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Functional effects of the muscarinic receptor agonist, xanomeline, at 5-HT_1 and 5-HT_2 receptors

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1 Xanomeline [3(3-hexyloxy-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine] has been reported to act as a functionally selective muscarinic partial agonist with potential use in the treatment of Alzheimer's disease. This study examined the functional activity of xanomeline at 5-HT_1 and 5-HT_2 receptors in native tissue and/or human cloned receptors.

2 Xanomeline had affinity for muscarinic receptors in rat cortical membranes where the ratio of the displacement affinity of [^{3}H]-Quinuclidinyl benzilate *vs* that of [^{3}H]-Oxotremorine-M was 16, indicative of partial agonist activity. Radioligand binding studies on human cloned receptors confirmed that xanomeline had substantial affinity for M_1, M_2, M_3, M_4, M_5 receptors and also for 5-HT_1 and 5-HT_2 receptor subtypes.

3 Carbachol and xanomeline stimulated basal [^{35}S]-GTPγS binding in rat cortical membranes with micromolar affinity. The response to carbachol was attenuated by himbacine and pirenzepine with pA_2 of 8.2, 6.9 respectively consistent with the response being mediated, predominantly, *via* M_2 and M_4 receptors. Xanomeline-induced stimulation of [^{35}S]-GTPγS binding was inhibited by himbacine with an apparent pK_b of 6.3, was not attenuated by pirenzepine up to 3 μM and was inhibited by the selective 5-HT_{1A} antagonist WAY100635 with an apparent pK_b of 9.4. These data suggest the agonist effect of xanomeline in this tissue is, in part, *via* 5-HT_{1A} receptors. Similar studies on human cloned receptors confirmed that xanomeline is an agonist at human cloned 5-HT_{1A} and 5-HT_{1B} receptors.

4 In studies using the fluorescent cytoplasmic Ca^{2+} indicator FLUO-3AM, xanomeline induced an increase in cytoplasmic Ca^{2+} concentration in SH-SY5Y cells expressing recombinant human 5-HT_{2C} receptors. Atropine antagonized this response, consistent with mediation *via* endogenously-expressed muscarinic receptors. In the presence of atropine, xanomeline antagonized 5-HT-induced cytoplasmic changes in Ca^{2+} concentration in cells expressing h5-HT_{2A}, h5-HT_{2B} and h5-HT_{2C} receptors with potencies similar to its affinity at these receptors.

5 These studies indicate that xanomeline is a potent agonist at 5-HT_{1A} and 5-HT_{1B} receptors and an antagonist at 5-HT_2 receptor subtypes.

Keywords: Xanomeline; muscarinic receptor agonist; rat brain; cultured cell lines; 5-HT receptors; radioligand binding; [^{35}S]-GTPγS binding; cytoplasmic [Ca^{2+}]

Introduction

Alzheimer's disease (AD) is one of the most common causes of mental deterioration in the elderly. It is thought to be due, at least in part, to the degeneration of acetylcholine releasing neurons (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). These brain areas are abundant with post-synaptic M_1 receptors (Levey *et al*., 1991; Flynn & Mash, 1993), thus suggesting that loss of stimulation of this receptor subtype may contribute to symptoms of the disease process. A number of research efforts for the treatment of AD have therefore been directed towards correcting for the loss of cholinergic transmission by enhancing the deficient synaptic levels of the neurotransmitter acetylcholine and/or producing selective post-synaptic M_1 receptor agonists.

Xanomeline [3(3-hexyloxy-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine] has been reported to be a selective M_1-muscarinic receptor agonist that crosses the blood-brain barrier (Sauerberg *et al*., 1992). From pre-clinical studies carried out in native tissue and cloned muscarinic receptors, xanomeline showed functional activity at, and selectivity for, M_1 receptors (Bymaster *et al*., 1994; Shannon *et al*., 1994; Eckols *et al*., 1995; Christopoulos & El Fakahany, 1997). This functional selectivity predicted a good side effect profile with few cardiovascular effects as these are mediated by activation of M_2 and M_3 receptors localized on the heart and vasculature, respectively (Wilffert *et al*., 1983; Clague *et al*., 1985). Xanomeline therefore has potential as a therapy for the treatment of AD and has been shown to improve both cognitive and disturbed behavioural symptoms of AD (Bodick *et al*., 1997a,b).

In addition to its activity at muscarinic receptors, xanomeline showed moderate/high affinity for a number of other neurotransmitter receptor subtypes, including 5-HT_1 and 5-HT_2 receptor subtypes (Shannon *et al*., 1994). During preliminary investigations of the effect of muscarinic agonists on [^{35}S]-GTPγS binding studies in rat cortex, we discovered that xanomeline was an agonist at non-muscarinic receptors in this preparation. In order to characterize this activity further we have investigated the functional effect of xanomeline both in rat brain and at recombinant human 5-HT_1 and human 5-HT_2 receptor subtypes expressed in cell lines, using radioligand binding studies, [^{35}S]-GTPγS binding studies and measurement of changes in cytoplasmic [Ca^{2+}].

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Methods

Cell culture

Chinese hamster ovary (CHO) cells expressing the human muscarinic receptor subtypes 1, 3, 4 and 5 were generated and cultured as described by Loudon *et al*., 1997. CHO cells expressing human M_2 receptors (Cat. No. M-195, 8.4 mg protein ml^{-1}) were obtained from RBI, Natick, U.S.A. HEK293 cells expressing the human 5-HT_{1A} receptor were obtained from the late Dr D Pritchard (The Children's Hospital, Philadelphia, U.S.A.) and CHO cells (ACC098) expressing the human 5-HT_{1B} receptor were generated and cultured as previously described by Watson *et al*., 1996. SH-SY5Y cells expressing human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors were generated and cultured as described by Kennett *et al*., 1997.

