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INHIBITION OF THE MUSCARINIC RECEPTOR BY DIBENAMINE

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Abstract—The rate of reaction of a group at the muscarinic receptor of isolated guinea-pig ileum and rat jejunum with the ethyleniminium ion derived from dibenamine is independent of pH over the range 6·9–8·9. This group may be either a carboxyl group or an imidazole residue. The muscarinic receptor is not identical with the active site of acetylcholinesterase as proposed since the enzyme reacts in a different manner with dibenamine over this pH range.

THE SUGGESTION[1] concerning the identity of AChE and the muscarinic receptor of animal tissue has been examined by other workers[2–6] and an accumulation of data has cast considerable doubt on the validity of the suggestion. We have approached this problem by comparing the kinetic parameters for the inhibition of AChE and the muscarinic receptor by dibenamine. Studies of the inhibition by dibenamine of the muscarinic receptor of isolated rat jejunum and guinea-pig ileum are described here. The studies with AChE have been described previously.[7]

EXPERIMENTAL AND RESULTS

Materials

The isolated preparations employed were the jejunum from male albino rats (150–200 g) and the terminal ileum from male albino guinea-pigs (300–500 g). Muscle contracture was recorded isotonically under a load of 1 g in most instances by means of a simple lever with a frontal writing point. In certain experiments with ileum recording was made by means of a linear differential transformer coupled to a chart recorder (Sanborn).

Effect of pH on the rate of inhibition of the muscarinic receptor by dibenamine

A 1-cm length of the tissue was suspended in a 5-ml glass tissue bath containing aerated Tyrode solution at a selected pH and rested for 30 min. A dose ("submaximal dose") of acetylcholine (ACh), contained in 0·1 ml, producing a submaximal response of the isolated tissue was employed, the contact time for ACh with the tissue being 25 sec. This was followed by three washes with the Tyrode solution lasting 90 sec *in toto*. The rest period between additions of agonist was 2·5 min. When the response to the submaximal dose of ACh was reproducible, the tissue was incubated for 2 min with a solution of dibenamine hydrochloride ($7{\cdot}5 \times 10^{-5}$ M) freshly prepared in the Tyrode solution. The bath was then washed out three times with Tyrode solution and

the dose–time cycle for ACh recommenced. The whole procedure of incubating with dibenamine and subsequent determination of the response to the submaximal dose of ACh was repeated between six and eight times (see Fig. 1). Replicate experiments were performed with Tyrode solution at each of the three pH values, 6·9, 7·9 and 8·9.

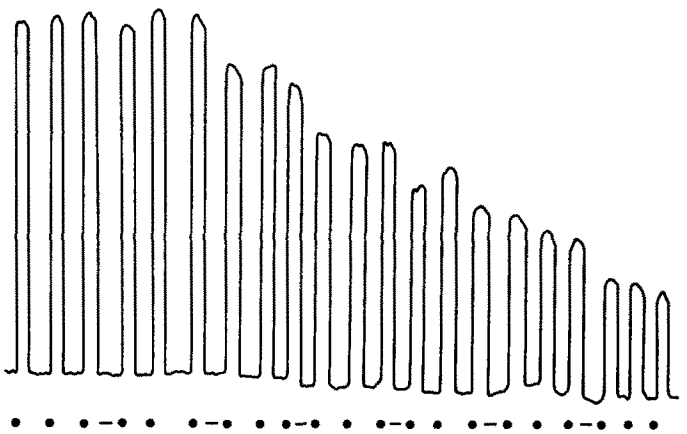

FIG. 1. Responses of the isolated guinea-pig ileum to ACh after incubation with dibenamine. ● ACh (0·08 μg) added to the bath. — Incubation for 2 min with Tyrode solution containing dibenamine ($7{\cdot}5 \times 10^{-5}$ M).

In contrast to guinea-pig ileum, rat jejunum proved to be an unsatisfactory preparation although it has been used previously for studies of this type.[8] In control experiments the response of the jejunum to ACh significantly decreased with time. Furthermore, after treatment with dibenamine there was a progressive increase in muscle tone which made accurate measurement of the response to ACh difficult.

The reaction between acetylcholinesterase and excess dibenamine follows first order kinetics[7] as expected for a pseudo-unimolecular reaction. The rate of the reaction is given by,

$$\frac{dx}{dt} = k_2\, a(b - x),$$

where k_2 is the second order rate constant, a and b are the initial concentrations of dibenamine and the functional group on the receptor respectively, and the concentration of product (i.e. alkylated enzyme) is x at time t. In the enzyme system, the amount of enzyme remaining, $b-x$, at time t is determined by assay with a suitable substrate and the initial rate of reaction is linearly proportional to the enzyme concentration.

