 © Macmillan Press Ltd, 1990

Rat hippocampal muscarinic autoreceptors are similar to the M_2 (cardiac) subtype: comparison with hippocampal M_1, atrial M_2 and ileal M_3 receptors

M.H. Richards

Merrell Dow Centre de Recherche, 67084 Strasbourg, France

1 Affinity constants for 15 non-selective or putatively selective muscarinic antagonists were determined at muscarinic autoreceptors and postsynaptic receptors (linked to phosphatidylinositol (PI) hydrolysis) in rat hippocampal slices, at muscarinic receptors mediating contractility in guinea-pig atria or ileal smooth muscle and at binding sites in rat cerebral cortical membranes labelled with [^{3}H]-1-quinuclidinyl benzilate or [^{3}H]-pirenzepine.

2 Comparison of the affinities of these antagonists at central M_1 receptors (inositol-monophosphate formation in rat hippocampal slices) with their affinities at peripheral M_1 receptors (inhibition by McN-A-343 of electrically stimulated twitches in rabbit vas deferens) provides support for the suggestion that these receptors may differ pharmacologically.

3 Comparison of affinity constants obtained by displacement of specifically bound [^{3}H]-pirenzepine from rat cerebral cortical membranes with those obtained in functional tests showed poor correlations between affinities for binding sites and for functional atrial receptors or for hippocampal autoreceptors. A significant correlation was found between affinities for [^{3}H]-pirenzepine binding and those determined at muscarinic receptors linked to PI turnover in rat hippocampus. A significant correlation was also obtained between the affinities for specific [^{3}H]-pirenzepine binding sites in cortical membranes and the affinities at ileal receptors.

4 Comparison of the affinity values for muscarinic autoreceptors in rat hippocampus with affinity values obtained from *in vitro* models of muscarinic receptor subtypes showed no significant correlations between these autoreceptors and either M_1 or M_3 receptors. A significant correlation was found between antagonist affinities for hippocampal autoreceptors and muscarinic receptors in the heart. Therefore, muscarinic autoreceptors in rat hippocampus are pharmacologically similar to the M_2 (cardiac) muscarinic receptor subtype.

Introduction

Late-onset Alzheimer's dementia appears to be a relatively pure cholinergic lesion confined to the hippocampus and temporal cortex (Rosser *et al.*, 1984). It is thus possible that antagonists of the acetylcholine autoreceptors in these brain regions may be therapeutically useful in the early stages of this disease. The synthesis of compounds to test this hypothesis, however, first requires a clear characterization of these autoreceptors.

Early studies found that atropine enhanced the stimulated release of tritium from cortical (Richardson & Szerb, 1974) or hippocampal (Nordstrom & Bartfai, 1980) tissues prelabelled with tritiated choline, suggesting that the autoreceptors in these tissues were muscarinic in character.

It is now evident that there are multiple types of muscarinic receptors. The non-classical antagonist, pirenzepine (PZ), differentiates muscarinic receptors in various tissues (Hammer *et al.*, 1980) and receptor subtypes were initially defined as M_1 with a high affinity for PZ, (pA_2 or pK_b between 7.5 and 8.0; see Eglen & Whiting, 1986) and 'M_2' (or non-M_1) with a lower affinity for PZ. Other compounds shown to display some selectivity between muscarinic receptors in different tissues included dicyclomine (M_1-selective; Potter *et al.*, 1984) and gallamine ('M_2'-selective; Mitchelson, 1984). All three antagonists have been used to characterize muscarinic autoreceptors in rat brain. PZ (Marchi & Raiteri, 1985; Schoffelmeer *et al.*, 1986; Roberts & Tutty, 1986) and dicyclomine (Marchi & Raiteri, 1985) were found to display low affinities for muscarinic autoreceptors, indicating that these receptors were not M_1. However, further efforts to characterize these autoreceptors produced ambiguous results. Gallamine blocked muscarinic autoreceptors in rat striatum at lower concentrations ($pEC_{50} = 5.5$; Schoffelmeer *et al.*, 1986) than were required to antagonize these autoreceptors in rat cerebral cortex ($pK_b < 4$; Roberts & Tutty, 1986). These observations are consistent with the findings of Ladinsky *et al.* (1987) that muscarinic autoreceptors in different brain regions may be differentially regulated and emphasize the importance of tissue variations within the same species.

More recently, data from the characterization of 'M_2' receptors in heart or smooth muscles suggests that they are not homogeneous. Himbacine (Anwar-ul *et al.*, 1986) and AF-DX 116 (Giachetti *et al.*, 1986a) were found to be more potent as antagonists of muscarinic receptors in heart than in smooth muscles. Conversely, hexahydrosila-diphenidol (HHSiD) was more potent in smooth muscles than in heart (Mutschler & Lambrecht, 1984). Results obtained with these and other putatively selective antagonists have led to proposals (see Eglen & Whiting, 1986; Birdsall *et al.*, 1987) that muscarinic receptors be divided into at least three categories: M_1 receptors, found in neuronal tissues, are selectively antagonized by PZ; M_2 receptors in heart are selectively blocked by himbacine and AF-DX 116; and M_3 receptors, located in smooth muscles or glandular tissues, are selectively antagonized by HHSiD.

The evidence for the existence of muscarinic receptor subtypes is based not only on pharmacological studies but also on results obtained from the application of molecular biology techniques to their study. There are currently at least five genes which encode for muscarinic receptor proteins (Bonner *et al.*, 1988). Although up to five tissue-dependent types of muscarinic receptor have been proposed (Mitchelson, 1988), the relationship between gene products and these putative receptor subtypes remains unclear. Moreover, the lack of selective antagonists for some of these gene products impedes definitive subdivision of tissue receptors. Until this deficit is filled, characterization of muscarinic receptors in tissues is confined to the above three categories.

By use of muscarinic antagonists that have been shown to display selectivity among the three pharmacologically defined receptor subtypes, a more refined characterization of muscarinic autoreceptors in rat hippocampus was undertaken. The formation of inositol-monophosphate (IP_1) in rat hippocampal slices in response to cholinoceptor agonists, which is blocked with low concentrations of PZ (Gil & Wolfe, 1985), was used as a model for postsynaptic (Fisher *et al.*, 1980; Smith *et al.*, 1989) muscarinic receptors. The affinities of these same compounds were also determined in a model of M_2 receptors (electrically contracted guinea-pig atria) and in a model of M_3 receptors (electrically contracted guinea-pig ileum).

In addition to the four *in vitro* assays, binding assays were set up using rat cerebral cortical membranes labelled with either [^{3}H]-QNB (l-quinuclidinyl [phenyl-4-^{3}H]-benzilate) or [^{3}H]-PZ. The former is a potent, non-selective, lipophilic antagonist that labels muscarinic receptors on or in the plasma membrane (El-Fakahany, 1985). [^{3}H]-PZ, a hydrophilic M_1-selective antagonist, labels a portion of the receptors that are selectively labelled by [^{3}H]-QNB (Watson *et al.*, 1986). The ratios of affinity values at the two binding sites obtained with the 15 compounds used in this study were calculated to determine if this information would be useful in predicting selectivity at M_1 or non-M_1 sites in the CNS.

Thus, there were three objectives in the present study: to compare central muscarinic autoreceptors and muscarinic postsynaptic receptors in the same tissue, to attempt to place these autoreceptors in the current scheme of three pharmacologically-defined subtypes, and to investigate the usefulness of [^{3}H]-PZ binding and [^{3}H]-QNB binding to define selective ligands. In addition, determination of the affinities of several antagonists of different putative selectivities in a model of central M_1 receptors made possible a comparison with their affinity values at peripheral (Eltze, 1988) M_1 receptors.