Radioligand binding assay in rat cortex

[^{3}H]-Oxotremorine-M acetate ([^{3}H]-OXO-M) and [^{3}H]-Quinuclidinyl benzilate ([^{3}H]-QNB) assays were performed in rat cortex following the method of Loudon *et al*., 1997. In brief, cerebral cortices were homogenized in ice-cold 50 mM Tris buffer, pH 7.7, followed by centrifugation at $28{,}000 \times g$ 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.

[^{3}H]-OXO-M (final concentration of 1.9 nM) binding was performed in 50 mM Tris, containing 2 mM $MgCl_2$ pH 7.7 using 100 μg protein per well. Oxotremorine (10 μM) was used to define non-specific binding. [^{3}H]-QNB binding (final concentration 0.3 nM) was 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% PEI followed by five washes with ice-cold 50 mM Tris buffer.

Radioligand binding assay in cloned cell lines

The binding affinity of xanomeline was determined at the following receptors; human (h) 5-HT_{1A}, h5-HT_{1B}, h5-HT_{1D}, h5-ht_{1E}, h5-ht_{1F}, h5-HT_{2A}, h5-HT_{2B}, h5-HT_{2C}, 5-HT_4 (piglet), h5-HT_6, h5-HT_7, hD_2, hD_3 and human adrenergic-alpha 1B (hα-1B), as described by Kennett *et al*., 1997. Studies on CHO cells expressing hM_1, hM_2, hM_3, hM_4 and hM_5 receptors were carried out according to Loudon *et al*., 1997.

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

Cerebral cortex from male Sprague Dawley rats was dissected and homogenized in 10 volumes of ice-cold 50 mM Tris HCl containing 1 mM dithiothreitol (DTT) and 1 mM EGTA, pH 7.4. The homogenate was centrifuged at $1{,}000 \times g$ for 5 min at 4°C and the resultant supernatant (S1) was removed and stored on ice. The pellet was resuspended in buffer, homogenized and centrifuged as before. The supernatant was combined with S1 and centrifuged at $11{,}000 \times g$ for 20 min at 4°C. The resultant pellet was resuspended in 20 volumes of buffer and centrifuged at $27{,}000 \times g$ 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 preincubated for 30 min at 30°C in 50 mM Tris HCl, pH 7.4 containing 1 mM DTT, 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 with 50 μM unlabelled GTPγS.

[^{35}S]-GTPγS binding studies in cloned cells lines

Membranes from CHO cells expressing h5-HT_{1B} receptors were prepared according to Thomas *et al*., 1995. Membranes from cells expressing h5-HT_{1A} receptors were prepared by the same procedure. [^{35}S]-GTPγS binding assays for both cell lines were carried out as described by Watson *et al*., 1996.

Measurement of changes in cytoplasmic [Ca^{2+}] in cloned cell lines

Cultured SH-SY5Y cells expressing recombinant h5-HT_2 receptors were plated onto Costar, black wall, clear base, 96 well plates at a density of 20,000 cells per well in Dulbecco's Minimum Essential Medium (DMEM) with dialysed FCS (5–10%) and Geneticin 418 (400–500 μg ml^{-1}). Twenty-four hours later cells were incubated in DMEM with the cytoplasmic Ca^{2+} indicator, FLUO-3AM (4 μM), and 2.5 mM probenecid to reduce dye leakage, at 37°C for 90 min in 5% CO_2/95% O_2. The cells were subsequently washed with 4×150 μl Tyrodes buffer (mM): NaCl 145, glucose 10, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10, pH 7.4, containing 2.5 mM probenecid. Finally, 150 μl of Tyrodes buffer was added to each well and the plate placed into a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Crawley, W. Sussex, U.K.) to monitor cell fluorescence ($\lambda_{Ex} = 488$ nm, $\lambda_{Em} = 540$ nm). Following exposure of individual wells to different agonist concentrations, peak fluorescence intensity (F.I.) was recorded and expressed as an increase over baseline fluorescence. Subsequently all results were expressed as a percent of the maximum 5-HT response performed in each plate. For antagonist studies, the cells were incubated for 30 min in the presence of antagonist after dye loading and prior to exposure to agonist.

Materials

Xanomeline [3(3-hexyloxy-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine] and SB-224289 (2,3,6,7-tetrahydro-1′-methyl-5-{2′-methyl-4′-[(5-methyl-1,2,4-oxadiazole-3-yl) biphenyl-4-yl]carbonyl}furo[2,3-f]indole-3-spiro-4′-piperidine oxalate) were synthesized by SmithKline Beecham Pharmaceuticals. Other drugs and reagents were purchased from Sigma (Poole, U.K.), Calbiochem (Nottingham, U.K.), Bio-Rad (Hemel Hempstead, U.K.). Fisons Scientific Equipment (Loughborough, U.K.), Research Biochemicals International (Poole, U.K.) and Gibco BRL (Paisley, U.K.). [^{3}H]-OXO-M and [^{3}H]-QNB were obtained from NEN DuPont (Hounslow, U.K.). [^{35}S]-GTPγS and [^{125}I]-SB-207710 were supplied by Amersham International (Little Chalfont, U.K.). Costar, black wall, clear base, 96 well plates were obtained from Corning Costar Corporation (Cambridge, U.K.) and

FLUO-3AM was purchased from TEFLABS (Texas, U.S.A.).

