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L-689,660, a novel cholinomimetic with functional selectivity for M_1 and M_3 muscarinic receptors

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1 L-689,660, 1-azabicyclo[2.2.2]octane, 3-(6-chloropyrazinyl)maleate, a novel cholinomimetic, demonstrated high affinity binding (pK_D (apparent) 7.42) at rat cerebral cortex muscarinic receptors. L-689,660 had a low ratio (34) of pK_D (apparent) values for the displacement of binding of the antagonist [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) compared with the displacement of the agonist [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M), in rat cerebral cortex. Low NMS/Oxo-M ratios have been shown previously to be a characteristic of compounds that are low efficacy partial agonists with respect to stimulation of phosphatidyl inositol turnover in the cerebral cortex.

2 L-689,660 showed no muscarinic receptor subtype selectivity in radioligand binding assays but showed functional selectivity in pharmacological assays. At M_1 muscarinic receptors in the rat superior cervical ganglion, L-689,660 was a potent (pEC_{50} 7.3 ± 0.2) full agonist in comparison with (±)-muscarine. At M_3 receptors in the guinea-pig ileum myenteric plexus-longitudinal muscle or in trachea, L-689,660 was again a potent agonist (pEC_{50} 7.5 ± 0.2 and 7.7 ± 0.3 respectively) but had a lower maximum response than carbachol. In contrast L-689,660 was an antagonist at M_2 receptors in guinea-pig atria (pA_2 7.2 (95% confidence limits 7, 7.4)) and at muscarinic autoreceptors in rat hippocampal slices.

3 The putative M_1-selective muscarinic agonist, AF102B (*cis*-2-methylspiro-(1,3-oxathiolane 5,3′)-quinuclidine hydrochloride) was found to have a profile similar to L-689,660 but had up to 100 times less affinity in binding and functional assays. RS-86 (2-ethyl-8-methyl-2,8-diazospiro[4,5]decan 1,3-dione hydrochloride) also had lower affinity than L-689,660, and had no binding selectivity for muscarinic receptor subtypes. RS-86 had a higher NMS/Oxo-M ratio than L-689,660 and was a full agonist at M_1, M_2 and M_3 receptors in the functional pharmacological assays.

4 The functional selectivity of L-689,660 in muscarinic pharmacological assays is consistent with the effects of a low efficacy partial agonist in tissues with different effective receptor reserves.

Keywords: Cholinomimetic; muscarinic receptor; low efficacy; functional selectivity; M_1 receptor agonist; M_3 receptor agonist; M_2 receptor antagonist

Introduction

Therapeutic approaches that aim to provide symptomatic relief in Alzheimer's disease have been directed by reports of specific cholinergic deficits in brain tissue when patients diagnosed as having Alzheimer's dementia were examined post-mortem (Davis & Maloney, 1976; Perry *et al.*, 1977). These observations gave rise to the cholinergic hypothesis of dementia (Bartus *et al.*, 1982) and the suggestion that cholinergic replacement therapy would provide relief from the deficits in cognition and memory function that occur in this disorder (Bartus *et al.*, 1985; Perry, 1986). The two most widely studied approaches to improvement of cholinergic function are the use of acetylcholinesterase inhibitors and a replacement strategy using directly acting cholinomimetic drugs (Hollander *et al.*, 1986; Gray *et al.*, 1989). Clinical trials with cholinergic replacement therapy have, however, been generally disappointing, often because of the incidence of side-effects at potential therapeutic doses.

Previous studies from our laboratories have described a series of azabicyclic oxadiazole compounds that are centrally active non-selective full muscarinic agonists (Freedman *et al.*, 1990). The use *in vivo* of such non-selective compounds that have high intrinsic activity is associated with a range of peripheral and central side effects, especially through activation of muscarinic receptors within cardiovascular systems (Pazos *et al.*, 1986; Sapru, 1989) and this prevented their use in the clinic.

Three distinct muscarinic receptor subtypes M_1, M_2 and M_3 have been distinguished from binding and functional studies (Hulme *et al.*, 1990) and these have been shown to have similar binding properties to the m1, m2 and m3 muscarinic receptors expressed in CHO cells transfected with the corresponding genes (Buckley *et al.*, 1989). Using *in situ* hybridization techniques these muscarinic receptor subtypes have been shown to be localized in discrete brain regions associated with distinct CNS functions. High levels of m1 and m3 receptor mRNA transcripts have been demonstrated in rat cortex and hippocampus (Brann *et al.*, 1988) whereas m2 transcripts appeared much rarer and were shown in the basal forebrain, thalamus and hindbrain (Buckley *et al.*, 1988). Since the cognitive and memory effects of muscarinic agents are thought to be localized to the cortex and hippocampus (Ridley *et al.*, 1985; 1986; 1989) and M_1 muscarinic receptors to be relatively preserved in Alzheimer's disease (Probst *et al.*, 1988; Araujo *et al.*, 1988), it was postulated that agonist drugs selective for this subtype may be of value in cognitive disorders as side-effects mediated by other muscarinic receptor subtypes would be minimized (Gray *et al.*, 1989; Quirion *et al.*, 1989).

Agents that are truly selective agonists at particular muscarinic receptor subtypes have not yet been reported. However, as Kenakin (1986) proposed, it may be possible to achieve functional receptor subtype selectivity by use of non-selective low efficacy agonists. These compounds could

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discriminate between target tissues and others, that could potentially be the sites of mediation for dose limiting side effects, on the basis of their effective receptor reserve.

The present studies describe the affinity for muscarinic binding sites and the pharmacological characterization of a novel low efficacy cholinomimetic, L-689,660 (1-azabicyclo[2.2.2]octane, 3-(6-chloropyrazinyl)maleate) (Baker *et al.*, 1991).

