Naunyn-Schmiedeberg's Arch Pharmacol (1986) 332:16–20

Naunyn-Schmiedeberg's
Archives of
Pharmacology

The cardio-selectivity of himbacine: a muscarine receptor antagonist

S. Anwar-ul, H. Gilani, and L. B. Cobbin

Department of Pharmacology, University of Sydney, NSW 2006, Australia

Summary. The antimuscarinic actions of himbacine were compared with those of atropine and/or homatropine on atria, ileum and trachea from guinea-pigs and rat uterus preparations.

1. The antagonism of acetylcholine or carbachol by all the antagonists was competitive on the preparations studied. The pA_2 values of himbacine in all smooth muscle preparations were similar (around 7.2) whereas in atria it exhibited about 10-fold higher affinity ($pA_2 = 8.2$).

2. In contrast, both atropine and homatropine had similar affinities for muscarine receptors (pA_2 values around 9.1 and 7.2 respectively) in both atria and smooth muscle.

3. It may be concluded from these results that cardiac and smooth muscle muscarine receptors are not homogeneous and that himbacine is a relatively potent and selective antagonist for cardiac receptors.

4. The cardio-selectivity of himbacine supports the concept of heterogeneity of muscarine receptors.

Key words: Himbacine – Cardio-selective antagonist – Cardiac and smooth muscle – Muscarine receptor heterogeneity

Introduction

It is accepted that muscarine receptors do not constitute a homogeneous population (Birdsall and Hulme 1983) and it appears that at least three sub-types exist although the exact number remains unknown. The recognition of muscarine receptor sub-types has been aided by the discovery of selective agonists and antagonists. More definitive classification of these receptors may depend upon the development of newer and more selective drugs which will interact with various muscarine receptors.

The experiments reported in this paper describe some antimuscarinic actions of himbacine which has approximately 10 times greater affinity for the muscarine receptors of atria than for those of smooth muscle.

Himbacine is an alkaloid which has been isolated from the bark of *Galbulimima* species (formerly *Himantandra*) and the structure of which is shown in Fig. 1 (Ritchie and Taylor 1967). The plants themselves are large trees reaching a height of 40 m, found in North Queensland (Australia) and Papua-New Guinea.

Send offprint requests to L. B. Cobbin at the above address

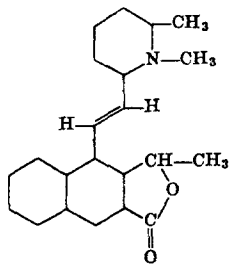

Fig. 1. Structure of himbacine

Methods

Guinea-pig ileum. Segments of ileum about 2 cm long obtained from guinea-pigs (500–700 g) were suspended in a 15 ml tissue bath filled with modified Tyrode's solution containing pempidine (3 μM), maintained at 37° C and aerated with 5% CO_2 in O_2. The composition of the Tyrode's solution was (mM): NaCl 136.9, KCl 2.7, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, D-glucose 5.6, $CaCl_2$ 0.9 (pH 7.4).

An initial loading of 0.7 g was applied to the tissue and isotonic contractions to acetylcholine (ACh) were recorded with a FME transducer (model P-1) on a potentiometric chart recorder (Heathkit model 1R-18M). The tissue was exposed for up to 20 s to a constant concentration of ACh which produced a sub-maximal response, then washed by overflow and the cycle repeated at 3 min intervals until constant responses were recorded (usually 15–25 contractions). Control concentration-response curves to ACh were then obtained by the addition of increasing concentrations of agonist and washing between doses. The concentration-response curves to ACh were then re-determined in the presence of a constant concentration of an antagonist after an initial equilibration period with the tissue of 15 min (himbacine) or 30 min (atropine). A minimum of two responses to each concentration of agonist was obtained to ensure that complete antagonism had been achieved. Usually, only one concentration of antagonist was used in a single preparation and in separate control experiments no significant change in tissue sensitivity to the agonist was found over the period of determination of three concentration-response curves.

