Br. J. Pharmac. (1973), **48**, 148–155.

Interaction of benzilylcholine mustard, benzilylcholine and lachesine with the histamine receptor in the longitudinal muscle of guinea-pig ileum

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Summary

1. The histamine receptors of longitudinal muscle strips from guinea-pig ileum were inactivated on extended exposure to concentrations of benzilylcholine mustard (BCM) above 10^{-6}M. These concentrations are 3 orders of magnitude greater than those required for inactivation of the muscarinic receptors. Mepyramine (3×10^{-8}M) afforded complete protection for the histamine receptor against the effects of BCM.

2. Recovery from block at 30° C was very slow with a first-order rate constant of approximately 10^{-7} s^{-1}.

3. The rate constant, k_2, for the alkylation reaction in which the reversible BCM-receptor complex is converted into an irreversible complex is much greater than the rate constant, k_{-1}, for dissociation of the reversible complex. The value of the rate constant, k_1, for formation of the reversible complex was $1{\cdot}1\pm0{\cdot}2\times10^{3}$ M^{-1} s^{-1}.

4. These observations suggested that k_{-1} was probably not much greater than for BCM acting on the muscarinic receptor, even though the value of k_1 was reduced by more than 2 orders of magnitude. This effect has been established for benzilylcholine and lachesine, reversible analogues of BCM. For both drugs k_1 for the histamine receptor was approximately 3 orders of magnitude less than for the muscarinic receptor, whereas k_{-1} was greater by a factor of only 6.

Introduction

Benzilylcholine mustard (BCM) was introduced by Gill & Rang (1966) as a specific long-acting inactivator of the muscarinic receptor and as such has proved to be a powerful experimental tool, particularly in the field of receptor labelling. By using radioactively labelled BCM or the *N*-propyl homologue it has been possible to determine the number of muscarinic receptors in longitudinal muscle strips from guinea-pig ileum (Rang, 1967; Young, Hiley & Burgen, in preparation), in subcellular fractions from rat cerebral cortex (Hiley, Young & Burgen, 1972) and in the chick amnion (Cuthbert & Young, 1973). In each case the specific, i.e. atropine-sensitive, uptake is accompanied by a certain amount of atropine-insensitive binding and in the course of an investigation into the origins of this non-specific binding we have examined the interaction of BCM with the histamine receptor in the longitudinal muscle of guinea-pig ileum.

In agreement with Gill & Rang (1966) the inactivation of this receptor required a concentration of BCM 500–1,000 times greater than that necessary for inactivation of the muscarinic receptor. Unexpectedly, however, the kinetics of the inactivation suggested that although there was a large drop in the magnitude of the rate constant for the formation of the reversible BCM-receptor complex, k_1, as compared to the interaction with the muscarinic receptor, the off-rate constant, k_{-1}, was probably relatively little altered. In this situation the value of k_{-1} is not easily obtained, but the same effect has been established for benzilylcholine and lachesine, reversible analogues of BCM, where k_{-1} could be measured directly. In this paper we describe these experiments and discuss the implications of the observation that the specificity of these antagonists for the muscarinic as opposed to the histamine receptor resides largely in the value of the rate constant for complex formation, k_1.

Methods

Longitudinal muscle strips from guinea-pig ileum were prepared essentially as described by Rang (1964) and suspended in a 10 ml organ bath in Krebs solution (composition, mM: NaCl, 118; KCl, 4·7; $MgSO_4$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 25; $CaCl_2$, 2·5 and D-glucose, 5·5) at 30° C bubbled with 5% CO_2 in oxygen. For ease of separation of the muscle layer, guinea-pigs weighing 500–600 g were employed, but in the experiments to determine k_{-1} values the strips were usually divided longitudinally. Histamine was applied for 15 s, or in some experiments 20 s, at a set time interval (60 or 90 seconds). Contractions were recorded isotonically and washing was carried out by overflow. Stable responses were usually obtained after the preparation had been allowed to equilibrate for 1 h and then taken through a cycle of dose-response curve, dose-response curve in the presence of a reversible inhibitor, wash and dose-response curve.

Affinity constants of reversible antagonists were determined from the shift of the log-dose-response curve after equilibration with Krebs solution containing the antagonist (K_a=(dose ratio−1)/[A]) and values of k_{-1} were obtained from the rate of recovery of the response after washing out the antagonist (Paton, 1961). Acetylcholine was used as the muscarinic agonist. Similar values were obtained, where comparison was made, when carbachol was employed.

