibition produced by benzhexol in the two preparations was variable. Comparison of the rate of equilibration of benzhexol with muscarinic receptors in intact and in distilled water pretreated muscle indicates that this inconsistency is unlikely to be due to incomplete equilibration.

The development of tritium-labelled muscarinic ligands of high specific activity and high specificity has opened the way to measurement of the receptor binding properties of muscarinic agonists and antagonists (reviewed by Birdsall & Hulme, 1976). The point of particular interest to emerge from these studies is that whereas the binding of antagonists appears to represent a simple reversible equilibrium between the drug and a single population of binding sites (Hill coefficient ~1), the binding of muscarinic agonists does not (Hill coefficients <1). The simplest explanation (Burgen & Hiley, 1974), and one which is consistent with the evidence currently available (Birdsall & Hulme, 1976), is that the curves represent binding to two independent sites for which agonists have differing affinities, but which are not distinguished by antagonists. The flattened binding curves for agonists are observed whether measurements are made on tissue homogenates (Birdsall & Hulme, 1976) or on intact muscle (Ward & Young, 1977).

In the course of an early study of the binding of [^{3}H]propylbenzilylcholine mustard (^{3}H-PrBCM) (Burgen, Hiley & Young, 1974a), the *N*-propyl homologue of benzilylcholine mustard (Gill & Rang, 1966), to longitudinal muscle strips from guinea-pig small intestine, it was observed that preexposure of the muscle strips to distilled water before measuring ^{3}H-PrBCM binding in Krebs-Henseleit solution in the usual way, increased the number of muscarinic receptors available to the mustard without any change in the inhibition produced by atropine (Burgen & others, 1974a). In view of this lack of effect on antagonist binding, we were prompted to examine the binding of an agonist, carbachol, to pretreated strips as a simple method of testing for any artifacts in intact strips resulting from contraction of the muscle cells. In this communication we describe these experiments and show that whereas hypotonic pretreatment has no effect on the binding of methylatropinium, it results in a parallel shift of the binding curve for carbachol to higher affinity.

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METHODS

[^{3}H]Propylbenzilylcholine mustard (^{3}H-PrBCM)

^{3}H-PrBCM (1·0 Ci mmol^{-1}) was prepared as described by Burgen & others, (1974a) and converted into the aziridinium ion derivative, the pharmacologically active form, by incubating a 0·13 mM solution in 10 mM phosphate buffer, pH 7·5, for 1 h at room temperature (approximately 22°). The reaction was stopped by diluting an aliquot 1:100 in ice-cold buffer. Under these conditions the yield of the aziridinium ion was 89%. For convenience in describing additions the solution added is termed ^{3}H-PrBCM, but this always refers to the cyclized reaction mixture and the concentration quoted is that of the aziridinium ion derivative.

Binding of ^{3}H-PrBCM to distilled-water pretreated intestinal muscle strips

Longitudinal muscle strips (6–12 per incubation;

each of 3–8 mg) from guinea-pig small intestine, prepared essentially as described by Rang (1964) were suspended in distilled water for 30 min at room temperature (approximately 22°) with occasional gentle agitation. They were then transferred to 400 or 800 ml Krebs-Henseleit solution bubbled with CO_2 in oxygen and incubated at 30° for 1 h before addition of ^{3}H-PrBCM (final concentration 2·4 nM). The reaction was terminated by transferring the strips briefly to 200 ml fresh Krebs and then to a further 200 ml at 30°. 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. A detailed description of the procedure has been given elsewhere (Burgen & others, 1974a).

Where appropriate atropine (final concentration 100 nM) was added 30 min before the ^{3}H-PrBCM.

Inhibition of ^{3}H-PrBCM binding by muscarinic ligands

The general procedure was as described above. Incubation with 2·4 nM ^{3}H-PrBCM was for 10 min at 30°. Antagonists were added 30 min, and agonists 1 min, before the ^{3}H-PrBCM, unless otherwise specified.

The methods of analysis of the inhibition curves have been described by Ward & Young (1977). The curve for methylatropinium was fitted by non-linear regression using a modified Marquardt method as implemented in the Harwell library routine VBO1A on the Cambridge IBM 370/165. Each point was weighed according to the reciprocal of the approximate variance associated with it. The actual equation fitted, assuming that inhibitor binding follows a Hill equation, was

$$\% \text{ of uninhibited binding of } ^3\text{H-PrBCM} = \frac{100 - NS}{A^n.Ka + 1} + NS$$

where A is the concentration of inhibitory ligand (methylatropinium), Ka is its affinity constant for binding to the muscarinic receptor, n is the Hill coefficient (n = 1 if binding follows a simple 1:1 drug-receptor equilibrium) and NS is the percentage of ^{3}H-PrBCM binding insensitive to inhibition by the competing ligand.