Should the active site of acetylcholinesterase constitute the muscarinic receptor as proposed[1] then the reaction between dibenamine and the muscarinic receptor would follow similar kinetics, provided that the concentration of dibenamine was in excess. However, in the tissue system the assay of the receptor content $b-x$ remaining as the reaction proceeds by measuring the height of contracture of the muscle to a fixed dose of acetylcholine presumes that the contracture (i.e. effect) is a direct measure of receptor occupation (i.e. stimulus) by acetylcholine which has not been established. Consequently, studies of this nature must be cautiously interpreted until further information is available regarding the relationship between effect and stimulus although in

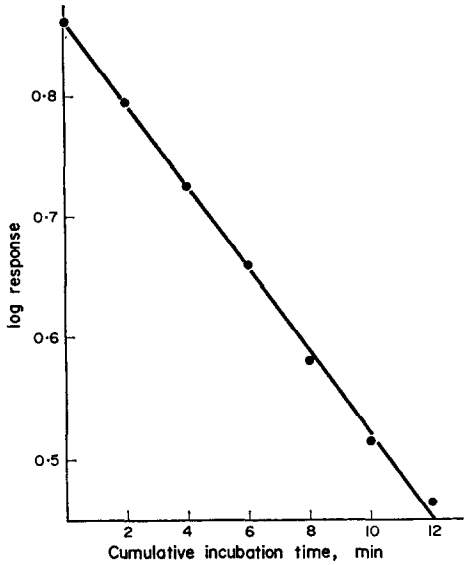

FIG. 2. Type (A) curve obtained for the first order plot of the reaction between the muscarinic receptors of isolated rat jejunum and dibenamine at pH 8·9.

certain systems a linear relationship has been noted.[9,10] To see whether a linear relationship existed in our system the experimental results with both tissues were plotted in accordance with first order kinetics, i.e. log response (height of contracture) vs. cumulative incubation time with dibenamine. Several of the graphs were non-linear to some degree and the shapes followed four basic forms (Figs. 2–5) linear (type A), sigmoid (type B), straight lines curved initially (type C) and straight lines curved finally (type D). The majority of the curves with both tissues were of type C.

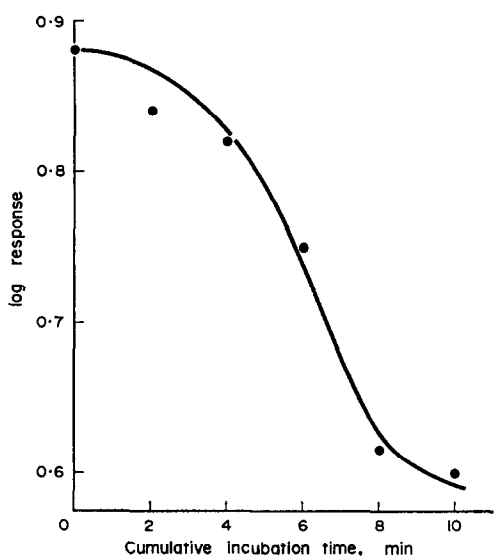

FIG. 3. Type (B) curve obtained for the first order plot of the reaction between the muscarinic receptors of isolated rat jejunum and dibenamine at pH 8·9.

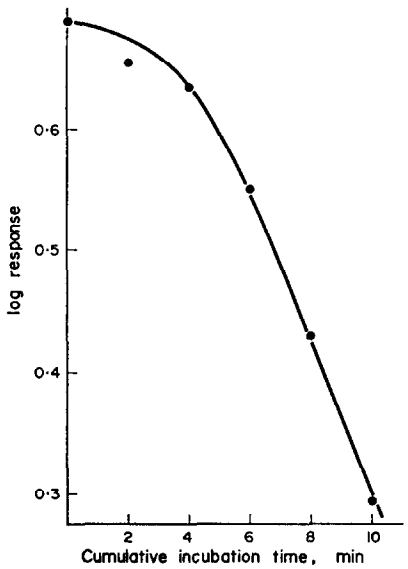

FIG. 4. Type (C) curve obtained for the first order plot of the reaction between the muscarinic receptors of isolated rat jejunum and dibenamine at pH 8·9.

The dose of ACh employed for most of the curves of type C had been accurately determined as a percentage of the maximum response, i.e. 70, 85, 89 per cent. Four standard inhibition experiments were conducted using doses of ACh which gave less than 50 per cent of the maximum contracture of the tissue. In all cases the curves were of type D, confirming that the form of the curve obtained is dependent upon the range of the E/E_{max} values covered.

