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Some Differential Effects of 4-Diphenylacetoxy-*N*-(2-chloroethyl)-piperidine Hydrochloride on Guinea-pig Atria and Ileum

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Abstract—4-Diphenylacetoxy-*N*-(2-chloroethyl)-piperidine hydrochloride (I) cyclizes at neutral pH to form an aziridinium salt. The formation and breakdown of the salt depend on the temperature (in the range 25 to 37°C). In solution at 30°C, peak levels, corresponding to 60–80% conversion, are reached after around 60 min and the half-life exceeds 100 min. In the presence of 0·9% NaCl conversion was reduced to 45–60%. I blocks muscarinic receptors in guinea-pig ileum and atria irreversibly and it is possible to produce dose-ratios on ileum with 10 nM I which are about 100 times those on atria. After about 30 min exposure to solutions of I (prepared 15–20 min previously so that formation of aziridinium ions is well-established) the graph of log (dose-ratio) against time is linear and similar plots were obtained with two different agonists, carbachol and ethoxyethyltrimethylammonium. With results for the ileum, extrapolation of the line suggests that it does not start from zero (dose-ratio = 1): this is because of an initial relatively rapid reversible block. This early phase is similar to that seen on ileum with 10 nM 4DAMP methobromide, which is a competitive antagonist, so is probably caused by competitive block by the aziridinium ion, which closely resembles 4DAMP metho-salts. The subsequent irreversible phase should be caused by alkylation of the receptors. I is easy to make and should be a valuable tool for the study of muscarinic receptors.

Many β-halo-ethylamines react irreversibly with receptors. The actions of dibenamine and related compounds, such as phenoxybenzamine, at adrenoceptors have been studied extensively (Nickerson 1949; Graham 1962). These compounds have also been used to block histamine receptors (Nickerson 1956) and even muscarinic acetylcholine receptors (Furchgott 1963) but a more specific irreversible muscarinic antagonist, benzilylcholine mustard, was described by Gill & Rang (1966). This is the chloro-ethyl analogue of the potent atropine-like compound lachesine (Ford-Moore & Ing 1947), from which it differs only in that the ethyl-dimethylammonium group is replaced by a chloro-ethyl-methylamine group. Homologues of benzilylcholine have been described by Young et al (1972) and isotopically labelled propyl-benzilylcholine mustard (Burgen et al 1974) is commercially available.

The compound diphenylacetoxy-*N*-methyl-piperidine methiodide (4DAMP methiodide) has greater affinity for muscarinic receptors in guinea-pig ileum than in guinea-pig atria (Barlow et al 1976) and although changes to the onium group reduce this selectivity (Barlow & Shepherd 1986) they do not abolish it. It seemed possible, therefore, that diphenylacetoxy-*N*-β-chloro-ethyl-piperidine (I, derived from 4DAMP and the classical mustards) might be an irreversible blocking agent at muscarinic receptors which retained some degree of selectivity, especially because it would be converted to a quaternary iminium (aziridinium) ion which would be very similar indeed to 4DAMP metho-salts, having an ethylene bridge in place of two separate methyl groups.

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This paper describes the synthesis of the compound, some experiments on its cyclization to form the aziridinium ion and some results obtained with it on guinea-pig ileum and atria.

Methods

Compounds

Carbachol chloride was obtained from Sigma. Ethoxyethyl-trimethylammonium iodide was the same sample as used in previous work (Barlow & Weston-Smith 1985).

4-Diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (I) hydrochloride was obtained by treating diphenylacetoxy-*N*-(2-hydroxyethyl)-piperidine (Barlow & Shepherd 1986) with a slight excess of thionyl chloride dissolved in benzene. The solid which formed was separated and recrystallized from butanone by addition of diethylether. The yield of 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine hydrochloride, m.p. 136°C, was greater than 50%.

Found C, 64·2; H, 6·33; N, 3·54; Cl, 17·5%
Theory C, 64·0; H, 6·39; N, 3·55; Cl, 18·0%

I hydrochloride dissolves easily in distilled water but there is a slow hydrolysis with the release of hydrogen and chloride ions (detectable with a pH meter and a chloride electrode): this can be stopped by adding dilute acetic acid to bring the pH to about 5. When diluted from 0·01 M in water to 0·8 mM in phosphate buffer (10 mM) at pH 7·5 the base comes out of solution, which turns cloudy; this does not occur with benzilyl choline mustard (Gill & Rang 1966) but does occur with the higher homologues (Young et al 1972). With I the base stays in solution if the phosphate buffer (10 mM) is made up with 50% (by volume) aqueous ethanol.