The binding of carbachol was analysed as binding to two independent sites, again using the VB01A routine. The binding to each site was assumed to be a simple mass-action equilibrium, the equation fitted being

$$\% \text{ of uninhibited binding of } ^3\text{H-PrBCM} = 100 - \frac{N1.A}{A+K1} - \frac{N2.A}{A+K2}$$

where A is the concentration of the inhibiting ligand (carbachol), K1 & K2 its dissociation constants for the two sites and N1 & N2 the perecentage of the binding of ^{3}H-PrBCM (after a 10 min incubation) associated with each site.

Where the data were insufficient for analysis by these methods the Hill coefficient was obtained as the slope of a Hill plot of log {(100 − % of uninhibited binding of ^{3}H-PrBCM)/(% of uninhibited binding − NS)} *vs* log [A], ignoring points within 5% of the 100% or non-specific (taken as 13%) limits. The best fit value of the slope and its error were determined by linear regression analysis.

Rate of equilibration with benzhexol and propylbenzilylcholine

The procedure for binding measurements described above was modified slightly in a series of experiments to investigate the rate of equilibration of benzhexol and propylbenzilylcholine with the receptor. Twenty longitudinal muscle strips were prepared from each of two guinea-pigs and alternate strips from each animal were incubated in Krebs or distilled water, the nature of the treatment being indicated by the colour of the cotton thread tied to the strip. For the subsequent incubation in Krebs and reaction with ^{3}H-PrBCM the strips were transferred to 4 incubation vessels (each containing 400 ml Krebs solution) so that there were 5 distilled water pretreated and 5 Krebs pretreated strips in each vessel. Benzhexol (10^{-7} M) or propylbenzilylcholine (5×10^{-8} M) was added to two of the incubations at a given time before the addition of ^{3}H-PrBCM. The advantage of this procedure was that it obviated any possibility that strips from differing pretreatments were exposed to different *free* concentrations of benzhexol, due to strips from one pretreatment, but not the other, having absorbed sufficient drug to lower the effective bath concentration. Any differences in the concentration of ^{3}H-PrBCM are similarly avoided. Values were averaged between the two similar concentrations.

There was no indication of any gross differences in the binding properties of the muscle strips from the pairs of guinea-pigs.

Organ-bath measurements

Intact longitudinal muscle strips were suspended in 10 ml Krebs-Henseleit solution gassed with 5% CO_2

in oxygen at 30° in a conventional organ bath. Carbachol was used as agonist and was normally in contact with the tissue for 30 s, with a dose-interval of 3 min. Contractions were recorded isotonically.

The binding affinities of competitive antagonists were determined from the shift of the dose-response curve to carbachol after equilibration with the antagonist, using the relation Dose Ratio—1 = A.Ka, where A is the concentration of the antagonist and Ka its affinity constant.

The dissociation rate constant, k_{-1}, was

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR$$

determined by measuring the decline of the occupancy with time (Paton, 1961), trying to work at as nearly constant response as possible. The occupancy, p, at any time t was obtained from the relation (Paton, 1961)

$$p = (\text{Dose Ratio} - 1)/\text{Dose Ratio}$$

and the rate constant k_{-1} determined from the slope of a plot of ln p *vs* t.

RESULTS

Binding of ^{3}H-PrBCM to muscle strips preexposed to distilled water

The effect on the morphology of the tissue of the exposure of longitudinal muscle strips to distilled water at *ca* 22° for 30 min followed by incubation in Krebs-Henseleit solution for 60 min is shown in Fig. 1. The most obvious effect is that in the hypotonic solution the smooth muscle cells swell and burst, thereby losing much of the cytoplasmic content staining with toluidine blue.

The time-course of the binding of 2·4 nM ^{3}H-PrBCM to distilled water pretreated strips is shown in Fig. 2a (●). In the presence of 100 nM atropine (■) the binding of ^{3}H-PrBCM increased linearly with time over the first 60 min, with possibly some decrease in the rate thereafter. The atropine-sensitive binding of ^{3}H-PrBCM, which is taken to represent muscarinic receptor binding, is saturable (Fig. 2b) and gives a value of 360 pmol g^{-1} wet weight for the number of muscarinic receptors in the tissue. This is an increase of nearly 50% on the number measured in intact muscle strips (Table 1), although the percentage increase is not as great as that observed in earlier and less extensive experiments (Burgen & others, 1974a). The time-course of the receptor-specific binding of ^{3}H-PrBCM approximates to an exponential and gives a value of 3·2 × 10^5 M^{-1} s^{-1} for k_1, the rate constant for formation of the initial reversible complex between ^{3}H-PrBCM and the receptor (cf. Gill & Rang, 1966), assuming that the drug-receptor interaction is rate-limiting. The probability that this assumption is not justified in the case of intact muscle strips has been discussed elsewhere (Ward & Young,

