Br. J. Pharmacol. (1990), **101**, 575–580

A novel series of non-quaternary oxadiazoles acting as full agonists at muscarinic receptors

[1]S.B. Freedman, E.A. Harley, S. Patel, N.R. Newberry, M.J. Gilbert, A.T. McKnight, J.K. Tang, J.J. Maguire, N.T. Mudunkotuwa, R. Baker, L.J. Street, A.M. MacLeod, J. Saunders & L.L. Iversen

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

1 A novel series of non-quaternary oxadiazole-based muscarinic agonists demonstrated high affinity for muscarinic receptors.

2 These agonists possessed high efficacy in the nanomolar range at muscarinic receptors in the superior cervical ganglion, atrium and ileum but did not show selectivity across the tissue preparations.

3 Two amino oxadiazoles, one from a quinuclidine series (L-660,863) and one from a 1-azanorbornane series (L-670,207) possessed a high ratio of potency for displacing the binding of [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) to potency for displacing the agonist [^{3}H]-oxotremorine-M ([^{3}H]-oxo-M) (NMS/oxo-M ratio) predictive of high efficacy in the cortex.

4 The two azanorbornane derivatives L-670,548 and L-670,207 stimulated the turnover of phosphatidyl-inositol in the cortex with a potency higher than that obtained with any other known muscarinic agonist (ED_{50} 0.26 and 0.18 μM respectively).

5 The maximum response obtained with L-670,207 was greater than that observed for carbachol but was comparable to that of the natural ligand acetylcholine.

6 These oxadiazole muscarinic agonists are among the most potent and efficacious non-quaternary muscarinic agonists ever described.

Introduction

Muscarinic cholinoceptors are present throughout the body in many peripheral organs, and are also found throughout the central nervous system (CNS) (Eglen & Whiting, 1986; Goyal, 1989). Interest in muscarinic pharmacology has been stimulated recently by the reports of specific cholinergic deficits in brain at autopsy from patients diagnosed as having Alzheimer's disease (Perry, 1986). This has led to the suggestion that enhancement of cholinergic neurotransmission would alleviate the symptoms of the disease, including the deficits observed in cognition and memory.

One approach to therapy in Alzheimer's disease has been the development and subsequent clinical trials of a number of directly acting muscarinic receptor agonists. These include such natural agents as arecoline (Christie *et al.*, 1981) and pilocarpine (Caine, 1980), and synthetic compounds such as RS-86 (Mouradian *et al.*, 1988). Clinical trials with such agents have generally been disappointing, at least partly due to the side effects associated with muscarinic receptor stimulation. Recent attention has focused on investigating the pharmacology of muscarinic receptor subtypes as a means of overcoming these problems. At least three distinct muscarinic receptors (M_1, M_2 and M_3) have been distinguished from both functional studies and binding studies (Mutschler *et al.*, 1989; Goyal, 1989). This work was made possible by the development of selective antagonists such as pirenzepine (Hammer *et al.*, 1980), AF-DX 116 (Hammer *et al.*, 1986), methoctramine (Melchiorre *et al.*, 1987) and hexahydrosiladiphenidol (Lambrecht *et al.*, 1984) and the identification of tissues containing a response mediated predominantly by a single receptor subtype. In complementary studies using a molecular biological approach, evidence has accumulated that at least five distinct muscarinic receptors can be identified (Bonner *et al.*, 1987; 1988). Using *in situ* hybridization it has been possible to identify all of these subtypes within the CNS and to identify a selective regional localisation (Buckley *et al.*, 1988).

The coupling of muscarinic receptors has been shown to vary between regions showing linkage to a variety of secondary messenger systems: adenylate cyclase, phosphatidyl-inositol (PI) turnover and ion channels (Brown *et al.*, 1984; Ehlert, 1985). There are also considerable differences in the efficiency of the coupling. The latter phenomenon is important since even in the absence of different receptors or selective agents, a functional selectivity that is based on differences in receptor reserve in different organs may be achieved by agents that are partial agonists (Kenakin, 1986).

In the cerebral cortex, the muscarinic receptors that are coupled to phosphatidyl-inositol turnover appear to lack an effective receptor reserve. Thus full agonists such as carbachol and muscarine have relatively low potency compared with that seen in parotid gland, and partial agonists such as pilocarpine and RS-86 produce a smaller maximal response relative to carbachol (Fisher *et al.*, 1983; Freedman, 1986; Freedman *et al.*, 1988). To date the only compounds with sufficient efficacy to stimulate phosphatidyl-inositol turnover in cerebral cortex have been quaternary agonists such as carbachol, muscarine, oxotremorine-M and acetylcholine itself. Although these compounds are highly efficacious at cortical muscarinic receptors they would be expected to have only limited ability to pass through the blood brain barrier and hence would not be able to activate effectively these cortical receptors in the whole animal.