Cyclization

The formation of the aziridinium ion was followed by adding excess sodium thiosulphate, with which it forms the alkyl thiosulphate ester (Bunte salt), and estimating the unreacted thiosulphate by titration with iodine. The method was similar to that used by Golumbic et al (1946), Chapman & James (1954), Gill & Rang (1966) and Young et al (1972). Samples (5 mL) were taken at selected times from 50 mL of 0·8 mM I and the cyclization stopped by adding 0·4 mL acetic acid (0·5 M). These were left for at least 15 min with 1mL sodium thiosulphate (10 mM) after which they were titrated with iodine (nominally 4 mM) with starch as the indicator. The amount of aziridinium ion formed was calculated from the difference between this volume and the volume of iodine solution equivalent to 1 mL sodium thiosulphate (10 mM).

The formation of the aziridinium ion was studied at 25, 30 and 37 °C in phosphate buffer (10 mM) made up with 50% (by volume) aqueous ethanol and at 30 °C in phosphate buffer (10 mM) made up with 30% (by volume) aqueous ethanol, also in aqueous phosphate buffer and in Krebs solution. The amount of aziridinium ion present at a particular time is the sum of an exponential rise (formation) offset by an exponential fall (breakdown), which is similar to the process governing the blood-level of a drug after oral absorption (Teorell 1937), and a direct fit of the amount of ion estimated by titration to time can be made using the program (DOSEFIT) for fitting blood-levels to time (Barlow 1983).

Animals

All preparations were obtained from male Dunkin-Hartley guinea-pigs, 500–700 g.

Guinea-pig isolated ileum

The guinea-pig ileum was prepared as described by Edinburgh Staff (1974). In some experiments each piece of ileum was cut longitudinally to produce two separate preparations (longitudinal sheets). The ileum was set up in Krebs solution, aerated with a mixture of 95% O_2 and 5% CO_2, with the responses recorded isotonically and a load of about 0·5 g. The agonists were allowed to act for 30 s and added once every 90 s by relays controlled from a PET microcomputer. As in previous work (Barlow & Shepherd 1986) the Krebs solution also contained norphenylephrine, 5 μM, and experiments were done at 29·8 ± 0·3 and 37·0 ± 0·1 °C.

Guinea-pig isolated atria

The atria were set up in Krebs solution aerated with a mixture of 95% O_2 and 5% CO_2 and containing 5μM norphenylephrine (Barlow & Shepherd 1986; Barlow et al 1988). The temperature was 29·8 ± 0·3 C and the spontaneous contractions were recorded isometrically with a load of about 0·2 g: action potentials were also recorded and the time required for 50 beats was continuously printed out (Barlow & Shepherd 1986). The agonist was added by relays operated from a microcomputer and allowed to act for 5 min: doses were given once every 15 min, with a second wash 10 min from the start of the cycle. The effects of the agonist were expressed as the percentage inhibition of the force of the contraction and the percentage increase in the time for 50 beats.

Preparation of I for use

Solutions were prepared in two ways:

(i) A 1 mM stock solution of the hydrochloride in water with a trace of acid (pH 5) was diluted to 10 μM, also in water with a trace of acid, before being finally diluted to 10 nM in Krebs solution immediately before use.

(ii) A 10 mM stock solution of the hydrochloride in ethanol was diluted to 100 μM in ethanol and then diluted to 10 nM in Krebs solution.

Experimental design

In the first experiments with atria and ileum, alternate small and large responses (roughly 25 and 75% of the maximum) were obtained with the agonist, carbachol or ethoxyethyltrimethylammonium iodide (EOE), and when these were regular the time was noted and the tissue was continuously exposed to 10 nM I, prepared by method (i). Solutions of I were prepared 15–20 min before they were applied to the preparations, so the formation of the aziridinium was well-established. As the antagonism progressed the concentrations of agonist were increased to try to obtain responses which lay within the range of the controls. When they did, the size was used to calculate the dose-ratio (DR) for that particular time as in a 3-point bioassay (Edinburgh Staff 1974).

In further experiments control responses were obtained with a range of concentrations of agonist but a fixed concentration, producing a medium sized response (roughly 50% of the maximum), was given between each of the control responses. This technique (Barlow et al 1963) ensures that each response used in the construction of the dose-response curve has been obtained after a similar response. When the responses were regular the time was noted and the tissue was continuously exposed to 10 nM I, prepared by method (ii). As the antagonism progressed the concentrations of agonist were increased. The control responses were fitted (by the method of least-squares) to dose using the logistic equation (Barlow 1983) and this was used to calculate the dose-ratio for any response obtained in the presence of the antagonist, provided it lay within the range of the control responses.

