ELSEVIER

European Journal of Pharmacology 370 (1999) 69–77

ejp

In vitro characterisation of the muscarinic receptor partial agonist, sabcomeline, in rat cortical and heart membranes

Jeannette M. Watson, A. Jaqueline Hunter, Anthony M. Brown, Derek N. Middlemiss *

Neurosciences Research, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, UK

Received 12 November 1998; received in revised form 25 January 1999; accepted 5 February 1999

Abstract

We have investigated the pharmacology of the functionally selective muscarinic M_1 receptor partial agonist, sabcomeline [SB-202026 (*R*-(*Z*)-(+)-α-(methoxyamino)-1-azabicyclo[2.2.2] octane-3-acetonitrile)], in rat cortex and heart using radioligand binding and functional studies. The Quinuclidinyl benzilate/Oxotremorine-M acetate ratio from radioligand binding studies suggested that sabcomeline and xanomeline [3(3-hexyloxy-1,25-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine] are muscarinic receptor partial agonists in cortical and heart membranes. In [^{35}S]GTPγS binding studies in rat cortex, carbachol stimulated binding via muscarinic M_2/M_4 receptors which could be blocked by sabcomeline with a pA_2 of 7.2. In rat heart membranes, carbachol also stimulated [^{35}S]GTPγS binding studies through muscarinic M_2 receptors. Sabcomeline caused a small stimulation of basal [^{35}S]GTPγS binding in both rat and heart tissues. Sabcomeline did not stimulate phosphoinositide hydrolysis in rat cortical slices, but did block the muscarinic M_1 receptor-mediated response caused by carbachol with apparent pK_b of 6.9. Xanomeline and milameline also had no effect on phosphoinositide hydrolysis up to 100 μM. In adenylyl cyclase studies in rat atria, sabcomeline inhibited forskolin-stimulated adenylyl cyclase activity to a similar extent to that of carbachol, xanomeline and milameline. The present study, using the techniques of radioligand binding, supports previous publications which have claimed that sabcomeline is a muscarinic receptor partial agonist. As expected, this study shows that the functional actions of this compound at muscarinic receptor subtypes and in different tissues will depend on receptor reserve.

Keywords: Sabcomeline; Cortex, rat; Heart, rat; Radioligand binding; [^{35}S]GTPγS binding; Phosphoinositide hydrolysis; Adenylyl cyclase

1. Introduction

Muscarinic receptors mediate most of the inhibitory and excitatory effects of acetylcholine on central neurons and the majority of effects in the periphery (Buckley and Caulfield, 1992). Currently, there are five muscarinic receptor subtypes, M_1–M_5, that have been cloned and expressed in various cell lines (Bonner et al., 1987, 1988), and have been pharmacologically characterised (Doods et al., 1987; Bonner, 1989; Lazareno et al., 1990; Caulfield and Birdsall, 1998). Distribution studies have shown that the predominant, post-synaptic receptor sites in central areas (cerebral cortex, hippocampus) are of the muscarinic M_1 receptor subtype (Levey et al., 1991; Flynn and Mash, 1993). Muscarinic M_2 receptors are more abundant in cardiac tissue (Levey, 1993) and, along with M_3 receptor sites, in gut tissue (Levey, 1993; Eglen et al., 1996; Ehlert et al., 1997). Muscarinic M_4 and M_5 receptor sites are found in both central and peripheral areas (Gross et al., 1997; Caulfield and Birdsall, 1998; Purkerson and Potter, 1998).

The degeneration of cholinergic neurons found in the human nervous system (CNS) is thought to be one of the critical processes contributing to impaired cognition in Alzheimer's Disease (Coyle et al., 1983), in particular, those projecting from the basal forebrain to the hippocampus and cortex (Bowen, 1983; Mash et al., 1985; Lehericy et al., 1993). As described above, the muscarinic M_1 receptor subtype is abundant in hippocampus and cerebral cortex, suggesting that muscarinic M_1 receptor numbers may be affected by the pathology of Alzheimer's Disease (Wesnes and Warburton, 1984). Thus, biochemical studies using receptor markers have shown severe depletion of pre-synaptic muscarinic receptors in Alzheimer's Disease (Davies and Maloney, 1976,) whereas post-synaptic receptor density has been reported as relatively normal (Davies

* Corresponding author. Tel.: +44-1279-622078; Fax: +44-1279-622230

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00081-3

and Verth, 1977). Therefore, it is reasonable to postulate that stimulating post-synaptic receptors in the CNS with muscarinic receptor agonists may compensate for the loss of pre-synaptic cholinergic neurons and so may alleviate impaired cognition in Alzheimer's Disease patients. However, if these agonists are not functionally selective, they may also have the ability to directly activate muscarinic receptors in the periphery with the potential for adverse side effects. A possible therapeutic target would, therefore, be a muscarinic M_1 receptor agonist acting with the minimal functional efficacy at CNS receptors required to alleviate the symptoms of Alzheimer's Disease but with little or no efficacy at peripheral receptors.

Sabcomeline [SB-202026 (*R*-(*Z*)-(+)-α-(methoxyamino)-1-azabicyclo[2.2.2] octane-3-acetonitrile)] is a novel muscarinic receptor partial agonist with functional selectivity for muscarinic M_1 receptors (Clark et al., 1996; Loudon et al., 1996, 1997). The purpose of this study was to investigate the in vitro pharmacology of sabcomeline in rat cortical and heart tissue using radioligand binding studies, [^{35}S]GTPγS binding studies, phosphoinositide turnover and adenylyl cyclase assays. In each series of experiments, we also investigated the responses to the non-selective muscarinic agonist, carbachol, the partial agonist, milameline (CI-979/RU35926) (Sedman et al., 1995), and the muscarinic M_1-selective agonist, xanomeline [3(3-hexyloxy-1,25-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine] (Shannon et al., 1994).

2. Materials and methods

2.1. Radioligand binding assay in rat cortex

[^{3}H]Oxotremorine-M acetate and [^{3}H]Quinuclidinyl benzilate binding assays were performed in rat cortex following the method of Loudon et al. (1997). In brief, cerebral cortices were homogenised in 50 mM Tris buffer, pH 7.7, followed by centrifugation at 28,000 × *g* for 15 min at 4°C. This procedure was repeated twice more and the homogenates were stored in 1 ml aliquots at −80°C until required.