In the present study we describe a novel series of non-quaternary muscarinic agonists that includes the most efficacious and potent muscarinic agonists known. These compounds possess the ability to activate fully muscarinic receptors in a range of pharmacological preparations, and in one particular example produce a maximal stimulation of cortical PI turnover comparable to that obtained with acetylcholine itself.

Portions of this work were presented at the subtypes of muscarinic receptor meeting (IV) at Wiesbaden, 20-22 July 1989 (Saunders, J. & Freedman, S.B., 1989).

[1] Author for correspondence.

Methods

Brain membrane preparation

Crude synaptosomal-mitochondrial membranes were prepared by homogenizing cerebral cortex from rat (250–300 *g*) in 0.32 M ice-cold sucrose (1/10, w/v) in a motor-driven teflon/glass homogenizer at 500 r.p.m. (10 strokes). The homogenate was centrifuged at 1000 *g* for 15 min and the resulting supernatant centrifuged at 17,000 *g* for 20 min. This yielded the crude synaptosomal mitochondrial pellet (P_2), which was used fresh or stored at −20°C before use.

Receptor binding studies

[^{3}H]-N-methylscopolamine binding ([^{3}H]-NMS) P_2 fractions were homogenized and resuspended at a final dilution of 1/600 (wet w/v) in ice-cold Krebs-HEPES buffer pH 7.4 (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 and HEPES 20). Binding of [^{3}H]-NMS was determined with 0.01–1.0 nM ligand and non-specific binding defined with 2 μM atropine. Displacing drugs were added in a volume of 10 μl to give a final assay volume of 1.0 ml. Incubations were initiated by adding 750 μl of membrane solution and were allowed to proceed for 60 min at 30°C. Assays were terminated by filtration using a Brandell Cell Harvester and Whatman GF/B filters with 2 × 10 ml rinses in ice cold saline (0.9% NaCl, w/v). Filters were then placed in 10 ml of scintillation fluid (Hydrofluor, National Diagnostics, New Jersey) and radioactivity estimated by liquid scintillation spectrometry. Kinetic analysis has been previously reported (Freedman *et al.*, 1988) with a dissociation constant (K_D) of 0.14 ± 0.02 nM and a maximum binding capacity (B_{max}) of 1400 ± 340 fmol mg^{-1} protein ($n = 5$).

[^{3}H]-oxotremorine-M binding ([^{3}H]-oxo-M) P_2 fractions were washed by resuspending in 10 ml of 20 mM HEPES buffer pH 7.4 and centrifuged at 17,000 *g* for 20 min. The washed membranes were homogenized and resuspended at a final dilution of 1/100 (wet w/v) in ice-cold 20 mM HEPES buffer pH 7.4. Binding of [^{3}H]-oxo-M was determined by use of 0.2–5 nM of ligand and non-specific binding defined with 2 μM atropine. Displacing compounds were added in a volume of 10 μl to give a final assay volume of 1.0 ml. Incubations were initiated by adding 750 μl of membrane solution and were allowed to proceed for 40 min at 30°C. Assays were terminated by filtration over Whatman GF/C filters presoaked in 0.05% polyethyleneimine, using a Brandell Cell Harvester. Samples were washed with 10 ml of ice-cold saline and filters placed in 10 ml of scintillation fluid (Hydrofluor, National Diagnostics, New Jersey, U.S.A.) and radioactivity estimated by liquid scintillation spectrometry. Scatchard analysis indicated a dissociation constant (K_D) of 0.68 ± 0.12 nM and a maximum binding capacity of 520 ± 160 fmol mg^{-1} protein.

Binding parameters were determined by non-linear, least-squares regression analysis using RS1 (BBN Research Systems, Cambridge, Mass.) and a computerised iterative procedure written by Dr A. Richardson, NRC Terlings Park.