Data-fitting

Previous work on the estimates of rate constants for offset and onset of antagonists (Paton 1961; Paton & Rang 1965; Rang 1966) and for the actions of irreversible antagonists (Gill & Rang 1966) has involved measuring dose-ratios and calculating the proportion of receptors occupied by the antagonist. If the proportion of receptors occupied by a competitive antagonist at equilibrium is Z_e, the proportion Z_t occupied at time, t, after washout is given by:

$Z_t = Z_e\, e^{-k_{-1}t}$, where k_{-1} is the offset rate constant.

For onset the receptor occupancy at time, t, $Z_t = Z_e (1 - e^{-k't})$, where the apparent rate constant, $k' = k_{+1}A + k_{-1}$: A is the concentration of the antagonist (the process is bimolecular).

For offset, the graph of log Z_t against time gives k_{-1}: for onset the graph of log $(Z_e - Z_t)$ gives k' (and hence k_{+1}). The actual dose-ratios are:

$DR = DR_{max}/(DR_{max} - (DR_{max} - 1)e^{-k_{-1}t})$ for offset and

$DR = DR_{max}/(1 + (DR_{max} - 1)e^{-k't})$ for onset,

where DR_{max} is the (maximum) dose-ratio obtained at equilibrium. A direct fit of values of DR to t is possible using procedures similar to those described by Barlow (1983): this avoids the need to know Z_e exactly in order to calculate k'.

With I, the action of the aziridinium ion should leave a total proportion, Z, of receptors occupied reversibly (p_1) and irreversibly (p_2) by the antagonist where $Z = p_1 + p_2$. There are four rate constants to be considered, involving the association and dissociation of the reversible complex, the reaction to form the irreversible complex and the breakdown of the irreversible complex. Gill & Rang (1966) showed that if this breakdown can be neglected.

$$Z = 1 + A \cdot e^{-qt} - B \cdot e^{-rt}$$

where A, B, q and r are terms involving the three rate constants (see Gill & Rang 1966).

The dose-ratio,

$$DR = 1/(1 - Z),$$
$$= 1/(-A \cdot e^{-qt} + B \cdot e^{-rt}).$$

However, many of the actual results (see below) can be satisfactorily fitted to $DR = 1/e^{-kt}$, where k is an empirical rate constant: the dose-ratio rises exponentially from 1 (so B = 1) and the graph of log (DR) plotted against time is a straight line. The significance of the empirical rate constant k is not clear but it is a useful experimental measure of the time course of blockade, which can be expressed as the time taken to double the dose-ratio.

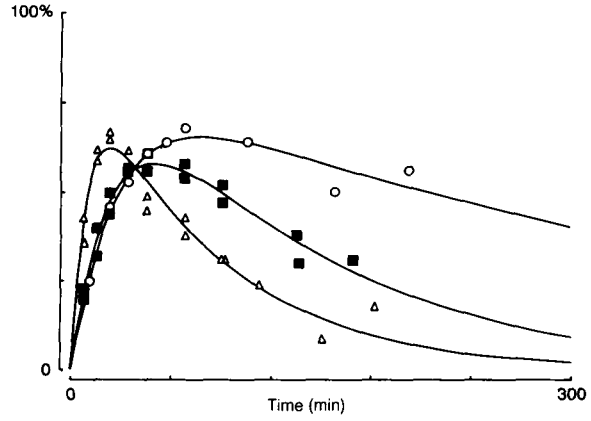

FIG. 1. Effects of temperature on the formation of aziridinium ions in 10 mM phosphate buffer made up in 50% (by volume) aqueous ethanol. The percentage conversion is plotted against time and the lines are the least-squares fit (Barlow 1983) to the relation:

$$C = B(e^{-kt} - e^{-Pt})/((p/k) - 1)$$

where k and p are the rate constants for formation and breakdown and B is a constant. The temperatures were 37° (△), 30° (■) and 25 °C (○).