Data analysis

In binding studies, pK_i values were calculated from IC_{50} values using the correction for radioligand concentration described by Cheng and Prussof (1973). In binding studies on CHO cells, the K_d value used for [^{3}H]-QNB binding was that reported by Bolden *et al*., 1991. Data from [^{35}S]-GTPγS binding and Ca^{2+} mobilization studies were fitted by a 4-parameter logistic equation using GRAFIT (Erithacus Software Ltd., Staines, U.K.) to yield values for maximum activity and EC_{50}. Antagonist activity of compounds was quantified by calculating their apparent pK_b value according to the Gaddum equation (Lazareno & Birdsall, 1993): 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. In studies measuring changes in cytoplasmic [Ca^{2+}], the inhibition curve design of the Gaddum equation was used to estimate apparent pK_b.

Results

Radioligand binding profile

In studies using [^{3}H]-OXO-M and [^{3}H]-QNB, xanomeline showed high affinity for muscarinic receptors in rat cortex (see Table 1). It has previously been reported that the ratio of the pIC_{50} obtained using [^{3}H]-OXO-M and [^{3}H]N-methylscopolamine is a reasonable prediction of whether a compound will show agonist or antagonist properties at muscarinic receptors (Freedman *et al*., 1988). From studies performed using [^{3}H]-OXO-M and [^{3}H]-QNB, it has been reported that a ratio close to unity predicts antagonism while full agonists have ratios >100 (Brown *et al*., 1988; Loudon *et al*., 1997). Intermediate ratios suggest varying degrees of partial agonism. In the present study the [^{3}H]-QNB/[^{3}H]-OXO-M pIC_{50} ratio for xanomeline was 16, suggesting xanomeline would be a partial agonist at muscarinic receptors.

From binding studies on cloned receptors, xanomeline showed similar affinities for hM_3, hM_4 and hM_5 receptor subtypes tested with a pK_i value of approximately 7.5 (Table 1) and a slightly lower pK_i value of 7.0 at hM_1 and hM_2 receptors. Xanomeline also showed high affinity for a number of h5-HT receptor subtypes, including h5-HT_{1A}, h5-HT_{1B}, h5-HT_{1D}, h5-HT_{2B} and h5-HT_{2C} and moderate affinity for h5-HT_{1F}, h5-HT_{2A}, 5-HT_4, h5-HT_7 and hD_3 (Table 1).

Table 1 Binding affinities of xanomeline for native and cloned neurotransmitter receptors

Receptor	*Ligand*	*pK_i*
Muscarinic		
Rat cortex	[^{3}H]-OXO-M	7.6±0.1
Rat cortex	[^{3}H]-QNB	7.1±0.2
hM_1	[^{3}H]-QNB	7.1±0.1
hM_2	[^{3}H]-QNB	6.9±0.1
hM_3	[^{3}H]-QNB	7.4±0.1
hM_4	[^{3}H]-QNB	7.7±0.1
hM_5	[^{3}H]-QNB	7.4±0.2
Non-muscarinic		
Piglet (5-HT_4)	[^{125}I]-SB-207710	6.6±0.1
h5-HT_{1A}	[^{3}H]-8-OH-DPAT	7.2±0.1
h5-HT_{1B}	[^{3}H]-5-HT	7.3±0.1
h5-HT_{1D}	[^{3}H]-5-HT	8.2±0.1
h5-HT_{1E}	[^{3}H]-5-HT	5.6±0.1
h5-HT_{1F}	[^{3}H]-5-HT	6.5±0.1
h5-HT_{2A}	[^{3}H]-Ketanserin	6.9±0.1
h5-HT_{2B}	[^{3}H]-5-HT	7.7±0.1
h5-HT_{2C}	[^{3}H]-Mesulergine	7.4±0.1
h5-HT_6	[^{3}H]-LSD	5.9±0.1
h5-HT_7	[^{3}H]-5-CT	6.9±0.1
hD_2	[^{125}I]-Iodosulpride	6.0, 6.2, <6.0
hD_3	[^{125}I]-Iodosulpride	6.4±0.1
hα-1B	[^{3}H]-Prazosin	5.8±0.1

pK_i values are means±s.e.mean from 3–5 individual experiments.

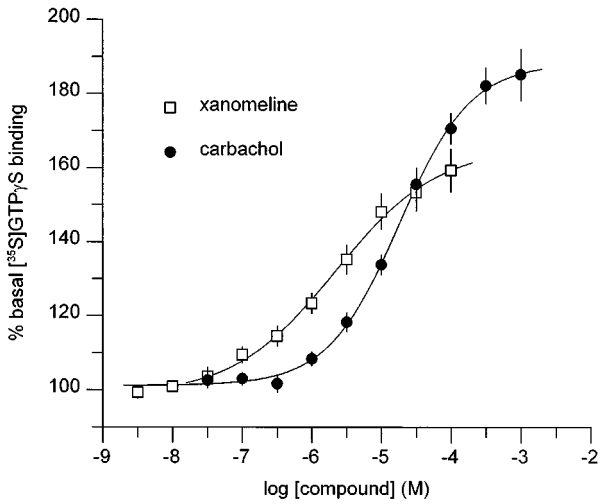

Figure 1 Effect of xanomeline and carbachol on [^{35}S]-GTPγS binding to rat cortical membranes. Data represent means±s.e.mean of eight individual experiments.

