Br. J. Pharmac. (1973), **49**, 498–505.

The number of muscarinic receptors in chick amnion muscle

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Summary

1. [^{3}H]-Propyl benzilylcholine mustard ([^{3}H]-PrBCM) an irreversible specific antagonist of muscarinic acetylcholine receptors, has been used to label the muscarinic receptors in the aneuronal muscle of isolated 11 day amniotic membranes of chick embryos.

2. This muscle was found to have about 9 fmol/mg dry weight of receptor material. This is only 1–2% of the amount found in intestinal muscle.

3. Pharmacological studies with isolated amniotic membranes using PrBCM show that labelling is to functional receptors and that the receptor reserve is small.

4. The significance of the difference in receptor reserve in innervated and aneuronal plain muscle is discussed.

Introduction

Benzilylcholine mustard (BCM) (Gill & Rang, 1966) and its *N*-propyl homologue (PrBCM) (Young, Hiley & Burgen, 1972) are highly specific and virtually irreversible antagonists of muscarinic receptors. In addition the tritium-labelled derivatives of these antagonists are of value as affinity labels for the muscarinic receptor (Rang, 1967 ; Fewtrell & Rang, 1971 ; Hiley, Young & Burgen, 1972). This paper reports on binding studies with [^{3}H]-PrBCM using the smooth muscle of the chick amnion.

The amnion exists as a fluid filled sac which surrounds the embryo during its *in ovo* development. Histologically it consists of a single layer of epithelial cells derived from ectoderm and a layer of smooth muscle derived from mesoderm (Romanoff, 1952). The muscle cells contain muscarinic acetylcholine receptors (Cuthbert, 1962a, 1963, 1964 ; Evans & Schild, 1959) but more importantly it is never innervated, so that there is no possibility of binding to nervous elements. The amnion is only 20–30 μm in thickness and hence diffusive delays are unlikely to create problems during labelling experiments. Furthermore there is a high proportion of smooth muscle cells, an essential feature for optimising the ratio of receptor-specific binding to non-specific binding.

The isolated amnion can be used both for recording contractions in response to drugs and for labelling studies. This means that the kinetics of uptake of the mustard can be compared with those for the onset of inhibition of the response to muscarinic drugs, providing valuable evidence that the receptor-specific binding is to functional receptors.

It is shown that 11 day amniotic membranes contain few muscarinic receptors when compared with the innervated longitudinal muscle of the guinea-pig ileum.

Methods

Tissues and solutions

Experiments were performed on amniotic membranes dissected from hens' eggs incubated at 40° C for 11 days. Longitudinal muscle strips from the small intestine of guinea-pigs were prepared as described by Rang (1964). Hanks' balanced salt solution (BSS) was used throughout. This solution had the following composition (mM): NaCl, 137; KCl, 5·4; $CaCl_2$, 1·26; $MgSO_4$, 0·4; $MgCl_2$, 0·5; Na_2HPO_4, 0·34; KH_2PO_4, 0·44; $NaHCO_3$, 0·35 and D-glucose, 5·55. The solution was bubbled with air and its pH was 7·65.

[^3H]-Propyl benzilylcholine mustard

The synthesis of [^{3}H]-PrBCM (*N*-2-chloroethyl-*N*-[2,3-$^3H_2$]-propyl-2-aminoethyl benzilate hydrochloride), specific activity 1·45 Ci/mmol, will be described elsewhere. The parent compound was converted into the pharmacologically active aziridinium ion by allowing a 0·13 mM solution of [^{3}H]-PrBCM in 10 mM phosphate buffer, pH 7·5, to stand for 1 hour at room temperature (22° C). This solution was then diluted 100-fold with ice-cold buffer and kept at 0° C until required. All additions denoted as [^{3}H]-PrBCM refer to this cyclised form and the concentrations quoted are those of the aziridinium ion calculated on the basis of a 91% yield.