Results

Formation of the aziridinium ion

The results are summarized in Table 1. Although the results are presented as half-times, the fitting process actually calculates rate constants and an estimate of their standard error, so the errors attached to the half-times will be logarithmic. The variation to be expected between experiments can be judged from the replicates shown for experiments. The volumes in the titrations are small and the variation must largely arise from the ability to perform replicate titrations. There is greatest uncertainty about the half-life of the ion (because the rate-constant is small) but it is clear that at 30 °C it is greater than 100 min. The effect of temperature, illustrated in Fig. 1, is similar to that observed by Young et al (1972) with the homologues of benzilylcholine mustard. The formation is reduced if I base is allowed to come out of solution: it is greater in solutions containing alcohol. It was not possible to assess the formation in Krebs solution because the calcium salts were precipitated if there was enough ethanol present to keep I base in solution but experiments were made in the presence of sodium chloride. This reduced the formation of aziridinium ions (Table 1), an effect which was also observed by Young et al (1972) who ascribed it to the increase in the ionic strength.

Time-course in biological experiments

The titrations demonstrate the formation of the aziridinium ion but do not give precise information about the time-course. Confirmation that the ion is formed is obtained from the biological experiments. In these the effect of the antago-

Table 1. Formation and breakdown of aziridinium ion in ethanolic aqueous buffers. Experimental data was fitted using DOSEFIT (Barlow 1983) to obtain the parameters shown.

Buffer (% ethanol)	Temp. (°C)	Half-time for formation (min)	Half-time for breakdown (min)	Time to maximum (min)	Maximum (%)
50	25	23·9	378	102	65·5
	30	23·2	112	66·5	58·4
	37	8·83	73·6	30·7	62·7
30	30	15·8	179	60·7	81·9
		15·6	214	63·7	66·7
30 + NaCl (50 mM)	30	13·4	1260	88·9	60·7
		12·9	331	62·9	54·8
30 + NaCl (154 mM)	30	13·1	543	72·1	59·4
		11·9	452	64·3	45·7

Table 2. Effects of I on guinea-pig atria and ileum at 30°C and on ileum at 37°C. Values of log (dose-ratio) (Log·DR) and time (t) after exposure to I (10 nM) were fitted by least squares to the straight line (Log·DR = Mt + C). The value of M (log·DR min^{-1}) has been used to calculate the time (min) taken for the dose-ratio to double (T = 0·301/M). The value of C should indicate log·DR for the initial (reversible) part of the block.

Temp. (°C)	Atria 30			Ileum 30			37		
	M	C	T	M	C	T	M	C	T
	0·00358	−0·027	84·9	0·0152	0·440	19·2			
				0·0141	0·440	21·3			
	0·00366	0·198	82·3						
	0·00210	0·108	142 }	0·0165	0·381	18·2			
	0·00315*	0·039	96·6 }	0·0192	0·416	15·7			
	0·00410	0·257	73·4	0·0237	0·154	12·7	0·0226	0·356	13·3
				0·0222	−0·079	13·5	0·0245	0·237	12·3
	0·00408*	−0·162	73·7	0·0197	0·043	15·3	0·0180	0·227	16·7
				0·0203	−0·049	15·0	0·0222	−0·052	13·6

Whenever possible experiments were made on atria and on two pieces of ileum simultaneously and are set out on the same line in the table (these are matched data, obtained from the same animal and with the same solutions). The asterisk indicates results for effects on atrial rate otherwise the effects were on atrial force and the brackets couple values obtained for the two effects (rate and force) on one pair of atria.

The means of log M (log·DR min^{-1} ± s.e., n) are −2·473 (±0·044, 6) for atria, −1·731 (±0·028, 8) for ileum at 30° and −1·664 (±0·029, 4) for ileum at 37°C.

