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Mechanism of Action of Benzilylcholine Mustard at the Muscarinic Receptor

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SUMMARY

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The irreversible antagonism exerted by benzilylcholine mustard (BCM) on acetylcholine-induced contractions of the isolated guinea pig ileum can best be characterized by multiple sites of alkylation. BCM (50 μM, for 15 min) completely abolishes the response to acetylcholine, but the maximum responsiveness of the tissue recovers with time according to a first-order process ($t_{1/2}$ of 45 min). Recovery of response is correlated with a first-order loss of tritium from tissues blocked with [^{3}H]BCM ($t_{1/2}$ of 38.9 min). The rightward shift of the dose-response curves observed after recovery of maximum response (acetylcholine dose ratio = 390) is stable over 8 hr, although the dose ratio would be expected to return toward control values if the receptor reserve model was applicable in this tissue. The data can best be interpreted on the basis that BCM alkylates two sites at the muscarinic receptor: an allosteric site which stabilizes an inactive form of the receptor, resulting in an apparent decrease in affinity for the agonist, and a second site which is postulated to be the agonist recognition site proper. Alkylation of the second site results in decreased maximum response.

INTRODUCTION

There have been several recent efforts to isolate and characterize the cholinergic muscarinic receptor through the use of radioactive alkylating agents, derived from muscarinic ligands (1–5) and radiolabeled competitive antagonists (6–9). A major criterion of such isolation procedures must be the establishment of some quantitative correlation between the pharmacological activities (pA_2 and pD_2 values) and the binding affinities of agonists and antagonists (10).

The existence of any significant receptor reserve for agonists will, however, generate substantial discrepancies between the "apparent" dissociation constants for agonists determined from dose-response curves and the "true" dissociation constants determined from binding studies (10, 11). Such discrepancies have been reported for a number of receptor systems, including the cholinergic muscarinic receptor (4, 8, 9). Thus Yamamura and Snyder (8) reported essential agreement between agonist dissociation constants deter-

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mined from binding in guinea pig ileal longitudinal muscle and dissociation constants determined from dose-response curves in which the receptor reserve had been eliminated by prior titration with an alkylating antagonist (10, 11).

Snyder *et al.* (9) have suggested recently, however, that the action of such alkylating antagonists may also be viewed in terms of an allosteric interaction in which the antagonist actually reduces the affinity of the receptor for the agonist rather than eliminating a receptor reserve. This proposal is in agreement with our previous work, in which we suggested that irreversible muscarinic antagonists act both at the agonist recognition site, to eliminate receptors, and at a second allosteric site, to modify agonist binding (12, 13).

The present paper provides additional evidence favoring an allosteric mode of interaction for benzilylcholine mustard, an irreversibly acting muscarinic antagonist.

MATERIALS AND METHODS

Preparation of longitudinal muscle. Isolated longitudinal muscles were prepared according to Rang (14) from male guinea pigs weighing 400–500 g. Segments of longitudinal strips 1.5–2.0 cm in length were mounted in a 10-ml organ bath containing Tyrode's solution maintained at 37° and gassed with 5% CO_2 in O_2. Each liter of the Tyrode's solution contained NaCl, 8 g; KCl, 0.2 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.26 g; $NaH_2PO_4 \cdot H_2O$, 0.05 g; $NaHCO_3$, 1.0 g; and glucose, 1.0 g. The Tyrode's solution also contained 1 μM esterine and 1.4 μM hexamethonium. Muscle contractions were recorded isotonically on smoked drums with a magnification factor of 15 and with 300 mg of tension on the muscle strip. Tissues were allowed to equilibrate for 1 hr with Tyrode's solution. Following equilibration, two maximum doses of agonist were administered 30 min apart to stabilize responses. After the maximum doses had been given, cumulative dose-response curves were determined at 30-min intervals with acetylcholine until superimposable curves were obtained. Dose-response curves in treated tissues were always compared with control dose-response curves constructed at similar times.