BCM was synthesized by the method described elsewhere (Young, Hiley & Burgen, 1972) and allowed to cyclize as a 0·8 mM solution in 10 mM phosphate buffer, pH 7·4, at room temperature for 40 minutes. The solution was then rapidly cooled to 0° C and stored at this temperature until required for use. The yield of the aziridinium ion, the pharmacologically active entity (Gill & Rang, 1966), was 80%. *All additions denoted as BCM refer to the cyclized solution and the concentration given is the concentration of the aziridinium ion calculated on the basis of an 80% yield.* In Krebs solution at 30° C the half-time of decay (presumably hydrolysis) of the aziridinium ion is 223 min (Young, Hiley & Burgen, 1972), i.e. at this temperature the extent of the decay during a 10 min incubation with the tissue is very small. This is important since a solution of BCM allowed to hydrolyze completely has an appreciable reversible affinity for the histamine receptor (K_a approx. 4×10^5 M^{-1}).

Acetylcholine bromide and histamine acid phosphate were obtained from B.D.H., lachesine chloride from Evans Medical and mepyramine maleate from May &

Baker. Benzilylcholine chloride was kindly prepared for us by Mr. B. Peck, following the method of Ford-Moore & Ing (1947).

Results

Inactivation of the histamine receptor by benzilylcholine mustard

Successive incubations of longitudinal muscle strips with concentrations of BCM above 10^{-6}M followed by extensive washing resulted first in a parallel shift of the log-dose-response curve for histamine to the right, followed eventually by a flattening of the curve. The concentration of BCM required was 3 orders of magnitude greater than that required to inactivate the muscarinic receptor, in agreement with the observations of Gill & Rang (1966). The course of an experiment in which 4×10^{-6}M BCM was employed is illustrated in Figure 1. These curves are similar to those for histamine obtained by Nickerson (1956) following treatment of guinea-pig ileum with the dibenamine analogue SY-14. Mepyramine, 5×10^{-8}M, added to the bath 20 min before 4×10^{-6}M BCM and the incubation then continued for a further 40 min, afforded complete protection against the effects of BCM, indicating that the mustard is acting at the histamine receptor rather than having some non-specific action elsewhere.

The recovery from block at 30° C was extremely slow with a rate constant, assuming the process to be first order, of the order of 10^{-7} s^{-1}. This may be somewhat slower than the recovery at the same temperature of the muscarinic receptor (k approx. 10^{-6} s^{-1}; Young, Hiley & Burgen, in preparation), but accurate measurement of rate constants of this magnitude is very difficult.

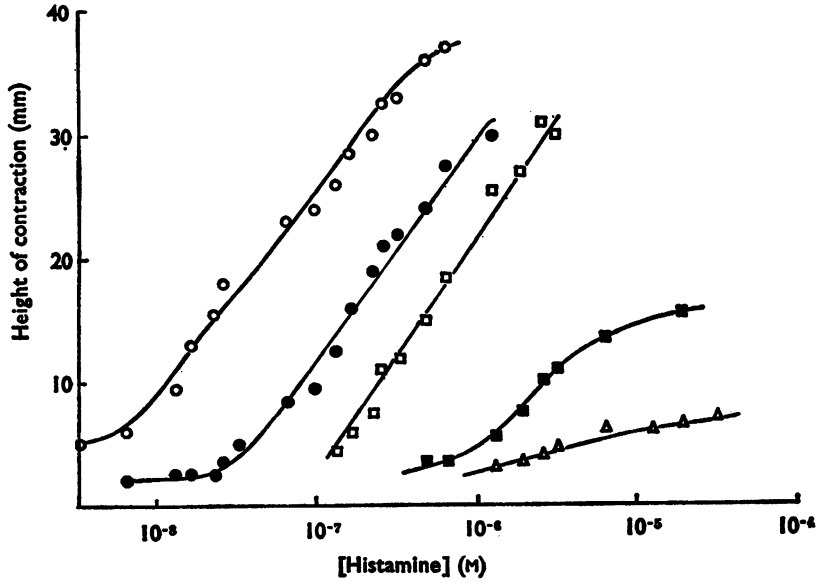

FIG. 1. Progressive inactivation of histamine receptors in guinea-pig ileum longitudinal muscle by benzilylcholine mustard (BCM). The muscle strip was exposed to 4×10^{-6}M BCM for successive intervals of 10 min, with extensive washing after each exposure prior to determination of a dose-response curve. ○——○, Control curve; ●——●, after 10 min exposure; □——□, after 20 min (in total); ■——■, after 30 min; △——△, after 40 min.