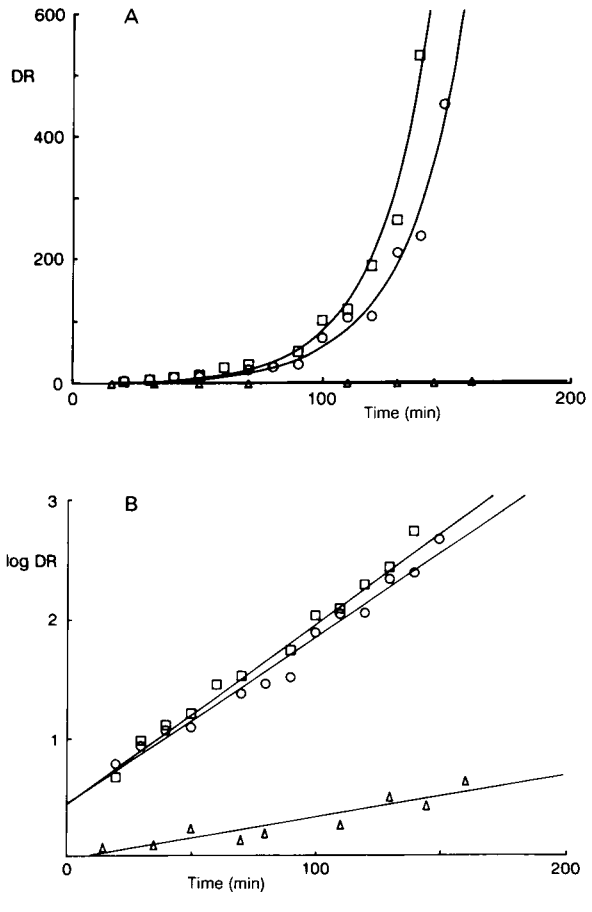

FIG. 2.A. Development of blockade with I (10 nM) on guinea-pig ileum and atria at 30°C. The agonist was carbachol and the dose-ratio is plotted against time (min). Results are shown for two experiments on ileum (○, □) and for effects on atrial force (△), all made at the same time. The lines are a least-squares fit of dose-ratio to time, t, where $DR = 1/e^{-kt}$. B. The same results with log·dose-ratio plotted against time. The lines are the least-squares fit of log·DR to time where log·DR = Mt + constant.

nist at a particular time was measured as the dose-ratio. The time-course of the blockade in one experiment with I (10 nM) is shown in Fig. 2A: duplicate tests were made on ileum at 30°C at the same time as one on atria. This provides matched data, with the tissues from the same animal and treated with the same solutions. After 120 min the dose-ratio on the atria was just over 2 compared with estimates of 115 and 197 for ileum. The lines shown in Fig. 2A represent the least-squares fit of dose-ratio to time with the relation $DR = 1/e^{-rt}$ where r is a rate-constant. In Fig. 2B the same results are shown with log (dose-ratio) plotted against time and can be seen to fit a straight line.

Although it is the rate constant which determines the time-course of the block, it is convenient (as with radioactive decay or drug kinetics) to think in terms of time and to use the time taken to double the dose-ratio as an experimental measure of the development of blockade. Values for a number of experiments are summarized in Table 2.

Results obtained with two different agonists, carbachol and ethoxyethyltrimethylammonium iodide, on the same piece of ileum at 37°C are shown in Fig. 3 and in Table 3. Statistical tests (paired *t*-test, signed-rank test) do not suggest any difference ($P > 0·1$).

Further experiments were made with I, starting from an alcoholic stock solution (method (ii)), to check on earlier work and to investigate possible recovery after the compound was removed. In these the control responses were obtained over a range of concentrations and fitted to the logistic equation and wherever possible a range of concentrations was also tested in the presence of the antagonist (with an interpolated dose producing a medium-sized response). The results (Table 4) confirm previous observations about the onset and show that the recovery is very slow at 37°C, similar to that with propylbenzilylcholine mustard (Burgen et al 1974). There is no recovery at 30°C (Fig. 4).

Reversible component of the block on ileum

For results on the ileum the graphs of log (DR) against time

Table 3. Effects of I on guinea-pig ileum at 37°C. Experiments with different agonists, carbachol (CC) and ethoxyethyltrimethylammonium iodide (EOE), tested on the same preparation. Values of log·dose-ratio (Log·DR) and time (t) after exposure to I (10 nM) were fitted by least squares to the straight line (Log·DR = Mt + C). The value of M (log·DR min^{-1}) has been used to calculate the time (min) taken for the dose-ratio to double, T, and the value of C should indicate log·DR for the initial (reversible) part of the block.

M (log·DR min^{-1})		C (log·DR)		T (min)	
CC	EOE	CC	EOE	CC	EOE
0·0109	0·0149	0·517	1·22	27·5	20·2
0·0126	0·0113	0·407	0·476	23·9	26·7
0·0109	0·0109	0·331	0·319	27·5	22·7
0·0114	0·0118	0·673	0·460	26·3	25·7
0·0119	0·0160	0·338	0·001	25·3	19·1
0·0116	0·0140	−0·018	−0·075	26·4	21·7

The means of log M (log·DR min^{-1} ± s.e., n) are −1·938 (±0·010, 6) for carbachol and −1·886 (±0·028, 6) for EOE and there is no significant difference ($P > 0·1$) with a paired t-test or a Wilcoxon signed-rank test.