Preparation of [^{3}H]benzilylcholine mustard. Benzilic acid was tritiated according to the Moravek process by Nuclear Dynamics Corporation. Using this as starting material, [^{3}H]benzilylcholine mustard was synthesized by the procedure of Gill and Rang (2), and the final purified product had a specific activity of 10 mCi/mmole. Unlabeled benzilylcholine mustard was synthesized by the same method.

Cyclization of aziridinium ions. The solutions of BCM[1] and [^{3}H]BCM were freshly prepared in pH 7.0 buffer and left at room temperature for 30 min to allow cyclization to the aziridinium ion.

Rate of pharmacological recovery with unlabeled BCM. Tissues were incubated with the indicated concentrations of BCM for 15 min and then washed several times with Tyrode's solution. The recovery of inhibition produced by BCM was followed by constructing cumulative dose-response curves at 30-min intervals after extensive washing with Tyrode's solution. The effects of the antagonist are expressed in terms of a dose ratio, the ratio of ED_{50} values of the agonist after and prior to treatment with the antagonist, or by percentage inhibition of the maximum response.

Rates of pharmacological recovery and ^{3}H washout after blockade by [^{3}H]benzilylcholine mustard. The response of ileal strips to a supramaximal concentration of acetylcholine (1 mM) was inhibited completely with a 15-min exposure to 50 μM [^{3}H]BCM. The tissues were washed exhaustively over the next 5 min, after which the organ bath was adjusted to exactly 10 ml with Tyrode's solution. Aliquots of 200 μl were taken every 15 min for the first 2 hr and every 30 min for an additional 5 hr in order to determine the rate of tritium appearance in the bath medium. The Tyrode's solution was changed every 120 min. Aqueous samples were counted as previously described (15), and longitudinal strips were dried, weighed, solubilized in Soluene, and counted in In-

[1] The abbreviation used is: BCM, benzilylcholine mustard.

stagel in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. Radioactivity was expressed as disintegrations per minute after correction for efficiency and quenching by the method of internal standardization (16). In control tissues, dose-response curves were determined at 30-min intervals over the first 2 hr and at hourly intervals thereafter.

The method described by Rose (17), as used by May *et al.* (15) and Moran and Triggle (12), was utilized to obtain the first-order rate constants for washout of the tritiated material. Briefly, the alternate values of total radioactivity in the bath are plotted against each other; i.e., the first count against the third, the second against the fourth, etc. The logarithm of the slope of the straight line obtained, multiplied by $2.303/\Delta t$, yields k in accordance with the equation

$$k = \frac{2.303}{\Delta t \cdot \log \text{slope}}$$

where Δt equals 30 min when samples are taken every 15 min. Based on the $t_{1/2}$ for radiochemical washout and the amount of radioactivity appearing in the bath during a specified interval, the total amount of material attributable to the first-order process can be calculated. The rate constants for recovery of response were determined as previously described (12, 15).

RESULTS

Pharmacological effects of BCM. Exposure of ileal strips to increasing concentrations of BCM for a standard time of 15 min resulted first in a parallel shift of the acetylcholine dose-response curve to the right, followed by a depression of the maximum response (Fig. 1). Dose ratios of 1085 ± 100 (n = 9) could be obtained with 30 nM BCM without any depression of maximum response. However, the dose ratio decreased dramatically with extensive washing over the first 15 min but remained stable at a dose ratio of approximately 30 at 75 min (Fig. 2). The greatest change in recovery occurred over the first 15 min of washing and probably represents a reversible competitive phase of inhibition: this phenomenon is generally observed with 2-halogenoethylamines and presumably results from competitive binding of the aziridinium ion (10, 12). Gill and Rang (2) observed a similar time for washout of the reversible antagonist benzilylcholine. Concentrations of BCM greater than 30 nM resulted in a depressed maximum response and, after extensive washing, in increased dose ratios. For example, 1 μM BCM resulted in a 30% depression of the maximum response, and after extensive washing for 2 hr the maximum response returned to control values and a stable shift of the dose-response curve remained,