These results were presented, in part, at the European Neuroscience Association meeting in Zurich, Switzerland, September, 1988.

Methods

Tissue preparation

Male Sprague-Dawley rats (Charles River France), 220–300 g, were stunned, decapitated and the brain quickly removed onto a glass plate. Transverse hippocampus slices (0.4 mm thick) were prepared as described by Teyler (1980). The slices weighed approximately 4 mg (wet weight) and the protein content was 0.21 ± 0.05 mg per slice ($n = 6$). The slices were kept in Krebs-Henseleit buffer (see below) which was continuously gassed with a mixture of 95% O_2 and 5% O_2 (carbogen) during all steps in the assays for muscarinic autoreceptors and muscarinic receptors linked to formation of inositol-monophosphate (IP_1).

Autoreceptor assay Hippocampus slices from one rat were preincubated in 10 ml of buffer containing 0.1 μM methyl-[^{3}H]-choline chloride for 15 min in a shaking bath at 37°C. The slices were then rinsed and placed in glass superfusion units fitted with platinum electrodes (for details, see Richards, 1985), two slices per unit. After 30 min of superfusion at 1.2 ml min^{-1}, the perfusion rate was reduced to 0.9 ml min^{-1} and a fraction collector, set to collect 2 min (1.8 ml) fractions, was started (time 0). At the start of fraction 2, all slices were stimulated (S1) for 2 min at 1 Hz with monophasic pulses of 2 ms duration. The tritium released by stimulation returned to basal values within 12 min (fraction 8). Test compounds were added to the superfusion media during the collection of fractions 8 and 20 and the slices were stimulated again during the collection of fractions 14 (S2) and 26 (S3). The superfusion was stopped after fraction 32 had been collected. The quantities of tritium remaining in the slices and that contained in the fractions were determined by liquid scintillation counting. Efflux coefficient values (% of tritium in a fraction as % of tritium in the slices at the start of collection of that fraction) were calculated. The areas under the curves (minus the basal level extrapolated from the first to last fraction under the curve) were determined by integration using the trapezium method. In each experiment the effects of the various treatments were determined in two slices. In most experiments, four concentrations of the agonist were tested at S2 and a given concentration of antagonist, sufficient to shift the concentration-response curve to carbachol to the right by 2 fold or more, was added to the buffer and to agonist-containing media before S3. The S2/S1 and S3/S1 ratios were calculated, plotted against the log of the concentration of agonist and the apparent pA_2 value calculated (Schlicker & Gothert, 1981). Values are expressed as means $\pm$ s.e.mean for n experiments.

Formation of inositol monophosphate The assay procedure of Brown *et al.* (1984) was used. Hippocampus slices from six rats were suspended in Krebs-Henseleit buffer continuously gassed with carbogen. During the 60 min preincubation period, the slices were in a container set in a shaking bath at 37°C and gently agitated to keep them from settling; the buffer was changed every 15 min. Individual slices were then placed in flat-bottomed 5 ml vials containing 5 mM lithium chloride in buffer. An aliquot of [^{3}H]-myo-inositol (purified just before use by passing through a small column of Dowex 1-X8 formate form) was added to each vial to a final concentration of 0.1 μM and the vials incubated for 30 min. Antagonists, when used, were added to the appropriate vials 25 min before addition of the agonist; an equal volume of buffer was added to the other vials. Different concentrations of carbachol were mixed with the contents of the vials to complete the incubation volume to 300 μl. After each addition, the vials were gently vortexed, flushed with carbogen, capped and returned in the shaking bath at 37°C. The reaction was stopped after 30 min incubation with carbachol by the addition of 940 μl chloroform/methanol (1:2 v:v) and the samples were vigorously vortexed. Additional chloroform and water (310 μl each) were added to each vial and the phases separated by centrifugation. An aliquot of 750 μl of the aqueous phase was taken to determine the quantity of [^{3}H]-inositol-monophosphate ([^{3}H]-IP_1) formed in each slice. [^{3}H]-IP_1 was separated from [^{3}H]-myo-inositol by ion exchange column chromatography. Radiolabelled standards were used to verify that the fraction eluted with 8 ml of 0.2 M ammonium formate in 0.1 M formic acid was inositol monophosphate. In each experiment, basal and blank values were determined in triplicate at the same time as the responses to agonists and antagonists. D.p.m. from blanks (no slice present) were subtracted from all samples (see below). The results were then expressed as % above basal and plotted vs log concentration. Three to seven concentrations of an antagonist, in the range that inhibited the reponse to 1 mM carbachol by 20 to 80%, were tested and the IC_{50} values determined graphically. pK_i values were calculated as described by Hawcock *et al.* (1986).

It became evident during the course of this work that a contaminant (or contaminants) in the stock solutions of [^{3}H]-myo-inositol passed through the purification column and was changed by incubation in buffer at 37°C (but not at 4°C) to another contaminant that co-eluted with IP_1. This gave rise to blank values ranging from less than 100 to more than 1000 d.p.m. depending on the specific activity and source of the [^{3}H]-myo-inositol. It was also noted that, although the stock solutions were stored as recommended by the manufacturers, blank values from a given solution increased with time. Moreover, solutions containing products to absorb radiodegradation contaminants (Amersham TRK.911 with PT6–271) gave blank values (after passage through a small purification column) of less than 100 to more than 250 d.p.m. Therefore, blank values were determined in each experiment and subtracted from experimental values before the effects of different treatments on IP_1 formation were calculated.

Atria

Left atrial preparations from male guinea-pigs (120–200 g) were set up in 10 ml isolated organ baths containing Tyrode solution (mM: NaCl 137, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 11) which was gassed with carbogen and maintained at 31°C. Resting tension was adjusted to 1 g and contractions measured under isometric conditions by Grass FTO3 transducers connected to a Grass 79D polygraph. The atrial preparations were stimulated via punctate electrodes at 0.2 Hz with square wave pulses of 3 ms duration. Voltage was adjusted to be just above threshold (1–3 V). These stimulation conditions did not appear to release catecholamines because the mechanical responses were unaffected by (±)-propranolol (1 μm). Compounds were tested for their ability to antagonise acetylcholine-induced responses. After construction of an initial cumulative concentration-effect curve to acetylcholine, atria were incubated in the presence or absence of the compound under test for 60 min before a second concentration-effect curve to acetylcholine was elicited. Further concentration-effect curves to acetylcholine were elicited using the same protocol in the presence of higher concentrations of antagonist. The pA_2 values were calculated by the method of Arunlakshana & Schild (1959).

Electrically stimulated guinea-pig ileum

Pieces of proximal ileum between 2 and 3 cm in length were removed from guinea-pigs (250–400 g) and suspended in Tyrode solution for isometric recording. Electrical field stimulation of the enteric cholinergic nerves was carried out by means of ring electrodes using the following parameters: 0.05 Hz, 2 ms, supramaximal voltage. Antagonist effects were quantified as the maximum inhibitory effect on electrically-induced contractions achieved during a 2 min contact period. Concentration-response curves were obtained, non-cumulatively, with a 30 min interval between each drug addition. Antagonist concentrations producing half-maximal inhibition (IC_{50}) were determined graphically. Mean pIC_{50} values ± s.e.mean were calculated from 4 to 6 experiments.