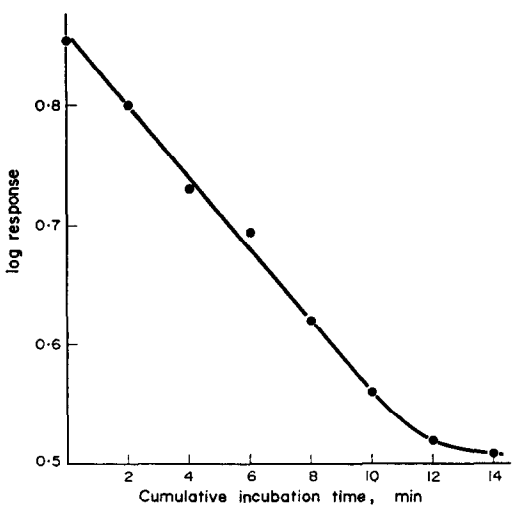

FIG. 5. Type (D) curve obtained for the first order plot of the reaction between the muscarinic receptors of isolated rat jejunum and dibenamine at pH 8·9.

TABLE 1. THE EFFECT OF pH ON THE RATE OF THE INHIBITION REACTION BETWEEN DIBENAMINE AND THE MUSCARINIC RECEPTORS OF THE ISOLATED RAT JEJUNUM

pH 8·9 $t_{0.5}$ (min)	pH 7·9 $t_{0.5}$ (min)	pH 6·9 $t_{0.5}$ (min)
5·3	6·1	7·4
10·9	6·0	3·5
5·7	13·4	3·1
5·4	5·1	4·0
10·3	5·1	9·0
8·9	12·1	6·1
5·1	6·7	5·1
4·1	5·6	5·7
	5·9	13·9
		7·5
		7·2
Mean 6·96	7·30	6·59
± Standard deviation 2·64	3·13	3·05

Each graph was examined and the points which lay on the linear portion were noted. The line of best fit through these points was determined by regression analysis and the slope of the line was conveniently expressed as the time required for the tissue response to be decreased to half its original value, i.e. $t_{0.5}$. The results at each pH studied are shown in Tables 1 and 2 for rat jejunum and guinea-pig ileum respectively. The data of each Table were subjected to an analysis of variance which revealed that the variance between groups (values at each pH) was not significantly different from that within

TABLE 2. THE EFFECT OF pH ON THE RATE OF THE INHIBITION REACTION BETWEEN DIBENAMINE AND THE MUSCARINIC RECEPTORS OF THE ISOLATED GUINEA-PIG ILEUM

pH 8·9 $t_{0.5}$ (min)	pH 7·9 $t_{0.5}$ (min)	pH 6·9 $t_{0.5}$ (min)
7·5	5·1	8·4
4·5	5·9	5·6
8·3	3·5	6·2
8·0	3·2	4·7
2·5	4·2	8·5
5·2	3·2	4·0
2·8	6·8	3·2
3·6	2·9	10·2
7·0	8·7	4·2
3·6	5·8	2·5
Mean 5·30	4·93	5·75
± Standard deviation 2·24	1·89	2·54

each group. (For P = 0·05, $F_{2,27} = 3{\cdot}35$, and $F_{2,25} = 3{\cdot}39$. The experimentally obtained values for guinea-pig ileum, $F_{2,27} = 0{\cdot}1369$ and for rat jejunum, $F_{2,25} = 0{\cdot}576$.) It is concluded that the inhibition of the muscarinic receptor by dibenamine is independent of pH over the range 6·9–8·9.

Preferential reaction of dibenamine at sites other than the muscarinic receptor

Preferential reaction of dibenamine with sites other than the muscarinic receptor in the initial stages of the experiments could lead to removal of the dibenamine which would influence the shape of the curve for the reaction with the receptor. This possibility was examined by the technique of cross- and self-protection.[11]

After selecting a submaximal dose of ACh the tissue was incubated with Tyrode solution containing either atropine sulphate ($1{\cdot}4 \times 10^{-6}$ M) or ACh ($4{\cdot}4 \times 10^{-5}$ M) for 5 min and then with Tyrode solution containing dibenamine ($7{\cdot}5 \times 10^{-5}$M) and either atropine sulphate ($1{\cdot}4 \times 10^{-6}$ M) or ACh ($4{\cdot}4 \times 10^{-5}$ M) respectively for 15 min. After this, the preparation was continuously washed with a slow flow of Tyrode solution and was tested periodically to determine whether the response of the muscle to ACh had returned, i.e. that the muscarinic receptors were free. The response to the sub-maximal dose of ACh returned to normal in about 3 hr. The inhibition of the muscarinic receptor by dibenamine was then studied by the previously described experimental procedure.