Rat uterus. Young female rats (150–200 g) pretreated with stilboestrol (0.1 mg/kg, SC, 24 h) were killed by a blow on the head and uterine horns were excised and separated from

fat deposits and ovaries. The middle 2 cm of the uterine horns were cut longitudinally and each strip was mounted in a 15 ml tissue bath containing De Jalon's solution at 31° C and gassed with a mixture of 95% O_2 and 5% CO_2. The composition of the De Jalon's solution was (mM): NaCl 154.0, KCl 5.6, D-glucose 2.8, $NaHCO_3$ 6.0, $CaCl_2$ 0.5 (pH 7.4). An initial loading of 0.5 g was applied and each tissue was allowed to equilibrate for 20 min before isotonic contractions to ACh were commenced. ACh was left in contact with the tissue for up to 30 s and contractions were induced at 4 min intervals. The rest of the procedure was similar to that described for ileum.

Guinea-pig trachea. The trachea obtained from guinea-pigs (400 – 600 g) was dissected free of surrounding tissues while immersed in Krebs solution, and then cut into a number of rings of 2 – 3 mm width, each containing 2 cartilages (a maximum of 4 preparations was used from each animal). Each ring was opened by a longitudinal cut on the ventral side opposite the smooth muscle layer thus producing a strip with a central region of smooth muscle attached to cartilage at either end. Each preparation was suspended in Krebs' solution containing pempidine (3 μM) in a 20 ml tissue bath maintained at 37° C and aerated with a mixture of 95% O_2 and 5% CO_2. The composition of Krebs solution was (mM): NaCl 118.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, and glucose 11.7 (pH 7.4).

Tension of the strips was recorded by a Grass force-displacement transducer (model FT03) on a Grass model 79 Polygraph. An initial tension of 1.5 g was applied to each preparation and contractile responses to a sub-maximal concentration of carbachol (CCh) were recorded (using 10 min agonist exposure time) until constant responses were obtained. Following this equilibration period of 2 – 3 h, cumulative concentration-response curves to CCh were obtained in all preparations from each animal using logarithmic increments in concentration (Van Rossum 1963). When a 3-fold increase in concentration produced no further increment in response, the tissue was washed at 10 min intervals until the base-line tension was re-established (usually 30 min). Using a different concentration of antagonist in each of three preparations, the concentration-response curves to CCh were repeated, and in the 4th preparation (which served as a control) a repeat concentration response relationship to CCh alone was determined to check for variation in tissue sensitivity to the agonist. In agonist-antagonist experiments, antagonist contact times of 60 min (atropine) and 30 min (himbacine and homatropine) were used prior to re-determination of concentration-response curves to CCh as these times were found be optimal in preliminary experiments.

Guinea-pig atria. Left atria were dissected from guinea-pigs (400 – 600 g) and suspended in 20 ml tissue baths containing Krebs solution at 32° C and bubbled with oxygen containing 5% CO_2. The Krebs solution contained pempidine (3 μM) and propranolol (1 μM) to block nicotinic effects of CCh (Barnett and Benforado 1966; Löffelholz 1970). Atria were stimulated with rectangular pulses of 3 ms duration, at 2.5 Hz from a Grass model SD 7 stimulator and the voltage was maintained at 10 – 20% above the threshold. Isometric responses were recorded as described for trachea. An initial diastolic tension of 1 g was applied to each tissue which was then allowed to equilibrate for 60 min before the determination of cumulative concentration-response curves to CCh commenced. Re-determination of CCh responses in the presence of antagonists was performed as described above for trachea with similar contact times for antagonists. Up to four concentration-response curves were obtained from each preparation. In a separate series of control experiments, four successive concentration-response curves to the agonist were found to be virtually super-imposable over a 5 h period. It was, therefore, not necessary to correct for diminution of tissue sensitivity during the time course of experiments.

Analysis of data. For determination of EC_{50}s, at least three concentrations of the agonist producing responses lying on the linear portion of the concentration-response curves were employed. Linear regression lines were fitted through the log concentration-response data by the method of least squares and the EC_{50}s were calculated as the concentrations of the agonist producing 50% of the maximal response in each curve. To assess the activity of the antagonist, dose-ratios were estimated by dividing EC_{50} values of agonists obtained in the presence of antagonist by the EC_{50}s obtained for the agonist alone.

