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THE IRREVERSIBLE BINDING OF ACETYLCHOLINE MUSTARD TO MUSCARINIC RECEPTORS IN INTESTINAL SMOOTH MUSCLE OF THE GUINEA-PIG

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1 Acetylcholine mustard (*N*-2-chloroethyl-*N*-methyl-2-acetoxyethylamine), a potent muscarinic agonist, binds virtually irreversibly to muscarinic receptors in longitudinal muscle strips from guinea-pig small intestine, as shown by the inhibition of the binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM), an affinity label for the muscarinic receptor.

2 A value for the apparent binding affinity of acetylcholine mustard and a value for the rate constant for the receptor alkylation reaction have been deduced from the rate of onset of the inhibition of [^{3}H]-PrBCM binding.

3 The kinetic constants obtained may refer largely to the interaction between acetylcholine mustard and the desensitized receptor.

4 At high concentrations acetylcholine mustard practically abolishes the contractile response to carbachol. At these concentrations acetylcholine mustard appears to have multiple actions on the tissue.

Introduction

The formation of a reversible complex between an agonist and its receptor is considered to be the first event in the chain which leads to the physiological response, but until recently methods for obtaining agonist binding affinities in intact tissues have been limited to indirect organ bath techniques (see e.g. Parker, 1972). The development of affinity labels for drug receptors has opened the way for more direct studies of agonist binding via the inhibition by the agonist of the binding of the affinity label. In the special case of an agonist which also binds irreversibly to the receptor there is in principle an alternative approach to the binding affinity from measurements of the rate of formation of the covalently-bound complex and in this paper we describe the application of this method to study the binding of acetylcholine mustard (*N*-2-chloroethyl-*N*-methyl-2-acetoxyethylamine) (Hanby & Rydon, 1947) to muscarinic receptors in longitudinal muscle strips from guinea-pig small intestine.

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Acetylcholine mustard in its cyclized aziridinium ion form (Figure 1) is a potent agonist at muscarinic receptors in guinea-pig ileum (Hirst & Jackson, 1972) and rat jejunum (Hudgins & Stubbins, 1972) but, surprisingly, has been reported as showing either no (Hirst & Jackson, 1972) or relatively weak (Hudgins & Stubbins, 1972) irreversible binding. There are, however, experimental difficulties involved in using the contractile response as a measure of the irreversible binding and to circumvent these we have compared the binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM), a specific and virtually irreversible muscarinic antagonist (Burgen, Hiley & Young, 1974a) before and after exposure of the tissue to acetylcholine mustard. By this method it is shown that acetylcholine mustard does bind irreversibly to muscarinic receptors in the longitudinal muscle from guinea-pig small intestine and that the rate of onset of block can be used to derive kinetic constants.

Some of these results have been communicated in preliminary form to the British Pharmacological Society (Robinson, Taylor & Young, 1974).

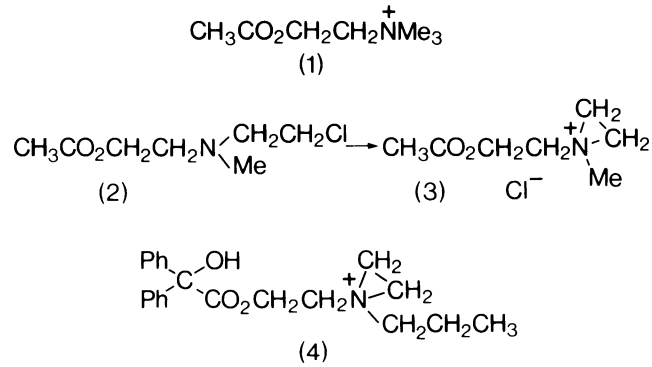

Figure 1 Structures of acetylcholine (1), acetylcholine mustard (2) and its aziridinium ion derivative (3), and the aziridinium ion from propylbenzilylcholine mustard (4).