[^{35}S]-GTPγS binding studies

Rat cortex In rat cortex, carbachol and xanomeline stimulated [^{35}S]-GTPγS binding with pEC_{50} of 4.8±0.1 and 5.6±0.2 respectively (Figure 1). The maximum response to carbachol was 88±5% above basal, whereas xanomeline produced a maximal stimulation of 59±6% above basal (Figure 1). The carbachol response was dose-dependently inhibited by the M_2/M_4-selective antagonist, himbacine, with $pA_2 = 8.2$ and the M_1-selective antagonist, pirenzepine, with $pA_2 = 6.9$, (Figure 2, Table 2). A component of xanomeline-induced stimulation of [^{35}S]-GTPγS binding was slightly inhibited by himbacine (1 μM) with apparent $pK_b = 6.3$ (Table 2) but pirenzepine (3 μM) produced no significant change in EC_{50}. In the presence of 100 nM atropine, to block muscarinic receptors, methiothepin (100 nM) and the 5-HT_{1A} receptor antagonist, WAY100635 (3 nM), attenuated the response to xanomeline with apparent pK_b of 7.7 and 9.4 respectively (Figure 3, Table 2). 5-HT and the selective 5-HT_{1A} agonist, (+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT), also stimulated [^{35}S]-GTPγS binding (Figure 4, Table 3). The response to 5-HT was attenuated by 1 nM WAY100635 with apparent pK_b of 9.9.

Cloned cell lines In HEK293 cell membranes expressing h5-HT_{1A} receptors and CHO cells expressing h5-HT_{1B} receptors, xanomeline stimulated [^{35}S]-GTPγS binding with pEC_{50} slightly lower than its binding affinities and a maximum

response comparable to that of 5-HT (Figure 5, Table 4). Carbachol had no effect on [^{35}S]-GTPγS binding on either cell line. The response to xanomeline in h5-HT$_{1A}$ receptor-expressing cells was inhibited by the selective 5-HT$_{1A}$ antagonist, WAY100635 (Figure 5a, Table 4), with an apparent pK$_b$ of 9.4. In h5-HT$_{1B}$ expressing cells, xanomeline-induced stimulation of basal [^{35}S]-GTPγS binding was inhibited by the selective 5-HT$_{1B}$ antagonist SB-224289 (Figure 5b, Table 4) with apparent pK$_b$ of 8.2. These data are consistent with xanomeline acting as a full agonist at cloned h5-HT$_{1A}$ and h5-HT$_{1B}$ receptors.

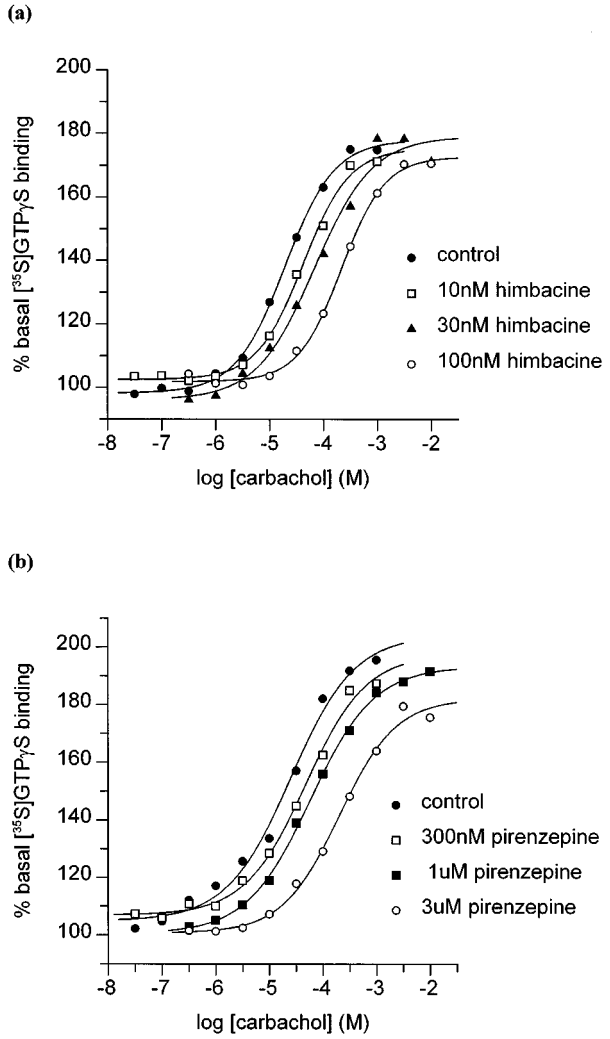

Figure 2 Inhibition of carbachol-induced stimulation of [^{35}S]-GTPγS binding to rat cortical membranes by muscarinic antagonists. (a) Shows the response to carbachol alone and in the presence of 10 nM, 30 nM and 100 nM himbacine. (b) Shows the response to carbachol alone and in the presence of 300 nM, 1000 nM and 3000 nM pirenzepine. Data represent a single experiment, typical of five giving similar results.

Table 2 Effect of muscarinic and 5-HT receptor antagonists against carbachol- and xanomeline-induced stimulation of [^{35}S]-GTPγS binding in rat cortical membranes

Compound	*pA$_2$ vs carbachol*	*Apparent pK$_b$ vs xanomeline*
himbacine	8.2±0.1	6.3±0.2
pirenzepine	6.9±0.2	<5*
methiothepin	ND	7.7±0.1
WAY100635	ND	9.4±0.2

Data represent mean value±s.e.mean from 3–5 individual experiments each performed in duplicate. Studies using methiothepin and WAY100635 were carried out in the presence of 100 nM atropine. ND: not determined. *No effect on xanomeline response at 3 μM.