Phosphatidyl-inositol turnover

Tissue slices of rat cerebral cortex (350 × 350 μm) were prepared with a McIlwain tissue chopper and were washed three times in Krebs bicarbonate buffer, followed by a 30 min preincubation in the presence of [^{3}H]-myo-2-inositol, 2 μCi, (Amersham International, TRK.807 13.8 Ci mmol^{-1}) and 10 mM lithium. Tissue slices were subsequently incubated in the presence of muscarinic agonists for 45 min in a volume of 250 μl. The reaction was terminated by addition of 940 μl of chloroform/methanol (1/2 v/v) and water-soluble inositol monophosphates were isolated by ion exchange chromatography. The methods have previously been described in detail by Brown and colleagues (1984). For studies with muscarinic antagonists, tissue slices were incubated with antagonists for 15 min before addition of agonist. All test compounds were added in a volume of 10 μl. Curves were fitted to data analysed by non-linear regression using the Allfit, four parameter logistic curve fitting programme (Delean *et al.*, 1978).

Rat superior cervical ganglion

Superior cervical ganglia from male Sprague-Dawley rats were superfused *in vitro* as previously described (Newberry & Priestley, 1987). The d.c. potential between the ganglion body and the internal carotid nerve was recorded across a greased gap with Ag/AgCl electrodes. Agonists were superfused with 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. Before determining the dose-response relationship, a reproducible response to 1 μM (±)-muscarine chloride was obtained on each ganglion. Subsequent responses were all related to that depolarizing response, given an arbitrary value of 1.0. The maximum response and the concentration required to evoke half of that response (EC_{50}) were determined on a number of ganglia, each from a different rat. It should be noted that the dose-response relationship was determined by increasing the agonist concentration until the response levelled off. However, it has recently been reported (Newberry & Gilbert, 1989) that the dose-response curve to muscarine is biphasic with higher concentrations of muscarine (>3 μM) causing the dose-response curve to rise to a second peak. We have preliminary evidence indicating that the dose-response curves of these novel compounds may also be biphasic. Given that the reasons for this phenomenon are not totally clear, the relative maximum values quoted in Table 3 correspond to the point where the dose-response curve first levelled off.

Guinea-pig isolated atria

Paired atria were removed from male guinea-pigs (300–400 g weight) and suspended under 1 g tension in Krebs bicarbonate solution containing 22 mM glucose. Preparations were paced by electrical field stimulation (3–4 Hz, 2–3 ms) with platinum electrodes. Following a 60 min equilibration period, non-cumulative dose-response curves to muscarinic agonists were constructed, allowing exposure to any one application of test compound until a maximum negative chronotropic effect was obtained. A period of at least 45 min was allowed between each dose-response curve. Agonist potency (EC_{50}) and the maximum response relative to the maximum response to carbachol or muscarine were determined using RS1 (BBN Research Systems, Cambridge, Mass.) and the computerised procedure described above.

Guinea-pig ileum, longitudinal muscle – myenteric plexus preparation

Preparations of longitudinal muscle with the myenteric plexus from the distal ileum of 300–400 g male guinea-pigs were obtained as described by Rang (1964). Preparations were washed and suspended under 1 g tension in glass organ baths containing 3 ml Krebs bicarbonate solution at 37°C and allowed to equilibriate for at least 60 min. Isometric contractions to muscarinic agents were measured for at least 30 s until a clear peak of response was obtained. A period of at least 45 min was allowed between each test compound. Potency and maximum response relative to that for carbachol or muscarine were determined as for the atrium. Tissue responses were measured as changes in isometric tension in the ileum.

The responses were then calculated as a percentage of the maximum response obtained relative to a dose of 1 μM carbachol. Agonist potency (EC_{50}) was determined by a non-linear iterative curve fitting procedure.

Materials

Compounds and reagents used in this study were obtained from the following sources. RS-86 (2 ethyl 8 methyl-2, 8 diazaspiro[4,5]decan-1,3-dion hydrobromide), Sandoz Ltd. Radioligands were purchased through New England Nuclear ([^{3}H]-N-methylscopolamine, NET 636, 85–90 Ci mmol^{-1}; [^{3}H]-oxotremorine-M, NET 671, 84.9 Ci mmol^{-1}). All other compounds were obtained from Sigma (Dorset). The detailed synthesis of the novel compounds reported here is published elsewhere (Saunders *et al.*, 1990; Street *et al.*, 1990).

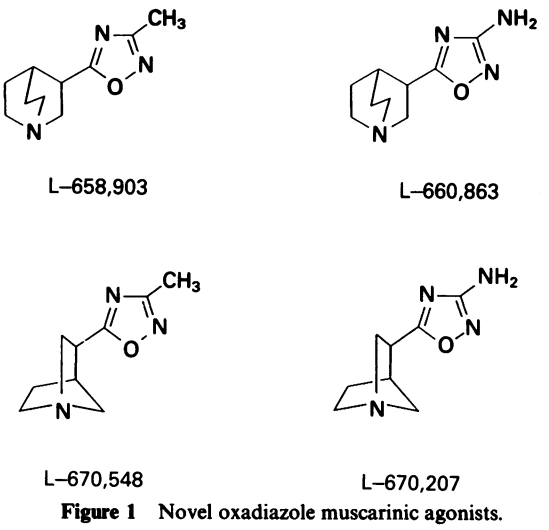

Figure 1 Novel oxadiazole muscarinic agonists.