Spare receptor ratio for histamine

The depression of the maximum response to histamine usually commenced at a dose-ratio between 50 and 80, i.e. a little less than the value of 100 observed by Nickerson (1956) after treatment with SY-14, but considerably less than the shift required (mean dose-ratio of 273) before the maximal response to carbachol is depressed (Burgen & Spero, 1968). The occupancy of BCM which produced dose-response curves that appeared to have started to flatten was determined by the method of Furchgott (1966), where the reciprocal of the dose of histamine required for a given response is plotted against the reciprocal of the dose required for the same response after treatment with an irreversible antagonist. The antagonist occupancy is obtained from 1–1/slope, and from such measurements it appears histamine must occupy 1–2% of the available receptors in order to produce a maximum response.

Kinetics of the inactivation of the histamine receptor by benzilylcholine mustard

The relatively high concentration of BCM required to inactivate the histamine receptor indicated that the affinity of the aziridinium ion for the receptor must be considerably less than for the muscarinic receptor and we had anticipated that the kinetics of the interaction would be complex. Gill & Rang (1966) have analysed the interaction of BCM with the muscarinic receptor in terms of a reaction scheme

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \xrightarrow{k_2} AR' \xrightarrow{k_3} A' + R$$

where a reversible antagonist-receptor complex, AR, is first formed, which is then converted into a covalently-bonded complex, AR′. AR′ is not completely stable and is hydrolyzed slowly to regenerate native receptor, R, and a hydrolysis product of the antagonist, A′ . A similar sequence of events may be presumed to occur with the histamine receptor and in both cases, since recovery is very slow, k_3 can be ignored. Gill & Rang (1966) have pointed out that if $k_2 \gg k_{-1}$, then a plot of ln [free receptor] *vs* time should be linear with a slope of $-k_1[A]$, where [A] is the concentration of the antagonist. If $K_{-1} \simeq k_2$ then, according to Gill & Rang (1966), double exponential kinetics would be experimentally obvious. A linear plot of ln [free receptor] *vs* time also results if $k_{-1} \ll k_2$ and the antagonist is removed prior to determination of the occupancy, but in this case the slope is a function of k_2, K_a and [A] (Kitz & Wilson, 1962).

A plot of log [free receptor] *vs* time for the inactivation of the histamine receptor by two concentrations of BCM is shown in Fig. 2 (open symbols). Both plots are linear. These experiments were carried out by addition of BCM to the bath for a specified time followed by wash out and establishment of a dose-response curve to histamine ; the irreversible occupancy, p, was then obtained from the relationship

$$p = \frac{\text{Dose-ratio} - 1}{\text{Dose-ratio}}$$

(Paton, 1961), which holds provided that the fraction of the available receptors occupied by the agonist is small. If $k_{-1} \gg k_2$, then this method clearly results in an