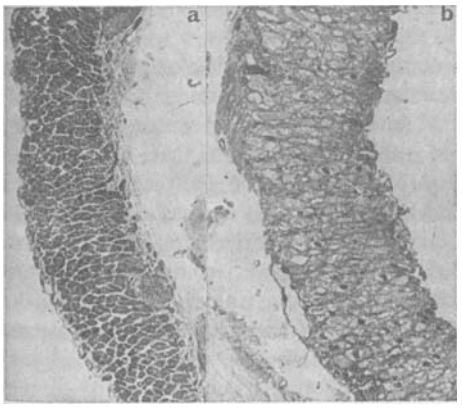

FIG. 1. Morphology of longitudinal muscle strips exposed to (a) Krebs-Henseleit solution only or (b) to distilled water, 30 min, followed by incubation in Krebs for 1 h. Strips were fixed in 5% glutaraldehyde for 2 h at room temperature; stain, toluidine blue. Magnification: (a) × 54 (b) × 41.

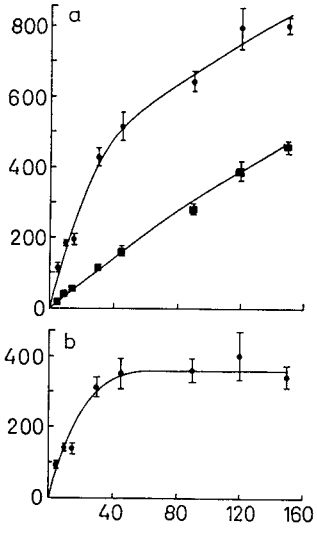

FIG. 2. (a) Time course of ^{3}H-PrBCM binding by distilled water pretreated muscle strips. Binding of 2·4 nM ^{3}H-PrBCM was measured as described under Methods in the absence (●) or presence (■) of 100 nM atropine, (b) Time-course of the atropine-sensitive binding. Points are means ± s.e., with 5–14 muscle strips per point. Ordinate: ^{3}H-PrBCM bound (pmol g^{-1} wet wt). Abscissa: Time (min).

Table 1. *^{3}H-PrBCM binding characteristics of longitudinal muscle strips.*

Muscle strip prep.	Atropine-sensitive ^{3}H-PrBCM binding (pmol g^{-1} wet wt)	Rate of atropine insensitive uptake (pmol g^{-1} wet wt h^{-1})	k_1* ($M^{-1} s^{-1}$)
Guinea-pig			
Intact†	260	72	$1{\cdot}4 \times 10^5$
Water-treated	360	210	$3{\cdot}2 \times 10^5$
Rat			
Intact‡	275	310	$5{\cdot}0 \times 10^5$

* Apparent rate constant for formation of ^{3}H-PrBCM receptor complex.
† Data from Burgen & others (1974a).
‡ Taylor, Cuthbert & Young (1975). Note that the values given in these references for pmol ^{3}H-PrBCM bound were based on an incorrect value for the specific activity of ^{3}H-PrBCM. The figures quoted above are corrected values.

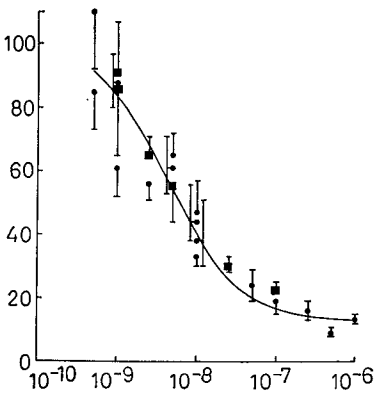

FIG. 3. Inhibition of ^{3}H-PrBCM binding by methylatropinium in intact and in distilled water pretreated strips. Binding measurements were made as described under Methods. The curve is a weighted non-linear fit to the pooled data. Error bars represent 69% confidence limits of the ratio (binding of ^{3}H-PrBCM in presence of methylatropinium) × 100/(binding in absence of methylatropinium). (●), intact strips, data taken from Ward & Young (1977), (■), distilled water pretreated. Ordinate: ^{3}H-PrBCM bound (% of uninhibited). Abscissa: [Methylatropinium] (M).