Radioligand binding studies using [^{3}H]Oxotremorine-M (final concentration of 1.88 nM) were performed in 50 mM Tris, containing 2 mM $MgCl_2$, pH 7.7 using 100 μg protein/well. Oxotremorine (10 μM) was used to define non-specific binding. [^{3}H]Quinuclidinyl benzilate (final concentration 0.27 nM) binding studies were performed in a similar manner except that $MgCl_2$ was omitted from the incubation buffer and 8 μg protein was added to each well. Atropine (1 μM) was used to define non-specific binding. Incubations with both radioligands were for 40 min at 37°C and were terminated by rapid filtration over Whatman GF/B glass fibre filters pre-soaked in 0.05% polyethylenimine.

2.2. Radioligand binding assay in rat heart

Rat heart membranes were prepared according to the method of Ehlert et al. (1989) with modifications. Whole rat heart was cut into small pieces and homogenised using a polytron tissue homogeniser, in 20 volumes of ice-cold 30 mM Na-HEPES containing 0.5 mM EGTA, 100 mM NaCl, pH 7.5. The homogenate was filtered through Millipore nylon net filters (pore size 180 μM) and subsequently centrifuged for 10 min at 30,000 × *g*. The resultant pellet was re-suspended in the above buffer, containing 1 mM dithiothreitol, pH 7.5 and stored in 1 ml aliquots (approximately 5 mg protein) at −80°C until required.

Radioligand binding assays were performed as described for rat cortex with modifications. Tissue protein (100 μg) was incubated in 0.5 ml of 30 mM HEPES, 100 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EGTA, pH 7.5 with 3 nM [^{3}H]Oxotremorine-M or 0.5 nM [^{3}H]Quinuclidinyl benzilate for 40 min at 25°C or 37°C, respectively. For [^{3}H]Oxotremorine-M binding, 10 mM $MgCl_2$ was present in the incubation buffer.

2.3. [^{35}S]GTPγS binding assay in rat cortex

Cerebral cortex from male Sprague–Dawley rats was dissected and homogenised in 10 volumes of ice-cold 50 mM Tris–HCl containing 1 mM dithiothreitol and 1 mM EGTA, pH 7.4. The homogenate was centrifuged at 1000 × *g* for 5 min at 4°C and the resultant supernatant (S1) was removed and stored on ice. The pellet was resuspended in buffer, homogenised and centrifuged as before. The supernatant was combined with S1 and centrifuged at 11,000 × *g* for 20 min at 4°C. The resultant pellet was resuspended in 20 volumes of buffer and centrifuged at 27,000 × *g* for 20 min at 4°C. The final membrane pellet was resuspended in the same buffer in 1 ml aliquots (approximately 4 mg protein) and stored at −80°C until required.

Cortical membranes (10 μg protein in a final volume of 500 μl) were pre-incubated for 30 min at 30°C in 50 mM Tris–HCl containing 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl and 100 μM GDP with or without test drugs. The reaction was started by the addition of 50 μl of [^{35}S]GTPγS (final concentration 0.25 nM) followed by a further 30 min incubation at 30°C. The reaction was stopped by rapid filtration over Whatman GF/B grade filters followed by five washes with ice-cold 50 mM Tris–HCl with 1 mM $MgCl_2$, pH 7.4. Radioactivity on filters was determined by liquid scintillation spectrometry. Non-specific binding was determined by 50 μM unlabelled GTPγS.

2.4. [^{35}S]GTPγS binding assay in rat heart

Rat heart membranes were prepared following the method of Hilf and Jakobs (1989) with modifications.

Whole heart was dissected into small pieces and homogenised, using a polytron tissue homogeniser, in 20 volumes of 20 mM Tris–HCl containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride, pH 8. The homogenate was centrifuged for 15 min at $400 \times g$, 4°C and the supernatant filtered through Millipore, nylon net filters (pore size 180 μM). The supernatant was centrifuged for 30 min at $40{,}000 \times g$, 4°C and the resultant pellet resuspended in the above buffer and stored at −80°C until required.

Rat heart membranes (20 μg protein in a final volume of 500 μl) were pre-incubated for 30 min at 30°C in 50 mM Tris–HCl, pH 7.5, containing 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl and 10 μM GDP with or without test drugs. The reaction was started by the addition of 50 μl of [^{35}S]GTPγS (final concentration 0.25 nM) followed by a 30-min incubation at 30°C. The reaction was stopped by rapid filtration over Whatman GF/B grade filters followed by five washes with ice-cold 50 mM Tris–HCl with 5 mM $MgCl_2$, pH 7.5. Radioactivity on filters was determined by liquid scintillation spectrometry. Non-specific binding was determined by 50 μM unlabelled GTPγS.

2.5. Phosphoinositide hydrolysis in rat cortex

The assay procedure used was adapted from Freedman et al. (1988), with modifications. Cross-chopped cerebral cortices (350×350 μm) were prepared from male Sprague–Dawley rats (250–300 g) and washed three times in ice-cold Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 11.7 mM glucose) saturated with 95% O_2/5% CO_2. Following pre-incubation for 15 min at 37°C, the tissue slices were washed with warm Krebs and subsequently incubated in Krebs containing 50 μCi [^{3}H]*myo*-2-inositol for 45 min at 37°C. Continuous gassing was maintained throughout. Pre-labelled tissue was then washed three times with warm Krebs and allowed to sediment. An amount of 50 μl of packed tissue was added to Krebs (final volume 300 μl) containing 10 mM LiCl, with or without test drug. Samples were gassed regularly, capped and incubated for 50 min at 37°C. The reaction was terminated by the addition of 600 μl of 9% perchloric acid solution and the samples placed on ice for 30 min. Following subsequent addition of a 1:1 mixture of trioctylamine/trichlorotrifluoroethane (1.3 ml) and vigorous mixing, the aqueous layer was separated by centrifugation at $1000 \times g$ for 5 min.

Separation of radiolabelled inositol phosphates was performed using columns containing 2 ml of Dowex resin. The aqueous samples were passed down primed columns (by addition of 20 ml H_2O), followed by a washing procedure of 2 ml H_2O and then 10 ml 60 mM sodium formate/5 mM borax to remove free [^{3}H]inositol. Radiolabelled inositol phosphates were eluted with 5 ml 1 M ammonium formate/0.1 M formic acid into scintillation vials and counted in a liquid scintillation counter. Columns were washed with 2 M ammonium formate/0.1 M formic acid and stored in 5% ammonium formate.