L-689,660 has been studied in muscarinic radioligand binding assays in the cerebral cortex and in assays that determine functional muscarinic receptor subtype selectivity. In the cortical binding assays, [^{3}H]-oxotremorine-M (Oxo-M) was used to label the high-affinity state of the muscarinic receptor in rat cerebral cortex and [^{3}H]-N-methylscopolamine (NMS) to label predominantly the low affinity state. Agonists such as carbachol recognise preferentially the high-affinity state of the receptor and so display high affinity in the agonist binding assay whereas antagonists such as atropine show similar affinity in both assays. The NMS/Oxo-M ratio has been shown previously to be correlated directly with the ability of compounds to stimulate phosphatidyl inositol turnover in the cerebral cortex and has been proposed for use as an index of efficacy at cortical muscarinic receptors (Freedman *et al.*, 1988; 1990).

The functional pharmacology of L-689,660 has been assessed at muscarinic receptors in the rat superior cervical ganglion (M_1), guinea-pig atria (M_2), guinea-pig ileum, myenteric plexus-longitudinal muscle (M_3) and guinea-pig trachea (M_3). The profile of L-689,660 has been compared with that of the putative M_1-selective muscarinic agonist AF102B (*cis*-2-methylspiro-(1,3-oxathiolane 5,3')-quinuclidine hydrochloride) (Fisher *et al.*, 1989), and of the non-selective muscarinic agonist RS-86 (2-ethyl-8-methyl-2,8-diazospiro [4,5]decan 1, 3-dione hydrochloride) (Palacios *et al.*, 1986). A preliminary account of these studies has been given to the British Pharmacological Society (Hargreaves *et al.*, 1991).

Methods

Receptor binding studies in vitro

[^{3}H]-NMS/[^{3}H]-Oxo-M binding ratio The preparation of a rat cerebral cortex membrane fraction and the assay conditions for [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M) radioligand binding studies have been described previously in detail (Freedman *et al.*, 1990). Binding parameters were determined by fitting a single site model to the data by a non-linear least squares regression analysis and an iterative procedure in the RS1 software package (BBN Software Products Corporation Cambridge MA).

Binding selectivity at subtypes of muscarinic receptors

Rat cerebral cortex (M_1) A crude preparation of cortical membranes was made by homogenizing the cerebral cortex from Sprague-Dawley rats (500 r.p.m. 10 strokes) in modified Krebs solution buffer (pH 7.4) with HEPES (composition, mM:NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 5, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11, HEPES, 20). The resulting homogenate was then diluted with the same buffer containing 100 μM Gpp(NH)p to a final concentration of 0.55% wet w/v. The membranes were preincubated for 10 min at 30°C to remove endogenous acetylcholine. Muscarinic binding was determined in 750 μl of homogenate (4.17 mg tissue) and 1 nM N-methyl-[^{3}H]-pirenzepine in a final assay volume of 1 ml. Non-specific binding was defined with 2 μM atropine. Incubation with radiolabel was for 60 min at 30°C and assays were terminated by filtering the membranes using a Brandel Cell Harvester onto GB/B filters pre-soaked in 0.1% polyethyleneimine. Samples were washed twice in 10 ml ice cold 0.9% w/v saline. Filters were then placed in 10 ml scintillation fluid and radioactivity estimated by liquid scintillation spectrophotometry. Data from this assay (and the M_2 and M_3 assay detailed below) were analysed in the manner described above for [^{3}H]-NMS and [^{3}H]-Oxo-M.

Rat heart (M_2) Sprague-Dawley rat hearts were perfused *in situ* with 10 ml of ice-cold modified Krebs solution buffer with HEPES (pH 7.4), cleared of connective tissue, removed and cut into pieces. The cardiac tissue was then transferred into plastic centrifuge tubes with 10 ml buffer containing 100 μM Gpp(NH)p. The tissue was disrupted by ultrasound (Polytron 2 × 20 s) and the resulting suspension homogenized (500 r.p.m., 20 strokes) and filtered coarsely by passing it through two layers of cheese cloth. This crude preparation of heart membranes was then preincubated for 10 min at 30°C and resuspended in buffer at a concentration of 0.83% wet w/v. Muscarinic binding was determined in 750 μl of homogenate (6.25 mg tissue) and [^{3}H]-NMS (0.1 nM) in the final assay volume of 1 ml. Incubation with radiolabel was for 60 min at 30°C. Non-specific binding was defined with 2 μM atropine. Assays were terminated and the samples washed and counted for radioactivity as before.

Rat lachrymal gland (M_3) Lachrymal glands were removed from male Sprague-Dawley rats, chopped with scissors, suspended in 10 ml modified Krebs solution buffer with HEPES (pH 7.4) and disrupted by ultrasound as described above. This crude preparation of lachrymal membranes was then homogenized and filtered as described for heart before centrifugation at 35 000 *g* for 20 min. The membrane pellet was resuspended in buffer containing 100 μM Gpp(NH)p at 0.05% wet w/v. Binding was determined in 750 μl homogenate (3.75 mg tissue) and [^{3}H]-NMS (0.1 nM) in a final assay volume of 1 ml. Incubation with radiolabel was for 60 min at 30°C. Non-specific binding was defined with 2 μM atropine. Assays were terminated and the samples washed and counted for radioactivity as before.

Functional pharmacological assays

In all pharmacological assays the drug effects on each tissue preparation have been normalized by relating the responses to drug to the effects of a reference compound as internal standard. In the rat superior cervical ganglion preparation (±)-muscarine was used as the standard to exclude nicotinic effects, whilst in the atria, ileum, and trachea, carbachol was used as the reference compound.