Binding assays

Membranes were prepared from cerebral cortices of male Sprague-Dawley rats. The tissues were homogenized at 4°C with a Polytron (Brinkman, setting 9) for 15 s in 50 mM sodium potassium phosphate buffer, pH 7.4. The homogenates were centrifuged for 10 min at 1000 *g* at 4°C, the resulting supernatant recentrifuged for 30 min at 48000 *g* and the pellet (P2) resuspended in the homogenizing buffer to a dilution of 70 times tissue wet weight for [^{3}H]-PZ binding and 280 times wet weight for [^{3}H]-QNB binding. The homogenates were incubated with 0.1 nM [^{3}H]-PZ or 0.2 nM [^{3}H]-QNB for 90 min at room temperature and the reactions terminated by filtration. Non-specific binding was defined as that in the presence of 1 μM atropine. IC_{50} values were determined graphically and corrected for the concentration of the tritiated ligand (Cheng & Prushoff, 1973). For [^{3}H]-PZ, the correction was $IC_{50}/1.05$; for [^{3}H]-QNB, the correction was $IC_{50}/2.05$. The corrected IC_{50} values were then expressed as the negative log (pIC_{50}).

Drugs and chemicals

Characterization of receptors depends a great deal on the ready availability of selective compounds and the generosity of individuals and enterprises in their exchange. Of the 15 compounds tested in these assays, 8 were gifts. I would like to thank the following: Dr R.B. Barlow and Dr M. Shepherd, University of Bristol, Bristol, U.K. for 4-diphenyl-acetoxy-N-piperidine methiodide (4–DAMP); Dr M. Eltze, Byk Gulden Lomberg Chemische Fabrik GmbH, Konstanz, F.R.G. (telenzepine); Dr R. Hammer, Boehringer Ingelheim Zentrale GmbH, Ingelheim, F.R.G. (pirenzepine and 11[[2-[(diethylamino)methyl] - 1 - piperidinyl]acetyl]5,11 - dihydro - 6H - pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX 116)); Dr M. Laduron, Rhone-Poulenc Sante, Vitry-sur-Seine, France (thiazinamium); Dr G. Lambrecht, Johann-Wolfgang Goethe Universität, Frankfurt-am-Rhein, F.R.G. (HHSiD); Dr W.C. Taylor, University of Sydney, Sydney, Australia (himbacine); Dr I. van Wijngaarden, Duphar B.V., Weesp, Holland (secoverine).

The following products were purchased from Sigma Chemical Co., St. Louis, MO. U.S.A.: atropine methyl nitrate, carbamylcholine (carbachol), gallamine triethiodide, hemicholinium-3 and (D, L)-trihexyphenidyl hydrochloride (THP; benzhexol). Amitriptyline was obtained from Produits Roche s.a., Neuilly, France. N-methyl scopolamine bromide (NMS) was from E. Merck, Darmstadt, F.R.G. Dicyclomine hydrochloride (DC) was obtained from Merrell-Dow, Cincinnati, OH, U.S.A.

Krebs-Henseleit buffer was prepared from analytical grade compounds and contained (in mM): NaCl 118, KCl 5.0, $CaCl_2$ 1.3, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose, 10. The pH was maintained at 7.4 with carbogen.

Dowex AG 1 X8 resin, formate form (100–200 mesh) was purchased from BioRad, Richmond, CA U.S.A. The resin was purified according to the procedure of Kakimoto & Armstrong (1962) except that 2 M formic acid was substituted for the 2 M HCl called for in the procedure. After the last wash (with acetone), the excess fluid was removed by filtration under vacuum. The resin was then weighed and a volume of 1 M formic acid equal to this weight was used to resuspend the resin. Aliquots of 1 ml suspension were used per column. One hundred columns (Econo-columns, 0.7 × 15 cm, BioRad, Richmond, CA, U.S.A.) were set up in a perspex holder. Before use, the resin in the columns was treated with 2 times 10 ml of 10 mM myo-inositol (Sigma Chemical Co., St. Louis, MO, U.S.A.) and one cycle of washing/eluting buffers to reduce variable recovery off the columns. The eluting mixtures were delivered to the columns with an Ismatec peristaltic pump set up to wash 20 columns simultaneously. The resin in the columns was regenerated after each experiment with 25 ml 1 M formic acid. The resin was renewed after approximately 20 experiments.

The following products were from New England Nuclear-Dupont, Dreieich, F.R.G.: methyl-[^{3}H]-choline chloride, 80 Ci mmol^{-1}; N-methyl-[^{3}H]-pirenzepine, 84.1 Ci mmol^{-1}; l-myo-1,2-[^{3}H]-(N) inositol, 47.1–61.2 Ci mmol^{-1}; myo-2-[^{3}H]-(N) inositol, 14 Ci mmol^{-1}; Aquasol-2 universal LSC cocktail. Amersham, International, Amersham, U.K., supplied l-quinuclidinyl(phenyl-4-[^{3}H]-benzilate, 39 Ci mmol^{-1}; myo-(2-[^{3}H])inositol, 16.6–17.9 Ci mmol^{-1} and l-myo-(U-[^{14}C]-inositol-1-phosphate, 55 mCi mmol^{-1}. American Radiolabelled Chemicals, Inc., St. Louis, MO, U.S.A., supplied myo-(2-[^{3}H]-(N))-inositol, 15 Ci mmol^{-1}.

Results

Binding studies with [^{3}H]-QNB and [^{3}H]-pirenzepine

Comparison of [^{3}H]-*QNB and* [^{3}H]-*pirenzepine binding* In this study, all the compounds tested in both binding assays

Table 1 Effects of muscarinic antagonists on binding in rat cerebral cortex, carbachol-induced formation of inositol monophosphate (IP_1) and carbachol-activated autoreceptors in rat hippocampus slices and electrically-induced contractions of guinea-pig atria and ileum

	Binding (corr. pIC_{50}) [^{3}H]-*QNB* (n = 3–4)	[^{3}H]-*PZ* (n = 3–4)	n_H	IP_1 *App. pK$_i$* (n = 3–7)	n_H	*Atria* pA_2 (n = 3–5)	*Ileum* pIC_{50} (n = 4–6)	*Autorec. app.* pA_2 (n = 3–6)
Non-selective antagonists								
Atropine	8.73	9.42	0.99	8.84	1.02	9.11	8.53	8.55
	±0.23	±0.07	±0.39	±0.03	±0.08	±0.23	±0.08	±0.10
NMS	ND	9.35	1.28	9.39	0.82	ND	ND	8.86
		±0.12	±0.06	±0.06	±0.04			±0.05
Putative M_1 selective antagonists								
THP	7.94	9.32	0.94	8.01	0.81	6.91	8.45	8.10
	±0.03	±0.12	±0.19	±0.14	±0.02	±0.20	±0.22	±0.12
Thiazinamium	7.96	8.50*	0.78	7.95	1.12	ND	8.20	7.82
	±0.14	±0.18	±0.15	±0.08	±0.09		±0.10	±0.18
DC	7.76	8.92	1.14	7.45*	0.72	6.22	8.05	6.54
	±0.06	±0.08	±0.09	±0.06	±0.03	±0.24	±0.31	±0.10
PZ	6.69	8.19	0.99	7.63*	0.56	6.94	6.79	6.63
	±0.07	±0.06	±0.07	±0.08	±0.06	±0.33	±0.06	±0.18
Telenzepine	ND	8.76	1.11	8.51	1.03	ND	ND	7.42
		±0.14	±0.08	±0.11	±0.12			±0.08
Putative ileal-selective (M_3) antagonists								
4-DAMP	8.54	9.17	1.09	8.13	0.91	7.80	8.89	8.18
	±0.20	±0.10	±0.03	±0.11	±0.06	±0.08	±0.04	±0.07
HHSiD	7.63	8.22	0.95	6.99*	0.72	ND	7.79	6.30
	±0.14	±0.03	±0.04	±0.06	±0.01		±0.15	±0.09
Secoverine	ND	8.71	1.02	7.83	ND	7.93	7.66	7.89
		±0.08	±0.06	±0.16		±0.28	±0.11	±0.13
Amitriptyline	7.52	8.28	1.09	6.97	1.23	6.74	7.66	5.94
	±0.35	±0.03	±0.09	±0.14	±0.06	±0.27	±0.09	±0.06
Putative atrial-selective (M_2) antagonists								
Himbacine	7.04	7.19	0.95	7.18*	0.71	7.42	6.94	8.28
	±0.19	±0.02	±0.06	±0.21	±0.09	±0.21	±0.04	±0.10
AF-DX 116	5.90	6.56	1.02	6.03	0.91	6.70	5.87	6.84
	±0.12	±0.03	±0.20	±0.17	±0.15	±0.16	±0.08	±0.05
Gallamine	ND	6.50*	0.77	<5	ND	5.01	4.62	5.35
		±0.15	±0.01			±0.42	±0.12	±0.20
Bret.tos	ND	5.16	0.92	<5	ND	ND	ND	5.66
		±0.03	±0.03					±0.13