To test for competitive antagonism, plots of log (dose ratio −1) against log concentration of antagonist were constructed using the least squares method of regression analysis (Arunlakshana and Schild 1959). The pA_2 values of an antagonist were derived from the intersection of the plot with the log antagonist concentration axis [i.e. where log (dose ratio −1) = 0]. The data obtained are expressed as mean ± SEM. All statistical comparisons were made by means of Student's *t*-test (two-tailed). *P* values smaller than 0.05 were considered to be significant.

Drugs. Drugs used were: acetylcholine perchlorate, atropine sulphate, carbamoylcholine chloride (carbachol) and diethylstilboesterol (Sigma, St. Louis, MO, USA), homatropine hydrobromide (Macfarlan-Smith, Edinburgh, UK), pempidine tartrate (May and Baker, Dagenham, England), (±)-propranolol hydrochloride (ICI, Alderley Park, UK) and himbacine hydrochloride (kindly donated by Professor W. C. Taylor, Department of Organic Chemistry, University of Sydney).

Results

In the concentration range studied (30 nM – 30 μM), himbacine had no agonist activity on smooth muscle preparations. In guinea-pig left atria, himbacine exhibited a small positive inotropic response at concentrations greater than 1 μM which was neither altered in the presence of propranolol (1 μM) nor by pretreatment of the animals with reserpine (5 mg/kg, 24 h). The maximum concentration of himbacine used in this study to antagonize the response to cholinomimetics was 3 μM, which produced only 9.1 ± 2.1% (mean ± SEM; $n = 6$) increase in basal force of contraction.

Responses to agonists were very rapid on ileum and uterus with plateau levels being achieved within 30 s, whereas the trachea responded more slowly and atria occupied an intermediate position. The effect of each concentration of agonist reached its peak within 10 min on trachea and 3 min on atria. Although tracheal preparations were found to be very slow in onset of contractions, the responses were very consistent in terms of reproducibility.