Methods

Acetylcholine mustard

Acetylcholine mustard was synthesized essentially as described by Hanby & Rydon (1947) and had b.p. 60-62° C/0.2 mm. The n.m.r. spectrum (in $CDCl_3$, measured on a Varian T-60A spectrometer at 60 MHz) was virtually identical with that reported by Hirst & Jackson (1972) for the pure liquid. Assignment of the line positions, made by comparison of the spectrum with that of mustine (see below), measurement of coupling constants and sequential spin-decoupling of the methylene resonances, provided proof of the structure. Assignments (line positions are given in parts/10^6 downfield from tetramethylsilane as internal standard): C-Me, 2.07, s; N-Me, 2.39, s; acetoxyethyl N-CH_2, 2.74, t (J 6.0 Hz); chloroethyl N-CH_2, 2.81, t (J 6.7 Hz); Cl-CH_2, 3.57, t (J 6.7 Hz) and 0-CH_2, 4.18, t (J 6.0 Hz).

Further structural evidence was provided by the dimerization of acetylcholine mustard in ethanol to give *N,N'*-dimethyl-*N,N'*-di(2-acetoxyethyl)-piperazinium dichloride, m.p. 221-222° C, literature m.p. 228° C (Hanby & Rydon, 1947), whose n.m.r. spectrum was identical with that given by Hirst & Jackson (1972).

The parent chloroethylamine-compound was converted into the active aziridinium ion form before use by maintaining a 0.7 mM solution in Krebs-Henseleit solution for 45 min at 30° C at which time the yield of the aziridinium ion, 70.5 ± 1% (4 measurements), is maximal. The cyclized solution was used immediately after preparation. *All concentrations of acetylcholine mustard quoted are those of the aziridinium ion form, calculated assuming a 71% yield.* Cyclization was carried out in Krebs solution to avoid any dilution of the Krebs solution constituents when large volumes of the mustard solution had to be added to the incubation medium, but a higher yield of the aziridinium ion (79 ± 0.5%, 3 measurements) can be achieved if cyclization is allowed to proceed in 10 mM phosphate buffer, pH 7.5. The decay of the aziridinium ion concentration, presumably due to hydrolysis, followed first order kinetics, the rate of the decay in Krebs solution at 30° C ($k = 2.3 \pm 0.2 \times 10^{-5}\ s^{-1}$, 4 measurements) being notably slower than in 10 mM phosphate buffer, pH 7.5 ($k = 6.0 \pm 0.1 \times 10^{-5}\ s^{-1}$, 3 measurements). A similarly slower decay in Krebs solution has been noted previously with the aziridinium ion from PrBCM (Young, Hiley & Burgen, 1972).

It should be noted that measurement of the yield of aziridinium ion is not a satisfactory test of the purity of acetylcholine mustard. In one preparation a second product, b.p. 40-41° C/0.2 mm, was isolated and identified as mustine, bis(2-chloroethyl)methylamine, by comparison of its i.r. and n.m.r. spectra with those of an authentic sample prepared by the method of Hanby & Rydon (1947). N.m.r. assignments (in $CDCl_3$, line positions in parts/10^6 downfield from tetramethylsilane): N-Me, 2.38, s; N-CH_2 methylenes, 4 protons, 2.82, t (J 6.7 Hz) and Cl-CH_2 methylenes, 4 protons, 3.57, t (J 6.7 Hz). The piperazinium dimers from both samples were likewise identical. Thus samples of acetylcholine mustard contaminated with mustine give a misleadingly high value of aziridinium ion (as measured by reaction with thiosulphate ion). As a rapid and reliable check of the purity of acetylcholine mustard samples, we have routinely used the relative integration values of the peaks in the n.m.r. spectrum (calculated from the number of protons involved in each resonance). In samples contaminated with mustine while there is almost no change in the line positions in the spectrum,

there is a clear discrepancy from the theoretical integration.

Acetylcholine mustard was stored as the pure liquid at -10°C. Under these conditions solid material (presumably the piperazinium dimer) was slowly deposited and was filtered off at intervals, but the purity of the supernatant liquid remained good, even after storage for a year.