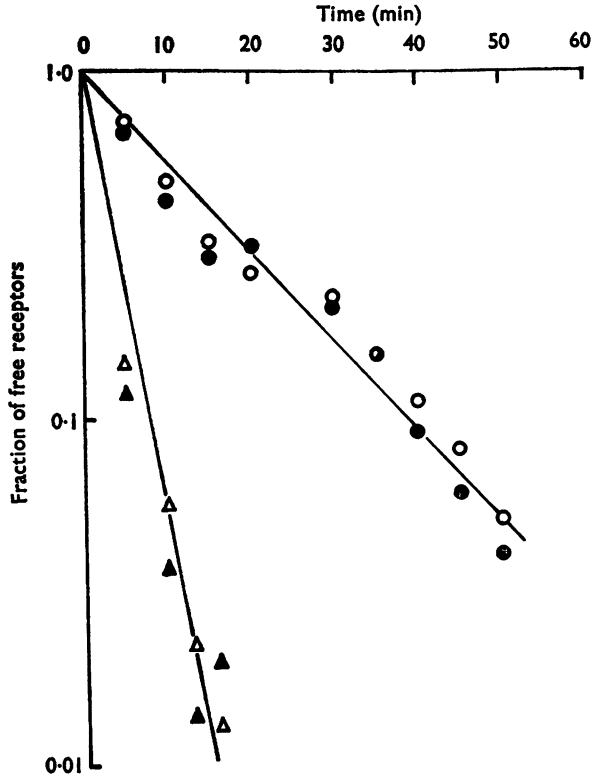

FIG. 2. Rate of onset of irreversible block of histamine receptors by benzilylcholine mustard (BCM). The free receptor fraction (1—p) was obtained from the dose-ratio as described in the text. For the open symbols (○ & △), the dose-ratio was determined from the dose-response curve established after washing out the BCM. The filled symbols (● & ▲) were derived from the dose-ratio calculated from the response to a single dose of histamine added immediately before the BCM was washed out. ○——○ & ●——●, $1{\cdot}1\times10^{-6}$M BCM; △——△ & ▲——▲, 4×10^{-6}M BCM.

overestimation of the free receptor availability during the time BCM is acting, since k_2 is rate limiting and there will be an appreciable concentration of the reversible complex, AR. However, when a dose of histamine was added immediately prior to washing out the BCM and the total antagonist occupancy thus obtained (filled symbols in Fig. 2), it was only slightly different from the irreversible occupancy (open symbols). This indicates that any of the reversible complex, AR, present when washing commences forms AR′ faster than it can dissociate, or in other words $k_2 \ll k_{-1}$ and hence $k_1[A]$ is rate determining. It also means that the irreversible occupancy after washing is a good measure of the total occupancy, AR+AR′, immediately before. Thus the slope of the plot of log [free receptor] *vs* time should be $-k_1[A]/2{\cdot}303$ and consistent with this the values of k_1 determined from experiments with varying concentrations of BCM were reasonably constant. The mean value of k_1 was $1{\cdot}1\times10^3$ M^{-1} s^{-1}.

Interaction of benzilylcholine and lachesine with muscarinic and histamine receptors

A minimum value of k_2 for the inactivation of the muscarinic receptor at 30° C is of the order of 10^{-2} s^{-1} and is not greatly different for homologues of BCM where

the *N*-methyl residue has been substituted for n-propyl or n-butyl (R. Hiley, personal communication). It seemed reasonable to suppose that the value would not be very different for the interaction with the histamine receptor and, since $k_2 \gg k_{-1}$, this suggested that the value of k_{-1} was not much greater for BCM acting on the histamine receptor than on the muscarinic receptor, even though the value of k_1 was lower by more than 2 orders of magnitude (Table 1).

TABLE 1. *Kinetic constants for the interaction of benzilylcholine mustard (BCM), benzilylcholine and lachesine with muscarinic and histamine receptors at 30° C*

Drug	Receptor	K_a (M^{-1})	k_{-1} (s^{-1})	k_1 ($M^{-1}\,s^{-1}$)
BCM	Acetylcholine	—	—	$2{\cdot}7\pm0{\cdot}6\times10^5$ (3)
	Histamine	—	—	$1{\cdot}1\pm0{\cdot}2\times10^3$ (3)
Benzilylcholine	Acetylcholine	$5{\cdot}3\pm0{\cdot}4\times10^8$ (3)	$1{\cdot}1\pm0{\cdot}2\times10^{-3}$ (6)	$5{\cdot}8\times10^5$
	Histamine	$1{\cdot}1\pm0{\cdot}1\times10^5$ (3)	$5{\cdot}9\pm0{\cdot}1\times10^{-3}$ (4)	$6{\cdot}5\times10^2$
Lachesine	Acetylcholine*	$1{\cdot}1\times10^9$	$1{\cdot}0\times10^{-3}$	$1{\cdot}0\times10^6$
	Histamine	$1{\cdot}0\pm0{\cdot}1\times10^5$ (4)	$6{\cdot}3\pm0{\cdot}4\times10^{-3}$ (4)	$6{\cdot}3\times10^2$

* Data of Paton & Rang (1966) measured at 30·5° C. Values are means ± standard error with the number of determinations in parentheses. Values of k_1 for benzilylcholine and lachesine have been obtained from $k_1 = K_a \times k_{-1}$.

The measurement of k_{-1} directly for the BCM-receptor interaction is experimentally very difficult (cf. Gill & Rang, 1966) and in order to test this hypothesis we have compared the values of the kinetic constants for benzilylcholine, which may be considered to be the reversible analogue of BCM and for which k_{-1} may be readily determined, interacting with the histamine and muscarinic receptors. The results (Table 1) strongly supported the hypothesis, since the affinity for the muscarinic receptor was 5×10^3 times greater than for the histamine receptor, but the values for k_{-1} differed by a factor of only 5. Similarly when values determined for the interaction of lachesine with the histamine receptor were compared with those of Paton & Rang (1966) for the muscarinic receptor, the change in k_{-1} was about 3 orders of magnitude less than the change in K_a.