Binding studies

The membranes were suspended by fine nylon threads in 800 ml of BSS and at 30° C for 1 hour. Where appropriate, atropine was added at the end of this time and the pre-incubation continued for a further 15 min before the [^{3}H]-PrBCM was added (final concentration 2·4 nM). After incubation the membranes were transferred to 200 ml fresh BSS which was then replaced after 2 min and washing was continued with several changes of solution for 1 hour. The membranes were then hung by their threads and dried in air overnight.

Each membrane was then weighed (usual range 4–9 mg) and placed in a vial to which water (0·02 ml) and soluene (0·7 ml, Packard) were added. After the tissues had dissolved (24 h) scintillator was added (ethoxyethanol, 1 ml; 0·4% butyl-PBD in toluene, 10 ml) and the tritium measured by liquid scintillation spectrometry. Ileal strips were treated by the same procedure.

Pharmacological studies

Whole 11 day amniotic sacs were suspended in a conventional organ bath (10 ml) in BSS at 30° C and bubbled with air. Contractions were recorded isotonically and washing was by overflow. Doses of acetylcholine given in random order were allowed to act for 20 seconds. The interval between doses was either 2 or 3 minutes.

Results

Binding of [^3H]-propyl benzilylcholine mustard

The usual criterion for measuring receptor-specific binding has been adopted as that portion of the uptake that is sensitive to atropine. The amount of [^{3}H]-

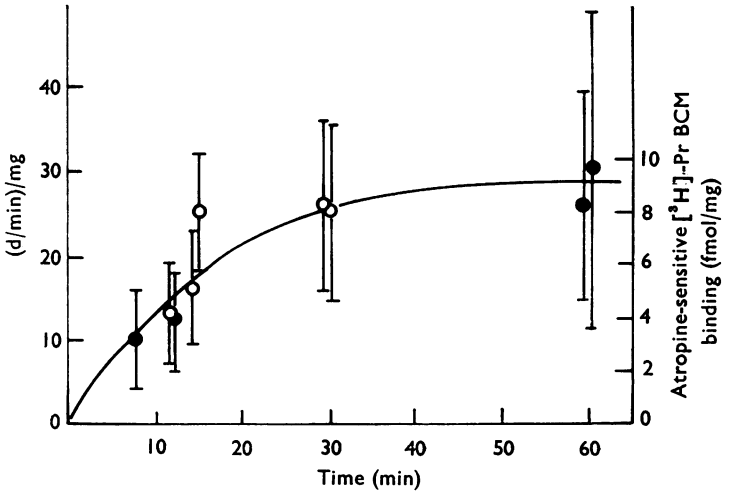

FIG. 1. Time-course of atropine-sensitive binding of [^{3}H]-propyl benzilylcholine mustard ([^{3}H]-PrBCM) by the isolated chick amnion. Points represent the difference ± S.E. between the uptake of tritium in the presence and absence of atropine, 10^{-7}M from groups of 5–8 membranes. Where the difference was statistically significant ($P \leq 0.05$) the points are shown as open circles (○).

PrBCM bound at various times of incubation in the presence and absence of atropine, 10^{-7}M, is shown in Figure 1. The amount of atropine-sensitive uptake was small and for some of the data shown was not significantly different from zero, especially at short incubation times and at 60 min when the amount of non-specific uptake had increased. However, the receptor-specific binding showed the essential property of saturating uptake. From the value at saturation the number of muscarinic receptors in the tissue is deduced as 9 fmol/mg dry weight. None of the [^{3}H]-PrBCM binding was sensitive to (+)-tubocurarine, 10^{-6}M.

