JPET Fast Forward. Published on May 12, 2004 as DOI: 10.1124/jpet.104.067140

JPET #67140

Functional Selectivity of Muscarinic Receptor Antagonists for Inhibition of M_3-Mediated Phosphoinositide Responses in Guinea-Pig Urinary Bladder and Submandibular Salivary Gland

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This work was financially supported by Pfizer Global Research and Novartis Pharma

JPET #67140

Running title: Tissue-selective effects of muscarinic antagonists

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Section Assignment: ***Cellular & Molecular***

Text pages:	31
Tables:	3
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Abstract:	248 words
Introduction:	642 words
Discussion:	1487 words
References:	40

Abbreviations: CCh, carbachol; CHO, Chinese hamster ovary; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; KHB, Krebs-Henseleit buffer; MCh, methacholine; mACh, muscarinic acetylcholine; [^{3}H]-NMS, [*N-methyl*-^{3}H]-scopolamine methyl chloride; OAB, overactive bladder; TCA, trichloroacetic acid.

ABSTRACT

Binding and functional affinities of the muscarinic acetylcholine (mACh) receptor antagonists darifenacin, tolterodine, oxybutynin and atropine were assessed in Chinese hamster ovary (CHO) cells expressing the human recombinant M_2 (CHO-m2) or M_3 (CHO-m3) receptors, and in guinea-pig bladder and submandibular gland. In [^{3}H]-NMS binding studies in CHO cells darifenacin displayed selectivity (14.8 fold) for the M_3 versus M_2 mACh receptor subtype. Oxybutynin was non-selective, while atropine and tolterodine were weakly M_2-selective (5.1 and 6.6 fold, respectively). Antagonist functional affinity estimates were determined by the inhibition of agonist-induced [^{3}H]-inositol phosphate accumulation in CHO-m3 cells, and antagonism of the agonist-induced inhibition of forskolin-stimulated cyclic AMP accumulation in CHO-m2 cells. Darifenacin was the most M_3-selective antagonist (32.4 fold), while oxybutynin, atropine and tolterodine exhibited lesser selectivity. Functional affinity estimates in guinea-pig urinary bladder and submandibular salivary gland using indices of phosphoinositide turnover revealed that oxybutynin, darifenacin and tolterodine each displayed selectivity for the response in the bladder, relative to that seen in the submandibular gland (9.3, 7.9 and 7.4 fold, respectively). In contrast, atropine displayed a similar affinity in both tissues. These data demonstrate that in bladder compared to submandibular gland from a single species the mACh receptor antagonists darifenacin, tolterodine and oxybutynin display selectivity to inhibit agonist-mediated phosphoinositide responses. It is proposed that both responses are mediated via M_3 mACh receptor activation and that differential functional affinities displayed by some, but not all, antagonists are indicative of the influence of cell background upon the pharmacology of the M_3 mACh receptor.

Introduction

The cholinergic nervous system is the major pathway by which bladder contraction is initiated in humans (Andersson, 1993). Molecular techniques have identified five muscarinic acetylcholine (mACh) receptor subtypes (m1-m5), while pharmacological data can distinguish only four subtypes, denoted as M_1-M_4 (Caulfield and Birdsall, 1998). Both M_2 and M_3 mACh receptors have been identified in bladder (detrusor) smooth muscle at the level of mRNA (Yamaguchi et al., 1996) and protein, with quantitative immunoprecipitation demonstrating a 75-90% predominance of the M_2 subtype in all species studied (Wang et al., 1995). Roles for the majority M_2 receptor population in the contraction of various smooth muscle types (including bladder detrusor) have been proposed, based upon observations in tissues treated with N-2-chloroethyl-4-piperidinyl diphenylacetate selectively to inactivate the M_3 receptor population. Under these conditions M_2 receptor activation may potentiate M_3-stimulated contraction (via activation of non-selective cation currents or inhibition Ca^{2+}-dependent K^+ efflux), in addition to reversing the relaxation mediated by agents that increase cytosolic cyclic AMP (Ehlert, 2003). However, pharmacological characterization of the mACh receptors mediating contraction in native detrusor smooth muscle indicate the predominant involvement of the minority M_3 receptor population in a variety of species including rat (Longhurst et al., 1995), guinea-pig (Wang et al., 1995), and human (Chess-Williams et al., 2001). Compounds with high affinity for the M_3 receptor have therefore been used in the management of overactive bladder (OAB) (Wallis and Napier, 1999). Indeed, the major current therapies for OAB, oxybutynin and tolterodine, both display high affinity antagonism at the M_3 receptor and potently inhibit mACh receptor agonist-induced urinary bladder bladder contractions *in vitro* and *in vivo* (Nilvebrant et al., 1997; Gillberg et al., 1998). However, the clinical utility of mACh receptor antagonists has been limited by lack of selectivity, which leads to classical anti-muscarinic side-effects, such as dry mouth,