1977) and adds weight to the evidence obtained by other groups for the presence of an access-limitation factor (Thron & Waud, 1968; Roberts & Stephenson, 1976). It seems very likely that the diffusion problem remains in the water-treated strips since the apparent rate constant is still much less than the values of 2–3 × 10^6 $M^{-1} s^{-1}$ observed in broken-cell systems (Burgen, Hiley & Young, 1974b; Hiley, 1974). The decrease in the diffusion barrier in the series guinea-pig intact - guinea-pig water treated - rat intact strips, as indicated by the increase in k_1 (Table 1) is mirrored, although the correspondence is not exact, by an increase in the rate of atropine-insensitive ^{3}H-PrBCM binding by the tissues.

Inhibition of ^{3}H-PrBCM binding by methylatropinium and carbachol

The inhibition of ^{3}H-PrBCM binding by methylatropinium bromide in distilled water pretreated strips (Fig. 3, ■), is closely similar to that in intact strips (●), in agreement with earlier observations with atropine (Burgen & others, 1974a). The good fit of both sets of points to the same curve, the percentage of non-specific binding of ^{3}H-PrBCM after 10 min incubation being very similar in both cases, taken with the evidence above, suggests that one effect of the hypotonic pretreatment is to make accessible areas of membrane otherwise occluded to ^{3}H-PrBCM in the intact muscle. The weighted best-fit curve to all the points in Fig. 3 has a Hill coefficient of 0·98, close to the value of unity expected from a simple mass-action equilibrium between methylatropinium and the receptor. However, the apparent affinity constant for methylatropinium binding, $1{\cdot}6 \times 10^8$ M^{-1}, is much lower than the value deduced from antagonism of the contractile response to agonists, $2{\cdot}1 \times 10^9$ M^{-1} (Paton & Rang, 1965), a discrepancy which is consistent with the near parallel shift of the binding curve along the concentration axis predicted by simple consideration of the effect of access-limitation (Ward & Young, 1977), although it is not certain that the discrepancy can be wholly explained in this way.

In contrast to the binding of methylatropinium, the curve of the inhibition by carbachol of ^{3}H-PrBCM binding to distilled water pretreated strips is not superimposable on that for intact strips (Fig. 4). The IC50 for the carbachol - sensitive binding of ^{3}H-PrBCM is decreased from 2×10^{-5} M in the intact strips to approximately 6×10^{-7} M in treated strips. However some caution must be exercised where agonist binding is concerned. In a previous series of experiments on oxotremorine binding by intact muscle strips we observed that with strips from one particular batch of guinea-pigs oxotremorine seemed to be less effective in inhibiting ^{3}H-PrBCM binding than in the general run (Ward & Young, 1977). The reasons for this behaviour are not clear but the possibility of variation between animals must be borne in mind. It seems very

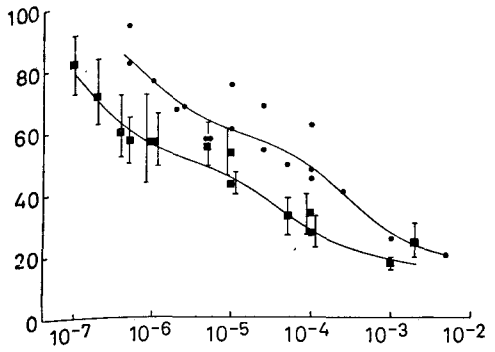

FIG. 4. Inhibition of ^{3}H-PrBCM binding by carbachol in intact and in distilled water pretreated muscle strips. Binding measurements were made as described under Methods. Error bars represent 69% confidence limits of the ratio (binding of ^{3}H-PrBCM in presence of carbachol) × 100/(binding in absence of carbachol) (●), intact strips, data taken from Ward & Young (1977) (the error bars have been omitted for the sake of clarity); (■), distilled water pretreated strips. The curves are weighted non-linear fits assuming that binding is to two independent sites. Ordinate: ^{3}H-PrBCM bound (% of uninhibited). Abscissa: [Carbachol] (M).

unlikely however that the difference between the carbachol inhibition curves (Fig. 4) can have arisen in this way. The points for the distilled water treated strips were determined on strips from 10 guinea-pigs over a period of 6 months, while the points on the intact strips curve were derived from 13 guinea-pigs, some of the measurements being made in the same period and using animals from the same batches as the water-treated strips.

The mean Hill coefficient of the binding curve for carbachol in intact strips is 0·36 (Ward & Young, 1977) indicating that the binding of carbachol does not correspond to a single simple mass-action equilibrium. The simplest explanation for the low Hill coefficients consistent with the evidence currently available (Birdsall & Hulme, 1976) is that the curve represents binding to two independent sites (Burgen & Hiley, 1974) and the curves drawn in Fig. 4 have been fitted on this model. The kinetic constants characterizing the two sites are set out in Table 2. The dissociation constants for both high and low affinity components are shifted to higher affinity by approximately the same factor (5·3 and 5·6, respectively) on going from intact to treated strips. The percentage of H-PrBCM binding insensitive to inhibition by carbachol is similar for both curves. The proportion of high affinity sites is numerically greater after hypotonic pretreatment, but the difference is not statistically significant.