[^{3}H]-propylbenzilylcholine mustard

[^{3}H]-PrBCM (specific radioactivity 1.8 Ci/mmol and 1.4 Ci/mmol) (Burgen *et al.*, 1974a) was cyclized as a 0.13 mM solution in 10 mM phosphate buffer, pH 7.5, at room temperature (approx. 23°C) for 1 h, as described previously (Burgen *et al.*, 1974a). Under these conditions the yield of the aziridinium ion was 89%.

Irreversible binding of acetylcholine mustard

Longitudinal muscle strips from guinea-pig small intestine were prepared essentially as described by Rang (1964) and suspended in Krebs-Henseleit solution gassed with 5% CO_2 in O_2 at 30°C. After 1 h physostigmine (10^{-6} M) was added and the preincubation continued for a further 30 minutes. At the end of this period the strips were transferred to fresh Krebs solution containing physostigmine 10^{-6} M and freshly cyclized acetylcholine mustard and incubated for a given time on a shaking water bath. Control strips were similarly treated except that the Krebs solution contained no acetylcholine mustard. At the end of the incubation all strips were transferred to fresh Krebs solution and washed, usually for 30-40 min, before treatment with [^{3}H]-PrBCM 2.4 nM for 10 minutes. The reaction was terminated and non-covalently bound tritium removed by washing for 75 min with several changes of Krebs solution. The strips were then blotted dry, transferred to scintillation vials, weighed, treated with 0.7 ml Soluene (Packard) and allowed to stand overnight at room temperature, by which time the strips had dissolved completely. Ethoxyethanol (1 ml) and 10 ml butyl-PBD in toluene (0.4%, w/v) were added and the tritium determined by liquid scintillation counting.

Determination of the binding affinity of acetylcholine mustard

The irreversible binding of benzilylcholine mustard to muscarinic receptors in the longitudinal muscle of guinea-pig ileum can be described in terms of a scheme where the reversible drug-receptor complex (AR) once formed is

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \xrightarrow{k_2} AR'$$

converted into the covalently-bound complex (AR′) faster than it dissociates, i.e. $k_2 \gg k_{-1}$ (Gill & Rang, 1966). For acetylcholine mustard, an agonist whose action is terminated rapidly on washing and which gives a contractile response at a concentration and for an exposure time which produces no apparent covalent binding, it is highly probable that $k_{-1} \gg k_2$. This is the usual situation for active-site-directed irreversible inhibitors of enzymes. Thus for acetylcholine mustard the free-receptor fraction should initially decline exponentially with time, the apparent rate constant, k_{app} being related to the concentration of acetylcholine mustard [A], by the expression (Kitz & Wilson, 1962):

$$\frac{1}{k_{app}} = \frac{1}{k_2} + \frac{1}{k_2 . K_a} \cdot \frac{1}{[A]}$$

where $K_a = k_1/k_{-1}$, the apparent binding affinity of acetylcholine mustard.

The time course of [^{3}H]-PrBCM binding by muscarinic receptors in muscle strips approximates to an exponential curve (Burgen *et al.*, 1974a). Assuming that the rate constant for uptake of [^{3}H]-PrBCM is the same before and after treatment with acetylcholine mustard, then the proportion of the total uptake achieved after a given time will be the same in both cases. Thus the percentage uptake of the treated strips as compared to the control strips provides a measure of the free-receptor fraction.

Organ-bath studies

Muscle strips were suspended in a conventional 10 ml organ bath in Krebs-Henseleit solution at 30°C bubbled with 5% CO_2 in O_2. Contractions were recorded isotonically and washing was by overflow. Agonists were in contact with the tissue for 20-40 s, with a dose interval of 2 or 3 minutes.

For measurement of the equipotent molar ratio of acetylcholine mustard to acetylcholine (2 x 2 dose assay) 'nerve-free' muscle strips were prepared by the technique of Paton & Zar (1968). The advantage of these strips is that the contraction produced by physostigmine, which was present at a concentration of 10^{-6} M, is very much diminished (Cox & Lomas, 1971). The considerably larger amount of tissue required for the binding experiments made the use of 'nerve-free' strips there impracticable.