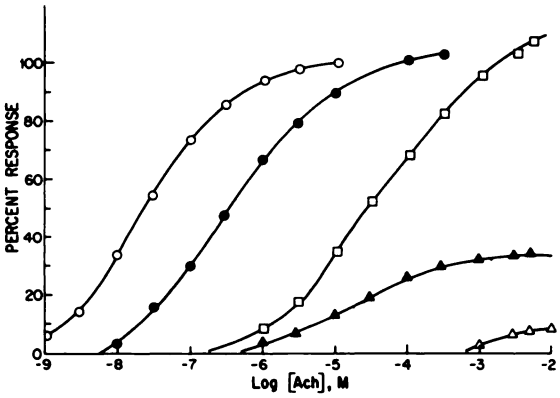

FIG. 1. *Progressive inhibition of acetylcholine (ACh) receptors by benzilylcholine mustard*

Muscle strips were exposed to increasing concentrations of BCM for 15 min: O——O, control curve; ●——●, 5 nM BCM; ■——■, 30 nM BCM; ▲——▲, 10 μM BCM; △——△, 30 μM BCM. Dose-response curves were constructed immediately following three successive washes after incubation with BCM.

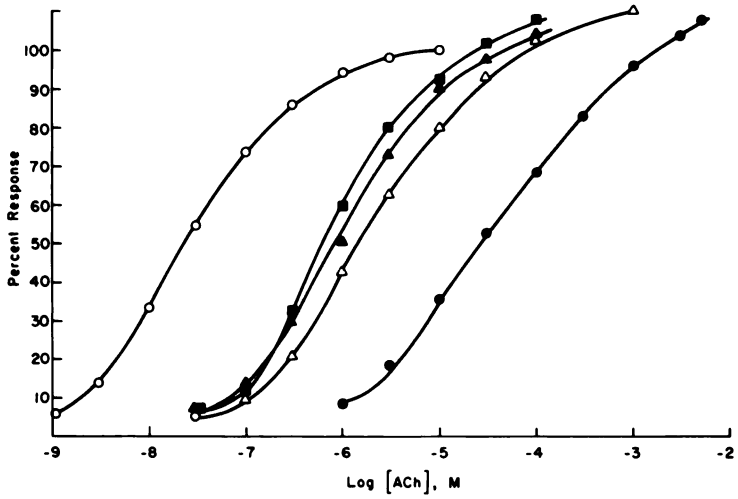

FIG. 2. *Recovery of response to acetylcholine (ACh) after incubation of ileal strips with 30 nM benzilylcholine mustard for 15 min*

●——●, zero time response; △——△, 15 min; ▲——▲, 45 min; ■——■, 75 min; ○——○, control. Tissues were washed extensively prior to construction of each dose-response curve.

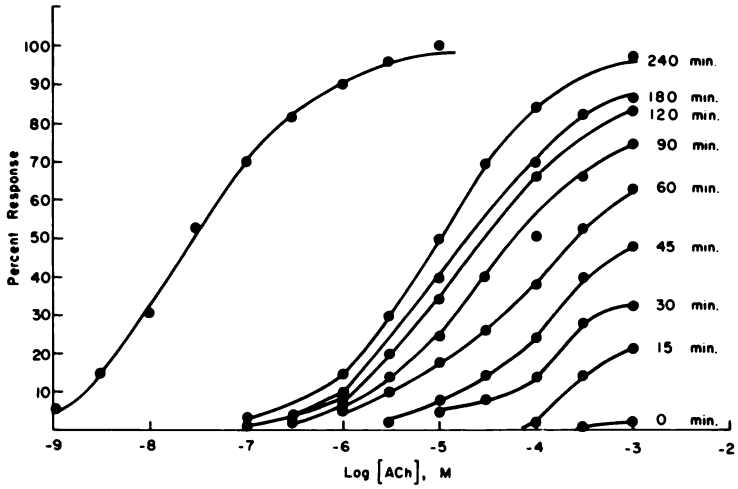

FIG. 3. *Recovery of acetylcholine (ACh) response at various time intervals following blockade by 50 μM benzilylcholine mustard for 15 min*

as shown by a dose ratio of 50.