Table 2. *Carbachol binding to longitudinal muscle strips preexposed to Krebs solution or distilled water analysed as binding to two independent sites.*

	Dissociation constants (M)		
Pretreatment	K_1	K_2	K_2/K_1
Krebs	$7·2 \pm 3·6 \times 10^{-7}$	$2·9 \pm 1·6 \times 10^{-4}$	403
Dist. water	$1·4 \pm 0·6 \times 10^{-7}$	$5·2 \pm 2·6 \times 10^{-5}$	371
	% of ^{3}H-PrBCM binding associated with each site		
	High affinity	Low affinity	Non-specific*
Krebs	41·7 ± 4·0	40·9 ± 4·6	17·4
Dist. water	50·6 ± 3·9	33·0 ± 3·8	16·4

Values are means ± estimated standard error obtained from non-linear regression analysis of the points shown in Fig. 4, using the Harwell Library routine VB01A (see Methods).
* Percentage of ^{3}H-PrBCM binding (after a 10 min incubation) insensitive to carbachol, calculated from 100 − (% high affinity site + % low affinity site).

Inhibition of ^{3}H-PrBCM binding by other agonists

In addition to carbachol we have examined, although not nearly so extensively, the effect of the hypotonic pretreatment on the binding of three other agonists, methylfurmethide, acetylcholine and hexyltrimethylammonium (C_6TMA). In no case was as large a shift observed as with carbachol. For methylfurmethide the factor for the shift to higher affinity after distilled water pretreatment (6 points on curve) compared to the curve for intact strips (10 points) was approximately 4. With the limited data it is not certain that the two curves are parallel, although the best fit values of the mean Hill coefficient for the treated curve, 0·68, was not significantly different from that for the intact strips, 0·58. The difference between the position of the two curves was highly significant ($P < 0·001$). For acetylcholine, in the presence of 10^{-6} M physostigmine as a cholinesterase inhibitor, the shift after hypotonic pretreatment appeared to be small, a factor of *ca* 1·5, but this value must be treated with some reserve since it is based on only 4 points for the treated and 7 for the intact muscle strips. The necessity of having a cholinesterase inhibitor present complicates the interpretation and has discouraged us from making a more detailed study of acetylcholine.

Some uncertainty also characterizes the effect of hypotonic pretreatment on the binding of C_6TMA (Fig. 5). C_6TMA was of particular interest for two reasons. Firstly it is usually regarded as a partial agonist, although in our experience this is not strictly in accord with the usual definition, since we have been able to obtain the same maximal contraction of intact strips with C_6TMA as with carbachol, and secondly it was an agonist for which there seemed to be some discrepancy between the Hill coefficients determined for the binding curve

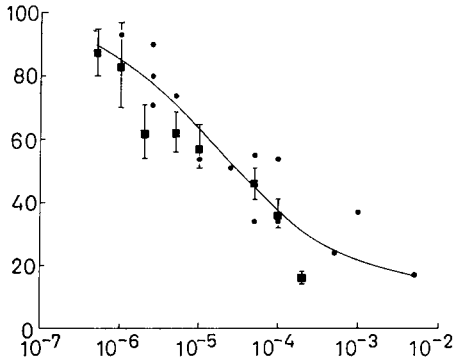

FIG. 5. Inhibition of ^{3}H-PrBCM binding by hexyltrimethylammonium (C_6TMA) in intact and in distilled water pretreated strips. Binding measurements were made as described under Methods. Error bars represent 69% confidence limits of the ratio (binding of ^{3}H-PrBCM in the presence of C_6TMA) × 100/(binding in absence of C_6TMA). (●), intact strips, data taken from Ward & Young (1977) (Error bars have been omitted for the sake of clarity); (■), distilled water pretreated strips. The curve is a weighted non-linear fit to the points for the intact strips only. Ordinate: ^{3}H-PrBCM bound (% of uninhibited). Abscissa: [C_6TMA] (M).