In experiments where the effect of extended

incubation with acetylcholine mustard on the contractile response to carbachol was investigated, the usual procedure was first to establish a dose-response curve to carbachol and then to expose the tissue to the same dose of acetylcholine and for the same time as was to be tested for the mustard. After exposure to acetylcholine the tissue was allowed to recover from desensitization until a stable dose-response curve to carbachol could be obtained and the cycle then repeated, but with freshly cyclized acetylcholine mustard instead of acetylcholine. Recovery from desensitization caused by acetylcholine was complete by 60-90 min, although in some experiments this treatment resulted in a small parallel shift to the right of the log dose-response curve for carbachol and in some cases there was a small decrease in the resting length of the muscle.

Results

Potency of acetylcholine mustard as a muscarinic agonist

Acetylcholine mustard had an equimolar potency ratio of 4.3 (95% confidence limits 3.8-4.8) compared with acetylcholine in producing a contractile response in 'nerve-free' muscle strips in the presence of physostigmine 10^{-6} M. The presence of physostigmine produced an approximately 8-fold increase in the sensitivity of the strips to acetylcholine, but in two experiments in which the potency ratio was measured on normal muscle strips in the absence of physostigmine the values were 3.1 and 3.9, indicating that the two drugs must be similarly sensitive to cholinesterase. This is consistent with the report that the mustard is a substrate for bovine erythrocyte acetylcholinesterase (Hirst & Jackson, 1972). Hexamethonium (5×10^{-6} M) had no effect on the sensitivity to either agonist and the piperazinium dimer (10^{-4} M), a by-product of the cyclization reaction (Hanby & Rydon, 1947), was similarly without effect. The potency ratio is similar to that reported for intact intestinal segments from the guinea-pig, 6.1 ± 0.1 (Hirst & Jackson, 1972) and the rat, approx. 5 (Hudgins & Stubbins, 1972), both measured in the absence of a cholinesterase inhibitor.

Irreversible binding of acetylcholine mustard

Exposure of muscle strips to acetylcholine mustard in the presence of physostigmine (10^{-6} M) for increasing times led to a progressive inhibition of the subsequent binding of [^{3}H]-PrBCM compared with control strips which had not been

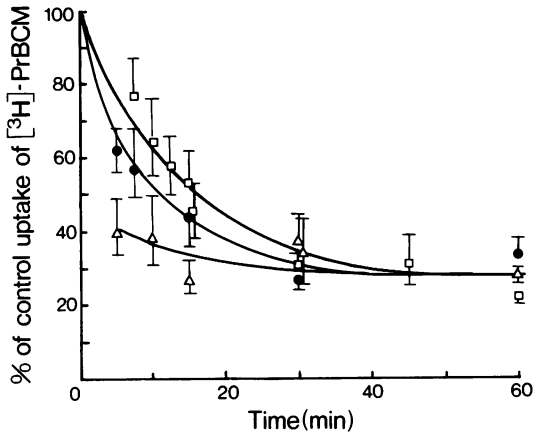

Figure 2 Progressive inhibition by acetylcholine mustard of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by muscle strips. The experimental conditions are described in the methods section. The time is the time of incubation with acetylcholine mustard. Each point is the ratio of the uptake of [^{3}H]-PrBCM by 5-7 strips previously exposed to acetylcholine mustard to the amount bound by 5-7 untreated strips from the same animal. The vertical bars are the approximate 69% confidence limits. Acetylcholine mustard concentrations (M): (□) 2.5×10^{-5}; (●) 4.9×10^{-5} and (△) 2.9×10^{-4}.

treated with acetylcholine mustard. Approximately 28% of the uptake could not be inhibited, even after prolonged incubation with acetylcholine mustard (Figure 2) and this is taken to represent the non-specific binding of [^{3}H]-PrBCM. This percentage is, however, larger than the percentage of [^{3}H]-PrBCM binding (about 15%) which cannot be inhibited by reversible muscarinic agonists or antagonists added at the same time as the [^{3}H]-PrBCM, e.g. acetylcholine (Figure 3), during a 10 min incubation period.