Rat superior cervical ganglion Superior cervical ganglia from male Sprague-Dawley rats were set up as described previously by Newberry & Priestley (1987) as an assay for activity at M_1 muscarinic receptors. Ganglia were excised, desheathed and submerged in a three compartment bath with the ganglion in the central compartment and the pre- and postganglionic nerve trunks projecting through greased gaps into the outer compartments. Each compartment contained 0.5 ml bathing medium (composition mM: NaCl 125, KCl 5, KH_2PO_4 1, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 25, glucose 10) equilibrated with 5% CO_2/95% O_2. The central compartment was continuously perfused with gassed medium at a temperature of 25°C and a flow rate of 1–2 ml min^{-1}. Potential difference between the ganglion body and the internal carotid nerve was recorded across the greased gap with Ag/AgCl electrodes and monitored by use of a d.c. amplifier connected to a chart recorder. Before determining the concentration-response relationship to the test compounds, a reproducible response to a superfusion of 1 μM (±)-muscarine chloride was obtained on each ganglion. The test compounds were then superfused at increasing concentrations for a 1 min period at 10 min intervals. Since the response did not return to baseline during the 9 min wash period, calculations were made from the extrapolated baseline. Responses were related to the depolarizing response to the 1 μM (±)-muscarine dose that was given an arbitrary value of 100.

Guinea-pig isolated tissues Isolated tissues (atria, ileum longitudinal muscle-myenteric plexus strips, spiral cut tracheal strips) were obtained from male Dunkin-Hartley guinea-pigs and used in assays for pharmacological activity at M_2 and M_3 muscarinic receptors. Unless otherwise stated, tissues were mounted for isometric tension recording (Dynamometer UFI transducer, Pioden Controls Ltd) in 3 ml siliconized organ baths containing Krebs Henseleit solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11) and gassed with 95% O_2 and 5% CO_2. The bathing solution for the atria contained 22 mM glucose. The temperature of the bathing medium was 30°C for the atrial and 37°C for the ileum and tracheal preparations. Tissues were allowed to equilibrate under a tension of 1 g for at least 1 h before exposure to test compounds and during this time were washed continuously by overflow of bathing medium.

The isolated atria used as the M_2 assay, were allowed to beat spontaneously during the equilibration period before being paced by electrical field stimulation (3–4 Hz frequency, pulse duration 2–3 ms) at supramaximal voltage using platinum electrodes. Pulses were given a biphasic component by placing a 2 μF condenser in series. On all atrial preparations, non-cumulative concentration-response curves were first constructed to carbachol, allowing exposure to any one application until the maximum negative inotropic effect was observed, before examining the activity of the test compounds.

The longitudinal muscle-myenteric plexus preparation and spiral strips of trachea were first exposed sequentially to increasing concentrations of carbachol. Dose cycles of 10–12 min were used. The contractile responses to the agonist were allowed to reach a clear peak (2–3 min), as shown by a sustained fall in tension from the maximum observed, before washout.

In all preparations to determine agonist potency, the initial concentration-response curve to carbachol was followed by a concentration-response curve to the test compound. A period of at least 45 min was allowed between the curves. With this protocol the initial carbachol concentration-response curve could be reproduced when repeated 45 min later in the tissues in which it was used as the standard agonist. Antagonist activity was examined after the initial concentration-response curve to carbachol by equilibration of the tissues with test compound for at least 30 min before repetition of the carbachol concentration-response curve in the continuing presence of antagonist.

Calculations

Agonist potency was determined by fitting the equation $Y = Y_{max}/1 + (EC_{50}/\text{agonist concentration})^{nH})$ to concentration-response curves by non-linear least squares regression analysis and an iterative procedure in RS1 software. In this equation EC_{50} is the concentration required to evoke a half maximal response (Y_{max}) and nH is the Hill coefficient. Potency was then expressed as the negative $\log_{10}$ EC_{50} (pEC_{50}) and is given for each agonist as the mean ± s.e.mean of the pEC_{50}'s in the preparations used. The activity of the compounds was also assessed by comparing the maximum response produced by the test compound to the maximum produced by the appropriate standard agonist ((±)-muscarine or carbachol) on the same preparation (relative maximum, RM × 100%). Data are given for each agonist as the mean ± s.e.mean of the RM's in the preparations used.

Antagonism was assessed from the degree of rightward shift of the log concentration-response curves to carbachol observed in the presence of antagonist with respect to the control curve (concentration-ratio CR). The CR was calculated at the response level at the EC_{50} in the control curve. Antagonist activity (pA_2) was obtained by the line of best fit (least squares – RS1 software) to the Schild regression (Arunlakshana & Schild, 1959) using four or more observations at each of four or more concentrations of antagonist.

Acetylcholine release from rat hippocampal slices

The methodology used has been described in detail by Nordstrom & Bartfai (1980). Coronal slices (400 μm) of rat hippocampus were prepared with a McIlwain chopper then loaded with 0.1 μM [^{3}H]-choline for 30 min in Krebs buffer (composition mM: NaCl 135, KCl 5, $CaCl_2$ 1.3, KH_2PO_4 1.25, $MgSO_4$ 1, $NaHCO_3$ 25, glucose 10) at 37°C. The slices were then washed 3 times with an equal volume of buffer and transferred to a superfusion apparatus. Slices were allowed to equilibrate at a superfusion rate of 1 ml min^{-1} for 50 min and then given two separate periods of field electrical stimulation (2 min, frequency 3 Hz, current 22 mA, pulse duration 2 ms) with platinum electrodes at 60 and 105 min after the start of superfusion. The superfusion buffer was Krebs containing 10 μM of both hemicholinium and physostigmine. Drugs were introduced into the superfusate 20 min before the second period of electrical stimulation. The effects of the drugs were assessed in terms of (S_2/S_1), the ratio of acetylcholine release in the presence of drug (S_2) to that in its absence (S_1).