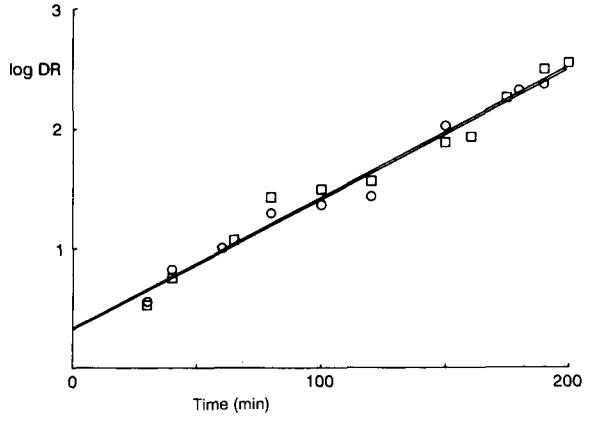

FIG. 3. Development of blockade with I (10 nM) on guinea-pig ileum at 37°C. Dose-ratios for carbachol (○) and ethoxyethyltrimethyl ammonium iodide (□) were obtained on the same piece of ileum and log·dose-ratio is plotted against time (min). The lines indicate the least squares fit as in Fig. 2B.

do not appear to start from zero (DR = 1). To see if this might be due to an initial reversible block by the aziridinium ion, tests were made with 4DAMP methobromide, which contains two methyl groups instead of the ethylene part of the aziridinium ring and acts competitively. Results obtained with 10 nM 4DAMP methobromide on ileum (Fig. 5) show that even at 30°C dose-ratios approaching 10 are obtained in less than 20 min, which could explain why the graphs of log (dose-ratio) against time for the mustard will not appear to start from zero. The results from several experiments are summarized in Table 5. From the logarithmic means k_{+1} (M^{-1} min^{-1}) is $(0·419 - 0·0762)/0·0762 = 4·50 \times 10^8$ at 30°C and $7·02 \times 10^8$ at 37°C. These give estimates of the equilibrium constants, however, which are 5·9 and $11·1 \times 10^9$ (L mol^{-1}), much higher than the values calculated from the dose-ratios at equilibrium.

Table 4. Time taken for dose-ratio to double (onset) or halve (offset) with I on guinea-pig ileum at 30° and 37°C. Values of log·dose-ratio (Log·DR) and time (t) after exposure to I (10 nM) were fitted by least squares to the straight line (Log·DR = Mt + C), as in Tables 2 and 3, and the value of M (log·DR min^{-1}) has been used to calculate the time, T (min), taken for the dose-ratio to double during onset or the half-time for recovery. In these experiments I was prepared from an ethanolic stock solution (method (ii)) and a range of concentrations of agonist (carbachol) was used which produced a range of responses.

Onset		Offset	
30 °C	37 °C	30 °C	37 °C
18·8	13·0		
16·5	8·6		
22·9	14·4		
19·7	13·7	no recovery	142
32·0	24·1	no recovery	107
29·3	15·7	no recovery	96·2
Mean ± s.e.			
23·2 ± 2·5	14·9 ± 2·1		115
Logarithmic mean			
22·5	14·2		113

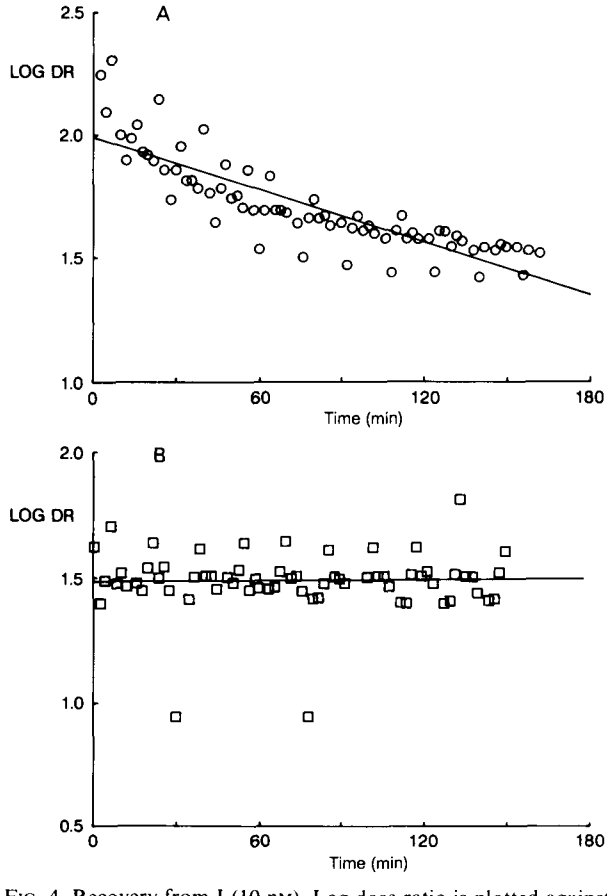

FIG. 4. Recovery from I (10 nM). Log dose-ratio is plotted against time for experiments at 37°C (A) and at 30°C (B). The lines are the least-squares fit of log·DR to time where log·DR = Mt + constant. In these experiments responses were obtained with a range of concentrations of agonist (see text).