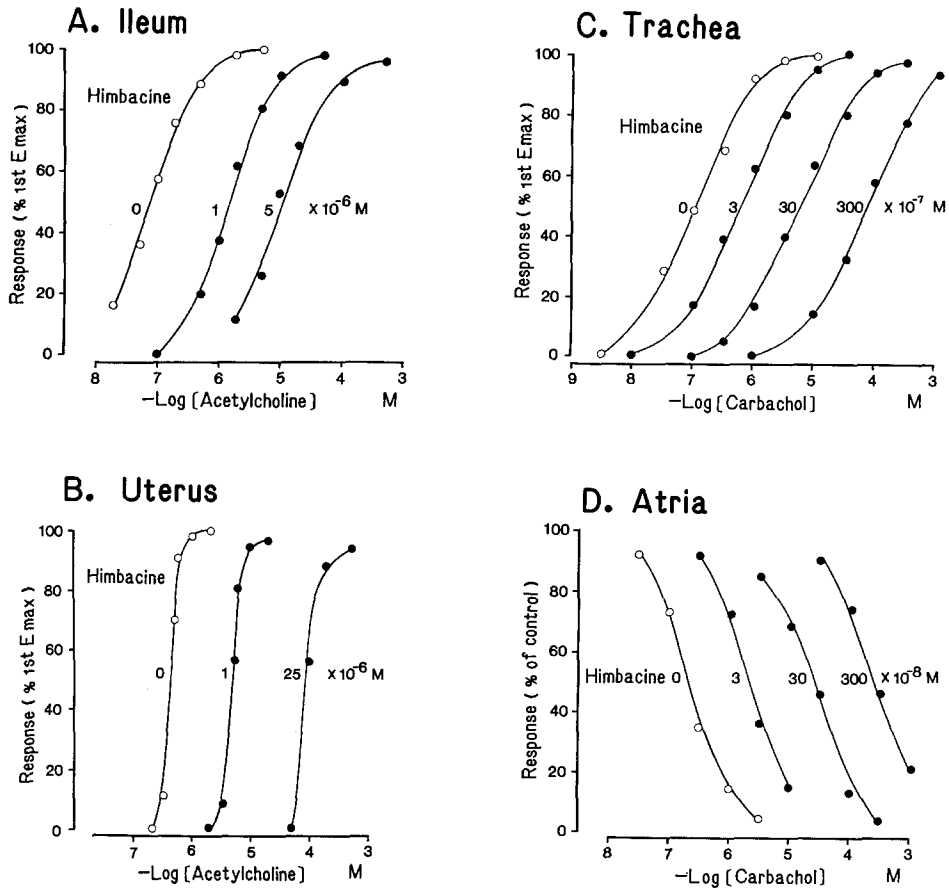

Fig. 2. Log concentration-response curves for cholinomimetics alone (○) and in the presence of himbacine (●) at concentrations indicated in cardiac and smooth muscle preparations from single experiments. Contractile responses to each concentration of agonist are expressed as percentages of the maximum response in the control curve (percent first E_{max}). The inhibitory responses in atria are expressed as percentages of the control force of contraction induced by electrical stimulation obtained immediately before application of CCh

Over a 12 h period, the maximum mean shift in the control preparations was 0.1 log unit to the right in the fourth determinations. The concentration-response curves for ACh in the uterus were found to be relatively steep (Fig. 2B). Similar results have been reported by Edinburgh Staff (1970).

Himbacine inhibited the responses to agonists in all preparations studied and inhibition was concentration-related. The results of typical experiments using himbacine as a muscarine receptor antagonist on each of the four tissues are shown in Fig. 2. In all cases, the presence of increasing concentrations of himbacine produced parallel displacements of the log concentration-response curves of the agonists progressively to the right and the magnitude of the maximum responses to agonists remained unchanged ($P > 0.05$).

Analysis of all results by linear regression analysis are shown in Fig. 3 where the values of log (dose ratio -1) are plotted against log molar concentrations of the antagonist.

In experiments using ileum and uterus, atropine was used as reference antimuscarinic drug and in experiments with trachea and atria, homatropine was used in addition to atropine. The experimental results are summarised in Table 1 which shows that in no case did the slope of the regression line differ significantly from 1 ($P > 0.05$).

Comparison of pA_2 values showed that atropine was the most potent of the muscarine receptor antagonists studied and exhibited a pA_2 value in all tissues of approximately 9.1. Homatropine was approximately 100 times less potent than atropine and had pA_2 values of the order of 7.2 on trachea and isolated atria. In all smooth muscles examined, himbacine had a pA_2 value close to 7.2 and thus had similar potency to homatropine. In atrial tissue, the effect of himbacine in antagonizing the negative inotropic action of CCh was much greater as shown by a pA_2 value of 8.2. Based on pA_2 values, himbacine was about 10 times more potent on cardiac tissue, whereas atropine and homatropine were each approximately equi-potent on the tissues studied.

Discussion

The experimental results show that himbacine is a moderately potent inhibitor of muscarine receptor-mediated responses induced by ACh or CCh on various smooth muscle preparations and electrically driven left atria. In the presence of himbacine, log concentration-response curves to the cholinomimetics are progressively displaced to the right in a parallel fashion, and by sufficiently increasing the agonist concentrations, the original maximum response could be achieved. These observations suggest that himbacine is acting as a competitive antagonist at muscarine receptors in these tissues and this interpretation was confirmed when Schild plots of the data were prepared. Here the plots of the log (dose ratio -1) against the molar concentrations of