Materials

Radioligands were purchased from New England Nuclear ([^{3}H]-N-methylscopolamine, NET 636, 70–87 Ci $mmol^{-1}$, methyl[^{3}H]-oxotremorine-M, NET 671 70–90 Ci $mmol^{-1}$; N-methyl-[^{3}H]-pirenzepine, NET 780, 70–87 Ci $mmol^{-1}$; [^{3}H]-choline chloride, 77.9 Ci $mmol^{-1}$). L-689,660 (1-azabicyclo [2.2.2]octane, 3-(6-chloropyrazinyl)maleate), AF102B (*cis*-2-methylspiro-(1,3,oxatriolane 5,3')-quinuclidine hydrochloride) and RS-86 (2-ethyl 8-methyl-2,8-diazaspiro [4,5]decan 1,3-dione hydrochloride) were synthesized in the Department of Chemistry at Merck Sharp and Dohme, Terlings Park, Harlow. All other compounds were obtained from Sigma Chemical Co.

Results

Receptor binding studies

Table 1 shows the binding profiles of L-689,660, AF102B and RS-86 in the NMS and Oxo-M binding assays in rat cerebral cortex. Data for carbachol, arecoline and atropine (Freedman *et al.*, 1990) are shown for comparison. The results indicate that L-689,660 had 100 fold higher affinity

Table 1 Binding profiles in N-methylscopolamine (NMS)/oxotremorine-M (Oxo-M) assays in rat cerebral cortex

Compound	*[^{3}H]-NMS* (pK_D(app) M)	*[^{3}H]-Oxo-M* (pK_D(app) M)	*NMS/Oxo-M ratio*
Carbachol*	4.66 (0.05)	8.32 (0.12)	4600
Arecoline*	5.21 (0.01)	7.97 (0.04)	580
Atropine*	9.00 (0.03)	9.32 (0.08)	2.1
RS-86	5.32 (0.05)	7.40 (0.07)	120
AF102B	5.51 (0.01)	7.11 (0.05)	40
L-689,660	7.42 (0.02)	8.95 (0.06)	34

Results are given as the $-\log_{10}$ of the apparent affinity constant (pK_D(app)) that has been corrected for ligand occupancy using the Cheng & Prusoff (1973) equation. The values given are arithmetic means of at least 3 separate determinations. Numbers in parentheses indicate the s.e.mean. Inhibition studies were carried out with 0.1 nM [^{3}H]-N-methylscopolamine and 3 nM [^{3}H]-oxotremorine-M. *Data from Freedman *et al.*, 1990.

than AF102B and RS-86 at cortical muscarinic receptors. The similarity of the NMS/Oxo-M ratios for L-689,660 and AF102B predicts that these compounds will have similar activity at cortical muscarinic receptors. The NMS/Oxo-M values for L-689,660 and AF102B are low compared to carbachol and arecoline. Low NMS/Oxo-M values are a characteristic of muscarinic agonists with low efficacy in the cerebral cortex (Freedman *et al.*, 1988). The NMS/Oxo-M ratio for RS-86 is intermediate in value suggesting that it will have greater activity at cortical muscarinic receptors than L-689,660 or AF102B. The NMS/Oxo-M ratio for RS-86 is similar to that reported previously for pilocarpine (Freedman *et al.*, 1988).

Muscarinic receptor subtype selectivity The binding selectivity of L-689,660, AF102B and RS-86 for cortical M_1, cardiac M_2 and glandular M_3 muscarinic receptors is shown in Table 2. The studies measured the low-affinity state of each receptor by the inclusion of 100 μM Gpp(NH)p in all assays. Reference data from concurrent experiments with the selective muscarinic antagonist pirenzepine and the muscarinic agonist McN-A-343 are shown for comparison.

L-689,660 displaced binding at all three muscarinic receptor subtypes with high affinity and showed slight selectivity (5 fold) for cortical M_1 muscarinic receptors, compared with cardiac or glandular muscarinic receptors; AF102B and RS-86 had much lower affinity (100 to 200 fold less) at muscarinic receptors than L-689,660, and less selectivity for the M_1 receptor subtype.

Pharmacological assays Cumulative concentration-depolarization curves for the effects of L-689,660, AF102B and RS-86 on M_1 muscarinic receptors in the rat superior cervical ganglion are shown in Figure 1. L-689,660 was a potent agonist with a maximum response equal to that to muscarine. The maximum responses to AF102B and RS-86 were also similar to those to muscarine but they were 200 fold and 20 fold less potent than L-689,660 respectively. The depolarizing responses to near maximum doses of L-689,660 (0.3 μM), RS-86 (3 μM) and AF102B (100 μM) were completely blocked by pretreatment with 0.1 μM pirenzepine (data not shown).

Figure 2 shows that RS-86 had full agonist activity at M_2 muscarinic receptors in guinea-pig atria and that weak agonist activity was detected with AF102B in this preparation. In contrast, L-689,660 had little agonist activity (maximum $<15\%$ of the maximum to carbachol at 10 μM). L-689,660 antagonized the responses to carbachol producing parallel shifts to the right of the concentration-response curve. The slope of the Schild regression was not significantly different from unity and this is consistent with a competitive interaction at M_2 atrial muscarinic receptors (Figure 3a). The pA_2 for L-689,660 was 7.2 indicating an affinity at M_2 muscarinic receptors that is commensurate with the binding data (Tables 1 and 2).