Discussion

There are two main uncertainties in this work; the extent to which the aziridinium has been formed and the correctness of the estimate of the dose-ratio. Although the titrations with sodium thiosulphate (Table 1) do not give precise values which can be applied to the biological experiments, they

Table 5. Onset and offset of effects of 10 nM 4DAMP MeBr: estimates of DR_{max}, k′ (apparent onset rate constant: $M^{-1}\,min^{-1}$) and k_{-1} (offset rate constant: min^{-1}) obtained by direct fit of dose-ratios to time.

Temp. (°C)	DR_{max}	k′	DR_{max}	k_{-1}
30	12·4	0·324	8·79	0·0679
	10·0	1·064	7·91	0·0752
	11·5	0·201	8·94	0·0583
	9·02	0·473	7·72	0·0925
	11·5	0·553	7·24	0·1213
	9·42	0·294	7·06	0·0711
	9·19	0·356	4·81	0·0744
	8·01	0·353	7·96	0·0643
	9·89	0·594		
Mean	10·1	0·468	7·55	0·0781
±s.e.	0·47	0·085	0·46	0·0071
logarithmic mean		0·419		0·0762
37	9·19	0·421	5·54	0·0517
	8·46	0·573	6·14	0·0837
	7·09	0·348	6·70	0·0610
	6·85	0·504	6·92	0·0824
	9·69	0·496	7·21	0·0556
	6·44	0·581	6·31	0·0623
	11·0	0·620	7·91	0·0405
	8·79	0·584	7·97	0·0847
Mean	8·44	0·516	6·84	0·0652
±s.e.	0·55	0·033	0·30	0·0059
logarithmic mean		0·508		0·0633

From the logarithmic means k_{+1} ($M^{-1}\,min^{-1}$) is (0·419 − 0·0762)/0·0762 = $4·50 \times 10^8$ at 30 °C and $7·02 \times 10^8$ at 37 °C: the corresponding equilibrium constants are 5·9 and $11·1 \times 10^9$ ($L\,mol^{-1}$), which are very much higher than the values calculated from the dose-ratios at equilibrium.

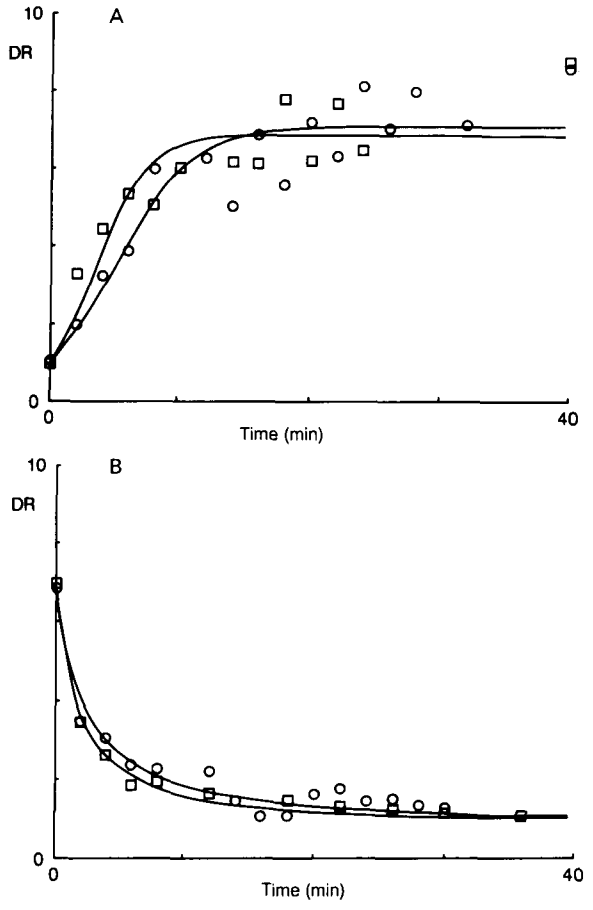

FIG. 5. Blockade by 4DAMP methobromide (10 nM) on guinea-pig ileum at 30 °C (○) and 37 °C (□) with the dose-ratio, DR, plotted against time, t (min),

A Onset. The line shows the least-squares fit where $DR = DR_{max}/(1 + (DR_{max} - 1)e^{-k't})$.
B Offset. The line shows the least-squares fit where $DR = DR_{max}/(DR_{max} - (DR_{max} - 1)e^{-k_{-1}T})$.