Recovery from block by acetylcholine mustard

The recovery from block by acetylcholine mustard was very slow. Muscle strips from three animals were exposed to acetylcholine mustard 2.5×10^{-5} M for 60 min at 30°C and then washed for periods ranging from 30 s to 2 h before labelling with [^{3}H]-PrBCM. To accentuate any recovery the washing after acetylcholine mustard was carried out at 37°C. No significant change in the level of the inhibition with time of washing was observed (Figure 4). If the difference between the 5 min and mean 120 min values were presumed to be real and assuming recovery follows first order kinetics, this would give a value for the

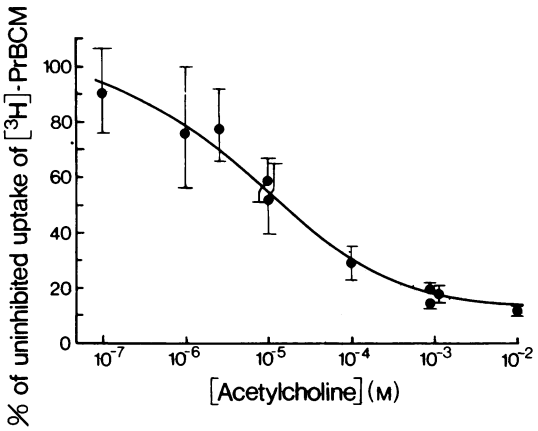

Figure 3 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by acetylcholine. Neostigmine (10^{-7} M) or physostigmine (10^{-6} M) (for 2 points at 10^{-3} M and the point at 10^{-2} M acetylcholine) were added 30 min before, and acetylcholine 1 min before, [^{3}H]-PrBCM 2.4 x 10^{-9} M. Incubation was for 10 min at 30°C. Each point is the percentage of the uptake of [^{3}H]-PrBCM in the absence of acetylcholine (4-7 strips in each group). The vertical bars are the approximate 69% confidence limits.

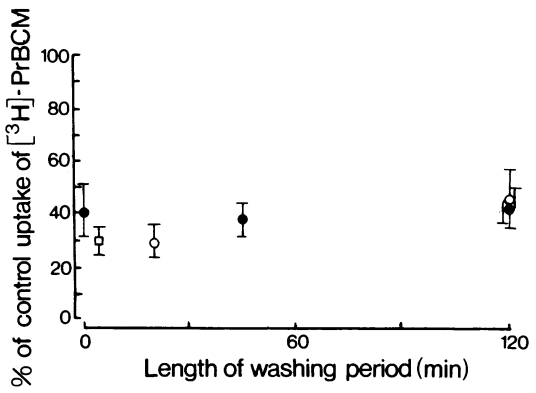

Figure 4 Rate of recovery from block by acetylcholine mustard: effect of varying the washing period at 37° C after exposure to acetylcholine mustard on the subsequent uptake of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM). Each point is the percentage binding of [^{3}H]-PrBCM by 4-5 muscle strips exposed to acetylcholine mustard 2.5 x 10^{-5} M for 60 min compared with the binding by 4-5 untreated strips from the same animals. The vertical bars are the approximate 69% confidence limits. The different symbols denote different animals.

rate constant of 3 x 10^{-5} s^{-1} and it may well be that the true value for acetylcholine mustard is not greatly different from that for [^{3}H]-PrBCM at 37° C, 6 x 10^{-6} s^{-1} (Burgen *et al.*, 1974a).

Rate of onset of block and the apparent binding affinity of acetylcholine mustard

The rate of decline of the free-receptor fraction with time for various concentrations of acetylcholine mustard is shown in Figure 5. The free-receptor fraction has been calculated from the receptor-specific uptake of [^{3}H]-PrBCM only, taking the non-specific uptake to be that portion, 28% (Figure 2), insensitive to acetylcholine mustard. The errors inherent in the measurements make a rigorous test of the predicted exponential relationship difficult, but the data for the three highest concentrations of the mustard suggest that it is obeyed, even though two of the points deviate markedly from the best straight line (fitted by inspection).