The plots of log response vs. cumulative incubation time were similar to those obtained without protection and it is concluded that the shape of the inhibition curve obtained by the standard technique is not influenced by reaction of dibenamine with sites other than at the muscarinic receptor.

Influence of sodium thiosulphate on the reaction of dibenamine with the muscarinic receptor

Using isolated guinea-pig ileum a submaximal dose of ACh producing less than 50 per cent response was selected and the tissue was then incubated for 2 min with Tyrode solution containing sodium thiosulphate ($6{\cdot}67 \times 10^{-2}$ M) and dibenamine hydrochloride ($7{\cdot}5 \times 10^{-5}$ M). The bath was washed out three times with Tyrode solution and the dosetime cycle for ACh was recommenced in the usual manner. The whole procedure of incubating with dibenamine and sodium thiosulphate, and subsequent determination of the response to the standard dose of ACh was repeated five times. The experiment was carried out in duplicate using tissue preparations from two guinea-pigs. A control experiment was also carried out in a similar manner but omitting the dibenamine.

In the control experiment the ileum contracted in the presence of sodium thiosulphate solution but relaxed again after washing. There was a loss in sensitivity as reflected by a low response to the original dose of ACh but on continued testing with ACh the preparation recovered and the response became slightly greater than the original contracture.

A similar contracture was observed in the test experiment on addition of Tyrode solution containing dibenamine and sodium thiosulphate and the response to ACh was also slightly greater than the initial contracture. Even after five incubation periods (2 min)with dibenamine in the presence of sodium thiosulphate there was no inhibition of the muscarinic receptor. The low submaximal dose of ACh was chosen so that the

inhibition curve would be of type A or D, i.e. the response would decrease after the first incubation period with dibenamine.

The effect of trypsin on the muscarinic receptor inhibited with dibenamine

Isolated guinea-pig ileum was incubated with Tyrode solution containing dibenamine hydrochloride ($7{\cdot}5 \times 10^{-5}$ M) until at least 50 per cent inhibition had occurred. The ileum was incubated for 1 min with a solution of trypsin (2×10^3 benzoyl arginine ethyl ester units/ml freshly prepared in Tyrode solution) and then, after washing out, the response of the ileum to the standard submaximal dose of ACh was recorded. Two further experiments were conducted by incubating the inhibited tissue with trypsin solutions containing 2×10^3 units/ml for 30 sec and 400 units/ml for 1–5 min.

TABLE 3. EFFECT OF TRYPSIN ON THE MUSCARINIC RECEPTOR (ISOLATED GUINEA-PIG ILEUM) INHIBITED WITH DIBENAMINE

Conc. of trypsin (units/ml)	Incubation time (min)	Effect on response of tissue to ACh
2×10^3	1·0	No response
2×10^3	0·5	Decreased response
4×10^2	1·0	No effect
4×10^2	$3 \times 1{\cdot}0$	No effect
4×10^2	5·0	Decreased response

The degree of irreversible inhibition of the muscarinic receptors by dibenamine was not affected by trypsin. The results are summarised in Table 3 and show that, depending on the concentration and incubation time, the effect of trypsin was either to cause further inhibition of the receptor, possibly by damaging the tissues contractile proteins, or to have no influence on the response.

DISCUSSION

A drug produces its biological response as the result of an interaction with a functional or organised group of atoms referred to as a receptor site. Various investigators[12–15] have tried to isolate receptors and study the interaction of drugs with such isolated receptor substances. However, the inevitable conclusion is that the receptor is the initiator of a series of events involving surrounding molecules and the integrity of the tissue as a whole is necessary for a response. Knowledge regarding the nature of the chemical moieties making up the receptor is scanty and, in general, ill-defined. Attempts to isolate these moieties from the muscarinic receptor using radioactively labelled alkylating agents[16,17] have proved unsuccessful. The most rewarding studies were conducted by Graham and Al Katib[18] using the α-adrenergic receptor of the guinea-pig vas deferens and dibenamine. Alkylation by dibenamine was associated with loss of response to noradrenaline which could be regenerated by trypsin. The known specificity of trypsin for hydrolysis of esters of arginine and lysine would indicate the presence of polypeptide material at the receptor.