Measurement of changes in cytoplasmic [Ca^{2+}]

5-HT stimulated an increase in cell fluorescence in cultured SH-SY5Y cells expressing h5-HT$_{2A}$, h5-HT$_{2B}$ and h5-HT$_{2C}$ receptors with pEC$_{50}$ of 7.7±0.1, 8.8±0.1 and 8.1±0.1 respectively (Figure 6). The muscarinic agonist, carbachol, also stimulated cell fluorescence in these cell lines, whereas xanomeline caused a small stimulation in h5-HT$_{2C}$ receptor-expressing cells only, with pEC$_{50}$ of 6.8±0.1 (Figure 6c). The responses to xanomeline were completely abolished by 100 nM atropine consistent with its activity being mediated *via* endogenous muscarinic receptors. In addition, the response to xanomeline in h5-HT$_{2C}$ expressing cells was not inhibited by the selective 5-HT$_{2C}$ receptor antagonist, SB-206553, up to a

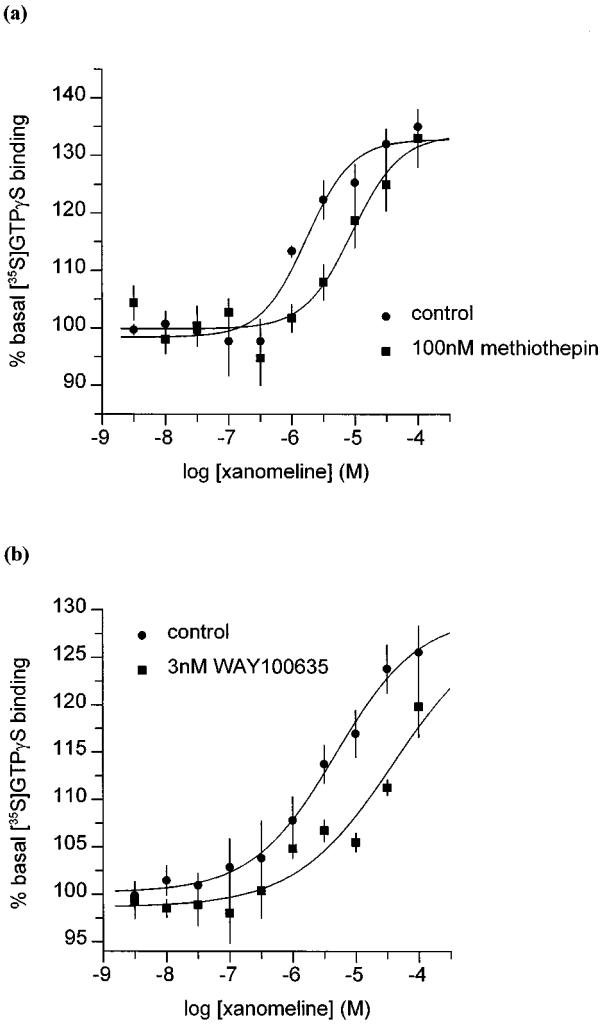

Figure 3 Inhibition of xanomeline-stimulated [^{35}S]-GTPγS binding to rat cortical membranes by non-muscarinic antagonists, in the presence of 100 nM atropine. Curves represent the response to xanomeline alone (a and b) and in the presence of 100 nM methiothepin (a) and 3 nM WAY100635 (b). Data represent means±s.e.mean of three individual experiments.

concentration of 100 nM. In the presence of atropine (100 nM) to prevent interference by muscarinic stimulation, xanomeline inhibited 5-HT-stimulated fluorescence in cells expressing h5-HT_{2A}, h5-HT_{2B} and h5-HT_{2C} receptors with potencies comparable with its binding affinity at each receptor (Table 5, Figure 7).

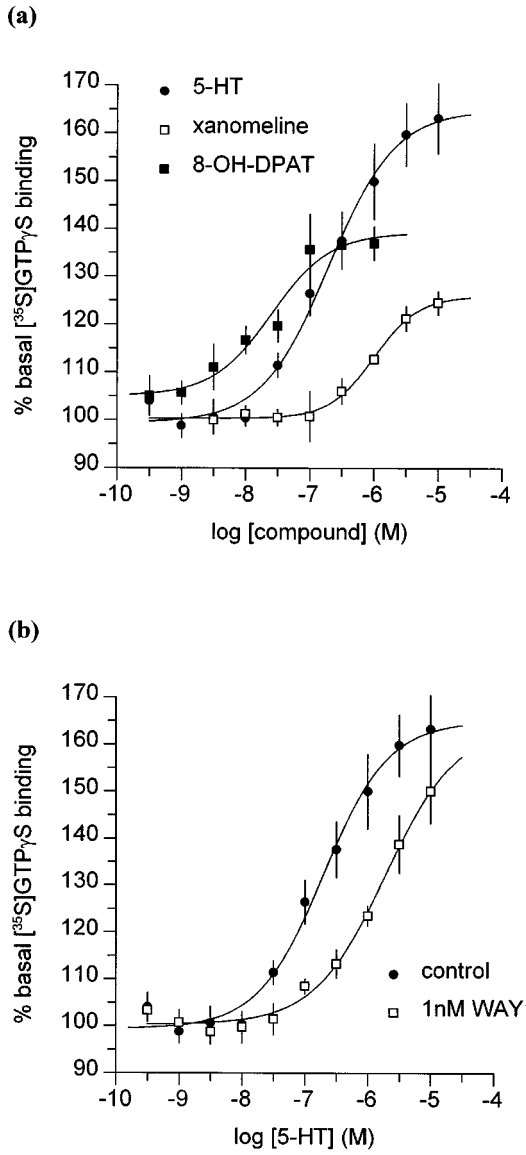

Figure 4 [^{35}S]-GTPγS binding to rat cortical membranes. (a) Shows the response to xanomeline (in the presence of 100 nM atropine), 5-HT and 8-OH-DPAT. (b) Shows the response to 5-HT alone and in the presence of 1 nM WAY100635. Data represent means$\pm$s.e.mean of 3–4 individual experiments.

