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The selectivity of the (−)-and (+)-forms of hyoscine methiodide and of hyoscyamine camphorsulphonate for muscarinic (M_2) receptors

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1 The affinities of (−)-S-hyoscyamine (+)-camphorsulphonate, (+)-**R**-hyoscyamine (−)-camphorsulphonate, (−)-S-hyoscine methiodide and (+)-**R**-hyoscine methiodide for muscarinic acetylcholine receptors in guinea-pig atria and ileum at 30°C and in ileum at 37°C have been measured in dose-ratio experiments. The agonists were carbachol, arecaidine propargyl ester (APE) and ethoxyethyl trimethylammonium iodide (EOE).

2 The effects produced by the agonists confirmed that, relative to carbachol, arecaidine propargyl ester is more active on atria than on ileum whereas ethoxyethyl trimethylammonium iodide is more active on ileum than on atria.

3 There was no striking difference between estimates of affinity based on the effects of agonists on atrial size and the effects on atrial rate nor was there any striking difference between the affinities measured with the different agonists.

4 With the isomers of hyoscyamine there was no striking difference between the affinity for receptors in atria and those in ileum, which is consistent with the low selectivity reported for atropine (racemic hyoscyamine). (−)-S-Hyoscine methiodide had greater affinity for muscarinic receptors in ileum than for those in atria, though the difference is smaller than has been previously observed. (+)-**R**-Hyoscine methiodide had no detectable selectivity. The phenomenon of selectivity cannot be wholly ascribed to differences in physicochemical properties of the antagonists: the three-dimensional structures with which the antagonists interact cannot be identical.

Introduction

The muscarine-sensitive acetylcholine receptors in guinea-pig ileum and atria are relatively insensitive to pirenzepine (Hammer *et al.*, 1980; Barlow *et al.*, 1981: reviews, Caulfield & Straughan, 1983, Birdsall & Hulme, 1983, Hammer & Giachetti, 1984) and so are classified as M_2. Estimates of the affinity constant are very similar for both tissues but there are other substances, such as 4-diphenylacetoxy *N*-methyl piperidine (4DAMP) metho-salts (Barlow *et al.*, 1976) and sila-procyclidine (Mutschler & Lambrecht, 1984) which have greater affinity for muscarinic receptors in ileum than for those in atria and it has been suggested that there may therefore be subclasses of M_2-receptors. There are slight differences in the relative activity of some agonists (Barlow & Weston-Smith, 1985) which support this idea. Arecaidine propargyl ester (APE), a potent muscarine-like compound which is a tertiary base (Mutschler & Hultzsch, 1973), was more active relative to carbachol on atria than on ileum, whereas ethoxyethyl trimethylammonium iodide (EOE; Ing *et al.*, 1952) was relatively more active on ileum than on atria.

There is considerable variation in the estimates of selectivity, however, and although pentamethylene *bis*-4DAMP bromide appears to be more selective that 4DAMP methobromide (Barlow & Shepherd, 1985), there has not yet emerged, even after 10 years, a compound which has say 100 times the affinity for ileum compared with atria. Is there perhaps, after all, only one type of M_2-receptor with the differences in affinity caused by the way the experiments have been done or perhaps attributable to some physical properties of the compounds which affect their distribution?

The recent appearance of a compound (AFDX-116: Hammer *et al.*, 1986; Giraldo *et al.*, 1986) with greater affinity for muscarinic receptors in atria than for those in ileum, makes this difficult to believe, though it, could still be argued that the physical properties of this compound account for its selectivity. An answer might be obtained by testing pairs of enantiomers, whose

physical properties, other than those involving polarized light, should be identical. This paper describes measurements of the affinity constants of (−)-S-hyoscine methiodide, (+)-**R**-hyoscine methiodide, (−)-S-hyoscyamine (+)-camphor sulphonate and (+)-**R**-hyoscyamine (−)-camphor sulphonate for receptors in guinea-pig ileum and atria. The effect of the agonist on the affinity constant was also studied by use of APE and EOE as well as carbachol as agonists.

(Hyoscine is also called scopolamine so hyoscine methiodide is N-methyl-scopolamine iodide: atropine is the racemic form of hyoscyamine).