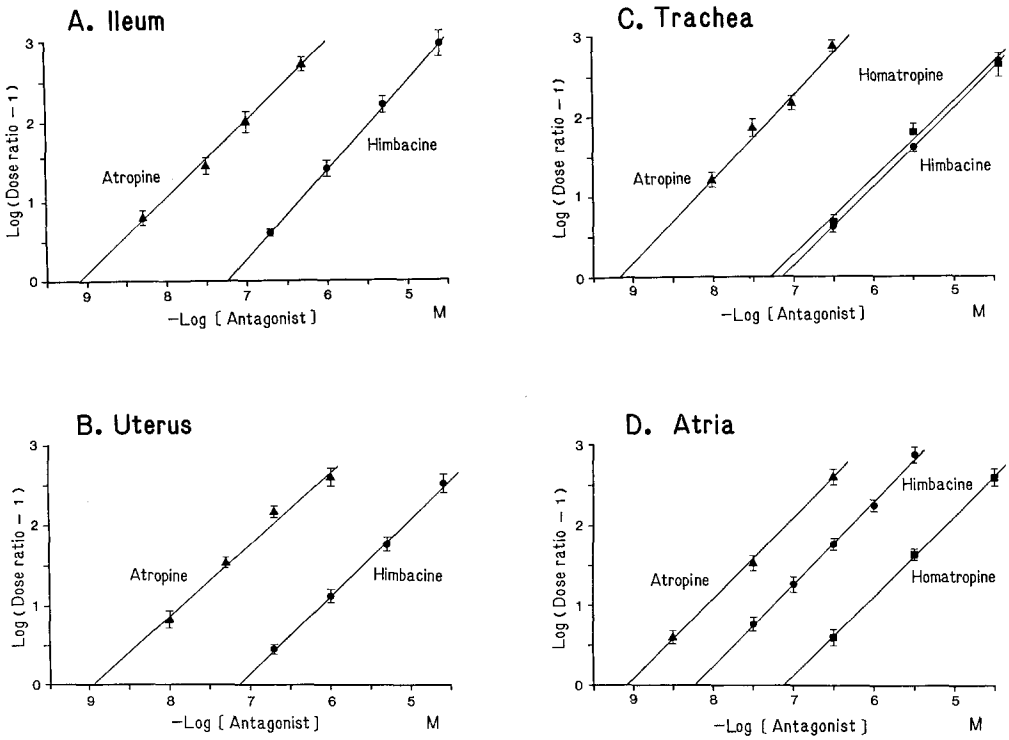

Fig. 3. Schild plots showing comparison of himbacine (●), atropine (▲) and homatropine (■) for the antagonism of cholinomimetics in cardiac and smooth muscle preparations. Each point is the mean ± SEM of 5 to 7 observations. Regression lines intersecting antagonist concentration axes indicate pA_2 values and detailed regression parameters are shown in Table 1

Table 1. Regression analysis of Schild plots for the antagonism of cholinomimetics by himbacine, atropine and homatropine in cardiac and smooth muscle preparations

Antagonist	Tissue	Agonist	PR	pA_2*	*n*	Slope*	SR
Himbacine	Ileum	ACh	1.0	7.27 ± 0.12	23	1.10 ± 0.07	0.11
	Trachea	CCh	1.0	7.13 ± 0.09	16	1.01 ± 0.05	0.08
	Uterus	ACh	1.0	7.14 ± 0.11	21	0.98 ± 0.06	0.08
	Atria	CCh	1.0	8.22 ± 0.10	24	1.04 ± 0.05	1.00
Atropine	Ileum	ACh	67.6	9.10 ± 0.14	14	0.96 ± 0.07	1.05
	Trachea	CCh	107.2	9.16 ± 0.17	18	1.05 ± 0.09	1.20
	Uterus	ACh	67.6	8.97 ± 0.15	17	0.90 ± 0.06	0.78
	Atria	CCh	7.2	9.08 ± 0.11	13	1.01 ± 0.06	1.00
Homatropine	Trachea	CCh	1.3	7.26 ± 0.15	12	0.98 ± 0.06	1.32
	Atria	CCh	0.1	7.14 ± 0.13	13	0.99 ± 0.07	1.00

PR Potency ratios of antagonists relevant to himbacine in that particular preparation

SR Selectivity ratios of antagonist for receptors in smooth muscle relative to those in atria = 1, i.e., antilog (pA_2 Smooth muscle − pA_2 Atria)

* The values shown represent mean ± SEM estimated from (*n*) number of data points. The pA_2 value of himbacine in atria is significantly different ($P < 0.001$) from those obtained in smooth muscle preparations whereas pA_2 values of atropine and homatropine are similar in all tissues ($P > 0.05$). None of the slopes is significantly different from unity ($P > 0.05$)

himbacine were shown to be linear over a wide range of antagonist concentrations and the slopes of the regression lines obtained did not differ significantly from unity ($P > 0.05$).

pA_2 values of himbacine for the antagonism of cholinomimetics determined from the Schild plots were found to be similar on all smooth muscle preparations regardless of whether ACh or CCh was used as the agonist. In atria, the pA_2 value of himbacine was considerably greater than those obtained in smooth muscle preparations ($P < 0.001$) which indicates that atria contain muscarine receptors for which himbacine has greater affinity than for those of smooth muscle.