2.6. Adenylyl cyclase studies in rat atria

Atrial tissue was dissected from male Sprague–Dawley rats (300–350 g) and prepared according to the method by Baumgold and Drobnick (1989). Adenylyl cyclase activity was determined by adding 50–100 μg of membrane protein to a reaction mixture, with or without test compound, containing a final concentration of 50 mM triethanolamine hydrochloride (pH 7.4), 50 mM $MgSO_4$, 100 μM ATP, 1 mM cAMP, 1 mM isobutylmethylxanthine, 100 μM GTP, 0.5 mM EGTA, 0.5 mM dithiothreitol, 240 mM sucrose, 0.5% bovine serum albumin, 5 mM creatine phosphate, 0.7 mg/ml creatine phosphokinase, 10 μM forskolin and 2 μCi [^{33}P]ATP. The mixture was incubated at 37°C for 10 min. The reaction was stopped by addition of l00 μl of 0.5 M HCl containing 10 mM cAMP and 40 mM ATP. Adenylyl cyclase activity was assessed by the production of [^{33}P]cAMP which was isolated using poly-prep column chromatography according to Salomon (1979) and counted in a liquid scintillation counter.

2.7. Materials

Sabcomeline (*R*-(*Z*)-(+)-α-(methoxyamino)-1-azabicyclo[2.2.2] octane-3-acetonitrile), xanomeline [3(3-hexyloxy-1,25-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine] and milameline (CI-979/RU35926) were syn-

Table 1
Affinities and ratios for selected muscarinic agonists in rat cortex using [^{3}H]Oxotremorine-M and [^{3}H]Quinuclidinyl benzilate

Compound	pIC_{50}		Quinuclidinyl benzilate/ Oxotremorine-M ratio
	[^{3}H]Oxotremorine-M	[^{3}H]Quinuclidinyl benzilate	
Carbachol	6.8 ± 0.1	3.5 ± 0.3	1995
Sabcomeline	7.7 ± 0.1	6.4 ± 0.1	20
Milameline	7.0 ± 0.1	4.6 ± 0.1	251
Xanomeline	7.5 ± 0.1	6.3 ± 0.2	16

Data represent mean value ± S.E.M. from three to five individual experiments each performed in duplicate. Quinuclidinyl benzilate/Oxotremorine-M ratio is calculated using the mean IC_{50} values.

Table 2
Affinities and ratios for selected muscarinic agonists in rat heart using [^{3}H]Oxotremorine-M and [^{3}H]Quinuclidinyl benzilate

Compound	pIC_{50}		Quinuclidinyl benzilate/ Oxotremorine-M ratio
	[^{3}H]Oxotremorine-M	[^{3}H]Quinuclidinyl benzilate	
Carbachol	6.8 ± 0.2	4.3 ± 0.2	316
Sabcomeline	8.0 ± 0.2	6.5 ± 0.2	32
Milameline	7.0 ± 0.1	5.3 ± 0.1	50
Xanomeline	8.0 ± 0.1	6.9 ± 0.1	13

Data represent mean value ± S.E.M. from three to five individual experiments each performed in duplicate. Quinuclidinyl benzilate/Oxotremorine-M ratio is calculated using the mean IC_{50} values.

thesised by SmithKline Beecham Pharmaceuticals. Other drugs and reagents were purchased from Sigma (Poole, UK), Calbiochem (Nottingham, UK), Bio-Rad (Hemel Hempstead, UK), Fisons Scientific Equipment (Loughborough, UK), Research Biochemicals International (Poole, UK) and GibcoBRL (Paisley, UK). [^{3}H]Oxotremorine-M acetate, [^{3}H]Quinuclidinyl benzilate, [^{33}P]ATP, [^{3}H]*myo*-2-inositol were obtained from NEN DuPont (Hounslow, UK). [^{35}S]GTPγS was supplied by Amersham International (Little Chalfont, UK).

2.8. Data analysis

Data from radioligand binding studies, [^{35}S]GTPγS binding, phosphoinositide and adenylyl cyclase studies were fitted by a four-parameter logistic equation using GRAFIT (Erithacus Software, Staines, UK) to yield values for maximum activity and IC_{50}/EC_{50}. Antagonist activity of compounds was quantified by calculating their apparent pK_b value according to the Gaddum equation: apparent $pK_b = \log[(EC_{50}$ in the presence of antagonist divided by the EC_{50} in its absence$) - 1] -$ log of the molar concentration of antagonist, or pA_2 values determined by Schild analysis. For phosphoinositide and adenylyl cyclase studies, the inhibition curve design was used to estimate apparent pK_b for antagonists and is described by Lazareno and Birdsall (1993a).

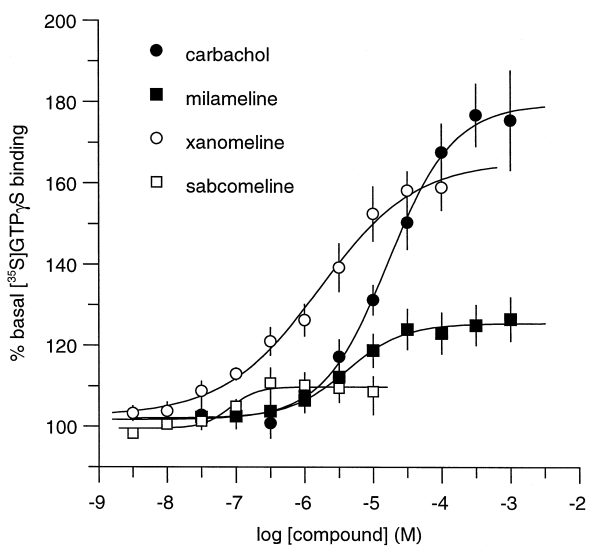


Fig. 1. [^{35}S]GTPγS binding to rat cortical membranes. Membranes were incubated in the presence or absence of increasing concentrations of carbachol, sabcomeline, xanomeline or milameline. Data represent mean ± S.E.M. of five individual experiments.

3. Results

3.1. Radioligand binding studies

Sabcomeline and xanomeline showed high affinity for muscarinic receptors in rat cortex, whereas carbachol and milameline were a half to three log units less potent at displacing binding, depending on the radioligand used (Table 1). Similar results were obtained in rat heart where both sabcomeline and xanomeline showed higher affinity for muscarinic receptors than carbachol and milameline (Table 2). It has previously been reported that the ratio of the binding affinities obtained using [^{3}H]Oxotremorine-M and [^{3}H]-*N*-methylscopolamine can predict whether a compound will show agonist or antagonist properties at muscarinic receptors (Freedman et al., 1988). This has been supported by studies on a range of muscarinic compounds using the radioligands employed in this study (Brown et al., 1988; Loudon et al., 1997) where it was reported that a ratio close to unity would predict antagonism and those > 100, full agonism. Intermediate ratios suggest varying degrees of partial agonism.