In tissues treated with 50 μM BCM, the response to supramaximal doses of acetylcholine was completely abolished. Atropine (50 nM), added to the bath 15 min prior to 50 μM BCM, afforded complete protection against the depression of the maximum response. In addition, this relatively high concentration of BCM did not affect the responses to KCl, indicating that the alkylating agent acts at the acetylcholine receptor as opposed to nonspecific actions elsewhere.

The maximum response to acetylcholine recovered with time (Fig. 3), this process being essentially complete in 4 hr. The recovery was a first-order process characterized by a $t_{1/2}$ of 45 ± 1.6 min (n = 20), corresponding to a rate constant of 1.5 × 10^{-2} min^{-1} (Fig. 4). A rightward shift of the dose-response curve of 390 ± 52-fold (n = 16) was observed in tissues which had

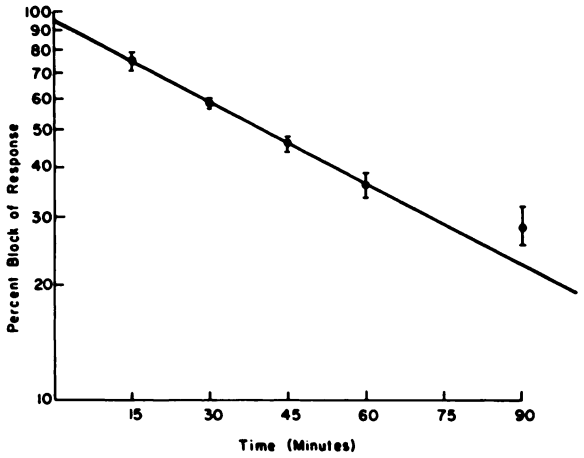

FIG. 4. *Plot showing first-order rate of recovery of maximum response to acetylcholine following blockade by 50 μM benzilylcholine mustard for 15 min*

A minimum of 20 observations was made at each time, and the standard errors are shown.

recovered to maximum response, and this ratio remained stable over the following 4 hr. It was not possible, however, to achieve a stable dose ratio of this magnitude without a simultaneous depression of the maximum response by varying either the concentration of BCM or the time of incubation of the tissue with a given concentration of BCM.

Recovery of response after treatment with 2-halogenoethylamines has been observed in other systems and has been attributed to intramolecular hydrolysis of esters formed at the receptor site by alkylation of carboxyl or phosphate functional groups (18, 19).

Loss of radioactivity in tissues treated with [^{3}H]BCM. After blockade of response to acetylcholine with 50 μM [^{3}H]BCM for 15 min, the rate of appearance of radioactivity in the organ bath was determined. The appearance of tritium was a first-order process characterized by $t_{1/2}$ of 38.9 min ($k = 1.77 \times 10^{-2}$ min^{-1}) (Fig. 5), which is in essential agreement with the $t_{1/2}$ determined for recovery of response, 45 min ($k = 1.5 \times 10^{-2}$ min^{-1}). The amount of radioactivity appearing in the first 15 min was more than could be accounted for on the basis of a first-order process and probably represents aziridinium ion bound in a competitive reversible manner, in agreement with the pharmacological results presented above. On the basis of the radioactivity appearing in the bath from 15 to 135 min, and the $t_{1/2}$ of the process, it can be calculated that a total of 1120 ± 279 ($n = 4$) dpm/mg of dry tissue are associated with the first-order process. This corresponds to 10,000 pmoles of BCM per gram of tissue, wet weight. During an additional 6 hr of monitoring the appearance of tritium in the organ bath, no additional radioactivity over and above that expected from the first-order process could be detected. At the end of 8 hr the tissue contained 960 ± 28 dpm/mg of tissue, dry weight, corre-