AF102B was shown also to antagonize the responses of the atria to carbachol. A Schild regression was used to explore

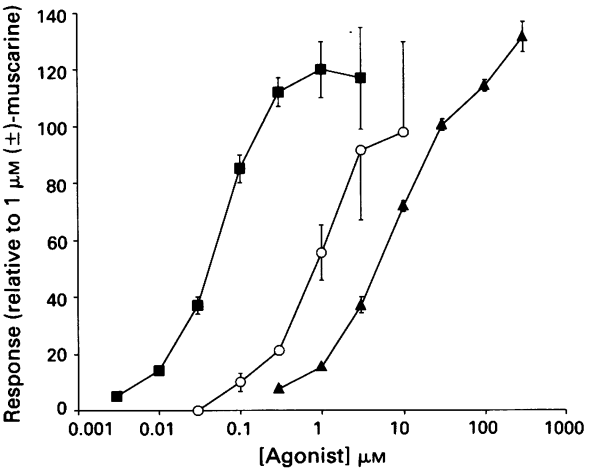

Figure 1 Depolarizing action of L-689,660, AF102B and RS-86 at M_1 muscarinic receptors in rat superior cervical ganglion. Cumulative log concentration-response curves for the effects of L-689,660 (■), AF102B (▲) and RS-86 (○) in the rat superior cervical ganglion. Responses are expressed relative to the response to 1 μM (±)-muscarine. Each point is the mean of four to six observations; s.e.mean shown by vertical bars.

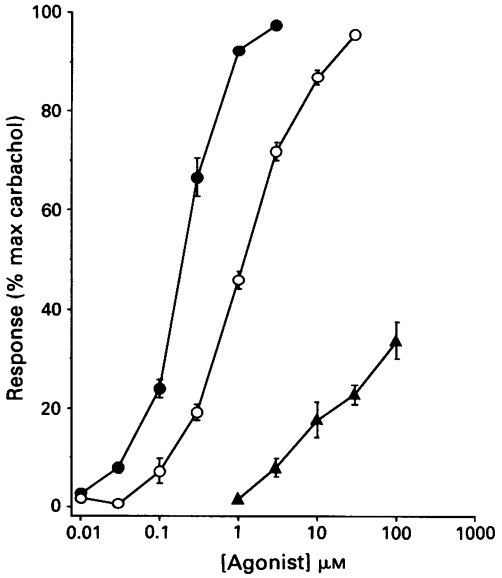

Figure 2 Agonist action of RS-86 and AF102B at M_2 muscarinic receptors in guinea-pig atria. Log concentration-response curves for the negative inotropic effects of carbachol (●), RS-86 (○) and AF102B (▲) on guinea-pig isolated atria. Responses are expressed in terms of the maximum response to carbachol in individual preparations. Each point is the mean of 4–6 separate observations; s.e.mean shown by vertical bars.

Table 2 Selectivity of L-689,660, AF102B, RS-86, McN-A-343 and pirenzepine for muscarinic receptor subtypes in rat tissues

Compound	*Cerebral cortex* M_1 (pK_D(app) M)	*Heart* M_2 (pK_D(app) M)	*Lachrymal gland* M_3 (pK_D(app) M)	*Selectivity* M_2/M_1	M_3/M_1
Pirenzepine	7.82 (0.03)	6.20 (0.05)	6.68 (0.06)	42	14
McN-A-343	5.26 (0.14)	4.39 (0.16)	4.85 (0.15)	7.4	2.6
L-689,660	7.68 (0.04)	6.96 (0.11)	7.04 (0.01)	5.2	4.4
AF102B	5.49 (0.11)	5.04 (0.02)	5.04 (0.07)	2.8	2.8
RS-86	5.41 (0.08)	4.81 (0.13)	5.51	4.0	0.8

Results are expressed as the $-\log_{10}$ of the apparent affinity constant (pK_d(app)) that has been corrected for ligand occupancy by use of the Cheng & Prusoff (1973) equation. The values are arithmetic means of at least 3 independent determinations except RS-86 in the M_3 assay. Numbers in parentheses indicate the s.e.mean.

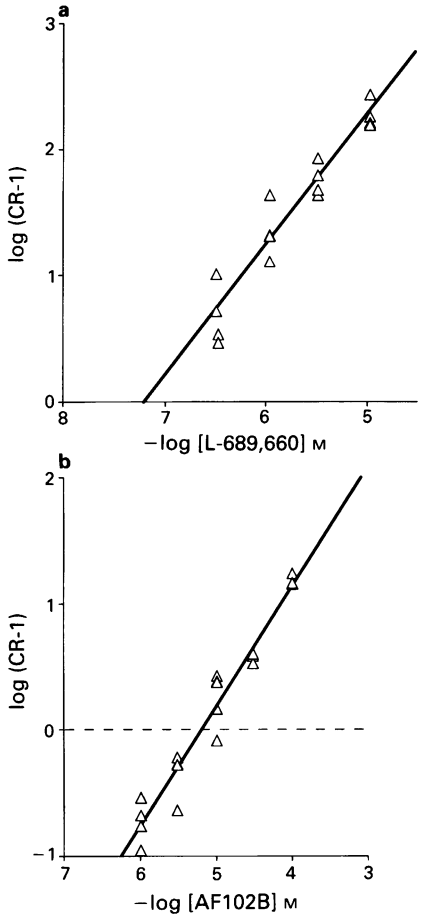

Figure 3 Antagonist action of (a) L-689,660 and (b) AF102B at M_2 muscarinic receptors in guinea-pig atria. Schild plot for the antagonism of carbachol by L-689,660 (△) or AF102B (△) in guinea-pig atria. Points are individual observations for log (CR − 1), $n = 4$ at each concentration. The concentration-ratio was calculated at the EC_{50} as a measure of the rightward shift of the log dose-response curve to carbachol by L-689,660 or AF102B. The lines are the best fit (least squares) to the data points and in neither case is the slope significantly different from unity.