In rat cortex, the ratio of [^{3}H]Quinuclidinyl benzilate:[^{3}H]Oxotremorine-M binding for sabcomeline and

Table 3
Effect of muscarinic agonists on basal [^{35}S]GTPγS binding in rat cortex and heart

	pEC_{50}		Percentage of stimulation	
	Cortex	Heart	Cortex	Heart
Carbachol	4.8 ± 0.1	4.9 ± 0.1	85 ± 5	35 ± 5
Sabcomeline	6.8 ± 0.2	6.8 ± 0.2	9 ± 5	10 ± 4
Milameline	5.6 ± 0.1	6.0 ± 0.2	22 ± 4	24 ± 5
Xanomeline	5.6 ± 0.1	7.2 ± 0.2	59 ± 6	15 ± 5

Data represent mean value ± S.E.M. from four to five individual experiments each performed in duplicate.

xanomeline was approximately 20, suggesting that these compounds act as partial agonists in this tissue, whereas the ratio for carbachol and milameline was indicative of full agonist activity (Table 1). Similar results were seen in rat heart, with the exception of milameline, which gave a [^{3}H]Quinuclidinyl benzilate:[^{3}H]Oxotremorine-M ratio indicative of partial agonism (Table 2).

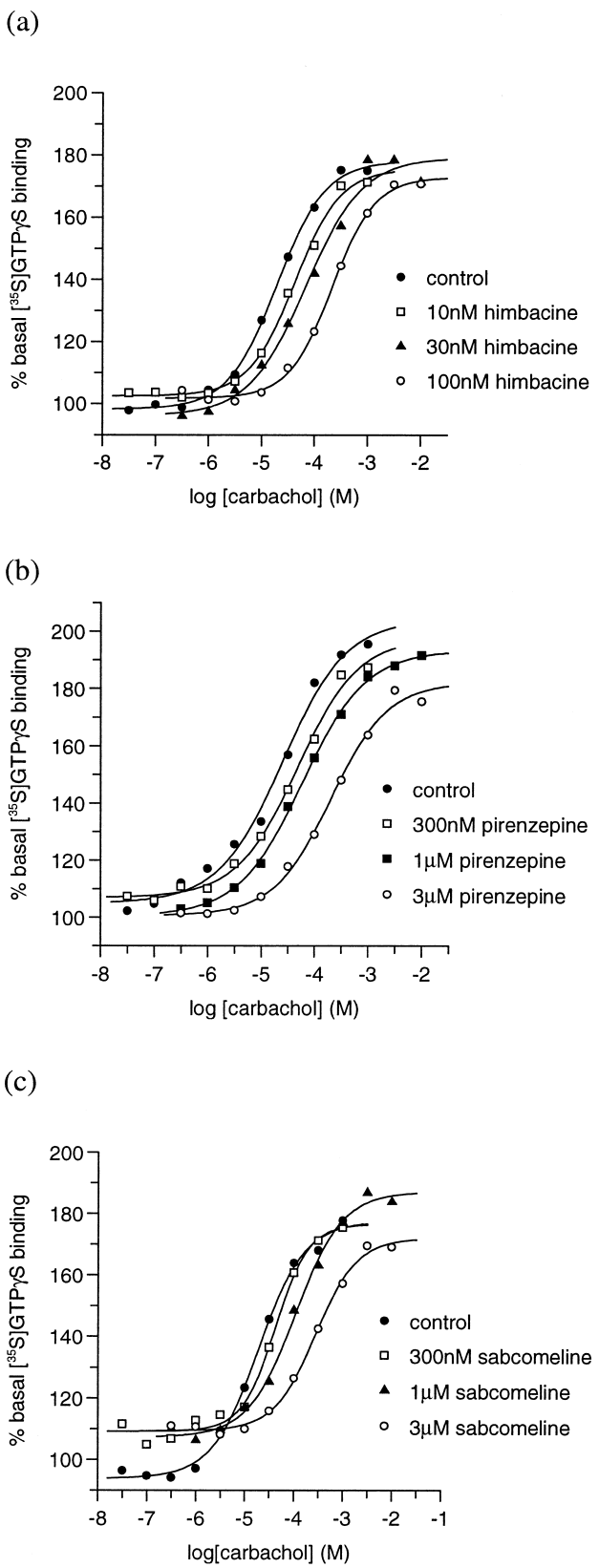


Fig. 2. Inhibition of carbachol-induced stimulation of [^{35}S]GTPγS binding to rat cortical membranes by muscarinic ligands. Panel (a) shows the response to carbachol alone and in the presence of 10 nM, 30 nM and 100 nM himbacine. Panel (b) shows the response to carbachol alone and in the presence of 300 nM, 1 μM and 3 μM pirenzepine. Panel (c) shows the response to carbachol alone and in the presence of 300 nM, 1 μM and 3 μM sabcomeline. Data represent a single experiment, typical of five giving similar results.