These results with himbacine provide further evidence to support the concept of heterogeneity of muscarine receptors in cardiac and smooth muscle which has been suggested by observations with gallamine for example, which exhibits cardio-selectivity (Brown and Crout 1970; Stockton et al.

1983; Nedoma et al. 1985) and with 4-diphenylacetoxy-N-methyl piperidine (4-DAMP) which is selective for ileum (Barlow et al. 1976).

New putative selective antagonists are often compared with atropine, the classical antimuscarinic agent, since atropine appears to have little selectivity for any known sub-types of muscarine receptors. In these experiments, the non-selectivity of atropine for cardiac and smooth muscle receptors was confirmed by similar pA_2 values in these tissues. Both atropine and homatropine were competitive antagonists and yielded pA_2 values around 9.1 and 7.2 respectively which are comparable to published values (see for example Arunlakshana and Schild 1959; Van Rossum 1963; Choo and Mitchelson 1978; Li and Mitchelson 1980).

When compared with atropine, himbacine exhibited a relative selectivity for cardiac muscarine receptors of 10 to 15-fold and a 13-fold selectivity for atria as compared to homatropine, which demonstrates clearly that himbacine has at least 10 times greater affinity for cardiac muscarine receptors than for those of smooth muscle.

Acknowledgements. We would like to thank Professor W. C. Taylor for supplies of himbacine. S. A. H. Gilani is on study leave from Zakariya University, Multan (Pakistan) and a recipient of a Sydney University Postgraduate Research Award.

References

Arunlakshana O, Schild HO (1959) Some quantitative uses of drug antagonists. Br J Pharmacol 14:48–58

Barlow RB, Berry KJ, Glenton PAM, Nikolaou NM, Soh KS (1976) A comparison of affinity constants for muscarinic sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C. Br J Pharmacol 58: 613–620

Barnett A, Benforado JM (1966) The nicotinic effects of choline esters and of nicotine in guinea-pig atria. J Pharm Exp Ther 152:129–136

Birdsall NJM, Hulme EC (1983) Muscarinic receptor subclasses. Trends Pharmacol Sci 4:459–463

Brown BR, Crout JR (1970) The sympathomimetic effect of gallamine on the heart. J Pharm Exp Ther 172:266–273

Choo LK, Mitchelson F (1978) Antagonism of cholinomimetics by troxypyrrolidinium in guinea-pig atria and longitudinal ileal muscle: comparison with hemicholinium-3. Eur J Pharmacol 52:313–322

Edinburgh Staff (1970) Pharmacological experiments on isolated preparations, 2nd edn. Churchill Livingstone, Edinburgh, p 95

Li CK, Mitchelson F (1980) The selective antimuscarinic action of stercuronium. Br J Parmacol 70:313–321

Löffelholz K (1970) Autoinhibition of nicotinic release of noradrenaline from postganglionic sympathetic nerves. Naunyn-Schmiedeberg's Arch Pharmak Exp Pathol 267:49–63

Nedoma J, Dorofeeva NA, Tucek S, Shelkovnikov SA, Danilov AF (1985) Interaction of the neuromuscular blocking drugs alcuronium, decamethonium, gallamine, pancuronium, ritebronium, tercuronium and d-tubocurarine with muscarinic acetylcholine receptors in the heart and ileum. Naunyn-Schmiedeberg's Arch Pharmacol 329:176–181

Ritchie E, Taylor WC (1967) The *Galbulimma* alkaloids. In: Manske RHF (ed) The alkaloids, vol. 9. Academic Press, New York, pp 529–543

Stockton JM, Birdsall NJM, Burgen ASV, Hulme EC (1983) Modification of the binding properties of muscarinic receptors by gallamine. Mol Pharmacol 23:551–557

Van Rossum JM (1963) Cumulative dose-response curves. II. Techniques for the making of dose-response curves in isolated organs and the evaluation of drug parameters. Arch Int Pharmacodyn 143:299–330

Received June 27/Accepted September 13, 1985