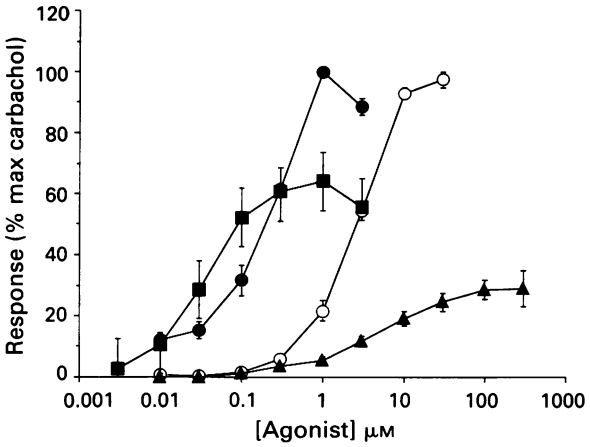

Figure 4 Agonist action of L-689,660, AF102B and RS-86 at M_3 muscarinic receptors in guinea-pig myenteric plexus-longitudinal muscle preparation. Log concentration-response curves for contractions of the ileum to carbachol (●), L-689,660 (■), AF102B (▲) and RS-86 (○). Each point is the mean for RS-86 ($n = 6$), AF102B ($n = 10$) and L-689,660 ($n = 4$); vertical bars show s.e.mean.

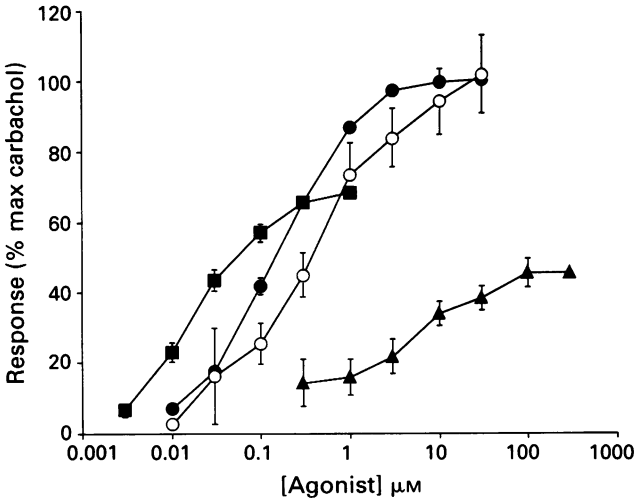

Figure 5 Agonist action of L-689,660, AF102B and RS-86 at M_3 receptors in guinea-pig trachea. Log concentration-response curves for contractions of the trachea spiral strip to carbachol (●), L-689,660 (■), AF102B (▲) and RS-86 (○). Responses are expressed in terms of the maximum response to carbachol in individual preparations. Each point is mean; L-689,660 ($n = 5$), RS-86 ($n = 4$), AF102B ($n = 7$); vertical bars show s.e.mean.

this interaction (see Discussion) and gave a line of best fit that had a slope that was not significantly different from unity. This analysis yielded an apparent pA_2 of 5.2 indicative of an affinity at M_2 muscarinic receptors some 100 times lower than L-689,660 (Figure 3b) in line with the binding data (Tables 1 and 2).

Figure 4 compares the agonist action of L-689,660, AF102B and RS-86 at M_3 muscarinic receptors in the guinea-pig ileum myenteric plexus-longitudinal muscle preparation. In this preparation L-689,660, was a potent agonist although its maximum response was less than could be obtained with carbachol. AF102B was again some 200 times less potent than L-689,660 and its maximum response was lower still than L-689,660. RS-86 was a full agonist with respect to carbachol but was less potent than L-689,660. In the ileum, as in the atria, antagonism of carbachol-induced contractions could be demonstrated with AF102B. Schild analysis (see Discussion) gave a line of best fit with slope of 0.5 and an apparent pA_2 of 5.5 suggestive of low affinity at M_3 muscarinic receptors (data not shown).

The agonist action of L-689,660, AF102B and RS-86 at M_3 receptors in the trachea is shown in Figure 5. The potency and maximum response relative to carbachol of L-689,660 on the trachea were similar to its agonist activity on the guinea-pig ileum. AF102B was again 100 times less potent than L-689,660 and had a lower maximum response. RS-86 was a full agonist at M_3 muscarinic receptors in this tissue although it was more potent than in the ileum.

The effects of L-689,660 or atropine on the release of acetylcholine from rat hippocampal slices is shown in Figure 6. L-689,660 and atropine caused a concentration-dependent increase in electrically-stimulated acetylcholine release from brain slices. Neither compound showed any effect on the basal release of acetylcholine. In the presence of an acetylcholinesterase inhibitor, acetylcholine released by electrical field stimulation is thought to interact with a terminal M_2 muscarinic autoreceptor (Richards, 1990) to reduce release and this is attenuated by muscarinic autoreceptor antagonists (James & Cubeddu, 1984). The present results therefore indicate that L-689,660 has antagonist properties at the muscarinic M_2 autoreceptor in the hippocampus in agreement with the activity detected in the guinea pig atria.