A comparison was made in identical conditions of the uptake of the mustard by ileal strips and by amniotic membranes. Table 1 shows that non-specific uptake was lower in the amnion by a factor of 8, while the atropine-sensitive uptake was an even smaller proportion of that in the ileum, even though the proportion of smooth muscle in each tissue is not greatly discrepant (approximately 70% in the amnion and 90% in the ileal strips (Rang, 1967)). The atropine-sensitive binding of PrBCM to ileum is not saturated at 30 minutes. The maximal level of atropine-sensitive binding of [^{3}H]-PrBCM (2·4 nM) in ileal strips is around 150 fmol/mg wet weight, (Burgen, Hiley & Young, unpublished), so allowing a factor of 3–4 for the conversion between wet and dry weight, the number of muscarinic receptors in the amnion is only 1–2% of that in ileal muscle.

TABLE 1. *Comparison of the binding of [^{3}H]-propyl benzilylcholine mustard ([^{3}H]-PrBCM) by chick amnions and longitudinal muscle strips from guinea-pig ileum*

	Amount of [^{3}H]-PrBCM bound (d/min)/mg dry weight			
	No additions	Atropine 10^{-7}M	Difference	*P* (Student's *t*)
Chick amnion	57± 9 (7)	31± 3 (7)	26±10	<0·05
Ileal muscle	600±39 (5)	246±23 (5)	354±45	<0·001

The tissues were exposed to 2·4 nM [^{3}H]-PrBCM for 30 min at 30° C. Values are the means ±S.E. The number of determinations are given in parentheses.

Affinity constant of atropine as an inhibitor of [^{3}H]-PrBCM uptake

Experiments were carried out to check that the atropine-sensitive uptake of [^{3}H]-PrBCM really did represent the uptake by binding to muscarinic receptors. The amount of atropine-sensitive uptake after 12 min incubation with the mustard was measured as a function of the atropine concentration. Ideally a shorter incubation period would have been preferred because the uptake of label should be only a small fraction of the maximal uptake if the reciprocal of the concentration giving 50% inhibition is to be an accurate estimate of the affinity constant. Experimentally this is impracticable but even so the error is not great. The results of these experiments are shown in Figure 2. From this it can be seen that the affinity constant of atropine is approximately $10^9 M^{-1}$. This value is in good agreement with the pA_2 value of 8·8 obtained by Evans & Schild (1959) for the inhibition of the pharmacological response of the amnion and with the affinity constant for atropine in other smooth muscle tissue, for example, $9 \times 10^8 M^{-1}$ at 37° C for longitudinal muscle strips of guinea-pig ileum (Paton & Rang, 1965).

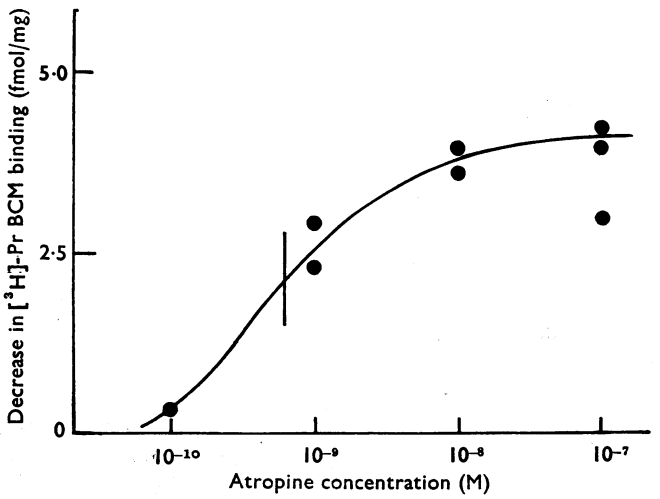

FIG. 2. The inhibition of binding of [^{3}H]-propyl benzilylcholine mustard ([^{3}H]-PrBCM) by atropine. Each point is the mean from 5–8 measurements. The tissues were incubated with the mustard for 12 minutes. The vertical bar indicates the point of 50% inhibition.

Rate of [^{3}H]-PrBCM uptake compared with the rate of onset of pharmacological blockade

Another method of testing the specificity of the atropine-sensitive uptake is to compare the rate of [^{3}H]-PrBCM uptake with the rate of onset of the pharmacological blockade by PrBCM. The effect of successive 10 min exposures to 2·4 nM PrBCM on the log-concentration response curve to acetylcholine is shown in Figure 3. The tissue was washed extensively after each exposure to the mustard. The first exposure resulted in a parallel shift of the curve to the right, but after a second treatment there was a distinct flattening of the curve and probably a reduction in the maximal response. Further exposure to the mustard caused increased flattening of the curves.