Methods

Guinea-pig isolated ileum

The guinea-pig ileum was set up as described by Edinburgh Staff (1974) with the responses recorded isotonically and a load of about 0.5 g. The agonist (see below) was allowed to act for 30 s and added once every 90 s by relays controlled from a PET microcomputer. The tissue was suspended in Krebs solution (Edinburgh Staff, 1974) aerated with a mixture of 95% O_2 and 5% CO_2, containing *nor*phenylephrine, 5 μM, and experiments were done at 29.8 ± 0.3°C and 37 ± 0.1°C. Alternate small and large control responses were obtained, usually to 0.1 and 0.2 μM carbachol, or to 0.015 and 0.030 μM APE, or to 1.0 and 2.0 μM EOE. When these responses were regular the agonist was usually changed and a set of responses was obtained with the second agonist. When these were regular the tissue was exposed to a solution of the antagonist and the concentration of agonist was increased to try to obtain responses which roughly matched the controls. When these were regular, the agonist was again changed so as to obtain responses for the second agonist in the presence of the antagonist. An approximate dose-ratio is given by the ratio of the concentrations of agonist used in the presence and in the absence of the antagonist and an exact dose-ratio was calculated from the size of the responses by a calculation similar to a 4-point assay (Edinburgh Staff, 1974; Barlow, 1983).

Guinea-pig isolated atria

The atria were set up in Krebs solution (Edinburgh Staff, 1974) aerated with a mixture of 95% O_2 and 5% CO_2, containing *nor*phenylephrine, 5 μM (the same solution as was used for the ileum). 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 & Kitchen, 1982: Barlow & Shepherd, 1985). The agonist was added by relays operated from a Commodore 128 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. As in the experiments on ileum, control responses were usually obtained with two agonists in each experiment, usually 0.1 and 0.2 μM carbachol, or 0.015 and 0.030 μM APE, or 2.0 and 4.0 μM EOE. The tissue was then exposed to the antagonist and the experiment continued as with the ileum.

These methods are similar to those previously described (Barlow *et al.*, 1976; Barlow & Kitchen 1982) except that, as described by Barlow & Shepherd (1985), Krebs solution, aerated with a mixture of 95% O_2 and 5% CO_2, was used for all tissues. No hexamethonium was present but in this work the solution contained 5 μM *nor*phenylephrine, which prevented the gradual slowing down of the spontaneously beating atria during the experiment. This concentration of the compound did not affect the responses of the ileum to carbachol or the dose-ratios obtained on either preparation.

When (+)-hyoscine methiodide was the antagonist, only carbachol was used as agonist but with all the other antagonists two agonists were tested in each experiment and these were arranged so that the agonists, and the order in which they were tested, were distributed evenly. Only one concentration of antagonist was tested, which was 0.020 μM for the (−)-isomers and 0.10 μM for the (+)-isomers.

Compounds

When atropine ((±)-hyoscyamine) is dissolved in acetone and treated with (+)-camphor sulphonic acid, the salt with the (−)-S-hyoscyamine crystallizes first (Werner & Miltenberger, 1960: Pearson, 1972). This was filtered off and recrystallized a further 3 times and the material had m.p. 160.3–160.3°C (yield 9%). When atropine was treated with (−)-camphor sulphonic acid the salt with (+)-**R**-hyoscyamine was obtained, which after 4 crystallizations had m.p. 160.1–161.2°C (yield 8%). Melting-points were taken with a Mettler FP-5 instrument, linked to a pen-recorder, with a rate of heating of 0.2°C per min. Werner & Miltenberger (1960) recorded m.p. 159°C for (−)-hyoscyamine (+)-camphorsulphonate.

As these compounds are mirror image forms, they should have the same m.p. and size of rotation (with opposite signs) and molar rotations at various wavelengths, measured with a Bellingham and Stanley spectropolarimeter, are shown in Table 1.