It is generally accepted that the muscarinic receptor carries a unit negative charge

for ion-pair formation with a parasympathomimetic drug, leading probably to a conformational or other change at the receptor[19,20] with the evoking of a response. Little or no change in the response of the tissue to drugs bearing a constant charge over the pH range 4–8 has been observed, leading to the conclusion that the unit negative charge at the receptor is either a carboxylate or phosphate ion.[21] Recently, it has been reported from studies with the alkylating agent, 4-(*N*-maleimido) phenyltrimethylammonium iodide (MPTA) that there is a disulphide bond at a distance of the order of 10 Å from the negatively charged group at the site.[22] Structure–activity studies with a large number of drugs have given some idea of the size and shape of the drug-binding surface of the receptor[23–25] and there is some agreement that the material making up the receptor is protein in nature.[26,17]

Triggle[9] has studied the regeneration of dibenamine-alkylated aortic strips by measuring the response with time of the tissue to noradrenaline. On the assumption that the tissue response was directly proportional to receptor occupation he obtained linear first-order plots for the regeneration process. In our work the curves obtained for the inhibition were mainly straight lines curved initially (type C) when the dose of ACh employed in the assay was 70–90 per cent of the dose required for maximum tissue response. At lower doses, straight lines curved finally (type D) were obtained indicating that the form of the curve is dependent on the degree of receptor occupancy selected for the assay. The shape of the curve was uninfluenced by reactions occurring between dibenamine and elsewhere on the tissue as shown by cross and self-protection experiments using atropine and acetylcholine respectively. In conclusion although the type C and D curves obey first-order kinetics for major part of the measured course of the reaction, our inability to account for their overall shape together with the paucity of information concerning the relationship between effect and receptor occupancy stipulates that the $t_{0.5}$ values derived here are only used in a qualitative manner and for comparative purposes.

The $t_{0.5}$ values for the reaction between dibenamine and the receptor were determined a number of times at each of the three pH values, 6·9, 7·9 and 8·9. The coefficient of variance (%) for the value was 34 (pH 8·9), 29 (pH 7·9) and 43 (pH 6·9) for rat jejunum and 45 (pH 8·9), 35 (pH 7·9) and 14 (pH 6·9) for guinea-pig ileum. The spread about the mean value for the $t_{0.5}$ values obtained here is not considered excessive for tissue experiments, especially since in the inhibition experiments on the enzyme, a more homogeneous biological material, the coefficient of variance for the second-order rate constant, k_2, was 6·1 (pH 6·5) and 8·9 (pH 9·5) and duplicate estimations for another enzyme–inhibitor system have been accepted[27] within $\pm$ 10 per cent. An analysis of variance showed that the variance between each group of results determined at a certain pH was not significantly different from the variance within each group. This result confirms that the group on the receptor which is alkylated by dibenamine does not change its state of ionisation to any appreciable extent over the pH range 6·9–8·9. Small changes in the degree of ionisation of the group would not be detectable within the system due to the overall error in the method, but large changes associated with a group with a pK_a within the pH range studied would be readily detected since the extent of ionisation is 10–90 per cent within the range ($pK_a + 1$) to ($pK_a - 1$). These results contrast with those obtained for the inhibition of acetylcholinesterase by dibenamine where it was shown that a group with pK_a 9·1 was alkylated in alkaline media, and the second-order rate constant increased tenfold when

the pH was raised from 7·3 to 8·9. We would conclude, in concurrence with the work of others (*loc. cit.*) that the enzyme does not constitute the muscarinic receptor as suggested but is present in the vicinity of the receptor for the removal of ACh.

The adrenergic blocking action of dibenamine may be prevented *in vivo* by the prior administration of sodium thiosulphate. This observation has been interpreted as reaction by ethyleniminium ion formed from dibenamine base with the receptor.[28,29] Sodium thiosulphate has been shown here to protect the muscarinic receptor *in vitro* from the alkylating action of dibenamine and it is similarly concluded that the alkylating species is the ethyleniminium ion.

The reaction between dibenamine and the muscarinic receptor is between the ethyleniminium ion and a group on the receptor which is either un-ionised or does not increase its ionisation to an appreciable extent over the pH range 6·9–8·9. Should the receptor consist of polypeptide material then the nature of the alkylated residue is restricted to either imidazole (pK_a 5·6–7·0, in proteins[30]) or carboxyl (α, 3·0–3·2; aspartyl, 3·0–4·7; glutamyl, ca. 4·4). The cross- and self-protection experiments with atropine and ACh respectively indicated that alkylation occurs either at or very close to the receptor but does not differentiate between these two possibilities in view of the bulky nature of the *N,N*-dibenzyl ethylamine residue.

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