Values are mean ± s.e.mean. ND—not determined.
* Indicates values which may not be true affinity constants because the compounds display low Hill coefficients (n_H).

had higher affinities for the binding sites labelled with [^{3}H]-PZ than for those labelled with [^{3}H]-QNB (Table 1). Atropine was 5 times more active in displacing specifically bound [^{3}H]-PZ than [^{3}H]-QNB. The putative M_2- and M_3-selective antagonists had affinity ratios (concentration displacing 50% of the specifically bound [^{3}H]-QNB divided by the concentrations displacing 50% of the specifically bound [^{3}H]-PZ; Table 2) similar to that of atropine. Of the four putative M_1-selective

Table 2 Ratios of affinity values of antagonists at different muscarinic receptors determined in binding or *in vitro* functional tests

Compound	[^{3}H]-*QNB* / [^{3}H]-*PZ*	IP_1 / [^{3}H]-*PZ*	A/M_1	A/M_2	A/M_3	M_2/M_1	M_3/M_1	M_2/M_3
Atropine	4.90	3.80	1.95	3.63	0.91	0.53	2.14	0.26
NMS	—	0.91	3.39	—	—	—	—	—
THP	23.98	20.42	0.81	0.06	1.86	12.59	0.44	34.67
Thiazinamium	3.47	3.55	1.35	—	2.14	—	0.63	—
DC	14.45	29.51	8.13	0.48	70.79	16.98	0.11	67.61
PZ	31.62	3.63	10.00	2.04	1.62	4.90	6.17	0.71
Telenzepine	—	1.78	12.30	—	—	—	—	—
4-DAMP	4.27	10.96	0.89	0.42	4.90	2.14	0.18	12.30
HHSiD	3.89	16.98	4.90	—	35.48	—	0.14	—
Secoverine	—	7.59	0.87	1.10	0.63	0.79	1.38	0.54
Amitriptyline	5.75	20.42	10.72	6.31	54.95	1.70	0.19	8.32
Himbacine	1.41	1.02	0.08	0.14	0.05	0.58	1.70	0.33
AF-DX 116	4.57	3.39	0.15	0.72	0.11	0.21	1.38	0.15
Gallamine	—	>32	>0.45	0.46	0.19	—	—	0.41
Bret. tos.	—	>0.7	>0.22	—	—	—	—	—

A = autoreceptor, M_1 = IP_1 response, both in rat hippocampus slices. M_2 = atrial response, M_3 = ileal contractions, both in guinea-pig tissues. — = not determined.

antagonists tested at the two binding sites, three- THP, DC and PZ were at least 14 fold more active in displacing [^{3}H]-PZ than [^{3}H]-QNB. The fourth compound, thiazinamium, had a ratio of affinities for the sites labelled by the two ligands similar to those of atropine. However, the Hill coefficient for this compound calculated from displacement experiments was less than unity when [^{3}H]-PZ was the ligand (Table 1), which may indicate a non-competitive interaction between displacer and ligand at the binding site.

Comparison of Hill coefficients between [^{3}H]-pirenzepine binding and (inositol monophosphate) formation It was of interest to compare not only affinity values but also Hill coefficients of compounds antagonizing IP_1 formation and specifically displacing [^{3}H]-PZ (Table 1). All compounds except thiazinamium and gallamine appeared to intereact competitively with [^{3}H]-PZ binding sites. In contrast to its low Hill slope determined from displacement studies of [^{3}H]-PZ, thiazinimium appeared to be a competitive antagonist at muscarinic receptors linked to IP_1 formation. There were four compounds that displaced [^{3}H]-PZ in an apparent competitive manner but displayed low Hill coefficients (<0.8) when tested for their ability to inhibit IP_1 formation -PZ, DC, HHSiD and himbacine.

Formation of inositol monophosphate in hippocampal slices induced by carbachol in the absence or presence of muscarinic antagonists

The number of d.p.m. isolated as IP_1 from hippocampal slices under basal or stimulated conditions varied depending on the specific activity and source of [^{3}H]-myo-inositol. In several series of experiments ($n = 9$ to 17 experiments per series for a total of 64 indepcndent observations), accumulation of IP_1 under basal conditions ranged from 386 ± 53 d.p.m. ($n = 14$) to 677 ± 80 d.p.m. ($n = 11$). In this same series of experiments, the maximum stimulation of IP_1 formation by 1 mM carbachol ranged from $1636 \pm 99\%$ ($n = 17$) to $2102 \pm 86\%$ ($n = 11$) above basal. The average of the means from these series was 1852% above basal. The pD_2 value (negative log of the EC_{50}) for carbachol ranged from 4.53 ± 0.05 ($n = 11$) to 5.00 ± 0.05 ($n = 14$). The average pD_2 value from these five series of experiments was 4.90.

The effects on basal accumulation of IP_1 were determined for the highest concentration of each antagonist used in this test. None except bretyllium tosylate and gallamine influenced basal IP_1 formation.

The fifteen antagonists used in this study inhibited, in a concentration-dependent manner, the formation of IP_1 induced by 1 mM carbachol. The pK_i values are listed in Table 1. The non-selective antagonists, atropine and NMS, displayed the highest affinities followed by the putative M_1-selective antagonists, telenzepine, THP, thiazinamium, PZ and DC. 4-DAMP, secoverine, and to a lesser degree, himbacine also antagonized IP_1 formation and had moderate to high affinities for the receptors. Compounds which displayed lower affinities ($pK_i < 7$) at this site included HHSiD, amitriptyline and AF–DX 116. Gallamine and bretyllium tosylate had very low affinities ($pK_i < 5$). However, in the presence of the high concentrations of these compounds required to block the carbachol response, basal formation of IP_1 was doubled, complicating the interpretation of these results.

The Hill coefficients were calculated for some of the antagonists that inhibited carbachol-induced IP_1 formation. Telenzepine and amitriptyline had Hill coefficients significantly greater than one. Increasing the incubation time with these antagonists to 60 min decreased the Hill coefficient to unity for telenzepine while that obtained with amitriptyline decreased from 1.44 ± 0.04 ($n = 3$) to 1.23 ± 0.06 ($n = 3$). Atropine, NMS, telenzepine, 4–DAMP, thiazinamium, amitriptyline and AF–DX 116 had Hill coefficients between 0.8 and 1.2, consistent with competitive antagonism at this receptor. Compounds with Hill coefficients of less than 0.8 included HHSiD, himbacine, DC and PZ. It is of interest to note the difference between PZ and its M_1-selective analogue, telenzepine.