(−)-S-hyoscine methiodide was obtained from (−)-hyoscine and methyl iodide and was recrystallized

Table 1 Molar rotations of S-(−)-hyoscyamine (+)-camphorsulphonate and **R**-(+)-hyoscyamine (−)-camphorsulphonate

Wavelength (nm)	S	R
589	− 40.43	39.23
550	− 41.63	43.50
500	− 44.10	44.77
475	− 43.13	42.27
450	− 37.83	36.77
425	− 24.00	22.87
400	7.70	− 7.68
390	27.80	− 28.87
380	59.40	− 59.87
370	103.6	− 103.7
360	169.6	− 169.2
350	272.2	− 269.8
340	445.7	− 442.2
330	766.9	− 760.7
320	1453	− 1435

Molar rotations were calculated from the means of triplicate measurements with 0.1 M solutions of each isomer in water and were corrected for solvent blanks. The temperature was 18–20°C and the path length was 5 cm. The actual angle measured in these conditions is 5/1000 times the molar rotation, i.e. 200 millidegrees for a molar rotation of 40 degrees.

several times from ethanol and water (to obtain crystals for an X-ray diffraction study) and had m.p. 207.6–208.1°C. The sample of (+)-**R**-hyoscine methiodide was prepared by Dr J.D.M. Pearson (1972; Barlow *et al.*, 1973).

Results

Because control responses were usually obtained with two different agonists in the experiments with the antagonists, it was possible to compare the relative potencies of the agonists. From the concentrations used and the size of the responses the concentrations producing the same responses were calculated (as in a four-point bioassay). These matching concentrations were used to calculate the equipotent molar ratio (e.p.m.r.) relative to carbachol: the results are shown in Table 2. If a concentration C_x of an agonist produces the same response as C_c of carbachol, the equipotent molar ratio is C_x/C_c and with a more active compound, such as APE, the number will be less than 1 (because it is active at a lower concentration). The results in Table 2 confirm the selectivity of the agonists already reported (Barlow & Weston-Smith, 1985): APE is relatively more active on atria (e.p.m.r. 0.070 for effects on rate and 0.085 for effects on force) than

Table 2 Equipotent molar ratios of APE and EOE relative to carbachol

Atria		*Ileum*	
rate	*force*	30°	37°
Arecaidine propargyl ester (APE)			
0.070 ± 0.008 (7)	0.085 ± 0.009 (7)	0.172 ± 0.013 (7)	0.149 ± 0.013 (7)
Ethoxyethyltrimethylammonium iodide (EOE)			
16.6 ± 1.79 (8)	16.8 ± 2.83 (8)	7.35 ± 0.91 (8)	5.65 ± 0.58 (7)

The figures indicate the ratio of the number of molecules of the compound to the number of molecules of carbachol producing the same response.

on ileum (e.p.m.r. 0.172): EOE is relatively more active on ileum (e.p.m.r. 7.35) than on atria (e.p.m.r. 16.6 and 16.8).

The results of the experiments with the antagonists are shown in Table 3 in which the dose-ratios have been converted into affinity constants and the mean estimate of log K is shown ± s.e. and number of estimates. There does not seem to be any difference between estimates obtained from effects on atrial rate and on atrial force, nor between estimates obtained with different agonists if the numbers are treated as independent samples and Student's t test is applied ($P = 0.05$). The results are mostly paired, however, and it should be more informative to examine the difference between the values of log K for atrial rate and atrial force in the same experiment, or the difference between log K obtained with one agonist and that obtained with another agonist in the same experiment.

The results of examining such differences are shown in Table 4. In section A the null hypothesis is that there is no difference between effects on rate and effects on force and the value of Student's t never exceeds that for $P = 0.05$, though the value for EOE and (−)-S-hyoscyamine (+)-camphorsulphonate exceeds that for $P = 0.1$. In 9 of the 10 comparisons, however the value for atrial rate is greater than that for atrial force, which suggests that the possibility that there is a difference cannot be ruled out.