Results

Receptor binding studies

We recently described (Freedman *et al.*, 1988) a receptor binding paradigm that can be used to measure affinity of compounds for the cortical muscarinic receptor population and also to predict their relative efficacy at cortical muscarinic receptors. This assay measures the ability of compounds to displace low concentrations of the potent muscarinic agonist [^{3}H]-oxo-M from the high affinity state of the receptor. In parallel studies the ability of these compounds to displace the non-selective antagonist [^{3}H]-NMS from the predominately low affinity states is measured. The ratio of affinities of compounds in these assays appears to correlate with the ability of compounds to stimulate the hydrolysis of phosphatidyl-inositol turnover. The results shown in Table 1 show that four broad categories of compounds can be identified. These range from the high affinity muscarinic antagonist atropine (NMS/oxo-M ratio of 2.1), the weak partial agonist pilocarpine (NMS/oxo-M ratio 100), the more efficacious partial agonist arecoline (NMS/oxo-M ratio of 560), to the full agonist carbachol (NMS/oxo-M ratio 4500).

It was previously shown in our laboratories that the ester functionality in benzodiazepines could be replaced by an oxadiazole group (Watjen *et al.*, 1989). This replacement appeared to produce an increase in the relative efficacy of benzodiazepines as measured by the relative shift in binding affinity observed in the presence and absence of GABA. We have recently shown that similarly for muscarinic agents the ester functionality of arecoline can be replaced by the oxadiazole moiety (Saunders *et al.*, 1990). In the present study four of the oxadiazole compounds have been extensively characterized. These include methyl oxadiazoles from the quinuclidine and 1-azanorbornane series, and two corresponding amino oxadiazoles (Figure 1).

Compared with the original natural agonist, arecoline, the methyl oxadiazole from the quinuclidine series, L-658,903, showed more than a 10 fold increase in affinity in the [^{3}H]-NMS assay and had a comparable NMS/oxo-M ratio (Table 1). The amino oxadiazole in this series, L-660,863, possessed similar affinity to the methyl oxadiazole but had a higher NMS/oxo-M binding ratio of 1300.

Replacement of the quinuclidine base by a 1-azanorbornane ring system produced a further 3 to 10 fold increase in binding affinity (Table 1) for the [^{3}H]-NMS binding site. Thus for the methyloxadiazole L-670,548 the apparent affinity in the [^{3}H]-NMS assay was 60 fold greater than that for arecoline, while for the amino oxadiazole (L-670,207) the affinity was 200 times that of arecoline. These compounds possess the highest binding affinity of any known muscarinic agonists; in addition both of the 1-azanorbornane derivatives display NMS/oxo-M binding ratios greater than either arecoline or pilocarpine with values of 720 and 1100 for the amino and methyl oxadiazole respectively.

Phosphatidyl-inositol turnover

Muscarinic agonists appear to show large differences in their efficacy for producing a stimulation of cortical phosphatidyl-inositol turnover (Table 2). The methyl oxadiazole L-658,903

Table 1 N-methylscopolamine/oxotremorine-M ratio in rat cerebral cortex

Compound	*[^{3}H]-NMS (K_{app} nM)*	*[^{3}H]-Oxo-M (K_{app} nM)*	*NMS/Oxo-M ratio*
Atropine	1.0 (0.94;1.1)	0.48 (0.40;0.57)	2.1
Pilocarpine	4000 (3000;5100)	40 (36;44)	100
Arecoline	6200 (4900;7800)	11 (9.7;11)	560
Carbachol	22000 (20000;24000)	4.9 (3.6;6.1)	4500
L-658,903	440 (420;450)	0.96 (0.82;1.1)	460
L-660,863	600 (550;660)	0.47 (0.40;0.54)	1300
L-670,548	100 (90;120)	0.090 (0.07;0.11)	1100
L-670,207	31 (27;36)	0.043 (0.039;0.049)	720*

Results are expressed as an apparent affinity constant (K_{app}) which has been corrected for ligand occupancy by use of the Cheng-Prussoff equation (1973). Each curve is typically 6–10 concentrations performed in triplicate. The values above are geometric means of at least 3 determinations performed on separate occasions. Numbers in parentheses indicate the range low and high error values of the geometric mean. Inhibition studies were performed with 0.1 nM [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and 3 nM [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M).