Effects of antagonists on muscarinic receptors in guinea-pig atria

Only atropine antagonized with high affinity the inhibition of atrial contractions induced by acetylcholine (ACh). Antagonists exhibiting moderate affinities (pA_2 between 6 and 7) at this site included secoverine, 4-DAMP and himbacine (Table 1). In the present studies, himbacine was less active than reported by Anwar-ul *et al.* (1986) due, perhaps, to different concentrations of calcium in the media and/or differences in the electrical stimulation parameters. Compounds with pA_2 values less than 7 included THP, PZ, amitriptyline, AF–DX 116 and DC. Gallamine had the lowest pA_2 value.

Effects of antagonists on guinea-pig ileal muscarinic receptors

In this test, 4-DAMP displayed the highest affinity followed by atropine, THP, thiazinamium and DC. Compounds with pIC_{50} values of between 7 and 8 were HHSiD, secoverine and amitriptyline. Antagonists with affinity values of less than 7 included PZ, himbacine, AF-DX 116 and gallamine.

Muscarinic autoreceptors in rat hippocampal slices

Rat hippocampal slices, stimulated three times at 1 Hz for 2 min, released tritium in a reproducible manner. For example, in one series of experiments, the areas under the curves (X 100) were: $S1 = 1.02 \pm 0.05$, $S2 = 1.00 \pm 0.11$, $S3 = 1.00 \pm 0.12$ ($n = 6$).

Carbachol 0.3 to 10 μM, concentration-dependently inhibited stimulated tritium efflux with no effect on basal tritium overflow. In ten series of experiments (to test antagonists, $n = 3$–6 experiments per series), the pD_2 value for carbachol was 6.03 ± 0.02 (range $= 5.90$ to 6.16) and the maximum inhibition, obtained with 10 μM carbachol, was $83.5 \pm 1.3\%$.

The antagonists tested in this study antagonized the carbachol-induced inhibition of stimulated release (apparent pA_2 values are listed in Table 1) with no effect on basal efflux of tritium. It is of interest to note that, at concentrations sufficient to antagonize activation of autoreceptors by the exogenous agonist, carbachol, none of the antagonists enhanced the stimulated overflow of tritium.

Antagonists with high affinity (apparent $pA_2 > 8$) in this test included NMS, atropine, THP, 4-DAMP and himbacine. Moderate affinities (apparent pA_2 values between 7 and 8) were displayed by thiazinamium, telenzepine and secoverine. Apparent pA_2 values between 6 and 7 were found with DC, PZ, HHSiD and AP-DX 116. Compounds with affinities of less than 6 included amitriptyline, gallamine and bretyllium tosylate.

Because himbacine exhibited the greatest selectivity for muscarinic autoreceptors compared to postsynaptic receptors linked to PI turnover (Table 2), additional concentrations of the antagonist were tested against carbachol-induced inhibition of stimulated tritium release and the data were plotted as a Schild regression. The pA_2 value was 7.94 and the slope was 0.8.

Regression analysis of correlations

Affinity values obtained with antagonists that did not display selectivity among the four *in vitro* functional tests were omitted from calculations of correlation coefficients. Defining selective compounds as those with ratios of less than 0.2 or greater than 5.0 in the *in vitro* tests in Table 2 eliminated atropine, NMS, thiazinamium, secoverine and bretylium tosylate.

Table 3 Correlation between affinity values of muscarinic antagonists, obtained by displacement of specifically bound [^{3}H]-pirenzepine ([^{3}H]-PZ) in rat cerebral cortical membranes or by antagonism of autoreceptors in rat hippocampus slices, and affinity values obtained from *in vitro* functional tests

	[^{3}H]-*PZ binding*			*Autoreceptor*		
	*df**	r	P	*df**	r	P
Functional Test						
Autoreceptor	9	0.420	0.226	—	—	—
Atria	7	0.469	0.241	7	0.827	0.011
IP_1 formation	8	0.815	0.007	8	0.480	0.191
Ileum	8	0.927	0.0003	8	0.566	0.113

* degrees of freedom

The significance of the correlations between affinity values obtained from any two tests was determined by regression analysis.

The level of significance of correlations between affinity values displayed by the ten selective antagonists for the displacement of [^{3}H]-PZ and their affinities in the four functional tests (Table 3) indicated which subtype model best described the binding data. There were poor correlations between affinities in the binding test with either those at atrial or those at hippocampal autoreceptors. As expected, a significant correlation was obtained between IP_1 formation, a model for M_1-receptors, and [^{3}H]-PZ binding in cerebral cortical membranes. However, the strongest correlation was observed between the affinities of these antagonists at [^{3}H]-PZ binding sites and their affinities at ileal M_3 receptors.

A comparison of affinities for the inhibition of carbachol-stimulated IP_1 formation in hippocampus with affinities at peripheral receptors showed a low correlation between atrial receptors and IP_1 formation ($r = 0.406$, $p = 0.37$, degrees of freedom (d.f.) = 6) while the correlation between affinities at ileal receptors and IP_1 formation was significant ($r = 0.799$, $P = 0.0175$, d.f. = 7).

The correlation between affinities at atrial receptors and those at ileal receptors was only slightly greater than the level accepted as significant ($r = 0.699$, $P = 0.0537$, d.f. = 7).

Regression analysis was also used to determine the significance of correlations between affinity values of these selective antagonists at muscarinic autoreceptors in rat hippocampus with their affinity values measured in functional models of M_1, M_2 or M_3 receptors. As shown in Table 3, the only correlation that attained significance was between muscarinic autoreceptors in rat hippocampus and muscarinic receptors in the heart.

Discussion

Although the affinities of many of these putatively selective antagonists have been previously obtained in two of the three models used in the present study, few have been determined for all three receptor subtypes tested in the same laboratory. Moreover, central M_1 receptors may differ from peripheral M_1 receptors (Lambrecht *et al.*, 1987; Bloom *et al.*, 1987). These considerations as well as the variable results presented in the literature (see, for example, Mitchelson, 1988) for some of these standard antagonists led us to set up these models in house.

A comparison of the ratios of affinity values of putatively selective antagonists for displacing [^{3}H]-PZ and [^{3}H]-QNB was of limited value for the prediction of M_1 versus non-M_1 selectivity in functional tests. Putative M_1 selective antagonists displayed affinity ratios of 3 to 32. If thiazinamium was eliminated from the comparison because it displayed no selectivity in functional tests (Table 2), the remaining putative M_1-selective compounds produced ratios of 14 or greater. The ratios of both M_2- and M_3-selective antagonists were less than 6. M_3 antagonists displaced both ligands and had high affinities at both binding sites whereas M_2 antagonists displaced both ligands but had low affinities. The highly significant correlation between affinities of M_3 antagonists for displacing [^{3}H]-PZ from binding sites in the cerebral cortex with their affinities for antagonizing ileal contractions could be a reflection of the homology demonstrated between m_1 and m_3 gene products (Bonner *et al.*, 1987) and suggests caution should be applied in interpreting binding data obtained by displacement of specifically bound [^{3}H]-PZ from rat cerebral cortical membranes (50% M_1, 15% M_2, 35% M_3; Giraldo *et al.*, 1987). The apparent lack of displacement of [^{3}H]-QNB by M_2 antagonists could be due to the small percentage of M_2 sites in this tissue.