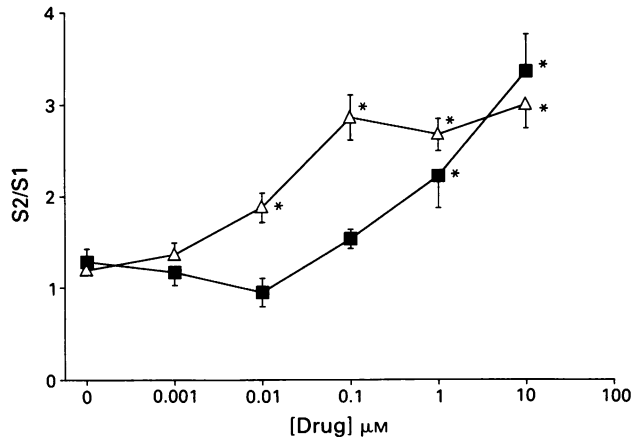

Figure 6 The effects of L-689,660 or atropine on the release of acetycholine from rat hippocampal slices. The actions of L-689,660 (■) and atropine (Δ) are shown as the ratio of electrically evoked acetylcholine release in the presence (S_2) and absence (S_1) of test compound. Results for S_2/S_1 are mean (± s.e mean vertical bars) for atropine ($n = 3-9$) or L-689,660 ($n = 5$). Points of significance are with respect to control period with no added drug ($P < 0.05$, paired t-test).

Discussion

L-689,660 had high affinity for muscarinic receptor binding sites in the rat cerebral cortex (pK_D (apparent) = 7.42). The NMS/Oxo-M ratio for L-689,660 is intermediate in value between that found for pilocarpine (ratio 100) and pirenzepine (ratio 2.2) in our earlier experiments (Freedman *et al.*, 1988). In studies on cortical phosphatidyl inositol turnover in rat cerebral cortex (Freedman *et al.*, 1988), pilocarpine was a partial agonist (pEC_{50} 5.23 with a maximum response relative to 1 mM carbachol of 11%) whilst pirenzepine was an antagonist (pIC_{50} 6.8). L-689,660 would therefore be expected to be a weak partial agonist with only small effects, if any, on phosphatidyl-inositol turnover in the cerebral cortex.

The binding assay values for pirenzepine at M_1, M_2 and M_3 receptors were in excellent agreement with the average affinity constants collated from the literature by Hulme *et al.* (1990) for binding studies using the same mammalian tissues as in the present experiments. The values also agree well, given likely differences in assay conditions, with the affinity of pirenzepine for cloned muscarinic m1, m2 and m3 receptors obtained by transfecting CHO cells with the appropriate muscarinic receptor gene (Buckley *et al.*, 1989).

Despite having no true selectivity for any particular muscarinic receptor subtype in the binding assays, L-689,660 was selective in functional pharmacological assays for tissues containing M_1 and M_3 muscarinic receptors (Table 3). This selectivity presumably reflects the low intrinsic activity of L-689,660 that confers an ability to exploit differences in effective muscarinic receptor reserve (Kenakin, 1986) between the preparations used in the functional studies. However, a further possibility that is not discounted by the present data is that the intrinsic activity of L-689,660 actually differs at the various muscarinic receptor subtypes.

The phenomenon of functional selectivity on the basis of effective receptor reserve was highlighted previously by Eglen & Whiting (1986) in their consideration of the pharmacology of the low efficacy muscarinic agonist McN-A-343. McN-A-343 was shown previously to have an NMS/Oxo-M ratio of 120 predictive of a partial agonist, and to elicit only modest (11%) increases in phosphatidyl inositol turnover in rat cerebral cortex (Freedman *et al.*, 1988). The present observations on L-689,660 together with those on McN-A-343 continue to support the hypothesis (Kenakin, 1986) that apparent receptor site specificity can be obtained with low efficacy partial agonist compounds. The findings also highlight the difficulty of using data from agonists, particularly those with low efficacy, as criteria in receptor classification since the relative potencies in different tissues are dependent critically upon the effective receptor reserve.

Previous studies have demonstrated a pharmacological profile similar to L-689,660 with an oxotremorine analogue, BM-5 (N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)acetamide), that was a low efficacy muscarinic agonist with no true muscarinic receptor subtype selectivity (Hawkins *et al.*, 1992). In the guinea-pig ileum, BM-5 was found to antagonize presynaptic muscarinic autoreceptors, but to be an agonist, with lower efficacy than carbachol, at postsynaptic muscarinic sites (Nordstrom *et al.*, 1983). Similar observations have been reported with other oxotremorine-based muscarinic partial agonists that stimulated guinea-pig ileum and blocked muscarinic responses in guinea-pig bladder (Ringdahl, 1987a,b; Ringdahl & Markovicz, 1987). The profile of L-689,660 in the present functional pharmacological assays suggests that the effective muscarinic receptor reserve in the various assay tissues used has the order: rat superior cervical ganglion > guinea-pig ileum = trachea > guinea-pig atria and hippocampal presynaptic receptors.

The present binding results failed to confirm the M_1 muscarinic receptor selectivity claimed (Fisher *et al.*, 1989) for AF102B (Table 2). Despite the much lower potency of AF102B compared to L-689,660, the similarity of their NMS/Oxo-M ratios (Table 1) predicts that these compounds would

Table 3 Summary of the effects of L-689,660, AF102B and RS-86 in functional pharmacological assays