Note that the rates of offset and onset are slightly higher at the higher temperature and that a steady state appears to have been reached in 20 min, even at 30 °C.

indicate that maximum formation is likely to be reached in 60 to 70 min and confirm that the ion has a half-life greater than 100 min. The experiments with the competitive antagonist 4DAMP methobromide indicate that the initial antagonism by I could well be due to a competitive action by the aziridinium ion. Subsequent antagonism appears to be quite well described by a single exponential process: this is irreversible and, from what is known about similar compounds, involves the alkylation of the receptor. The results with ethoxyethyltrimethylammonium confirm that it is a muscarinic receptor which is being affected. The blockade seems to develop much more slowly than with benzilylcholine mustard which, in 16 nM solutions, appears to have a half-time for onset of about 30 s (Gill & Rang 1966). The process is bimolecular, however, so the apparent rate constant includes the concentration (see above) and the difference may be partly because the solutions of I are weaker, with the concentration of aziridinium ion much less than the nominal concentration (10 nM).

In most experiments the rates were faster at 37 °C than at 30 °C and the block was greater. In contrast the equilibrium dose-ratio with 4DAMP methobromide is slightly larger at the lower temperature (Table 5) and these results confirm that I is involved in a reaction with the receptor. This is shown directly by the finding that at 30 °C there is no recovery after washout (Fig. 4B) and this also rules out the possibility that the hydroxyethyl-compound, formed by hydrolysis of the aziridinium ion, contributes to the block. This is unlikely anyway because the hydroxyethyl-analogue of 4DAMP methobromide has only about 10% of its affinity for muscarinic receptors in ileum and behaves competitively (Barlow & Shepherd 1986). Even at 37 °C recovery is extremely slow (Fig. 4A): perhaps this involves the synthesis of fresh receptor protein. It seems likely that the selectivity seen in Table 2 and Fig. 2 originates from the lower affinity of the aziridinium ion for receptors in atria, rather than from lack of ability to react with the receptor.

In the calculation of rate-constants for blockade with a competitive blocking agent (4DAMP methobromide) a direct fit of dose-ratio to time, such as has been used here, seems preferable to using receptor occupancy (Z) because this is calculated from (DR-1)/DR and errors in DR will affect both numerator and denominator. It is doubtful whether the experimental rates of onset and offset really indicate rates of association with and dissociation from the receptors. The equilibrium constant calculated from these rate constants is higher than would be expected from the dose-ratios obtained at equilibrium. Possibly the rate-constant for offset has been underestimated and diffusion of the compound from the tissue is very slow.

With an irreversible blocking agent there are the same reasons for using dose-ratio rather than receptor occupancy (Z) to calculate rate-constants but it is preferable to fit log(DR) to time, to avoid giving more weight to results obtained later in the experiment, when the dose-ratios are large. The variation in the results in Tables 2–4 could arise from differences in the measurement of dose-ratios, as well as from differences in the formation of the aziridinium ion. The variation in the responses of ileum has long been known and the response produced by a dose of agonist following a large response can be quite different from that obtained with the same dose after a small response. In experiments where antagonism is being followed against time, the repeated application of the same dose of agonist may show a regular decline until such time as it becomes necessary to increase the concentration; in plotting either receptor occupancy or dose-ratio against time there are often discontinuities when this has been done. By careful selection it is possible to produce elegant records but there is the danger that their consistency may be artificial and different results may be obtained in subsequent experiments. It seems preferable to make experiments with a range of concentrations of agonist, as has been done in the latter part of this work (e.g. Fig. 4).

In spite of the variations in dose-ratio the general trends are clear and the results obtained indicate the range of dose-ratio to be expected with guinea-pig ileum and atria at a particular time and temperature, when treated with I (10 nM) prepared as described.

It remains to be seen how far an irreversible blocking agent like I will be selective in-vivo but, as a tool for investigating muscarinic receptors, it may be preferable to those currently available, being more specific than phenoxybenzamine and much easier to make than benzilylcholine mustard.

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