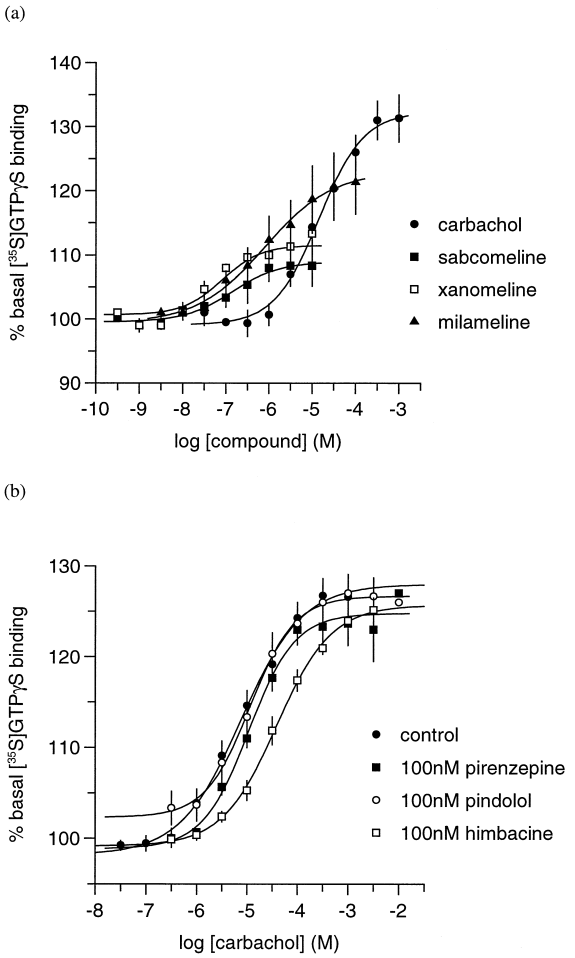


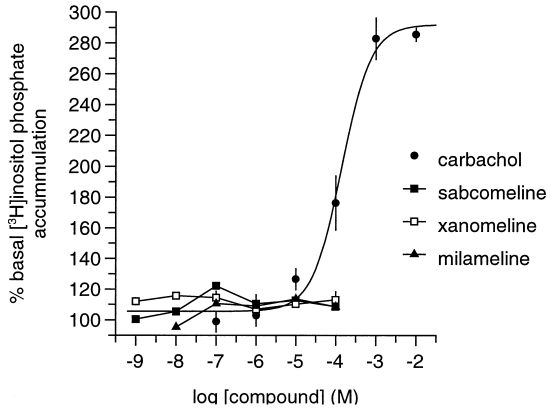
Fig. 3. [^{35}S]GTPγS binding to rat heart membranes. Membranes were incubated in the presence of increasing concentrations of carbachol, sabcomeline, xanomeline or milameline (panel a). Panel (b) shows the response to carbachol alone and in the presence of 100 nM of pirenzepine, himbacine or pindolol. Data represent mean ± S.E.M. of three individual experiments, each performed in duplicate.

3.2. [^{35}S]GTPγS binding studies

3.2.1. Rat cortex

In rat cortex, carbachol stimulated [^{35}S]GTPγS binding with a pEC_{50} of 4.8 and a maximum response of 85%

(a)



(b)

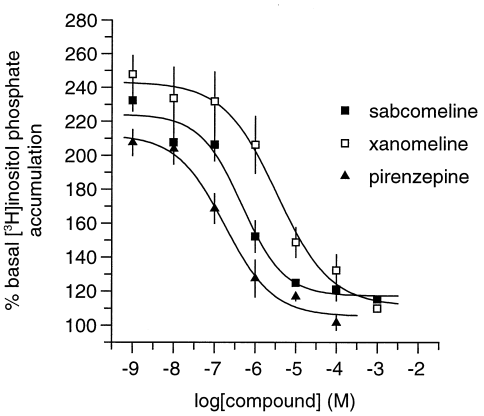


Fig. 4. Measurement of inositol phosphate accumulation in rat cortical slices. Panel (a) shows the response to increasing concentrations of carbachol, xanomeline, sabcomeline and milameline. Panel (b) shows the response to increasing concentration of pirenzepine, sabcomeline or xanomeline in the presence of 1 mM carbachol. Data represent mean ± S.E.M. of four to five individual experiments, each performed in duplicate.

above basal (Fig. 1; Table 3). Xanomeline and milameline also stimulated basal binding with an equivalent pEC_{50} of 5.6, whereas sabcomeline produced a small stimulation (9%) of basal binding with pEC_{50} of 6.8. The carbachol response was dose-dependently inhibited by the muscarinic M_2/M_4-selective receptor antagonist, himbacine, with a pA_2 of 8.2 ± 0.1 and the muscarinic M_1-selective receptor antagonist, pirenzepine, with a pA_2 of 6.9 ± 0.2 (Fig. 2a and b). Sabcomeline also attenuated the carbachol-induced stimulation of [^{35}S]GTPγS binding, with a pA_2 of 7.2 ± 0.2 (Fig. 2c).

3.2.2. Rat heart

In rat heart, carbachol stimulated basal binding to a lesser extent than that seen in the cortex, but with a similar potency (Table 3; Fig. 3a). Sabcomeline produced a small

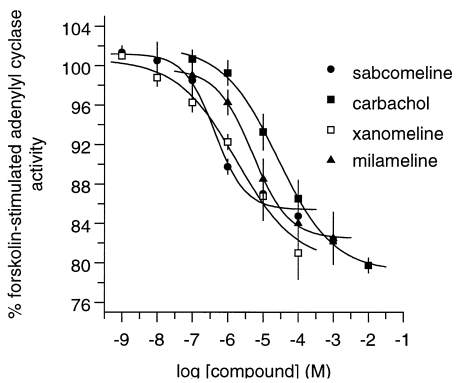
Table 4
Inhibition of carbachol-induced phosphoinositide hydrolysis in rat cortex

Compound	pIC_{50}	Apparent pK_b
Sabcomeline	6.2 ± 0.1	6.9 ± 0.2
Xanomeline	5.6 ± 0.1	5.9 ± 0.2
Pirenzepine	6.7 ± 0.2	7.7 ± 0.2

Data represent mean value ± S.E.M. from four to five individual experiments each performed in duplicate. pIC_{50} values determined from increasing concentration of test compound in the presence of 1 mM carbachol and used to calculate apparent pK_b using the Gaddum equation.

stimulation of [^{35}S]GTPγS binding (10% above basal) with a pEC_{50} of 6.8. Similar results were obtained with xanomeline (pEC_{50} 7.2, 15% stimulation above basal). Carbachol-induced stimulation of [^{35}S]GTPγS binding was inhibited by himbacine with apparent pK_b of 7.5 ± 0.1

(a)



b)

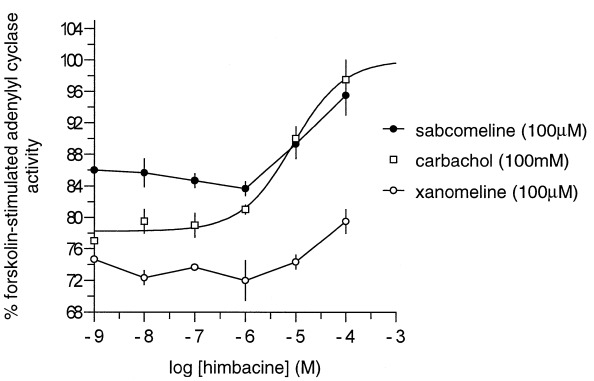


Fig. 5. Adenylyl cyclase studies in rat heart membranes. In all studies, membranes were incubated in the presence of 10 μM forskolin to stimulate adenylyl cyclase activity. Panel (a) shows the inhibition of forskolin-stimulated adenylyl cyclase activity by carbachol, sabcomeline, xanomeline and milameline. Panel (b) shows the response to increasing concentration of himbacine in the presence of sabcomeline (100 μM), carbachol (10 mM) and xanomeline (100 μM). Data represent mean ± S.E.M. of four individual experiments, each performed in duplicate.

(Fig. 3b). Pirenzepine (100 nM) and the β-adrenoreceptor antagonist, pindolol (100 nM), had no significant effect on the carbachol response.