Discussion

The observations presented above lead to the conclusion that the affinity of benzilylcholine and lachesine for muscarinic as compared to histamine receptors in longitudinal muscle strips from guinea-pig ileum is determined mainly by the rate constant for complex formation, k_1. However, in any such study of drug-tissue interaction under non-equilibrium conditions a special difficulty exists, namely to know to what extent the method employed for measuring k_1 or k_{-1} really measures the rate of association or dissociation from the receptor rather than diffusion through some barrier to or away from the site of action. We have tried to avoid any gross diffusional problems due to folding of the tissue on being tied up by using as narrow a strip as possible, but the problem of a diffusional barrier within the tissue remains and Thron & Waud (1968) have advanced arguments based on comparisons between intact ileum and longitudinal muscle strips for access limited kinetics, at least in intact ileum. Rang (1966) has studied this problem in the muscle strips in some detail and has described experiments in which undecyltrimethylammonium bromide, a rapidly acting antagonist, was added to a preparation already equilibrated

with atropine or hyoscine. A transient overshoot of receptor occupancy occurred, the extent of which was consistent with a slow dissociation of atropine or hyoscine from the receptor. Conversely, on washing out the undecyltrimethylammonium a transient undershoot was observed, the extent and kinetics of which were again in much better agreement with dissociation of hyoscine from the receptor being the rate-limiting step, rather than a 'limited biophase' model in which diffusion is rate-limiting. Thron & Waud have pointed out that even this experiment is not entirely conclusive, but until more specific experimental evidence to the contrary is advanced it provides, for this preparation, some grounds for confidence that the values of the kinetic constants obtained represent reasonable approximations to the true values.

In view of the importance of k_1, it is satisfying that the values obtained (from $k_1 = K_a \times k_{-1}$) for benzilylcholine are of the same order of magnitude as those determined directly for its irreversible analogue, BCM. How exact a correspondence ought to be expected is in any case uncertain since the relative binding affinities are unknown and it is of interest in this respect that acetylcholine mustard has recently been shown to have only approximately a fifth of the potency of acetylcholine as a muscarinic agonist (Hirst & Jackson, 1972 ; Hudgins & Stubbins, 1972), although this does not necessarily mean that the binding affinities differ. The value for k_1 for BCM acting on the muscarinic receptor is in good agreement with the value obtained, $1{\cdot}8 \pm 0{\cdot}2 \times 10^5$ M^{-1} s^{-1}, from an earlier and more extensive series of experiments (Young, Hiley & Burgen, 1972).

The factors which might lead to the affinities of a series of antagonists being determined largely by k_1 have been discussed by Taylor, King & Burgen (1970) with reference to the interaction of a number of sulphonamides with carbonic anhydrase and in many ways the present case is analogous, although here we are interested in the same antagonist interacting with different receptors. Clearly on the basis of the present limited study we can do no more than speculate on the molecular basis for the large fall in the value of k_1 compared to the relatively modest change in k_{-1}. It is possible that the topography of the histamine receptor is such that the penetration of the antagonist to its binding site is difficult, e.g. if the site were in a cleft, but diffusion away from the site is also hindered. In this case both k_1 and k_{-1} would be affected and the similarity between the k_{-1} values for the histamine and muscarinic receptors need not be indicative of structural similarities. Alternatively, the two receptors may be closely related and the histamine receptor may largely retain the site involved in binding antagonists such as benzilylcholine. Certainly the possession of atropine-like side-effects is a common property of antihistamine drugs, particularly those of the dialkylaminoethyl ether type such as diphenhydramine. The similarity of the k_{-1} values would then not be fortuitous ; the lower value of k_1 on the histamine receptor resulting from kinetic factors, e.g. the inability to form weak preliminary complexes with the incoming antagonist which can reorientate to the more stable complex before dissociation occurs—an effect which would be particularly important if access to the more stable binding site was sterically hindered.

On the basis of our present evidence we cannot choose between these possibilities, but the idea of a close relationship between the histamine and muscarinic receptors, which may well have evolved from some common prototype, is an attractive one.

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(*Received November* 20, 1972)