in intact muscle strips (Ward & Young, 1977) and that reported for cortical homogenates (Birdsall & Hulme, 1976). There is a considerable scatter on the points from the intact strips, but even so most of the points from the distilled water pretreated strips lie near to the weighted best-fit curve for the intact muscle. The two points at 2×10^{-6} and 2×10^{-4} M C_6TMA which diverge from the general trend of the distilled water values came from strips from the same guinea-pig, in which the contractile response of the muscle was particularly sensitive to carbachol. Even with the large weight attached to the point at 2×10^{-4} M C_6TMA the best-fit value of the Hill coefficient for the distilled water pretreated curve, obtained from weighted non-linear regression analysis (setting the C_6TMA insensitive binding of ^{3}H-PrBCM to 14%) was only 0·70 (cf. 0·55 for intact strips under similar incubation conditions; Ward & Young, 1977). The slope of a conventional unweighted Hill plot (see Methods) was 0·65 ± 0·12, significantly different from unity. If the hypotonic pretreatment does not flatten ligand binding curves, and the limited evidence that we have so far suggests that it doesn't, then this would seem to provide some support for the belief that there may be a discrepancy between the binding of C_6TMA to intact muscle strips and to homogenates of rat cerebral cortex, where the Hill coefficient is reported to be 1·0 (Birdsall & Hulme, 1976).

Inhibition of ^{3}H-PrBCM binding by benzhexol

The data presented above suggest that for antagonists distilled water pretreatment may have no effect on either the slope or position of binding curves while for agonists the curves may be shifted but without any change in slope. However our data are too limited to be able to suggest that this is a general rule and the problems associated with working with muscle strips have discouraged us from making a more extensive survey at the present. The only other antagonist we have investigated is benzhexol. There were again two particular reasons for investigating this drug. Firstly, benzhexol was the only antagonist examined by Burgen & Spero (1968) which distinguished between the carbachol-stimulated contractile response in longitudinal muscle strips and the efflux of potassium and rubidium produced by higher concentrations of the agonist, the latter being more sensitive by a factor of 8·5 to inhibition by benzhexol. Secondly the discrepancy between the apparent binding affinity of benzhexol for muscarinic receptors deduced from the inhibition of ^{3}H-PrBCM binding to intact muscle strips and the values obtained from antagonism of the contractile response was much larger than might have been expected from simple considerations of the possible effect of an access-limitation factor in the kinetics of binding of ^{3}H-PrBCM (Ward & Young, 1977). The results of experiments on the binding of benzhexol to distilled water pretreated compared to intact strips were variable. In one series of measurements (5 points) the curve was shifted to higher affinity by a factor of approximately 2 compared with intact strips, but in subsequent experiments the difference was smaller or non-existent. Depletion of benzhexol from the solution by partitioning into lipid phases in the tissue was apparently not responsible for the variability since differences were observed in experiments where the medium was replenished or where treated and intact strips were incubated in the same vessel with ^{3}H-PrBCM.

Rate of equilibration of benzhexol and propylbenzilylcholine with muscarinic receptors in intact and distilled water treated strips

One possibility we considered as an explanation for the behaviour of benzhexol was that its relatively lipophilic character might greatly slow equilibration with the tissue in some intact strip preparations so that even after 30 min it was not complete. To investigate this possibility the affinity constant, K_a, the rate constant for the dissociation of benzhexol

from the receptor, k_{-1}, and hence the rate constant for complex formation, k_1, were determined from the equilibrium dose-ratio for inhibition of the contractile response of muscle strips to carbachol and the rate of decline of inhibition on washout. For comparison, measurements were also made with propylbenzilylcholine, a quaternary compound and consequently less lipophilic, whose affinity for the muscarinic receptor estimated from its mydriatic effect in mice relative to that of atropine (2×10^8 M^{-1}; Ford-Moore & Ing, 1947) or determined from inhibition of [^{3}H]propylbenzilylcholine binding ($1{\cdot}0 \times 10^8$ M^{-1}; Birdsall & Hulme, 1976) was similar to that expected for benzhexol. Affinity constants ranging from $1{\cdot}6 \times 10^8$ to $7{\cdot}7 \times 10^9$ M^{-1} (calculated from measurements made in a variety of ways) have been reported for benzhexol but a more recent determination from inhibition of the contractile response of guinea-pig ileum to carbachol, $2{\cdot}3 \pm 0{\cdot}1 \times 10^8$ M^{-1} (Barlow, Franks & Pearson, 1972) suggests that the true value lies at the lower end of the range.