In section B the null hypothesis is that there is no difference between the pooled values for atria and the value for ileum at the same temperature. There is a clear difference with (−)-hyoscine methiodide and all the agonists but not with any of the other compounds. The difference does not appear to depend on the agonist, with the possible exception of the combination of carbachol and (−)-hyoscine methiodide. It would be expected that any differences between agon-

Table 3 Estimates of affinity

	Atria		*Ileum*	
	rate	*force*	30°	37°
(−)-Hyoscyamine (+)-camphorsulphonate				
CC	9.163	9.126	9.259	9.022
	± 0.167 (5)	± 0.110 (5)	± 0.167 (5)	± 0.022 (4)
APE	9.084	8.657	9.014	8.982
	± 0.151 (4)	± 0.495 (4)	± 0.107 (4)	− 0.207 (4)
EOE	9.269	8.987	9.266	9.155
	± 0.114 (5)	± 0.144 (5)	± 0.122 (5)	± 0.137 (5)
(+)-Hyoscyamine (−)-camphorsulphonate				
CC	7.018	6.994	6.913	6.914
	± 0.034 (4)	± 0.022 (4)	± 0.051 (4)	± 0.091 (4)
APE	6.962	6.951	7.021	6.953
	± 0.156 (4)	± 0.050 (4)	± 0.037 (4)	± 0.061 (4)
EOE	7.167	6.933	6.995	7.038
	± 0.065 (4)	± 0.116 (4)	± 0.057 (4)	± 0.067 (4)
(−)-Hyoscine methiodide				
CC	9.919	9.897	10.206	10.035
	± 0.071 (6)	± 0.106 (6)	± 0.088 (6)	± 0.096 (6)
APE	9.920	9.927	10.453	10.116
	± 0.102 (5)	± 0.069 (5)	± 0.102 (5)	± 0.126 (6)
EOE	9.878	9.852	10.388	10.194
	± 0.071 (7)	± 0.056 (7)	± 0.113 (6)	± 0.101 (6)
(+)-Hyoscine methiodide				
CC	8.361	8.216	8.272	8.209
	± 0.081 (4)	± 0.019 (4)	± 0.025 (4)	± 0.036 (4)

The numbers show the mean estimate of the value of log affinity constant ± s.e. and number of results. The agonists were carbachol (CC), arecaidine propargyl ester (APE) or ethoxyethyltrimethyl ammonium iodide (EOE).

ists would involve APE, which is more atrial selective, or EOE, which is more ileal selective: possibly the smaller difference observed with carbachol (0.298 compared with 0.545 or 0.529 log units) is a sampling error.

The values in section C were obtained by pooling estimates of log K for all agonists (usually two) used in any one experiment. These confirm the findings in section B and give an estimate of the mean difference in log K and its standard error, which can be used as an overall assessment of selectivity. The value for (−)-hyoscine methiodide is considerably less than the value (0.93) observed by Barlow *et al.* (1976). The value for (−)-hyoscyamine, 0.131 log units, is also lower than would be expected from results with atropine ((±)-hyoscyamine). This is 50% (−)-hyoscyamine and Barlow *et al.* (1981) obtained differences of over 0.3 log units.

The values in section D investigate the effects of temperature on affinity. There is a definite decrease in affinity with a rise in temperature for the isomers of hyoscine methiodide. The rise is considerably bigger with the (−)-S-enantiomer. With this compound the change in log K for 1°C (0.03) is lower than the value (0.06) previously recorded for ileum but bigger than that recorded for binding experiments with rat cortex (0.01: Barlow *et al.*, 1979).

Discussion

The use of enantiomeric pairs to try to decide whether differences involve physicochemical properties or actions at receptors depends upon having the enantiomers stereochemically pure. With naturally occurring compounds, such as hyoscine and hyoscyamine, it is not unreasonable to hope that the form occurring in nature is sterochemically pure, though it may be partly racemised during its extraction and isolation. If the other isomer can be obtained with a rotation of equal size but opposite sign it is probable that something approaching stereochemical purity has been attained.

With compounds that do not occur in nature there is no means of knowing this. However, if they are competitive antagonists the biological activity may be used to set limits to the degree of optical purity (Barlow *et al.*, 1972). If the results in Table 3 for (−)-S- and (+)-**R**-hyoscyamine on ileum at 30°C are

Table 4 Differences in affinity (paired experiments)