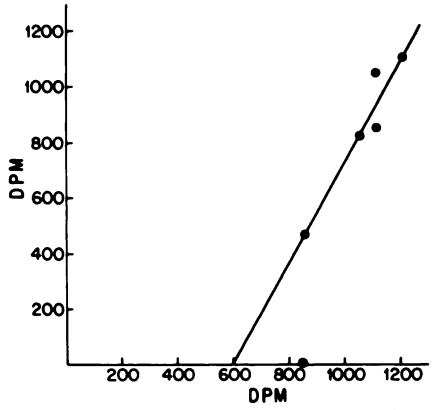

FIG. 5. *Rose plot of loss of radioactivity from longitudinal muscle of guinea pig ileum exposed to 50 μM [^{3}H]benzilylcholine mustard for 15 min*

Each point is the average of four determinations. See the text for experimental details.

sponding to 8800 pmoles of BCM bound per gram of tissue, wet weight.

DISCUSSION

According to the receptor reserve hypothesis, the production of a rightward shift of the agonist dose-response curve prior to any depression of response by irreversible antagonists represents the fractional occupancy requirements of the agonist. The effects of BCM on the acetylcholine dose-response curve (Fig. 1) are consistent with this proposal. However, although the dose-response curve determined with acetylcholine immediately after washout of 30 nM BCM is characterized by a dose ratio of approximately 1000, a large part of this shift is rapidly reversible on further washing, and the dose ratio becomes stable at a value of 30 (Fig. 2).

Increasing the concentration of BCM beyond 30 nM or increasing the time of exposure to BCM to more than 15 min did not increase the dose ratio immediately after washout without simultaneously depressing the maximum response. These observations are consistent with those reported by Gill and Rang (2), who found some flattening of the log dose-response curve when dose ratios of 50 were approached. The large parallel shifts of the log dose-response curves observed prior to prolonged washing of the tissues arise from a reversible competitive phase of action of BCM, due to bound aziridinium ion, and an irreversible phase, which is stable (18, 19). It is generally recognized that 2-halogenoethylamines are initially competitive, and this component of BCM action has been observed at high concentrations of BCM (2). After prolonged washing, the competitive phase of action is minimized through either dissociation of the reversible complex or covalent bond formation. The residual stable shift of 30 (Fig. 2) can be attributed to receptor alkylation and represents 97% occupancy by BCM (occupancy = dose ratio-1/dose ratio).

Complete inhibition of the response to acetylcholine is observed only when ileal strips are exposed to 30 μM BCM for 15 min, and the effect is specific, since this concentration does not affect the response to 80 mM KCl and the depression of maximum response is protected by prior incubation with atropine. However, the maximum response recovers with time (Fig. 3) in a first-order process with a $t_{1/2}$ of 45 min ($k = 1.5 \times 10^{-2}$ min^{-1}). After 4 hr, which represents five half-lives or 97% completion of the reaction, a stable shift corresponding to a dose ratio of 390 ± 52 is observed, and this ratio remains stable over the following 4 hr. Since this dose ratio, according to the receptor reserve hypothesis, corresponds to an occupancy of 99.74%, only 0.26% of the receptors is required for maximum response. Based on this assumption, the observed recovery of maximum response would correspond to a rate constant of 2.8×10^{5} min^{-1}, and over the 8-hr time course of these experiments a total of 1.35% of the receptors should recover. Although this is a very small percentage, recovery of this fraction should result in an observed dose ratio of 70. A change in dose ratio of this magnitude, although easily measurable compared to the observed ratio of 390 ± 52, was never observed in over 100 experiments. This observation, together with the finding that dose ratios of this magnitude (390) cannot be obtained by titration of receptors without simultaneous depression of the maximum response, suggests that at least two sites undergo alkylation at the cholinergic receptor. Alkylation of the first site causes perturbation of the binding site for acetylcholine, resulting in a decrease in affinity for acetylcholine, whereas alkylation of the second site produces a depression of the maximum response. It is known that alkylation of carboxyl or phosphate groups by 2-halogenoethylamines results in labile esters which undergo spontaneous hydrolysis, whereas alkylation of amino, hydroxyl, or sulfhydryl groups yields stable products (18, 19). Thus the formation of labile and stable alkylated species accommodates the short duration of action of BCM with respect to maximum response and the stable parallel shifts of the log dose-response curves.