Table 3 Effect of 5-HT receptor agonists on [^{35}S]-GTPγS binding in rat cortical membranes

Compound	*pEC_{50}*	*% stimulation*	*Apparent pK_b WAY100635*
5-HT	6.7$\pm$0.1	67$\pm$9	9.9$\pm$0.2
8-OH-DPAT	7.4$\pm$0.2	37$\pm$5	ND
xanomeline	5.8$\pm$0.1	31$\pm$2	9.4$\pm$0.2

Data represent mean value$\pm$s.e.mean from 3–4 individual experiments each performed in duplicate. Studies using xanomeline were carried out in the presence of 100 nM atropine. ND: not determined.

Discussion

In this study we have investigated the functional effects of xanomeline on native tissue and cloned receptors and have characterized the receptors involved. From radioligand binding studies in whole rat cortex, xanomeline showed high affinity for muscarinic receptors and the QNB/OXO-M ratio predicted partial agonism according to previous studies with muscarinic ligands (Brown *et al*., 1988; Loudon *et al*., 1997).

Data from radioligand binding studies using cloned cell lines expressing a variety of different receptors revealed that xanomeline is not particularly selective for any of the five

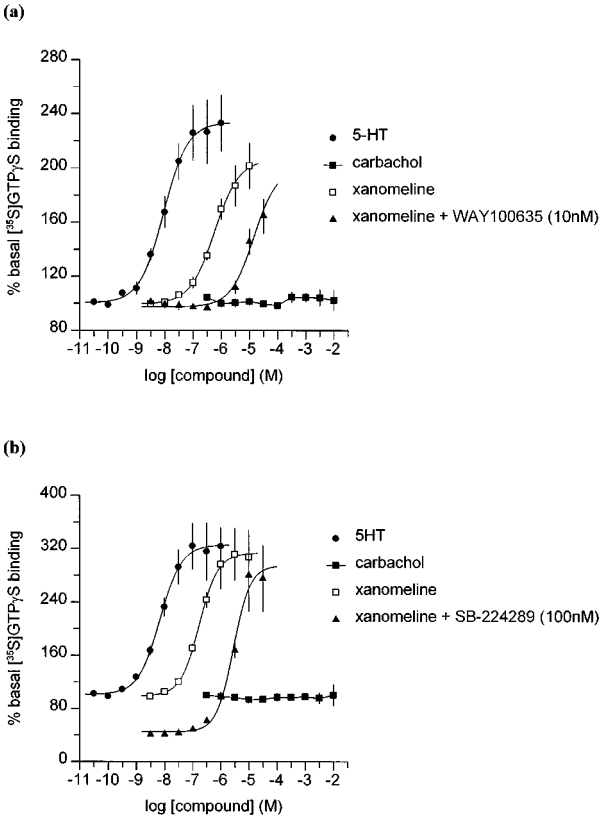

Figure 5 Effect of 5-HT, xanomeline and carbachol on [^{35}S]-GTPγS binding to HEK293 cell membranes expressing h5-HT_{1A} receptors (a) and CHO cell membranes expressing h5-HT_{1B} receptors (b). For antagonist studies, membranes were incubated with xanomeline in the absence or presence of 10 nM WAY100635 or 100 nM SB-224289. Data represents means$\pm$s.e.mean of 3–4 individual experiments.

Table 4 Xanomeline-induced stimulation of [^{35}S]-GTPγS binding in h5-HT_{1A} or h5-HT_{1B} receptor-expressing cells and inhibition by selective antagonists

Receptor	*pEC_{50}*	*I.A.*	*Apparent pK_b WAY100635*	*SB-224289*
h5-HT_{1A}	6.4$\pm$0.1	0.8$\pm$0.1	9.4$\pm$0.1	ND
h5-HT_{1B}	6.8$\pm$0.1	0.9$\pm$0.1	ND	8.2$\pm$0.1

I.A.: intrinsic activity compared to 5-HT. Data represent mean value$\pm$s.e.mean from three individual experiments each performed in duplicate. ND: not determined.

muscarinic receptor subtypes and has substantial affinity at 5-HT_1 and 5-HT_2 receptors. Data on affinities for 5-HT receptors have previously been reported by Shannon *et al*., 1994 but their affinity values are 3–10 fold lower than those reported in this study. The discrepancies seen could reflect receptor species variability or different experimental conditions.

The functional effect of xanomeline at these 5-HT receptors was investigated using [^{35}S]-GTPγS binding which provides a measure of agonist-stimulated receptor-G-protein coupling. In rat cortical membranes, both xanomeline and the muscarinic agonist carbachol produced concentration-related stimulations of basal [^{35}S]-GTPγS binding with micromolar potency. The slope of the response to xanomeline was less than unity suggesting the involvement of more than one receptor. Antagonist studies were carried out to elucidate the receptors by which carbachol and xanomeline produced their effect. The selective 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 receptors (Dorje *et al*., 1990; Lazareno *et al*., 1990; Lazareno & Birdsall, 1993). The M_1 selective antagonist, pirenzepine, inhibited the response with pA_2 of 6.9 which is consistent with its affinity at M_2 and M_3 receptors but is over 10 fold less than its affinity at M_1 receptors (Dorje *et al*., 1990; Lazareno & Birdsall, 1993). It is reported that M_2/M_4 receptors inhibit adenylyl cyclase *via* G_i/G_o (Peralta *et al*., 1988; Novotny & Brann, 1989) and that $M_1/M_3/M_5$ receptors are linked to phosphoinositide hydrolysis *via* G_q (Brann *et al*., 1988; Peralta *et al*., 1988; Liao *et al*., 1989). The affinity of [^{35}S]-GTPγS for G_i/G_o is higher than for G_q (Blank *et al*., 1991) and so it is reasonable to assume that carbachol stimulated [^{35}S]-GTPγS binding, predominantly, through M_2/M_4 receptors as compared to G_q linked receptors. A similar finding for