* Evidence of depletion was obtained for this compound resulting in an underestimation of the affinity of this compound (see Discussion).

Table 2 Stimulation of phosphatidyl-inositol turnover in rat cerebral cortex

	Phosphatidyl-inositol turnover	
Compound	EC_{50} (μM)	% maximum response
Pilocarpine	13 (6.6,25)	12 (11,14)
Arecoline	16 (9.4,29)	19 (6,25)
Carbachol	220 (150,330)	130 (110–170)
L-658,903	4.3 (2.4,7.6)	20 (17–21)
L-660,863	2.5 (2.1,3.1)	58 (51–66)
L-670,548	0.26 (0.21,0.32)	93 (69–120)
L-670,207	0.18 (0.15–0.22)	160 (150–170)

EC_{50}: Potency of compounds in eliciting breakdown of labelled inositol-phospholipids.
% maximum response: results have been expressed as a % of the maximum response to 1 mM carbachol included in all experiments. The maximum response was equivalent to approximately an 8–10 fold increase over unstimulated controls.
Each experimental value is calculated from between 2–5 independent determinations. Curves were fitted to data and analysed by non-linear regression using the Allfit, four parameter logistic curve fitting programme (DeLean *et al.*, 1978). The EC_{50} value was calculated from the individual maximum response of each compound. Results are expressed as the geometric mean with low and high error margin. The % maximum response is the median value (low, high range).

showed only a limited ability to stimulate phosphatidyl-inositol turnover in rat cerebral cortex and like arecoline produced a maximum response of 20% of that for carbachol. The amino oxadiazole L-660,863 which had a higher binding ratio, stimulated PI turnover dose-dependently (EC_{50} 2.5 μM) with a maximum response 58% that of carbachol (Figure 2a).

The two 1-azanorbornane derivatives were potent agonists for producing a stimulation of cortical PI turnover with EC_{50} values of 0.26 μM for the methyl oxadiazole L-670,548 and 0.18 μM for the amino oxadiazole L-670,207. These two compounds are the most potent muscarinic agonists that we have tested in this assay. The maximum response for L-670,548 was 93% of that observed with carbachol (1 mM) while the amino oxadiazole L-670,207 produced an increased response greater than that of carbachol (160%) (Figure 2b). These effects were comparable with those observed with acetylcholine, the natural ligand for the muscarinic receptor. However the potency of acetylcholine was over 100 fold weaker with an EC_{50} value of 30 μM.

All of the responses to the novel muscarinic agonists were dose-dependently blocked by atropine (1 μM) which was added 15 min before addition of the agonist. Very high concentrations of L-660,863 (1000 to 30,000 μM) produced an additional stimulation (250%) which was not completely reversed by atropine. Solubility of L-660,863 prevented detailed study of this additional component.

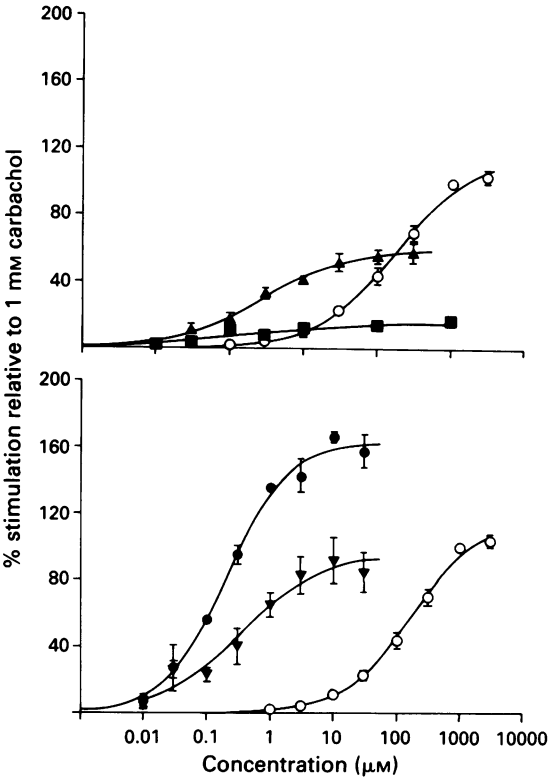

Figure 2 Effect of novel muscarinic agents upon phosphatidyl-inositol turnover in rat cerebral cortex. Methods were as described in Methods section. (a) Effects of quinuclidine derivatives L-658,903 (■) and L-660,863 (▲). (b) Effects of 1-azanorbornane derivatives L-670,548 (▼) and L-670,207 (●). Each curve is the cumulative results of three to five individual experiments, each of which was performed in triplicate. Each curve is the mean with s.e.mean shown by vertical bars. Carbachol (○) is included as a reference compound. Curves were fitted by Allfit as described previously (see Methods section).