In experiments with [^{3}H]BCM, the recovery of maximum response is accompanied by the appearance of tritiated hydrol-

ysis product in the organ bath according to the first-order process, having a $t_{1/2}$ of 38.9 min ($k = 1.77 \times 10^{-2}$ min^{-1}), which is in excellent agreement with the $t_{1/2}$ of 45 min ($k = 1.5 \times 10^{-2}$ min^{-1}) determined for recovery of the maximum response to acetylcholine. An approximately equal amount of BCM remains bound to the tissue and does not dissociate over the time course of the experiments (8 hr), which parallels the observation that the dose ratio of 390 is stable over this time course. Approximately 10,000 and 8800 pmoles of tritiated material per gram of longitudinal muscle are associated with the first-order process and the stable phase, respectively. These values are 50–75 times those observed by others for the concentration of receptors per gram of longitudinal muscle determined in studies on the binding of atropine, quinuclidinyl benzilate, and irreversible analogues of benzilylcholine (6, 8, 5, 20). Clearly, there has been substantial alkylation of non-receptor material by BCM. However, the parallel kinetic behavior of both the recovery of tissue response and the loss of radioactivity suggests a basic similarity in the chemical reactivity of the groups alkylated at the receptor and non-receptor sites.

Analogously to the closely related compound benzhydryl mustard (12, 13), BCM alkylates two sites at the muscarinic receptor. Occupation of an allosteric site causes an apparent decrease in affinity for acetylcholine, resulting in a parallel rightward shift of the dose-response curve. This alkylation is stable, at least over the time course of these experiments. The apparent decrease in affinity can best be explained using the Monod-Wyman-Changeux two-state allosteric model (21),

$$T \overset{L}{\rightleftharpoons} R$$

Alkylation of an allosteric site serves to stabilize the T state and increases the allosteric constant, L. Thus more agonist is required to convert the receptor to the active R state. Edelstein (22) has presented a quantitative treatment of the Monod-Wyman-Changeux model for the nicotinic receptor from *Electrophorus*. Changeux *et al.* (23) and Levitzki (24) have proposed models in which the receptors are arranged in clusters. Binding of a ligand to one receptor in the cluster induces a conformational change which is propagated to neighboring receptor molecules. This would account for graded changes in L and the progressive rightward shifts of the dose-response curves with increasing alkylation by irreversible agents.

With increasing concentrations of BCM, a second site is alkylated which causes the loss of response to acetylcholine. This second site of alkylation may represent the agonist recognition site proper but is clearly distinct from the allosteric site, since recovery of response occurs from this site whereas no measurable recovery of response occurs from the allosteric site.

However, the allosteric site and agonist recognition site do not show sufficient differences in affinity and/or reactivity toward BCM to permit their selective titration, and the maximum shift of the acetylcholine dose-response curve (390-fold) can only be realized following recovery of the maximum response after complete elimination of response.

These results lend further experimental support to our previous suggestion that alkylating agents have an allosteric component of action (12, 13). Clearly, reversible agents such as benzilylcholine, which are structurally analogous to BCM, can act by a similar mechanism. However, in the case of reversible agents, it is experimentally difficult to distinguish directly between a purely competitive and an allosteric inhibitor, as pointed out by Ariëns and Simonis (25), who first suggested an allosteric mechanism of action for cholinergic antagonists.

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