The rate constants (k_{app}) for each acetylcholine mustard concentration, obtained from the lines in Figure 5, are replotted in Figure 6 as $1/k_{app}$ against 1/[acetylcholine mustard]. The slope and intercept of this plot (cf. methods section) give a value for k_2 of 1.6 x 10^{-3} s^{-1} (95% confidence limits. 1.1-3.0 x 10^{-3}) and for K_a 1.2 x 10^5 M^{-1} (0.6-1.7 x 10^5).

Effect of acetylcholine mustard on the contractile response to carbachol

The irreversible binding of acetylcholine mustard to muscarinic receptors demonstrated by the inhibition of the subsequent binding of [^{3}H]-PrBCM is in marked contrast with the report (Hirst & Jackson, 1972) that exposure of segments of guinea-pig ileum to acetylcholine mustard 10^{-4} M for 1 h (but in the absence of a cholinesterase inhibitor) produced no irreversible antagonism of the contractile response to acetylcholine or pilocarpine. In view of this discrepancy a number of organ bath experiments were carried out in which the effect of exposure of the longitudinal muscle strips to acetylcholine mustard (9.8 x 10^{-6} M for 20 min, 2.5 x 10^{-5} M for 30 min and 4.9 x 10^{-5} M for 30 min) on the subsequent response to carbachol was compared with exposure to the same dose of acetylcholine. Physostigmine (10^{-6} M) was added approximately 3 min before the acetylcholine or acetylcholine mustard, but was washed out at the end of the incubation and was not present during measurement of the response of the tissue to carbachol (cf. methods section).

The effect of exposure to acetylcholine

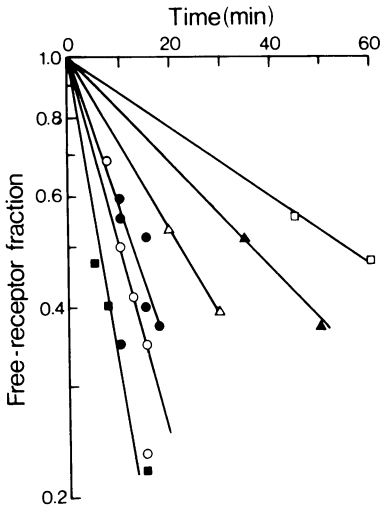

Figure 5 The rate of decline of the free-receptor fraction as a function of acetylcholine mustard concentration. The derivation of the free-receptor fraction and the experimental conditions are described in the methods section. In calculating the free-receptor fraction the non-specific component of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) uptake was taken as 28%. The error bars have been omitted for clarity, but they were generally comparable with those of a similar level of inhibition in Figure 2. The data represent measurements on a total of 261 muscle strips. Acetylcholine mustard concentrations (M): (□) 1.23×10^{-6}; (▲) 2.2×10^{-6}; (△) 4.9×10^{-6}; (●) 9.8×10^{-6}; (○) 2.5×10^{-5} and (■) 4.9×10^{-5}.

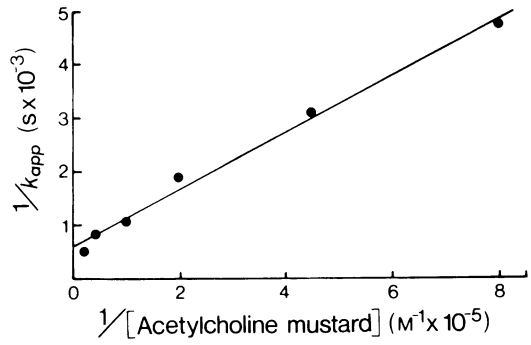

Figure 6 Plot of $1/k_{app}$ against 1/[acetylcholine mustard]. The values of k_{app} were derived from the slopes of the lines in Figure 5. The line is a least mean squares regression line.