3.3. Phosphoinositide hydrolysis in rat cortex

Carbachol increased basal [^{3}H]inositol phosphate accumulation with pEC_{50} of 3.8 ± 0.1 and a maximum stimulation of 195 ± 14% (Fig. 4a). Sabcomeline, xanomeline and milameline had no effect up to 100 μM. To investigate antagonist potencies in this assay system, we used the inhibition curve design which involved the assessment of the effects of increasing concentration of antagonist in the presence of a maximal concentration of carbachol (1 mM). pIC_{50} values were calculated which were converted into an estimated pK_b value using the Gaddum equation. The muscarinic M_1-selective receptor antagonist, pirenzepine, inhibited carbachol-induced accumulation of inositol phosphates with an apparent pK_b of 7.7 (Fig. 4b; Table 4). Sabcomeline and xanomeline produced an apparent pK_b of 6.9 and 5.9, respectively.

3.4. Adenylyl cyclase studies in rat atrial membranes

Forskolin (10 μM) stimulated basal adenylyl cyclase activity by 8–10-fold in rat atrial membranes. This response was concentration-dependently inhibited by sabcomeline, carbachol, xanomeline and milameline with different potencies but by a similar maximum inhibition of approximately 20% (Fig. 5a; Table 5), which was not significantly different from each other (student's *t*-test: $p < 0.05$). Muscarinic M_2/M_4 receptors are reported to be negatively linked to adenylyl cyclase (Peralta et al., 1988; Novotny and Brann, 1989). Therefore, to determine whether the above compounds were acting through these receptor subtypes, we investigated the effect of increasing concentration of himbacine in the presence of maximal concentrations of sabcomeline (100 μM), carbachol (10 mM) and xanomeline (100 μM). Himbacine fully attenuated the carbachol-induced inhibition of forskolin-stimulated adenylyl cyclase activity with an apparent $pK_b = 7.7 \pm 0.1$ (Fig. 5b). Sabcomeline- and xanomeline-induced inhibition of forskolin-stimulated adenylyl cyclase activity appeared to be attenuated by himbacine at higher concentrations of 10–100 μM (Fig. 5b).

Table 5
Effect of muscarinic agonists on forskolin-stimulated adenylyl cyclase activity in rat atria

Compound	pEC_{50}	Percentage of inhibition of forskolin-stimulated adenylyl cyclase activity
Sabcomeline	6.4 ± 0.2	17 ± 2
Carbachol	4.6 ± 0.3	21 ± 1
Xanomeline	6.1 ± 0.3	17 ± 2
Milameline	5.2 ± 0.1	16 ± 2

Data represent mean value ± S.E.M. from four individual experiments each performed in duplicate.

4. Discussion

In this study, we have investigated the functional effects of the muscarinic agonist, sabcomeline, in native rat tissue, and compared its activity against other known muscarinic agonists. In radioligand binding studies, sabcomeline showed high affinity for muscarinic receptors in rat cortical and heart membranes. The Quinuclidinyl benzilate/Oxotremorine-M ratio for sabcomeline in each tissue was comparable and predicted low intrinsic activity in agreement with previous studies performed with these muscarinic radioligands (Brown et al., 1988; Loudon et al., 1997). Similar results were also obtained with the muscarinic M_1-selective receptor agonist, xanomeline, where the ratios predicted partial agonist activity in both rat cortical and heart membranes but milameline appeared to be a full agonist in cortex and a partial agonist in heart. Although we have used the Quinuclidinyl benzilate/Oxotremorine-M ratio as a simple way to predict the intrinsic activity of these muscarinic agonists, it must be noted that, in rat cortex, it is possible that different receptor subtypes are labelled by [^{3}H]Quinuclidinyl benzilate and [^{3}H]Oxotremorine-M. This may be a problem if the agonists discriminate greatly between muscarinic receptor subtypes but is not such a concern in rat heart as only the muscarinic M_2 receptor predominates.

The functional effects of sabcomeline at muscarinic receptors were investigated using [^{35}S]GTPγS binding which provides a measure of agonist-stimulated receptor G-protein coupling. In rat cortex, sabcomeline produced a small stimulation of basal [^{35}S]GTPγS binding and attenuated the carbachol response with a pA_2 of 7.2, which is comparable with its binding affinity at muscarinic receptors (Loudon et al., 1997). The selective muscarinic M_2/M_4 receptor antagonist, himbacine, blocked the response to carbachol with a pA_2 of 8.2 which is consistent with its affinity at these receptor subtypes (Dorje et al., 1990; Lazareno et al., 1990; Lazareno and Birdsall, 1993b), suggesting that carbachol stimulated [^{35}S]GTPγS binding, predominantly, via muscarinic M_2/M_4 receptors. This conclusion is further supported by antagonist studies using pirenzepine, which inhibited the response to carbachol with a potency intermediate to that of muscarinic M_2 and M_4 receptors (Dorje et al., 1990; Pedder et al., 1991; Lazareno and Birdsall, 1993b; Esqueda et al., 1996). These data suggest that, in this model, sabcomeline, which is selective for muscarinic receptors (Loudon et al., 1997), has slight intrinsic activity at muscarinic M_2/M_4 receptors and is acting as an antagonist at these receptors. Xanomeline- and milameline-induced stimulation of [^{35}S]GTPγS

binding in rat cortex may also be attributed to activation of muscarinic M_2/M_4 receptors; however, previous studies have shown that the response to xanomeline is, in part, due to activation of 5-HT_{1A} receptors (Watson et al., 1998).

Studies in rat heart membranes showed that carbachol-induced stimulation of [^{35}S]GTPγS binding was also blocked by himbacine with a potency comparable to its binding affinity at muscarinic M_2/M_4 receptors. The muscarinic M_2 receptor predominates in heart and so, assuming this tissue is a model system for these receptors, the results suggest that carbachol exerted its effect through muscarinic M_2 receptors. Similar to results obtained in rat cortex, sabcomeline produced a small stimulation of [^{35}S]GTPγS binding in rat heart. This response is most likely due to activation of muscarinic M_2 receptors because, as mentioned, these receptors are predominant in heart and it is unlikely that we could measure [^{35}S]GTPγS binding through muscarinic M_1, M_3 or M_5 receptors due to reasons previously discussed (Watson et al., 1998).

This would suggest that, compared to carbachol, sabcomeline has greater intrinsic activity at muscarinic M_2 receptors in rat heart compared to rat cortex. This phenomenon has previously been observed with other partial agonists, e.g., pindolol which acts as a 5-HT_{1A} agonist in the dorsal raphe nucleus (Clifford et al., 1998) and an antagonist at 5-HT_{1A} receptors in hippocampus (Sharp et al., 1993).