A

Log K atrial rate − log K atrial force (30°C)

		t
(−)-Hyoscyamine (+)-camphorsulphonate		
CC	0.037 ± 0.074 (5)	0.51
EOE	0.281 ± 0.122 (5)	2.30
APE	0.426 ± 0.344 (4)	1.24
(+)-Hyoscyamine (−)-camphorsulphonate		
CC	0.018 ± 0.022 (4)	0.81
EOE	0.234 ± 0.143 (4)	1.64
APE	0.010 ± 0.106 (4)	0.10
(−)-Hyoscine methiodide		
CC	0.022 ± 0.089 (6)	0.25
EOE	0.027 ± 0.073 (7)	0.36
APE	− 0.008 ± 0.099 (5)	0.08
(+)-Hyoscine methiodide		
CC	0.145 ± 0.076 (4)	1.92

B

Mean log K for effects on atria − log K ileum (30°C)

		t
(−)-Hyoscyamine (+)-camphorsulphonate		
CC	− 0.115 ± 0.123 (5)	0.93
EOE	− 0.138 ± 0.090 (5)	1.54
APE	− 0.144 ± 0.360 (4)	0.40
(+)-Hyoscyamine (−)-camphorsulphonate		
CC	0.093 ± 0.068 (4)	1.37
EOE	0.054 ± 0.062 (4)	0.87
APE	− 0.066 ± 0.080 (4)	0.82
(−)-Hyoscine methiodide		
CC	− 0.298 ± 0.077 (6)	3.89*
EOE	− 0.545 ± 0.129 (6)	4.23**
APE	− 0.529 ± 0.078 (5)	6.81**
(+)-Hyoscine methiodide		
CC	0.015 ± 0.050 (4)	0.30

C

Log K atria − log K ileum (30°C), based on means for all agonists.

	t
(−)-Hyoscyamine (+)-camphorsulphonate	
− 0.131 ± 0.105 (14)	1.25
(+)-Hyoscyamine (−)-camphorsulphonate	
0.027 ± 0.042 (12)	0.64
(−)-Hyoscine methiodide	
− 0.453 ± 0.061 (17)	7.41**
(+)-Hyoscine methiodide	
0.015 ± 0.050 (4)	0.30

D

Log K ileum (30°C) − log K ileum (37°C), based on means for all agonists.

	t
(−)-Hyoscyamine (+)-camphorsulphonate	
0.135 ± 0.098 (13)	1.37
(+)-Hyoscyamine (−)-camphorsulphonate	
0.008 ± 0.048 (12)	0.17
(−)-Hyoscine methiodide	
0.211 ± 0.031 (17)	4.92**
(+)-Hyoscine methiodide	
0.063 ± 0.016 (4)	4.01**

The numbers show the mean ± s.e., the number of results and the value of Student's t test which can be used to test the null hypothesis (that the mean = 0). *$P<0.05$; **$P<0.01$

compared, the average difference, 2.2 log units, indicates that the stronger isomer is 158 times as active as the weaker on (the stereospecific index = 158). This indicates that the stereochemical purity, assuming that the (+)-**R**-isomer is completely inactive (which is highly unlikely), should be better than 158/159, i.e. 99.4%. This agrees well with the rotations shown in Table 1. If the values of (+)-**R**-hyoscyamine (−)-camphorsulphonate (Y) are plotted against those for the (−)-**S**- isomer (X), ignoring the signs, the least-squares fit to a straight line has the equation $Y = 0.988X + 0.739$. If the natural isomer is absolutely pure, the (+)-**R**- isomer should be $(1.988/2) \times 100 = 99.4\%$ stereochemically pure.

The rotation (at 300 nm) of the sample of (+)-**R**-hyoscine methiodide used (Barlow *et al.*, 1973) suggested that it might be 96.7% stereochemically pure. If the (+)- compound were completely inactive the effects produced would be from the 3.3% (−)- isomer present and the sample should show the same tissue selectivity as the (−)- isomer and be more active on the ileum than the atria. That this fortunately does not happen indicates that (+)-**R**-hyoscine methiodide does have some affinity for muscarinic receptors and that this predominates over the effects due to the (−)- isomer present. In fact the results on ileum at 30°C (Table 3) suggest that the rotations may be inaccurate. The stereospecific index for effects on ileum at 30°C is over 100 suggesting a stereochemical purity of over 99%.

The work emphasizes the need to consider the stereochemical purity of optical enantiomers when these are used as tools to try to distinguish between physicochemical and receptor processes, and also that the biological results in some instances are a sensitive means of setting limits to this.

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