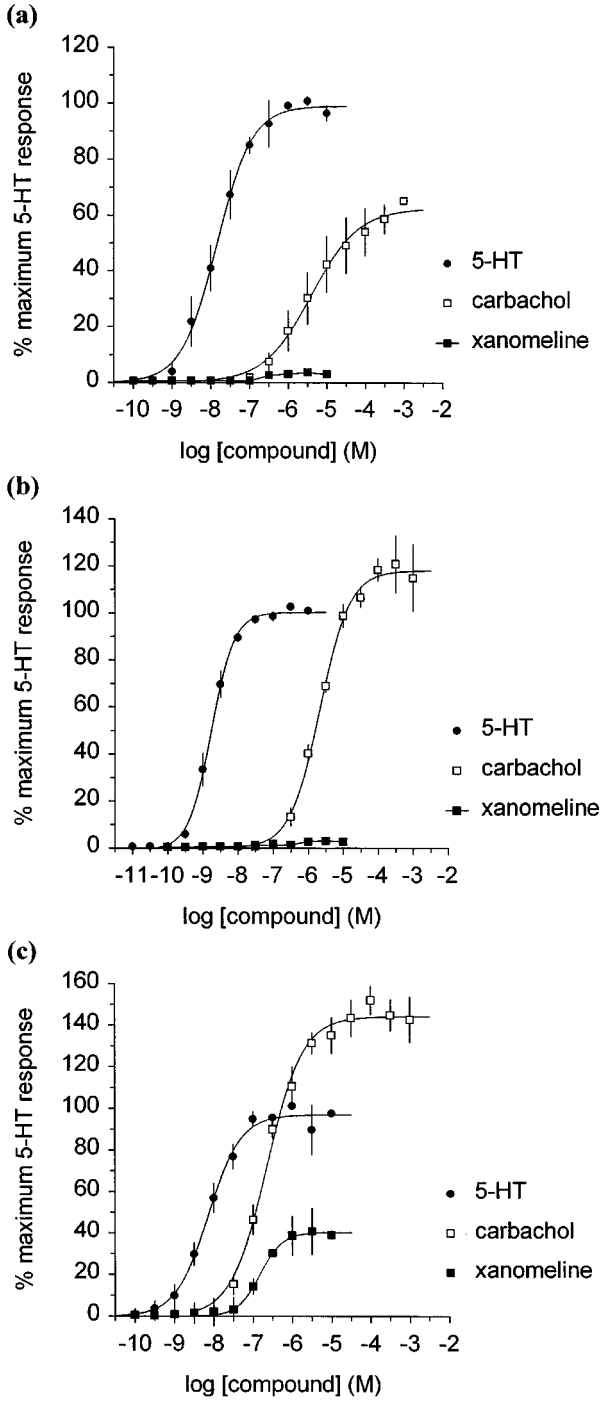

Figure 6 Measurement of changes in cytoplasmic [Ca^{++}] in SH-SY5Y cells expressing h5-HT_{2A} (a), h5-HT_{2B} (b) and h5-HT_{2C} (c) receptors. Cells were incubated in the presence of increasing concentrations of 5-HT, carbachol or xanomeline. Data show peak FLUO-3AM cell fluorescence expressed as per cent of the maximum 5-HT response performed in the same plate and represent means ± s.e.mean of 3–5 individual experiments.

Table 5 Effect of xanomeline on 5-HT-induced Ca^{2+} mobilization in cultured SH-SY5Y cells expressing human 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors

Receptor	*pIC_{50}*	*Apparent pK_b*
5-HT_{2A}	5.4 ± 0.3	6.4 ± 0.1
5-HT_{2B}	6.4 ± 0.1	6.9 ± 0.2
5-HT_{2C}	6.0 ± 0.2	7.0 ± 0.2

Data represent mean value ± s.e.mean from 3–5 individual experiments each performed in duplicate. pIC_{50} values were determined from concentration-response curves to xanomeline in the presence of 10 or 100 nM 5-HT and used to calculate apparent pK_b using the Gaddum equation.

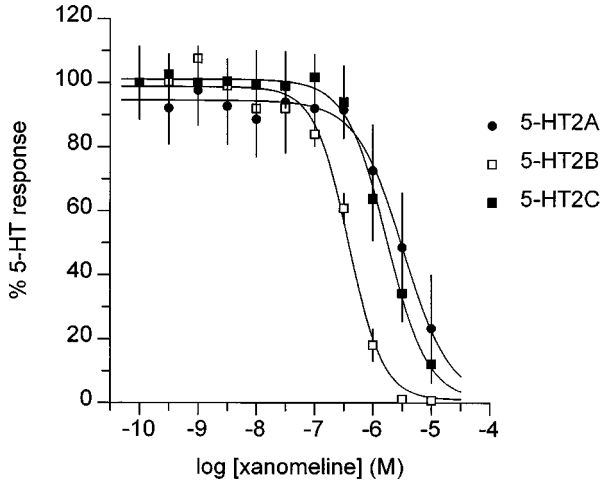

Figure 7 Inhibition of 5-HT-induced stimulation of cell fluorescence, by xanomeline, in SH-SY5Y cells expressing h5-HT_{2A}, h5-HT_{2B} or h5-HT_{2C} receptors. Cells were incubated with increasing concentrations of xanomeline in the presence of 10 nM 5-HT (h5-HT_{2B} receptor-expressing cells) or 100 nM 5-HT (h5-HT_{2A} and 5-HT_{2C} receptor-expressing cells). Data show peak FLUO-3AM fluorescence intensity expressed as per cent of the 5-HT response and represent means ± s.e.mean of 3–5 individual experiments.