Phosphoinositide studies were also performed to investigate the functional activation of muscarinic receptors by sabcomeline. In this study, we employed the use of rat cortical slices as this tissue is abundant in muscarinic M_1 receptors (Levey et al., 1991; Flynn and Mash, 1993) which are linked to G_q and phosphoinositide hydrolysis (Brann et al., 1988; Peralta et al., 1988; Liao et al., 1989). Carbachol produced a substantial stimulation of inositol phosphate accumulation which was attenuated by pirenzepine with apparent pK_b of 7.7, which is consistent with its affinity at muscarinic M_1 receptors (Dorje et al., 1990; Lazareno and Birdsall, 1993b). Sabcomeline, xanomeline and milameline had no effect on inositol phosphate accumulation up to 100 μM but sabcomeline and xanomeline inhibited the response to 1 mM carbachol in a concentration-related manner. The potency of sabcomeline in antagonising the effects of carbachol was comparable to its binding affinity at muscarinic receptors, whereas that of xanomeline was lower (Shannon et al., 1994). The lack of intrinsic activity displayed by sabcomeline in this assay system is likely to be due to insufficient muscarinic M_1 receptor reserve, although sabcomeline could still bind to and block muscarinic M_1 receptors.

It is known that muscarinic M_2/M_4 receptors inhibit adenylyl cyclase via G_i/G_o and that, in heart tissue, this response is mediated via muscarinic M_2 receptors (Ehlert et al., 1989; Wei and Wang, 1990). We therefore used this system to further investigate the functional effects of sabcomeline at muscarinic M_2 receptors.

In rat heart atria, sabcomeline produced a small inhibition of forskolin-stimulated adenylyl cyclase activity which was less than but not significantly different from the response caused by carbachol, xanomeline and milameline. The response to carbachol was attenuated by himbacine with an apparent pK_b of 7.7, suggesting that this response is mediated via muscarinic M_2 receptors. However, we could not estimate a potency value for sabcomeline- and xanomeline-induced inhibition of forskolin-stimulated adenylyl cyclase activity using this antagonist. Thus, it is an open question as to whether sabcomeline and xanomeline act at muscarinic M_2 receptors at high concentrations (100 μM) in this assay system.

In summary, we have investigated the functional effects of the muscarinic agonist, sabcomeline, in a series of pharmacological assays in native rat tissue. Radioligand binding assays suggested that sabcomeline is a muscarinic partial agonist in rat cortex and heart membranes. Sabcomeline did not display muscarinic M_1 receptor agonist activity in phosphoinositide hydrolysis assays in rat cortex but studies investigating muscarinic M_2 receptor function did reveal sabcomeline to possess some muscarinic M_2 receptor agonist activity. A similar profile was observed for the other muscarinic agonists, xanomeline and milameline, evaluated in this study.

It is important to note that these results have been obtained from in vitro experiments on a laboratory animal tissue preparation and so the relationship to functional effects at brain and cardiac muscarinic receptors in man would depend on the degree of receptor reserve present, as is commonly observed with partial receptor agonists.

Acknowledgements

The authors would like to thank Dr. G.W. Price for his help in the preparation of the manuscript.

References

Baumgold, J., Drobnick, A., 1989. An agonist that is selective for adenylate cyclase-coupled muscarinic receptors. Mol. Pharmacol. 36, 465–470.

Bonner, T.I., 1989. The molecular basis of muscarinic receptors. Trends Neurosci. 12, 149–151.

Bonner, T.I., Buckley, N.J., Young, A.C., Brann, M.R., 1987. Identification of a family of muscarinic acetylcholine receptor genes. Science (Washington, DC) 237, 527–532.

Bonner, T.I., Buckley, N.J., Young, A.C., Brann, M.R., 1988. Cloning and expression of the human and rat M_5 muscarinic acetylcholine receptor genes. Neuron 1, 403–410.

Bowen, D.M., 1983. Biochemical assessment of neurotransmitter and metabolic dysfunction and cerebral atrophy in Alzheimer's Disease. In: Katzman, R. (Ed.), Banbury Report 15. Cold Spring Harbour, pp. 219–232.

Brann, M.R., Conklin, B., Dean, N.M., Collins, R.M., Bonner, T.I., Buckley, N.J., 1988. Cloned muscarinic receptors can couple to different G-proteins and second messengers. Soc. Neurosci. 14, 600, Abstr.

Brown, F., Clark, M., Graves, D., Hadley, M., Hatcher, J., McArthur, R.,

Riley, G., Semple, J., 1988. Variations of muscarinic activities of oxotremorine analogues. Drug Dev. Res. 14, 343–347.

Buckley, N.J., Caulfield, M., 1992. Transmission: acetylcholine. In: Burnstock., G., Hoyle, C. (Eds.), Autonomic Neuroeffector Mechanisms. Harwood, Reading, pp. 257–332.

Caulfield, M.P., Birdsall, N.J.M., 1998. International union of pharmacology: XVII. Classification of muscarinic acetylcholine receptors. Pharmacol. Rev. 279–290.

Clark, M.S.G., Brown, F., Harries, M., Hatcher, J., Hawkins, J., Loudon, J.M., Noy, G., Riley, G., 1996. SB-202026: a muscarinic partial agonist with functional selectivity for M_1 receptors. Neurobiology of Aging 17 (4F), S161.

Clifford, E.M., Gartside, S.E., Umbers, V., Cowen, P.J., Hajos, M., Sharp, T., 1998. Electrophysiological and neurochemical evidence that pindolol has agonist properties at the 5-HT_{1A} autoreceptor in vivo. Br. J. Pharmacol. 124, 206–212.

Coyle, J.T., Price, D.L., Delong, M.R., 1983. Alzheimer's Disease: a disorder of cortical cholinergic innervation. Science 219, 1185–1190.

Davies, P., Maloney, A.J.F., 1976. Selective loss of central cholinergic neurons in Alzheimer's Disease. Lancet 2, 1403.

Davies, P., Verth, A., 1977. Regional distribution of muscarinic acetylcholine receptors in normal and Alzheimer's-type dementia brain. Brain Res. 138, 385–392.

Doods, H.N., Mathy, M.-J., Davidesko, D., van Charldorp, K.J., De Jonge, A., van Zwieten, P.A., 1987. Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors. J. Pharmacol. Exp. Ther. 241 (1), 257–262.

Dorje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E., Brann, M.R., 1990. Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J. Pharmacol. Exp. Ther. 256, 727–733.

Eglen, R.M., Hegde, S.S., Watson, N., 1996. Muscarinic receptor subtypes and smooth muscle function. Pharmacol. Rev. 48, 531–565.