The kinetic constants measured for benzhexol and propylbenzilylcholine are set out in Table 3. The affinity constant for benzhexol is in agreement both with value of Barlow & others (1972) and with the affinity deduced from binding studies with [^{3}H]-atropine, $1{\cdot}4 \times 10^8$ M^{-1} (Paton & Rang, 1965) and [^{3}H]propylbenzilylcholine, $1{\cdot}4 \times 10^8$ M^{-1} (Birdsall & Hulme, 1976). The close correspondence of Ka for propylbenzilylcholine (Table 3) with the value estimated from the mydriatic effect (Ford-Moore & Ing, 1947) is also very satisfactory. The similarity in the values of Ka for the two compounds was not, however, observed with the rate constant for the decline of occupancy on washing out the antagonist, k_{-1}. The values of k_{-1} determined in this way almost certainly have a large diffusional element in them (Roberts & Stephenson, 1976), but the difference clearly implies that benzhexol will equilibrate more slowly with intact muscle strips than propylbenzilylcholine. Even so the apparent onset rate constant (k_1 [A] + k_{-1}) at 10^{-7} M benzhexol, a concentration which gives approximately 50% inhibition of ^{3}H-PrBCM binding, is $9{\cdot}5 \times 10^{-3}$ s^{-1} and is not slow enough to account for any noticable shift in the curve on going to distilled water pretreated strips, since after 30 min incubation the benzhexol should have achieved complete equilibration, and even after 5 min the receptor occupancy should have reached 94% of the final equilibrium level.

This was borne out by a series of experiments comparing distilled water pretreated and normal strips, where in place of the usual 30 min pre-incubation of the benzhexol with the tissue before addition of ^{3}H-PrBCM, the time was varied between 0 and 120 min. The incubation with ^{3}H-PrBCM was for the normal 10 min. The results are shown in Fig. 6. Equilibration with the distilled water pretreated strips is clearly much more rapid than with the intact muscle, indeed even when the benzhexol is added 5 s (zero time point) before the ^{3}H-PrBCM the degree of inhibition achieved is the same as with much longer incubation times. Even with the intact strips the rate of equilibration is sufficiently fast that by 5 min there is as predicted, no significant difference between the two sets of

Table 3. *Kinetic constants for the interaction of benzhexol and propylbenzilylcholine with muscarinic receptors in intact muscle strips, obtained from measurement of the inhibition of the contractile response to carbachol.*

	Ka (M^{-1})	k_{-1} (s^{-1})	k_1 (M^{-1} s^{-1})
Benzhexol	$1{\cdot}3 \pm 0{\cdot}1 \times 10^8$ (3)	$6{\cdot}6 \pm 0{\cdot}4 \times 10^{-4}$ (3)	$8{\cdot}8 \times 10^4$
Propylbenzilylcholine	$2{\cdot}3 \pm 0{\cdot}4 \times 10^8$ (3)	$1{\cdot}4 \pm 0{\cdot}1 \times 10^{-3}$ (3)	$3{\cdot}3 \times 10^5$

Values are means ± s.e. (number of determinations). The method for determination of Ka, the affinity constant and k_{-1}, the rate constant for dissociation of the drug-receptor complex are given under Methods. The rate constant for complex formation, k_1, was calculated from $k_1 = Ka.k_{-1}$.

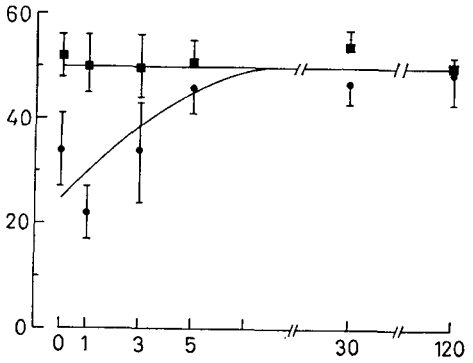

FIG. 6. Rate of equilibration of benzhexol with ^{3}H-PrBCM binding sites in intact and in distilled water pretreated muscle strips. The curves show the percentage inhibition of 2·3 nM ^{3}H-PrBCM binding (10 min incubation) by 100 nM benzhexol as a function of the time of incubation with benzhexol before addition of ^{3}H-PrBCM. (●), intact strips; (■), distilled water pretreated. The experimental procedure is described under Methods. Error bars represent the 69% confidence limits of the ratio (binding of ^{3}H-PrBCM in presence of benzhexol) × 100/(binding in absence of benzhexol). Ordinate: Inhibition of ^{3}H-PrBCM binding (%). Abscissa: Time (min).

strips. In parallel experiments with propylbenzilylcholine the difference had disappeared after 2 min preincubation.

DISCUSSION

The observation of particular interest described above is that the muscarinic receptor binding of methylatropinium, an antagonist, does not have the same properties as that of carbachol, an agonist. Our data are too limited to allow any general rule to be formulated, although methylfurmethide appears to behave like carbachol and atropine (Burgen & others, 1974a) like methylatropinium. Benzhexol is problematical, but any shift is at the most small.