Evaluation in pharmacological preparations

The identification of muscarinic receptor subtypes has been possible by the characterization of tissue preparations containing responses mediated by a single receptor subtype. These include the depolarizing response on the rat superior

Table 3 Pharmacological evaluation of novel oxadiazole muscarinic agonists

	Rat superior cervical ganglion M_1			*Guinea-pig atrium* M_2			*Guinea-pig ileum* M_3		
Compound	EC_{50} (nM)	RM	*n*	EC_{50} (nM)	RM	*n*	EC_{50} (nM)	RM	*n*
Muscarine	90 (70;100)	1.2 (1.0–1.8)	10	200 (150;230)	1.0 (0.9–1.1)	8	120 (110;140)	1.0 (0.9–1.1)	18
Carbachol	—	—	—	400 (280;450)	1.0 (0.9–1.1)	7	130 (110;150)	1.0 (0.9–1.1)	10
L-658,903	20 (10;30)	1.2 (0.9–1.2)	4	40 (30;50)	0.95 (0.9–1.0)	5	16 (13;19)	1.0 (0.9–1.1)	8
L-660,863	10 (9.9;2.0)	0.7 (0.5–1.1)	6	17 (14;20)	1.0 (0.9–1.1)	6	10 (80;11)	1.0 (0.9–1.1)	6
L-670,548	2.0 (1.0;3.0)	0.9 (0.9–1.2)	6	3.0 (2.0;3.4)	0.95 (0.9–1.0)	6	1.2 (1.0;1.5)	0.95 (0.9–1.0)	5
L-670,207	10 (7.0;20)	1.3 (0.6–1.6)	5	2.0 (1.0;3.0)	0.8 (0.6–0.9)	4	0.9 (0.8;1.1)	0.8 (0.75–0.85)	3

Detailed methods are described in the Methods section.
EC_{50}: EC_{50} is the concentration required to produce half of RM. Geometric mean (−s.e.mean, +s.e.mean)
RM: relative maximum i.e. amplitude of the response at which the dose-response curves exhibit a plateau relative to the amplitude of a response to 1 μM muscarine (see Methods) or to the maximum response to muscarine or carbachol (atrium and ileum) on the same preparation. Results are expressed as median (range).

cervical ganglion (mediated by M_1 receptors, Brown *et al.*, 1980; Newberry & Priestley, 1987), the negative chronotropic effect on electrically driven guinea-pig atria (mediated by M_2 receptors) and the contraction of the guinea-pig myenteric plexus preparation (mediated by M_3 receptors). The four oxadiazole derivatives were assessed for their relative potencies and efficacies at the muscarinic receptor in these preparations in order to identify any possible selectivity between the M_1, M_2 and M_3 subtypes. The results are shown in Table 3. All four compounds were similarly potent muscarinic agonists in the three pharmacological preparations with a relative maximum response that was indistinguishable from muscarine. The two quinuclidine compounds were up to 10 fold more potent than muscarine whereas the two 1-azanorbornane derivatives were up to 100 fold more potent than muscarine. None of the compounds exhibited any appreciable degree of selectivity between the pharmacological preparations.

Discussion

In cerebral cortex the muscarinic receptors coupled to PI turnover appear to lack an effective receptor reserve. Before the start of these studies it appeared that only quaternary agonists could possess the necessary efficacy to stimulate maximally cortical PI turnover. We now report on the design of non-quaternary compounds that have higher efficacy than natural non-quaternary agonists. In recent studies we have reported that the distribution of the electrostatic charge on a quaternary methylammonium group could be closely mimicked by a protonated 1-azabicyclic ring system exemplified by a quinuclidine ring system (Saunders *et al.*, 1990). This approach led to the development of L-658,903 in which affinity and potency at muscarinic receptors in cortex were increased 10 fold compared with arecoline. Removing a carbon atom from one of the bridges of the quinuclidine ring gave the target compound L-670,548 in which the correct 3 dimensional topography was retained. This derivative showed a further 3 fold increase in affinity and in potency at muscarinic receptors. These results suggested that removal of one methylene group from a bridge of the quinuclidine ring was associated with an increased ability to interact within the binding pocket in the muscarinic receptor pharmacophore.