The kinetics of inactivation of muscarinic receptors in ileal muscle strips with BCM have been shown (Gill & Rang, 1966) to be consistent with the formation of

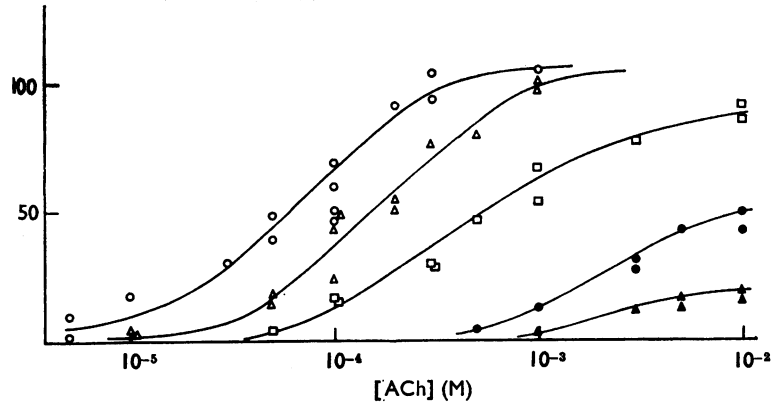

FIG. 3. Log concentration-response curves for acetylcholine on the responses of the isolated chick amnion. After the initial curve (○) was determined the preparation was subjected to successive 10 min incubations with propyl benzilylcholine mustard (2·4 nM). Total times of incubation with the mustard were 10 (△), 20 (□), 30 (●) and 40 (▲) minutes. The temperature was 30° C.

a reversible antagonist receptor complex, AR, followed by covalent bond formation.

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

If $k_2 \gg k_{-1}$ then the free receptor fraction, 1-p where p is the total antagonist occupancy, declines exponentially with time. A plot of ln (1-p) against time is linear with a slope equal to $-k_1$ [A], where [A] is the concentration of the mustard (Gill & Rang, 1966).

The same kinetic behaviour is observed for the chick amnion. From the curves shown in Fig. 3 receptor occupancy by PrBCM after 10 and 20 min exposure was calculated using the double reciprocal plot technique developed by a number of workers (for example, Furchgott & Bursztyn, 1967). Excessively flattened curves introduce errors and were not used for this analysis. The results from two other experiments were similarly treated. The logarithm of the free receptor fraction, 1-p, declined linearly with time (Fig. 4, filled points).

The open points shown on Fig. 4 have been calculated from the mean atropine-sensitive binding of [^{3}H]-PrBCM at given times (Figure 1). The occupancy at any time is calculated from the ratio of the amount of [^{3}H]-PrBCM bound at any time to the amount bound at saturation. The agreement between the two sets of points is good and provides strong grounds for believing that the atropine-sensitive binding of [^{3}H]-PrBCM is by functional receptors. The value of k_1 derived from Fig. 4 is 6×10^5 M^{-1} s^{-1}.

The extent of the receptor reserve for the contractile response to acetylcholine

The small parallel shift to the right of the log-concentration response curve to acetylcholine before the maximum response is depressed after treatment with the mustard (Fig. 3) indicates there is a small receptor reserve in the amnion for