Assay	*L-689,660* (R-enantiomer)	*AF102B* (Racemic)	*RS-86* (Achiral)
M_1 Rat ganglion (pirenzepine-sensitive depolarization)			
pEC_{50}[a]	7.3 ± 0.1	5.1 ± 0.1	6.0 ± 0.2
RM(%)[b]	120 ± 10	130 ± 6	100 ± 24
M_2 Guinea-pig paced atria (negative inotropy)			
pA_2[c]	7.2 (7,7.4)	5.2 (5,5.3)	–
pEC_{50}	–	5.0 ± 0.6	6.0 ± 0.1
RM(%)[d]	–	34 ± 4	98 ± 3
M_3 Guinea-pig ileum (contraction)			
pA_2		5.5 (5.3, 5.6)[e]	
pEC_{50}	7.5 ± 0.2	5.3 ± 0.3	5.6 ± 0.1
RM(%)[d]	64 ± 8	29 ± 5	98 ± 3
M_3 Guinea-pig trachea (contraction)			
pEC_{50}	7.7 ± 0.3	5.4 ± 0.7	6.4 ± 0.2
RM(%)[d]	65 ± 3	44 ± 5	100 ± 10

[a]pEC_{50} = -log EC_{50} ± s.e.mean; [b]relative to the depolarizing responses to 1 μM (±)-muscarine; [c]pA_2 (95% confidence limits); [d]relative to the maximum response of carbachol; [e]apparent pA_2 (slope 0.5).

have similar activity at muscarinic receptors and therefore that their muscarinic activity profiles in isolated tissues would be the same. AF102B was found to be an agonist in M_1 and M_3 assays (confirmed recently by Boddeke & Buttini, 1991) and to antagonize the effects of carbachol on the atria (Table 3).

The weak agonist activity of AF102B detected in the atria complicated assessment of its antagonist profile at these M_2 muscarinic receptors. It is acknowledged that the underlying assumption of the Schild analysis that equal response to agonist in the presence and absence of antagonist represents equal receptor occupancy by the agonist are not strictly met when assessing a partial agonist. Nonetheless, the responses to the addition of the full agonist, carbachol, in the presence of increasing concentrations of AF102B were shifted to the right in a parallel fashion. Plots of the CR − 1 (concentration-ratio − 1) against AF102B concentration yielded graphs that were best fitted by a line with a slope not significantly different from unity, suggesting a competitive interaction (Figure 3b). The apparent pA_2 for AF102B estimated by this analysis was 5.2 indicating an apparent affinity that was commensurate with the binding data for AF102B (Tables 1 and 2). The apparent affinity of AF102B is therefore 100 times lower than L-689,660 ($pA_2 = 7.2$) at M_2 muscarinic receptors in atria when antagonism in M_2 functional assays is measured by Schild analysis. This ratio of 100 is similar to the ratio of 83 for their relative affinities in the M_2 radioligand binding studies (Table 2).

Interpretation of the pharmacological profile of AF102B is further complicated by its low potency and the consequent need to use relatively high concentrations in pharmacological assays thereby incuring a risk of provoking non-specific effects. Furthermore, AF102B is a racemic mixture and the presence within the compound of two different chemical enantiomers, that may have differing agonist profiles adds to the uncertainty in the definition of its activity.

In functional pharmacological assays, RS-86 was a full agonist with similar potency at M_1, M_2 and M_3 receptor subtypes. The potency estimates for RS-86 in rat ganglia and guinea-pig ileum (Table 3) confirm earlier observations with this compound (Palacios *et al.*, 1986). The present binding studies show that RS-86 had no selectivity for muscarinic receptor subtypes and a higher NMS/Oxo-M ratio than L-689,660 (NMS/Oxo-M ratio 120 compared with 34 respectively) that is predictive of greater activity at cortical muscarinic receptors (Tables 1 and 2). Studies *in vivo* with RS-86 (Pazos *et al.*, 1986) have shown that non-selective muscarinic agonists with this level of efficacy have the capacity to induce a range of side effects at CNS-active doses particularly on the cardiovascular system (Brezenoff & Guiliano, 1982; Pazos *et al.*, 1986; Sapru, 1989; Palacios *et al.*, 1990).

The clinical value of functional receptor selectivity achieved by controlling efficacy is difficult to predict. The approach is limited by the relative agonist potency in target versus side-effect tissues being dependent upon their effective receptor reserves and these cannot be manipulated. In the early stages of Alzheimer's disease, M_1 receptors are thought to be relatively preserved (Probst *et al.*, 1988; Araujo *et al.*, 1988; Quirion *et al.*, 1989) whereas M_2 autoreceptors are lost progressively as nerve terminals degenerate (Sims *et al.*, 1985; Mash *et al.*, 1985; Araujo *et al.*, 1988; Palacios *et al.*, 1990). The effective receptor reserve in target tissues relative to others that mediate side-effects may therefore alter when pathological changes occur (Kenakin, 1990). As a result, the pharmacological profile of a partial agonist may change with the progression of a disorder.

There are a number of questions that remain to be answered *in vivo* with L-689,660. Is its efficacy set at a level that will target the abundant m1 and m3 muscarinic receptors in cortex and hippocampus (Buckley *et al.*, 1988; Brann *et al.*, 1988) but remain relatively inactive at cardiovascular M_2 (central and peripheral) and peripheral M_3 receptors that could mediate side-effects? Is there a difference *in vivo* between the apparent effective receptor reserve of postsynaptic and prejunctional M_2 receptor-mediated responses that would favour increased acetylcholine release over M_2-mediated side-effects?

Preliminary accounts of studies *in vivo* with L-689,660 indicate that the potential therapeutic window for low efficacy muscarinic agonists differs from that of higher efficacy compounds. L-689,660 has been shown to produce hypothermia and antinociception through central cholinergic mechanisms (Dawson *et al.*, 1991; Freedman *et al.*, 1991) and to have smaller cardiovascular side-effects at CNS-active doses than the higher efficacy muscarinic agonist RS-86 (Freedman *et al.*, 1991). Studies on the effects of L-689,660 in behavioural tests of long term and working memory are in progress.

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