Both of these methyl-oxadiazole analogues were potent and efficacious agonists on the pharmacological preparations with no evidence of selectivity for any of the three muscarinic receptor subtypes in the pharmacological models. These compounds are therefore amongst the most potent muscarinic agonists known and support the results from the NMS/oxo-M binding assays that suggested that the compounds were potent agonists at the muscarinic receptor in cortex. The findings from the peripheral tissues also support the view that in all three of the pharmacological preparations there is a relatively high receptor reserve for muscarinic receptor stimulation since all of the compounds had a similar maximum response relative to carbachol or muscarine. In contrast when the compounds were examined on the cortical PI model, L-658,903 was a partial agonist with a response similar to that of arecoline though with a substantial increase in potency. Replacement of the methyl oxadiazole in both series by amino oxadiazole resulted in large increases in the efficacy in both chemical series. This can be correlated with the electron donating properties of the amino group resulting in enhancement of the hydrogen bond acceptor properties of the oxadiazole ring system (Saunders *et al.*, 1990). These compounds are some of the most efficacious compounds known and are the first examples to be reported of non-quaternary muscarinic agonists able to stimulate PI turnover in rat cerebral cortex to a level seen with carbachol.

The amino oxadiazole in the 1-azanorbornane series, L-670,207, displayed significantly greater efficacy than either carbachol or muscarine in the cortical slice assay. This response to L-670,207 was entirely sensitive to antagonism by atropine and was the same as that seen with the natural ligand acetylcholine (170% relative to 1 mM carbachol). Acetylcholine was however considerably less potent with an EC_{50} value of 30 μM, even when tested in the presence of the cholinesterase inhibitor, physostigmine (1 μM). This greater relative efficacy was somewhat different from that seen in the results obtained in both the binding studies and the results in the functional studies. The very high affinity of L-670,207 for muscarinic receptors posed a number of technical problems in studying its effects. In the binding studies evidence of significant ligand depletion was observed, an effect that was more pronounced at low concentrations in the agonist binding assay because of the higher protein concentrations that are routinely used for this assay. In order to compensate for this depletion, corrections were made to the displacement curves for L-670,207 by approximating the amount of free drug remaining after depletion by the receptor content of the assay. When this correction was made, the K_{app} was found to be 0.035 (0.030, 0.040) nM which corresponded to an NMS/Oxo-M ratio of 890. In other experiments, lowering the protein content of this assay did also result in higher binding affinity and correspondingly higher ratios; however, there were practical limits in how far this could be followed since lowering the protein concentration reduced the numbers of counts in the assays.

In the functional assays it seems likely that there was a much larger receptor reserve than that present in the cortical PI assay. Under these circumstances even compounds with relatively low NMS/oxo-M ratios, such as RS-86, can be shown to be relatively efficacious agonists. Correspondingly the maximum responses observed for the more efficacious agonists were all similar.

These results describe four oxadiazole based muscarinic agonists that are amongst the most potent muscarinic agonists known. These compounds are highly efficacious agonists at all three of the muscarinic receptor subtypes examined and are the first reports of non quaternary agonists with sufficient intrinsic efficacy to stimulate maximally cortical PI turnover. These compounds will provide useful tools in the study of muscarinic receptors and our understanding of their involvement in central processes.

The authors would like to thank Mrs E. Brawn and Mrs S. Burton for manuscript preparation.

References

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., 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.

BROWN, D.A., FORWARD, A. & MARSH, S. (1980). Antagonist discrimination between ganglionic and ileal muscarinic receptors. *Br. J. Pharmacol.*, **71**, 362–364.

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

BUCKLEY, N.J., BONNER, T.I. & BRANN, M.R. (1988). Localisation of a family muscarinic receptor mRNA's in rat brain. *J. Neurosci.*, **8**, 4646–4652.

CAINE, R.D. (1980). Cholinomimetic treatment fails to improve memory disorder. *N. Engl. J. Med.*, **303**, 585–586.

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

CHRISTIE, J.E., SHERING, A., FERGUSON, J. & GLEN, A.I.M. (1981). Physostigmine and arecoline. Effects of intravenous infusions in Alzheimer presenile dementia. *Br. J. Psychiat.*, **138**, 46–50.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–102.

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.

EHLERT, F.J. (1985). The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rat myocardium. *Mol. Pharmacol.*, **28**, 410–421.

FISHER, S.K., KLINGER, R.D. & AGRANOFF, B.W. (1983). Muscarinic agonist binding and phospholipid turnover in brain. *J. Biol. Chem.*, **258**, 7358–7363.