In evaluating the data it must be borne in mind that there are difficulties inherent in the use of intact or semi-intact tissues. The major problem arises from the almost certain presence of an access-limitation factor in the binding of ^{3}H-PrBCM to intact muscle strips, since the binding measurements with ^{3}H-PrBCM are necessarily rate assays. We have discussed this problem elsewhere (Ward & Young, 1977) and showed that if simplifying assumptions are made then it might be expected that the effect of the diffusion barrier would be to produce a near parallel shift of inhibition curves to higher ligand concentrations, without any marked effect on the shape of the curve. The observed ligand inhibition curves (Ward & Young, 1977) on the whole followed this pattern well, although the extent of the shift, compared with curves measured on tissue homogenates, was variable. Carbachol was unusual in that there was no displacement in the position of the curve from that reported for cortical homogenates. However, there was no general difference between the behaviour of agonists or antagonists in the extent of the shift.

It seems likely that there is still an access-limitation factor in the kinetics of ^{3}H-PrBCM binding to distilled water pretreated muscle strips since k_1, the apparent rate constant for ^{3}H-PrBCM-receptor reversible complex formation, is still less than in tissue homogenates (cf. Table 2 in Taylor, Cuthbert & Young, 1975). Clearly the diffusional barrier has been diminished, as evidenced both by the increase in k_1 over the value in intact muscle and by the more rapid equilibration of benzhexol. In spite of these changes there is no difference in the position of the methylatropinium inhibition curve in normal or pretreated strips. This is consistent with ^{3}H-PrBCM reacting rapidly with easily accessible receptors and more slowly with others where the concentration increases only gradually. The increased rate constant k_1 means that after a given time reaction will have taken place with more receptors, but the proportions of fast and slow are apparently not much altered. This is also in accord with the observation that there was no difference in the position of the curve measured on guinea-pig intestine or on rat intestine, although in the rat muscle k_1 was also greater than in the guinea-pig (Taylor & others, 1975).

The fact that methylatropinium binding is not affected by the changes produced by distilled water pretreatment provides a useful control and makes the effect on carbachol binding all the more striking. Similarly the lack of any marked effect of hypertonic pretreatment on the inhibition curve for C_6TMA, which like carbachol was added 1 min before the ^{3}H-PrBCM, argues strongly against the shift in the carbachol curve being due to incomplete equilibration in the intact muscle. The question then remains whether the position of the inhibition curve for carbachol is influenced in intact muscle strips by the contraction that the drug produces. In this respect C_6TMA does not provide a clear control. In two independent experiments the ED50 for C_6TMA-induced contraction of longitudinal muscle strips was 6×10^{-6} M and 2×10^{-5} M, i.e. in the same range as the IC50, *ca* 2×10^{-5} M (Fig. 5), for inhibition of ^{3}H-PrBCM binding. In contrast, 10^{-6} M carbachol which usually gives a maximal contraction produces only *ca* 25% inhibition in intact strips and a similar situation exists for methylfurmethide.

The possible mechanical effects of contraction on ^{3}H-PrBCM diffusion in the intact tissue are not easy to predict. Whereas shortening of the muscle and contraction of the muscle cells might be expected to lead to greater inhibition of ^{3}H-PrBCM binding by hindering access into the tissue or onto the receptors, electron micrographs suggest that the intercellular channels are wider in the contracted tissue. It must also be noted that after distilled water pretreatment the binding curve for carbachol lies at lower carbachol concentrations than in homogenates of cerebral cortex (Birdsall & Hulme, 1976), where there is no possibility of effects due to contraction. A mechanical effect thus seems unlikely, but the possibility is not excluded that following large doses of carbachol metabolic effects accompanying contraction influence receptor binding of the drug in intact tissue.

Recently, Birdsall, Burgen & others (1977) reported that if binding to muscarinic receptors in

cerebral cortex is measured at low ionic strength, then the affinity of *both* agonists and antagonists is increased. The two binding sites for carbachol were affected unequally, there being a greater increase in the affinity of the lower than the higher affinity site, without any change in the proportions of the two sites. Our conditions differ in that binding to the hypotonically-shocked muscle strips was always measured in Krebs solution, the ^{3}H-PrBCM being added not earlier than 1 h after transfer from the distilled water, but the contrast in effects is notable. The carbachol binding was altered but not that of atropine or methylatropinium and the slope of the carbachol curve was not affected. It may be noted that the slope of the carbachol curve in intact muscle can seemingly be altered by pretreatment of tissue with chlorpromazine (Haigh & Young, 1975) or high concentrations of carbachol (Young, 1974) but the mechanisms of these effects are not known.

In their study of binding at low ionic strength Birdsall & others (1977) made the interesting observation that the changes in ligand affinity could be reversed by the addition of various ions, including Ca^{2+}. It is quite conceivable that changes in Ca^{2+} binding or mobilization could be involved in all these effects on carbachol binding.

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