Ehlert, F.J., Delen, F.M., Yun, S.H., Friedman, D.J., Self, D.W., 1989. Coupling of subtypes of the muscarinic receptor to adenylate cyclase in the corpus striatum and heart. J. Pharmacol. Exp. Ther. 251, 660–671.

Ehlert, F.J., Ostrom, R.S., Sawyer, G.W., 1997. Subtypes of the muscarinic receptor in smooth muscle. Life Sci. 61, 1729–1740.

Esqueda, E.E., Gerstin, E.H. Jr., Griffin, M.T., Ehlert, F.J., 1996. Stimulation of cyclic AMP accumulation and phosphoinositide hydrolysis by M_3 muscarinic receptors in the rat peripheral lung. Biochem. Pharmacol. 52, 643–658.

Flynn, D.D., Mash, D.C., 1993. Distinct kinetic binding properties of *N*-[^{3}H]-methylscopolamine afford differential labelling and localisation of M_1, M_2 and M_3 muscarinic receptor subtypes in primate brain. Synapse 14, 283–296.

Freedman, S.B., Harley, E.A., Iversen, L.L., 1988. Relative affinities of drugs acting at cholinoreceptors 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.

Gross, J., Mutschler, E., Lambrecht, G., 1997. Evidence for muscarinic M_4 receptors mediating nonadrenergic noncholinergic relaxations in rabbit anococcygeus muscle. Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 505–516.

Hilf, G., Jakobs, K.H., 1989. Activation of cardiac G-proteins by muscarinic acetylcholine receptors: regulation by Mg^+ and Na^+ ions. Eur. J. Pharmacol. 172, 155–163.

Lazareno, S., Birdsall, N.J.M., 1993a. Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng–Prusof equations. Br. J. Pharmacol. 109, 1110–1119.

Lazareno, S., Birdsall, N.J.M., 1993b. Pharmacological characterisation of acetylcholine-stimulated [^{35}S]GTPγS binding mediated by human muscarinic M_1–M_4 receptors: antagonist studies. Br. J. Pharmacol. 109, 1120–1127.

Lazareno, S., Buckley, N.J., Roberts, F.F., 1990. Characterisation of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. Mol. Pharmacol. 38, 805–815.

Lehericy, S., Hirsch, E.C., Cervera-Pierot, P., Hersh, L.B., Bakchine, S., Piette, F., Duyckaerts, C., Hauw, J.-J., Javoy-Agid, F., Agid, Y., 1993. Heterogeneity and selectivity of the degeneration of cholinergic neurons in the basal forebrain of patients with Alzheimer's Disease. J. Comp. Neurol. 330, 15–31.

Levey, A.I., 1993. Immunological localisation of M_1–M_5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci. 52, 441–448.

Levey, A.I., Kitt, C.A., Simonds, W.F., Price, D.L., Brann, M.R., 1991. Identification and localisation of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J. Neurosci. 11, 3218–3226.

Liao, C.F., Themmen, A.P., Joho, R., Barberis, C., Birnbaumer, M., Birnbaumer, L., 1989. Molecular cloning and expression of a fifth muscarinic acetylcholine receptor. J. Biol. Chem. 264, 7328–7337.

Loudon, J.M., Hatcher, J., Storey, V., Clark, M.S.G., 1996. SB-202062, a functionally selective M_1 partial agonist, enhances cognition at doses which do not induce side effects. Neurobiology of Aging 17 (4F), S30.

Loudon, J.M., Bromidge, S.M., Brown, F., Clark, M.S.G., Hatcher, J.P., Hawkins, J., Riley, G.J., Noy, G., Orlek, B.S., 1997. SB-202026: a novel muscarinic partial agonist with functional selectivity for M_1 receptors. J. Pharmacol. Exp. Ther. 283, 1059–1068.

Mash, D.C., Flynn, D.D., Potter, L.C., 1985. Loss of M_2 muscarinic receptors in the cerebral cortex in Alzheimer's Disease and experimental cholinergic denervation. Science 228, 1115–1117.

Novotny, E.A., Brann, M.R., 1989. Agonist pharmacology of cloned muscarinic receptors. Trends Pharmacol. Sci. 4, 116, Suppl.

Pedder, E.K., Eveleigh, P., Poyner, D., Hulme, E.C., Birdsall, N.J., 1991. Modulation of the structure–binding relationship of antagonists for muscarinic acetylcholine receptor subtypes. Br. J. Pharmacol. 103, 1561–1567.

Peralta, E.G., Ashkenazi, A., Winslow, J.W., Ramachandran, J., Capon, D.J., 1988. Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature (London) 334, 434–437.

Purkerson, S.L., Potter, L.T., 1998. Use of antimuscarinic toxins to facilitate studies of striatal M_4 muscarinic receptors. J. Pharmacol. Exp. Ther. 284, 707–713.

Salomon, Y., 1979. Adenylate cyclase assay. Adv. Cyclic Nucleotide Res. 10, 35–55.

Sedman, A.J., Bockbrader, H., Schwarz, R.D., 1995. Preclinical and phase 1 clinical characterisation of CI-979/RU35926, a novel muscarinic agonist for the treatment of Alzheimer's Disease. Life Sci. 56, 877–882.

Shannon, H.E., Bymaster, F.P., Calligaro, D.O., Greenwodd, B., Mitch, C.H., Sawyer, B.D., Ward, J.S., Wong, D.T., Olesen, P.H., Sheardown, M.J., Swedberg, M.D.B., Suzdak, P.D., Sauerberg, P., 1994. Xanomeline: a novel muscarinic receptor agonist with functional selectivity for M_1 receptors. J. Pharmacol. Exp. Ther. 269, 271–281.

Sharp, T., McQuade, R., Bramwell, S., Hjorth, S., 1993. Effect of acute and repeated administration of 5-HT_{1A} receptor agonists on 5-HT release in rat brain in vivo. Naunyn-Schmiedeberg's Arch. Pharmacol. 348, 339–346.

Watson, J., Coldwell, M.C., Gager, T., Ho, M., Hunter, A.J., Jerman, J., Middlemiss, D.N., Riley, G., Brown, A.M., 1998. Functional effects of the muscarinic receptor agonist, xanomeline, at 5-HT_1 and 5-HT_2 receptors. Br. J. Pharmacol. 125, 1413–1420.

Wei, J.-W., Wang, M.-C., 1990. Muscarinic M_2 receptors coupled to inhibition of adenylate cyclase in rat heart. Chin. J. Physiol. 33, 315–327.

Wesnes, K., Warburton, D.M., 1984. Effects of scopolamine and nicotine in human rapid information processing performance. Psychopharmacology 82, 147–150.