FREEDMAN, S.B. (1986). Regional differences in muscarinic receptor linked inositol-phospholipid hydrolysis. *Br. J. Pharmacol.*, **87**, 29P.

FREEDMAN, S.B., HARLEY, E.A. & IVERSEN, L.L. (1988). Relative affinities of drugs acting at cholinoceptors in displacing agonist and antagonist radioligands: the NMS/Oxo-M ratio as an index of efficacy at cortical muscarinic receptors. *Br. J. Pharmacol.*, **93**, 437–445.

GOYAL, R.K. (1989). Muscarinic receptor subtypes. Physiology and clinical implications. *N. Engl. J. Med.*, **321**, 1022–1029.

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

HAMMER, R., GIRALDO, E., SCHIAVI, G.B., MONFERINI, E. & LADINSKY, H. (1986). Binding profile of a novel cardioselective muscarinic receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci.*, **38**, 1653–1662.

KENAKIN, T.P. (1986). Receptor reserve as a tissue misnomer. *Trends Pharmacol. Sci.*, **7**, 93–95.

LAMBRECHT, G., MOSER, U., MUTSCHLER, E., WESS, J., LINOH, H., STRECKER, M. & TACKE, R. (1984). Hexahydrosilafenidol a selective antagonist on ileal muscarinic receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325** (Suppl), R62.

MELCHIORRE, C., CASSINELLI, A. & QUAGLIA, W. (1987). Differential blockade of muscarinic receptor subtypes by polymethylene tetraamines. Novel class of selective antagonists of cardiac M-2 muscarinic receptors. *J. Med. Chem.*, **30**, 201–204.

MOURADIAN, M.M., MOHR, E., WILLIAMS, J.A. & CHASE, T.N. (1988). No response to high-dose muscarinic agonist therapy in Alzheimer's disease. *Neurology*, **38**, 606–608.

MUTSCHLER, E., MOSER, U., WESS, J. & LAMBRECHT, G. (1989). Muscarinic receptor subtypes: agonists and antagonists. In *Prog. Pharmacol. Clin. Pharmacol.*, Vol. 7/1, pp. 13–31. Stuttgart, New York: Gustav Fischer Verlag.

NEWBERRY, N.R. & GILBERT, M.J. (1989). Biphasic dose-response curve to muscarine on the rat superior cervical ganglion. *Eur. J. Pharmacol.*, **163**, 237–244.

NEWBERRY, N.R. & PRIESTLY, T. (1987). Pharmacological differences between two muscarinic responses of the rat superior cervical ganglion *in vitro*. *Br. J. Pharmacol.*, **92**, 817–826.

PERRY, E.K. (1986). The cholinergic hypothesis – ten years on. *Br. Med. Bull.*, **42**, 63–69.

RANG, H.P. (1964). Stimulant actions of volatile anaesthetics on smooth muscle. *Br. J. Pharmacol. Chemother.*, **22**, 356–365.

SAUNDERS, J., CASSIDY, M., FREEDMAN, S.B., HARLEY, E.A., IVERSEN, L.L., KNEEN, C., MACLEOD, A., MERCHANT, K., SNOW, R.J. & BAKER, R. (1990). Novel quinuclidine based ligands for the muscarinic cholinergic receptor. *J. Med. Chem.*, **33**, 1128–1138.

SAUNDERS, J. & FREEDMAN, S.B. (1989). The design of full agonists for the cortical muscarinic receptor. *Trends Pharmacol., Sci. Suppl.*, 70–75.

STREET, L.J., BAKER, R., BOOK, T., KNEEN, C.O., MACLEOD, A.M., MERCHANT, K.J., SHOWELL, G.A., SAUNDERS, J., HERBERT, R.H., FREEDMAN, S.B. & HARLEY, E.A. (1990). Synthesis and biological activity of 1,2,4-oxadiazole derivatives: Highly potent and efficacious agonists for cortical muscarinic receptors. *J. Med. Chem.* (in press).

WATJEN, F., BAKER, R., ENGELSTOFF, M., HERBERT, R., MACLEOD, A., KNIGHT, A., MERCHANT, K., MOSELEY, J., SAUNDERS, J., SWAIN, C.J., WONG, E. & SPRINGER, J.P. (1989). Novel benzodiazepine receptor partial agonists; Oxadiazolylimidazobenzodiazepines. *J. Med. Chem.*, **32**, 2282–2291.

(Received March 23, 1990
Revised June 15, 1990
Accepted July 3, 1990)