carbachol has also been reported in rat olfactory bulb membranes (Olianas & Onali, 1996). Xanomeline-induced stimulation of [^{35}S]-GTPγS in rat cortex binding was not measurably inhibited by pirenzepine at a concentration, 3 μM, which should have been adequate to block all muscarinic receptor subtypes (Dorje *et al*., 1990; Lazareno & Birdsall, 1993). Himbacine attenuated part of the response to give an apparent $pK_b = 6.3$, a potency 100 fold lower than its affinity for M_2/M_4 receptors. Although this is similar to the affinity of himbacine for M_1 and M_3 receptors, the lack of effect of 3 μM pirenzepine together with the findings discussed above make it unlikely that xanomeline-induced stimulation of [^{35}S]-GTPγS binding reflected M_1/M_3 activation. These data suggest, instead, that xanomeline was acting, in part, through non-muscarinic receptors.

In order to determine the receptors mediating xanomeline-induced [^{35}S]-GTPγS binding we initially investigated the effect of the non-selective 5-HT receptor antagonist methiothepin, since we had demonstrated the high affinity of xanomeline for 5-HT receptors. In the presence of 100 nM atropine, to block muscarinic activity, the maximal response to xanomeline was reduced by approximately 50% and was attenuated by methiothepin with a potency comparable to its affinity at 5-HT_1 receptors. Further studies indicated that the selective 5-HT_{1A} receptor antagonist, WAY100635, inhibited xanomeline-induced stimulation of [^{35}S]-GTPγS binding with an apparent pK_b of 9.4, similar to its affinity at h5-HT_{1A} receptors (Fletcher *et al*., 1996), although it did appear that there may be a small component of the response that was not blocked by WAY100635. These data suggest that, in rat cortex, xanomeline stimulated basal [^{35}S]-GTPγS binding, in part, *via* 5-HT_{1A} receptors. To support this conclusion we confirmed that both 5-HT and the selective 5-HT_{1A} agonist, 8-OH-DPAT, stimulated basal [^{35}S]-GTPγS binding in rat cortex. The response to 5-HT was greater than that to xanomeline and was blocked by WAY100635 with a potency of 9.9 consistent with activation at 5-HT_{1A} receptors. These data suggest that both 5-HT and xanomeline stimulated 5-HT_{1A} receptors and that xanomeline acts as a partial 5-HT_{1A} agonist in rat cortex.

Consistent with these findings in rat cortex, we showed that xanomeline stimulated [^{35}S]-GTPγS binding in cultured HEK293 cells expressing h5-HT_{1A} receptors. In this system, xanomeline displayed full agonism compared to 5-HT which presumably reflects the higher 5-HT_{1A} receptor expression compared to rat cortex. The response to xanomeline was blocked by WAY100635, indicative of 5-HT_{1A} receptor activation. We also investigated the effect of xanomeline on basal [^{35}S]-GTPγS binding in CHO cells expressing h5-HT_{1B} receptors. Similarly, xanomeline produced a maximal stimulation of binding, compared to 5-HT, which could be blocked by the selective 5-HT_{1B} receptor antagonist, SB-224289, with a potency similar to its binding affinity at h5-HT_{1B} receptors (Selkirk *et al*., 1998). Carbachol, at concentration up to 1 mM, had no effect on [^{35}S]-GTPγS binding in either h5-HT_{1A} or h5-HT_{1B} receptor-expressing cells, consistent with xanomeline exerting its agonist effect through these 5-HT receptors and not *via* endogenous muscarinic receptors.

From radioligand binding studies, xanomeline also displayed moderate affinity at h5-HT_2 receptors and to investigate function at these receptors we used a Fluorometric Imaging Plate Reader to measure changes in cytoplasmic [Ca^{2+}] using the Ca^{2+} indicator dye, FLUO-3AM. Xanomeline produced a small stimulation of cell fluorescence intensity in cultured SH-SY5Y cells expressing h5-HT_{2C} receptors. This response could be blocked by atropine and not by the selective 5-HT_{2C} receptor antagonist, SB-206553 (Kennet *et al*., 1996), suggesting xanomeline elicited its effect *via* muscarinic receptors endogenously expressed by the cells. Surprisingly, xanomeline did not display agonist activity in SH-SY5Y cells expressing h5-HT_{2A} or h5-HT_{2B} receptors. Further studies are underway to determine whether this reflects supression of endogenous muscarinic receptor expression by concomitant expression of h5-HT_{2A} and h5-HT_{2B} receptors or stimulation by h5-HT_{2C} receptor expression. Consistent with the latter explanation, carbachol displayed greater efficacy and potency in h5-HT_{2C} receptor-expressing cells compared to cells expressing h5-HT_{2A} or h5-HT_{2B} receptors, suggestive of larger muscarinic receptor expression. In studies to examine antagonist activity of xanomeline on h5-HT_2 receptors, atropine was included to prevent interference of muscarinic receptor agonism by xanomeline. Using these conditions, xanomeline inhibited the 5-HT response, at each receptor subtype, with potencies similar to its respective binding affinities. These data suggest that xanomeline acts as an antagonist at all h5-HT_2 receptor subtypes. In conclusion, we have shown that the muscarinic agonist, xanomeline, is an agonist at 5-HT_{1A} and 5-HT_{1B} receptors in native tissue and/or cloned cell lines and acts as an antagonist at h5-HT_2 receptors.

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