The results obtained with eleven of the antagonists used in this study allowed another question to be addressed, that of possible differences between central and peripheral M_1 receptors. Affinities of compounds determined at M_1 receptors of rat hippocampus were compared with their affinities at M_1 receptors in rabbit vas deferens obtained by Eltze (1988), which were found to be similar to the ganglionic M_1 receptor (Eltze *et al.*, 1988). Himbacine, THP and AF-DX 116 were about 6 times more active at antagonizing the latter than the former while both 4-DAMP and HHSiD were 8 tmes more active. Thus, three antagonists in addition to HHSiD (Lambrecht *et al.*, 1987) and AF-DX 116 (Bloom *et al.*, 1988) appear to present some selectivity between central and peripheral M_1 receptors.

Of the 15 antagonists tested in this study against carbachol-induced IP_1 formation, 11 had Hill coefficients of about 1, compatible with competitive antagonism at this site. Low Hill coefficients were found with himbacine, HHSiD, DC and PZ. This has been previously observed for PZ on IP_1 formation in rat CNS (Rooney & Nahorski, 1986) and in guinea-pig cortex (Kunyzs *et al.*, 1988; but see Ek & Nahorski, 1988). Thus, these four compounds may not be competitive antagonists or there may be multiple muscarinic sites in hippocampal slices linked to PI turnover, possibly on different cell types. *In situ* hybridization experiments have demonstrated the presence of m1 and m3 mRNA in rat hippocampus (Buckley *et al.*, 1988) whose corresponding receptors have both been found to be linked to phosphatidylinositol turnover (Peralta *et al.*, 1988; where HM4 = m3). Recently, the affinity values and Hill coefficients for PZ, HHSiD and DC were determined in binding experiments using CHO-K1 cells expressing m1 or m3 genes (Buckley *et al.*, 1989). The former two antagonists displayed n_H values of approximately unity for their interactions with both gene products whereas the n_H values obtained from the interaction of DC with either m1 or m3 gene products was less than 0.7. These data suggest, therefore, that the low Hill coefficients calculated from data obtained with PZ and HHSiD for antagonizing carbachol-induced IP_1 formation in rat hippocampus may be due to interactions with multiple receptors linked to this second messenger, while the low n_H value calculated from data obtained with DC may be due to an allosteric interaction of this latter antagonist with one or both receptors.

In peripheral tissues DC potently inhibited electrically-induced contractions of guinea-pig ileum, being 70 times more active in this tissue than in blocking atrial receptors. Doods *et al.*, (1987) demonstrated that the antagonist was 28 times more active in inhibiting salivation (M_3) than it was in heart (M_2). In rat frontal cortex DC was 250 times more active than PZ in blocking muscarinic receptors modulating [^{3}H]-dopamine release (March & Raiteri, 1985), implicating M_3 receptors in this response. The lack of antagonism by gallamine on striatal muscarinic receptors influencing dopamine release (Schoffelmeer *et al.*, 1986) would indirectly support this suggestion. It would be of interest to determine the effects of other M_3-selective antagonists on muscarinic modulation of dopamine release.

While the determination of affinity values of muscarinic antagonists in four *in vitro* functional receptor systems was to provide a basis for the characterization of hippocampal muscarinic autoreceptors, comparison of the affinities of these antagonists among the other tests was also of interest. For example, the significant correlation between the affinities of the selective antagonists for receptors mediating hippocampal IP_1 formation with those at ileal receptors mediating contractility lends support to the suggestion that this second messenger may play a role in ileal smooth muscle contractions (see Goyal, 1988, and references therein). The low correlation between affinity values of these antagonists at IP_1 formation and muscarinic receptors in atria is in accord with findings that muscarinic receptors inducing negative inotropic responses are not linked to PI hydrolysis (Eglen *et al.*, 1988). The relatively high correlation coefficient between affinities at heart receptors with affinities at ileal receptors is consistent with the lack of a high degree of selectivity of the antagonists used in these models.

The primary goal of this work was to determine affinity values of putatively selective or non-selective antagonists at blocking activation of muscarinic autoreceptors in electrically stimulated rat hippocampal slices. The activities of these antagonists were also determined in different *in vitro* models of muscarinic receptor subtypes and the affinity values in each test were then correlated with their affinity values for antagonizing muscarinic autoreceptors. In the four *in vitro* functional tests, the classical muscarinic antagonist, atropine, displayed high but differential affinities, being 3.8 times less active in atrium than ileum. If atropine is assumed to be a non-selective antagonist, then differences of greater than 0.6 log units will be necessary (but perhaps not sufficient) to distinguish receptor subtypes.

In binding studies, THP (Tien & Wallace, 1985; Nivelbrant & Sparf, 1986) and thiazinamium choride (Muth *et al.*, 1985) were shown to display M_1 selectivity. Functional tests *in vivo* and *in vitro* have shown that THP was more active at blocking peripheral M_1 than M_2 receptors (Giachetti *et al.*, 1986*b*; Eltze, 1988) whereas we and others (Eglen & Whiting, 1987) found THP to be most active at antagonizing M_3 receptors. In the present study, thiazinamium also displayed high affinity at ileal receptors. This may explain, in part, its bronchodilator activity (Muth *et al.*, 1985) which could be due to antagonism of M_3 receptors on airway smooth muscle (Barnes *et al.*, 1988). However, both THP and thiazinamium were as active at central M_1 sites as at ileal M_3 receptors and both compounds displayed high affinity for the muscarinic autoreceptors in rat hippocampus. These results are inconsistent with these compounds acting as selective antagonists.

Muscarinic antagonists other than PZ that have been shown to be M_1-selective include telenzepine (Eltze *et al.*, 1985) and DC (Potter *et al.*, 1984). In hippocampus slices both of these compounds, as well as PZ, were 8 to 10 times more active in inhibiting the PI response than in blocking activation of the autoreceptor. This supports the conclusion of Raiteri *et al.* (1984), that muscarinic autoreceptors in rat hippocampus are not likely to be M_1 and accords with similar conclusions based on data obtained with muscarinic autoreceptors in rat striatum (Schoffelmeer *et al.*, 1986) and cerebral cortex (Marchi & Raiteri, 1985; Roberts & Tutty, 1986).

Antagonists suggested to be selective for the M_3 ileal muscarinic receptor include 4-DAMP (Barlow & Shepherd, 1985), HHSiD (Mutschler & Lambrecht, 1984) and secoverine (Zwagemakers & Claassen, 1980). Amitriptyline was shown to be 29 times more active in blocking striatal muscarinic receptors negatively linked to adenylate cyclase than cortical muscarinic receptors linked to PI turnover (Nomura *et al.*, 1987). In the present study, secoverine showed no selectivity in any of the functional tests. Amitriptyline was more active on ileal than atrial receptors indicating some M_3 selectivity. Consistent with their M_3 selectivity, both 4-DAMP and HHSiD were most active in antagonizing muscarinically-mediated ileal contractions. Comparison of the affinities of these three compounds on ileal receptors versus muscarinic autoreceptors in hippocampus showed greater activity at the former. 4-DAMP displayed the smallest degree of selectivity (5 fold) while HHSiD and amitriptyline were 35 and 55 times, respectively, more active at blocking ileal receptors than hippocampal autoreceptors. These data indicate that the muscarinic autoreceptors in rat hippocampus are not likely to be of the M_3 subtype.

Putative cardioselective agents bretylium tosylate (Schreiber & Sokolovsky, 1985), gallamine (Mitchelson, 1984), himbacine (Anwar-ul *et al.*, 1986) and AF-DX 116 (Giachetti *et al.*, 1986a) were also tested in the four *in vitro* assays. These compounds were more active as antagonists at hippocampal autoreceptors than at postsynaptic receptors and all four antagonized muscarinic autoreceptor activation at similar concentrations to those required to antagonize cardiac receptors. The selectivity ratios between pre- and postsynaptic hippocampal receptors were small for gallamine and bretylium tosylate whereas the ratios for AF–DX 116 and himbacine were 6 and 13, respectively. Moreover, the analysis of correlation coefficients between autoreceptors and the three *in vitro* models of muscarinic receptor subtypes showed a significant correlation only between autoreceptors and cardiac receptors. These data indicate that muscarinic autoreceptors in rat hippocampus are pharmacologically similar to muscarinic receptors in the heart.