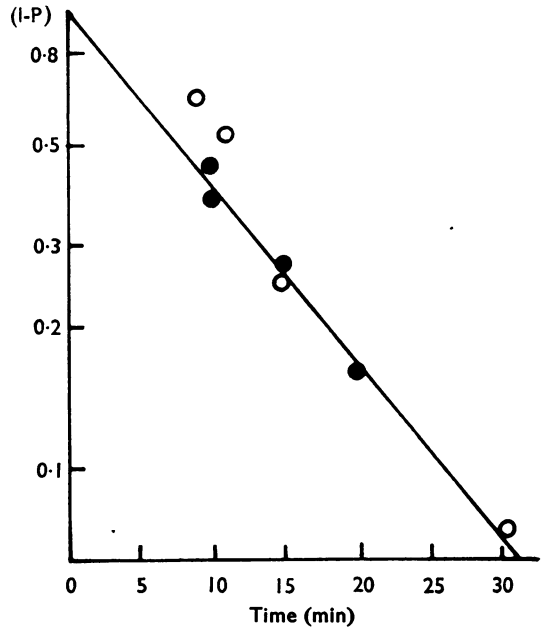

FIG. 4. Decline of the free receptor fraction (1-p) with time determined from the binding of [^{3}H]-propyl benzilylcholine mustard [^{3}H]-PrBCM (○) and from the blockade of the contractile response of acetylcholine (●) from three separate experiments. The antagonist occupancy was determined in each instance as described in the text.

acetylcholine. From experiments of this type it appears that approximately 25% of the receptors must be available to acetylcholine for the maximum response to be achieved.

Discussion

These results show that the amount of atropine-sensitive binding of [^{3}H]-PrBCM in the 11 day chick amnion is very small and indeed if the non-specific uptake had not been low it would have been experimentally very difficult to measure. There can be little doubt that the atropine-sensitive binding does represent binding to functional receptors, since the uptake curve shows saturation kinetics, atropine has the anticipated affinity constant as an inhibitor and the rate of uptake of [^{3}H]-PrBCM is the same as the rate of onset of pharmacological blockade. The amount of receptor material in the 11 day chick amnion is 9 fmol/mg dry weight, and is considerably less than that in longitudinal muscle strips of the guinea-pig ileum, which has 150 fmol/mg wet weight as determined from [^{3}H]-PrBCM binding (Burgen, Hiley & Young, unpublished). Although a comparison based on tissue weight is not entirely satisfactory it can be calculated that the amnion muscle has only 1–2% of the receptors present in intestinal muscle. This figure is of the same order as the ratio of the apparent receptor reserve in the two tissues. In the amnion a factor of 2 has been found for acetylcholine compared to a factor of about 65 for acetylcholine and methylfurmethide (Gill & Rang, 1966) and over 400 for carbachol (Burgen & Spero, 1968) in ileal muscle strips.

Since in the pharmacological studies we have used acetylcholine our results can be objected to on the grounds that the receptor reserve observed may be

limited by action of cholinesterase. The error is probably negligible since the amount of enzyme in the tissue is low (Cuthbert, 1962b ; Blaber & Cuthbert, 1962) and at the same time the concentrations of acetylcholine used are very high.

The simplest hypothesis that we can formulate for our results is that the muscarinic receptors of the amnion have the same properties as those in intestinal muscle strips, the difference in the amount in the two tissues reflecting the minimal receptor reserve in the former. As the major difference in the two muscles is that the amnion is aneuronal, the postulate might be carried a stage further to the tentative proposition that the existence of a large receptor reserve in the ileal muscle is a consequence of innervation. Functionally this is entirely reasonable since as Paton (1970) has pointed out there are problems of synthesizing and storing transmitters in the limited space of the nerve terminations, together with those of termination of action, which make it economically advantageous for drugs to work at low occupancies. At the neuromuscular junction the receptor reserve is also low (Paton & Waud, 1967) but here there is a much superior structural organization leading to high ' capture ' of the transmitter. The difficulty in rationalizing the differences between the two types of plain muscle which have been examined is that there is no known function in the amnion for receptors which are accessible to external acetylcholine.

The relatively low non-specific uptake of [^{3}H]-PrBCM in the amnion may prove significant and in spite of the small number of receptors the tissue may be experimentally valuable where changes in this number are of interest, for example, receptor induction.

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(*Received February* 14, 1973)