mustard varied, depending on the dose employed, although the time course of the fade from the contraction produced was very similar to that after the same dose of acetylcholine. After the lowest dose (9.8×10^{-6} M for 20 min) there was a decrease in the resting length (to approx. 50% of previous) which was not influenced either by washing or by atropine (10^{-5} M). Carbachol produced small contractions superimposed on the raised baseline. In one experiment there was also an increase in the spontaneous activity. This became more marked where the treatment with acetylcholine mustard was 2.5×10^{-5} M for 30 minutes. Washing at 1 min intervals minimized the fluctuations without reducing the raised baseline (approx. 55% of previous maximum contraction), but with 3 min intervals the periodicity of the mechanical stimulus caused by washing produced slow oscillations of relaxation and recovery to the raised baseline. Carbachol still produced a response and indeed in one experiment this seemed to be much greater than would have been anticipated from the binding experiments, but the erratic baseline and the spontaneous slow oscillations made quantitative interpretation impossible. The spontaneous activity and raised baseline remained to some extent in two experiments where acetylcholine mustard 4.9×10^{-5} M for 30 min was employed, but in two other experiments after this dose of the mustard there was no change in the baseline and the response to carbachol was practically abolished.

The contrast between these observations and those of Hirst & Jackson (1972) is readily explained by the presence of physostigmine in the present experiments. This is supported by an experiment in which two muscle strips from adjacent segments of the intestine were set up in separate organ baths and both exposed to acetylcholine mustard 4.9×10^{-5} M for 30 min, one with and one without physostigmine 10^{-6} M. With physostigmine present the response to carbachol was practically abolished, but in its absence the result was similar to that reported by Hirst & Jackson (1972). After an initial instability the baseline returned to its original level and carbachol produced contractions not much smaller than the control responses, although the log dose-response curve was somewhat flattened.

Discussion

The inhibition of the binding of [^{3}H]-PrBCM to longitudinal muscle strips by acetylcholine mustard leaves little doubt that acetylcholine mustard does bind irreversibly to muscarinic receptors though its complex effects on the contractile properties of the tissue indicate that at the concentrations necessary for the inhibition of uptake it most probably has multiple actions on

the tissue. This is not surprising, since the three-membered aziridinium ring is a powerful electrophile and there is likely to be a wide choice of nucleophilic reaction partners, such as thiol or amino groups, present in the tissue. The lack of specificity of other aziridinium ion-forming drugs, e.g. dibenamine, when used at concentrations above 10^{-6} M is well known and at 10^{-5} to 5×10^{-5} M, as required for acetylcholine mustard, the binding affinity for secondary sites need not be high if covalent bond formation is efficient, i.e. if k_2 is reasonably large. Thus although it is tempting to suppose that the increased tone which follows exposure to acetylcholine mustard might represent a permanent activation of the receptor, such as Silman & Karlin (1969) have observed for bromoacetylcholine bound to the electroplax of *Electrophorus electricus*, it seems more likely that this is the result of some other action of the mustard. In particular while there is no evidence that the electroplax shows the phenomenon of desensitization (Higman, Podleski & Bartels, 1963; Weber & Changeux, 1974), in intestinal smooth muscle permanent activation would be expected to lead to receptor desensitization, effectively producing irreversible antagonism. It may also be noted that whereas permanent activation of the electroplax by bromoacetylcholine is sensitive to (+)-tubocurarine (Silman & Karlin, 1969), the decreased resting length of the muscle strips after acetylcholine mustard was not influenced by atropine.