Thus the evidence presented does not support classification of muscarinic autoreceptors in rat hippocampus as M_1 or M_3 but does suggest that these autoreceptors are similar to the M_2 subtype. However, the homology between m2 and m4 gene products (Bonner, 1989) and the lack of selective antagonists for the latter do not allow a firm conclusion, based on pharmacological evidence alone, to be drawn. Again, supporting evidence may be obtained from *in situ* hydridization results. The presence of m2 mRNA and the apparent absence of mRNA for other muscarinic receptors, including m4 mRNA, in medial septal nuclei (Buckley *et al.*, 1988) is consistent with hippocampal autoreceptors being of the M_2 subtype. This also suggests that muscarinic autoreceptors in rat hippocampus may be a relatively pure population and that the variability obtained with muscarinic antagonists is indicative of possible multiple receptors being recognized by these compounds in the other *in vitro* tests.

In conclusion, comparison of 15 muscarinic antagonists in four *in vitro* functional tests and two binding assays provided additional evidence for at least three pharmacologically defined muscarinic receptor subtypes. There may be pharmacological differences between peripheral and central M_1 receptors. Muscarinic autoreceptors in rat hippocampus appear to be pharmacologically similar to the M_2 (cardiac) receptor subtype.

I would like to thank Ms C. Berg, N. Edel and M. Host for the ileal, binding and atrial data, respectively. I would also like to thank Ms M. Schneider for expert technical assistance. The helpful comments and criticisms of Dr R.C. Miller and an anonymous referee of the *Br. J. Pharmacol.* is acknowledged with pleasure.

References

ANWAR-UL, S., GILANI, H & COBBIN, L.B. (1986). The cardioselectivity of himbacine: a muscarinic receptor antagonist. *Naunyn-Schmeidebergs Arch. Pharmacol.*, **332**, 16–20.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARLOW, R.B. & SHEPHERD, M.K. (1985). A search for selective antagonists at M_2 muscarinic receptors. *Br. J. Pharmacol.*, **85**, 427–435.

BARNES, P.J., MINETTE, P. & MACLAGAN, J. (1988). Muscarinic receptor subtypes in airways. *Trends Pharmacol. Sci.*, **9**. 412–415.

BIRDSALL, N.J.M., HULME, E.C., KROMER, W., PECK, B.S., STOCKTON, J.M. & ZIGMOND, M.J. (1987). Muscarinic receptor subclasses. In *New Concepts in Alzheimer's Disease*, ed. Briley, M., Kato, A. & Weber, M., *Pierre Fabre Monograph Series*, Vol. **1**, pp. 103–121.

BLOOM, J.W., HALONEN, M., SEAVER, N.A. & YAMAMURA, H.I. (1987). Heterogeneity of the M_1 muscarinic receptor subtype between peripheral lung and cerebral cortex demonstrated by the selective antagonist AF-DX 116. *Life Sci.*, **41**, 491–496.

BONNER, T.I., YOUNG, A.C., BRANN, M.R. & BUCKLEY, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron*, **1**, 403–410.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science*, **237**, 527–532.

BONNER, T.I. (1989). The molecular basis of muscarinic receptor diversity. *Trends Neurosci.*, **12**, 148–151.

BROWN, E., KENDALL, D.A. & NAHORSKI, S.R. (1984). Inositol phospholipid hydrolysis in rat cerebral cortical slices: I. Receptor classification. *J. Neurochem.*, **42**, 1379–1387.

BUCKLEY, N.J., BONNER, T.I. & BRANN, M.R. (1988). Localization of a family of muscarinic receptor mRNAs in rat brain. *J. Neurosci.*, **8**, 4646–4652.

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469–476.

CHENG, Y.-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

DOODS, H.N., MATHY, M.-J., DAVIDESKO, D., VAN CHARLDORP, K.J., DE JONGE, A. & VAN ZWEITEN, P.A. (1987). Selectivity of muscarinic antagonists in radioligand and *in vivo* experiments for the putative M1, M2 and M3 receptors. *J Pharmacol. Exp. Ther.*, **242**, 257–262.

EGLEN, R.M., MONTGOMERY, W.W. & WHITING, R.L. (1988). Negative and positive inotropic responses to muscarinic agonists in guinea pig and rat atria *in vitro*. *J. Pharmacol. Exp. Ther.*, **247**, 911-917.

EGLEN, R.M. & WHITING, R.L. (1986). Muscarinic receptor subtypes: a critique of the current classification and a proposal for a working nomenclature. *J. Auton. Pharmacol.*, **5**, 323–346.

EGLEN, R.M. & WHITING, R.L. (1987). Competitive and non-competitive antagonism exhibited by 'selective' antagonists at atrial and ileal muscarinic receptor subtypes. *Br. J. Pharmacol.*, **90**, 701–707.

EK, B. & NAHORSKI, S. (1988). Muscarinic receptor coupling to inositol phospholipid metabolism in guinea-pig cerebral cortex, parotid gland and ileal smooth muscle. *Biochem. Pharmacol.*, **37**, 4461–4467.

EL-FAKAHANY, E. (1985). Additional complexities in the binding of muscarinic receptor antagonists. *Trends Pharmacol. Sci.*, **6**, 429–430.

ELTZE, M. (1988). Muscarinic M1- and M2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.*, **151**, 205–221.

ELTZE, M., GONNE, S., RIEDEL, R., SCHLOTKE, B., SCHUDT, C. & SIMON, W.A. (1985). Pharmacological evidence for a selective inhibition of gastric acid secretion by telenzepine, a new antimuscarinic drug. *Eur. J. Pharmacol.*, **112**, 211–224.

ELTZE, M., GMELIN, G., WESS, J., STROHMANN, C., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1988). Presynaptic muscarinic receptors mediating inhibition of neurogenic contractions in rabbit vas deferens are of the ganglionic M1-type. *Eur. J. Pharmacol.*, **158**, 233–242.

FISHER, S.K., BOAST, C.A. & AGRANOFF, B.W. (1980). The muscarinic stimulation of phospholipid labeling in hippocampus is independent of its cholinergic input. *Brain Res.*, **189**, 284–288.

GIACHETTI, A., MICHELETTI, R. & MONTAGNA, E. (1986a). Cardioselective profile of AF-DX 116, a muscarinic M_2 receptor antagonist. *Life Sci.*, **38**, 1663–1672.

GIACHETTI, A., GIRALDO, E., LADINSKY, H. & MONTAGNA, E. (1986b). Binding and functional profiles of the selective M_1 muscarinic receptor antagonists trihexyphenidyl and dicyclomine. *Br. J. Pharmacol.*, **89**, 83–90.

GIL, D.W. & WOLFE, B.B. (1985). Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. *J. Pharmacol. Exp. Ther.*, **232**, 608–616.

GIRALDO, E., HAMMER, R. & LADINSKY, H. (1987). Distribution of muscarinic receptor subtypes in rat brain as determined in binding studies with AF-DX 116 and pirenzepine. *Life Sci.*, **40**, 833–840.

GOYAL, R.K. (1988). Identification, localization and classification of muscarinic receptor subtypes in the gut. *Life Sci.*, **43**, 2209–2220.