The use of the inhibition of [^{3}H]-PrBCM binding as a criterion for irreversible binding to muscarinic receptors circumvents the difficulties of multiple sites of action of acetylcholine mustard but it does, of course, demand that the binding of [^{3}H]-PrBCM which can be inhibited by acetylcholine mustard should represent muscarinic receptor binding. Evidence has been presented elsewhere that the uptake of [^{3}H]-PrBCM sensitive to atropine and other muscarinic drugs in intestinal muscle strips (Burgen *et al.*, 1974a), mammalian cerebral cortex (Burgen, Hiley & Young, 1974b) and chick amnion (Cuthbert & Young, 1973) is receptor-specific. However, there is a discrepancy between the extent of the maximum inhibition of [^{3}H]-PrBCM uptake by acetylcholine mustard (approximately 70%) and that by atropine or acetylcholine (approx. 85%). The reason for this difference is not clear and at present there is little evidence to support an explanation that the discrepancy reveals two populations of receptors in the proportion 70:15, both of which bind acetylcholine, but one of which does not react readily with acetylcholine mustard. It is still an open question whether all the receptor sites bound by [^{3}H]-PrBCM are involved in mediating the contractile response, but there is no good evidence to suggest that the 15% could represent such a fraction with which acetylcholine mustard forms no covalent bond. The behaviour of the tissue after large doses of acetylcholine mustard compromises any quantitative comparison of the contractile response before and after treatment and it has been shown that the discrepancy with previously published work (Hirst & Jackson, 1972) can be ascribed to the absence of a cholinesterase inhibitor. Similarly it seems likely that Hudgins & Stubbins (1972) would have observed a greater effect on the rat jejunum had they protected the mustard against enzymatic hydrolysis.

Taking the figure of 28% of the uptake of [^{3}H]-PrBCM to represent the effective non-specific binding, it has been shown that from an analysis of the decline of the free-receptor fraction values can be obtained for the rate constant of the alkylation reaction, k_2, and for the apparent binding affinity of acetylcholine mustard, which appears to correspond well with the concentration of acetylcholine required (approx. 10^{-5} M) for half-maximal inhibition of [^{3}H]-PrBCM binding. However, there are difficulties in interpreting these values. The prime difficulty is the lack of knowledge of receptor mechanisms, a problem which has recently been discussed by Rang (1973). Interpretation is further complicated by the fact that binding studies with agonists require the agonist to be present in relatively high concentrations for appreciable periods of time, conditions which favour receptor desensitization. It has recently been demonstrated (Young, 1974) that the pre-exposure of longitudinal muscle strips to a high concentration of carbachol followed by a brief wash changes both the position and the slope of the curve relating inhibition of [^{3}H]-PrBCM binding to carbachol concentration. Thus ongoing desensitization during exposure to the agonist may be the reason why binding curves for agonists obtained from the competitive inhibition of [^{3}H]-PrBCM binding have Hill coefficients of approx. 0.4-0.5 (Burgen & Hiley, 1974; Young, 1974), instead of the value of unity expected from a simple mass-action equilibrium. The curve for acetylcholine (Figure 3) has a Hill coefficient of 0.54. It is thus of particular interest that the kinetics of the irreversible binding of acetylcholine mustard appear to fit well with a scheme involving a simple drug-receptor equilibrium as the first step.

The reason for this contrast is uncertain. It is, however, notable that predesensitization of muscle strips results in an increase in the Hill coefficient for carbachol binding from 0.4 to 0.9 (Young, 1974), i.e. after desensitization the binding curve approximates to the theoretical curve for a

mass-action equilibrium. The concentrations of acetylcholine mustard employed and the period of incubation with the tissue are such that it is very likely that at least some desensitization will have occurred and it is possible that it is extensive. If this is the case then the binding affinity, K_a, derived for acetylcholine mustard, 1.2×10^5 M^{-1}, may well be a measure of the affinity for the desensitized receptor.

In interpreting K_a it must in any event be borne in mind that the observation that a plot of $1/k_{app}$ against 1/[acetylcholine mustard] is linear does not provide proof that the simple reaction scheme adopted (cf methods section) is correct, since the introduction of further reversible complexes between AR and AR′ does not alter the linear relationship, provided that the alkylation reaction is still the slowest step, although the constants become combinations of the various rate constants. Consequently K_a must be regarded as a kinetic constant whose meaning, just as with K_m in the normal Michaelis-Menton treatment of enzyme kinetics, will only become clear when the exact mechanism is known.

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