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature*, **283**, 90–92.

HAWCOCK, T., LAZARENO, S. & ROBERTS, F. (1986). Assessment of the muscarinic subtype selectivity of (±)-3-quinuclidinyl xanthene-9-carboxylate hemioxalate hydrate (QNX). *Br. J. Pharmacol.*, **88**, 357P.

KAKIMOTO, Y. & ARMSTRONG, M.D. (1962). The phenolic amines of human urine. *J. Biol. Chem.*, **237**, 208–214.

KUNYSZ, E.L., MICHEL, A.D. & WHITING, R.L. (1988). Functional and direct binding studies using subtype selective muscarinic receptor antagonists. *Br. J. Pharmacol.*, **93**, 491–500.

LADINSKY, H., VINCI, R., WANG, J.-X., PALAZZI, E., CICIONI, P. & CONSOLO, S. (1987). On the functional role of muscarinic receptor subtypes in the regulation of cholinergic neurotransmission in striatum and hippocampus of the rat. In *Cellular and Molecular Basis of Cholinergic Function*, ed. Dowdall, M.J. & Hawthorne, J.N. pp. 193–199. Chichester: Ellis Horwood Publishers.

LAMBRECHT, G., MOSER, U., RIOTTE, J., WAGNER, M., WESS, J., GMELIN, G., TACKE, R., ZILCH, H. & MUTSCHLER, E. (1987). Heterogeneity in muscarinic receptors: evidence from pharmacological and electrophysiological studies with selective antagonists, In *International Symposium on Muscarinic Cholinergic Mechanisms*, ed. Cohen, S. & Sokolovsky, M. p. 245. London: Freund Publishing House, Ltd.

MARCHI, M. & RAITERI, M. (1985). On the presence in the cerebral cortex of muscarinic receptor subtypes which differ in neuronal localization, function and pharmacological properties. *J. Pharmacol. Exp. Ther.*, **235**, 230–233.

MITCHELSON, F. (1984). Heterogeneity in muscarinic receptors: evidence from pharmacologic studies with antagonists. *Trends Pharmacol. Sci.*, **5** (Suppl.) 12–16.

MITCHELSON, F. (1988). Muscarinic receptor differentiation. *Pharmacol. Ther.*, **37**, 357–423.

MUTH, E., LEWIS, A.J., DERVINIS, A., HAND, J.M., ROTHBERG, K. & DOUGLAS, J.S. (1985). A comparison of the muscarinic and histamine H_1 receptor binding activities of thiazinamium chloride and its major metabolite. *Br. J. Pharmacol.*, **86**, 452P.

MUTSCHLER, E. & LAMBRECHT, G. (1984). Selective muscarinic agonists and antagonists in functional tests. *Trends Pharmacol. Sci.*, **5** (Suppl.), 39–44.

NILVEBRANT, L. & SPARF, B. (1986). Dicyclomine, benzhexol and oxybutynine distinguish between subclasses of muscarinic binding sites. *Eur. J. Pharmacol.*, **123**, 133–143.

NOMURA, S., ZORN, S.H. & ENNA, S.H. (1987). Selective interaction of tricyclic antidepressants with a subclass of rat brain cholinergic muscarinic receptors. *Life Sci.*, **40**, 1751–1760.

NORDSTROM, O. & BARTFAI, T. (1980). Muscarinic autoreceptors regulate acetylcholine release in rat hippocampus: *in vitro* evidence. *Acta Physiol.*, **108**, 347–353.

PERALTA, E.G., ASHKENAZI, A., WINSLOW, J.W., RAMACHANDRAN, J. & CAPON, D.J. (1988). Differential regulation of PI hydrolysis and adenylate cyclase by muscarinic receptor subtypes. *Nature*, **334**, 434–437.

POTTER, L.T., FLYNN, D.D., HENCHETT, H.E., KALINOSKI.. D.L., LUBER-NAROD, J. & MASH, D.C. (1984). Independent M1 and M2 receptors: ligands, autoradiography and functions. *Trends Pharmacol. Sci.*, **5** (Suppl.), 22–31.

RAITERI, M., LEARDI, R. & MARCHI, M. (1984). Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release from rat brain. *J. Pharmacol. Exp. Ther.*, **228**, 209–214.

RICHARDS, M.H. (1985). Efflux of (^{3}H)-5-hydroxytryptamine from rat hypothalamus slices by continuous electrical stimulation: frequency-dependent responses to serotonergic antagonists and 5-hydroxytryptamine. *Naunyn-Schmeidebergs Arch. Pharmacol.*, **329**, 359–366.

RICHARDSON, I.W. & SZERB, J.C. (1974). The release of labelled acetylcholine and choline from cerebral cortical slices stimulated electrically. *Br. J. Pharmacol.*, **52**, 499–507.

ROBERTS, F. & TUTTY, C. (1986). Quantification of the effects of muscarinic antagonists on the release of (^{3}H)-acetylcholine from rat cerebral cortex slices. *Br. J. Pharmacol.*, **88**, 358P.

ROONEY, T.A. & NAHORSKI, S.R. (1986). Regional characterization of agonist and depolarization-induced phosphoinositide hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.*, **239**, 873–880.

ROSSER, M.N., IVERSEN, L.L., REYNOLDS, G.P., MOUNTJOY, C.Q. & ROTH, M. (1984). Neurochemical characteristics of early and late onset types of Alzheimer's disease. *Br. Med. J.*, **288**, 961–964.

SCHLICKER, E. & GOTHERT, M. (1981). Antagonist properties of quipazine at presynaptic serotonin receptors and alpha-adrenoceptors in rat brain cortex slices. *Naunyn-Schmeidebergs Arch. Pharmacol.*, **317**, 204–208.

SCHOFFELMEER, A.N.M., VAN VLIET, B.J., WARDEH, G. & MULDER, A.H. (1986). Muscarine receptor-mediated modulation of (^{3}H)dopamine and (^{14}C) acetylocholine release from rat neostriatal slices: selective antagonism by gallamine but not pirenzepine. *Eur. J. Pharmacol.*, **128**, 291–294.

SCHREIBER, G. & SOKOLOVSKY, M. (1985). Muscarinic receptor heterogeneity revealed by interaction with bretylium tosylate. Different ligand-receptor conformations versus different receptor subclasses. *Mol. Pharmacol.*, **27**, 27–31.

SMITH, C.J., COURT, J.A., KEITH, A.B. & PERRY, E.B. (1989). Increases in muscarinic stimulated hydrolysis of inositol phospholipids in rat hippocampus following cholinergic deafferentation are not paralleled by alterations in cholinergic receptor density. *Brain Res.*, **485**, 317–324.

TEYLER, T.J. (1980). Brain slice preparation: hippocampus. *Brain Res. Bull.*, **5**, 391–403.

TIEN, X.-Y. & WALLACE, L.J. (1985). Trihexylphenidyl-further evidence for muscarinic receptor classification. *Biochem. Pharmacol.*, **34**, 588–590.

WATSON, M., ROESKE, W.R. & YAMAMURA, H.I. (1986). (^{3}H)-Pirenzepine and (−)-(^{3}H)-quinuclidinyl benzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. II. Characterization and regulation of antagonist binding to putative muscarinic subtypes. *J. Pharmacol. Exp. Ther.*, **237**, 419–427.

ZWAGEMAKERS, J.M.A. & CLAASSEN, V. (1980). Pharmacology of secoverine, a new spasmolytic agent with specific antimuscarinic properties. *Arzneim. Forsh. Drug. Res.*, **30**, 1517–1526.

(Received October 13, 1989
Accepted December 11, 1989)