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tachycardia and blurred vision. This has led to the search for mACh receptor antagonists with greater selectivity for the M_3 receptor and in particular for mACh receptor agonist-mediated bladder contraction.

The mACh receptor antagonist darifenacin displays both high affinity (pK_i = 9.12) and selectivity for the human M_3 receptor (Wallis and Napier, 1999). Intriguingly, darifenacin has been reported to inhibit urinary bladder contraction at concentrations lower than those required to influence salivary secretion *in vivo* in both rat (Wallis and Napier, 1999) and dog (Gupta et al., 2002) despite both responses being predominantly or exclusively M_3-mediated. In contrast, atropine inhibited both responses equipotently, while oxybutynin has been shown to be either non-selective (Newgreen and Naylor, 1996; Ikeda et al., 2002), or slightly selective for the salivation response (Nilvebrant et al., 1997).

Similar tissue-specific selectivity had earlier been reported between different smooth muscle types for other muscarinic receptor antagonists, including zamifenacin (Watson et al., 1995) and *p*-fluorohexahydrosiladifenidol (*p*-FHHSiD) (Eglen et al., 1990). However, tissue-dependent selectivity may not only be a property of subtype-selective antagonists. Thus, tolterodine, a compound widely used in the clinical management of OAB, has been reported to display a greater functional affinity for urinary bladder than salivary gland, despite failing to exhibit selectivity between mACh receptor subtypes (Nilvebrant et al., 1997). It has been proposed that such 'functional selectivity' could lead to a superior clinical side-effect profile in the management of OAB and may at least in part explain why tolterodine is equally as effective as oxybutynin for improving the symptoms of OAB, yet is better tolerated by patients (Malone-Lee et al., 2001).

The aim of this study was to establish whether a range of subtype-selective and non-selective mACh receptor antagonists display *in vitro* selectivity for M_3-mediated responses between

urinary bladder and salivary gland, within the same species, at the level of second messenger generation. Darifenacin, along with the current OAB therapies oxybutynin and tolterodine and the classical non-selective muscarinic antagonist atropine, were investigated for their ability to inhibit mACh receptor agonist-mediated phosphoinositide turnover in guinea-pig urinary bladder and submandibular salivary gland.

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Materials and Methods

Materials. Radiolabeled compounds were obtained from Amersham Pharmacia (Little Chalfont, UK), with the exception of [^{3}H]-Ins(1,4,5)P_3 which was from NEN Life Science (Zaventem, Belgium). Darifenacin and tolterodine were synthesized in the laboratories of Pfizer Global Research and Development (Sandwich, UK). MT-7 was purchased from Peptides Int. (Kentucky, USA). All other reagents were purchased from Sigma-Aldrich (Poole, U.K.) or Fisher Scientific (Loughborough, UK).

Cell culture. Chinese Hamster Ovary lines stably expressing cloned human m2 or m3 receptors (CHO-m2 and CHO-m3, respectively) were grown in Minimum Essential Medium-α (MEM-α) supplemented with 10% newborn calf serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B. Cells were maintained at 37^{o}C in a humidified atmosphere of O_2/CO_2 (19:1) and were routinely split 1:5 every 3-4 days, using trypsin-EDTA.

Animals and tissue slice preparation. Male Dunkin Hartley guinea-pigs (David Hall guinea-pigs; Burton-on-Trent, U.K.), 300-500 g in weight, were euthanized by injection of 1 g kg^{-1} pentobarbitone. For each experiment, urinary bladder and submandibular glands were dissected from four animals. The tissue was cleared of connective tissue and immediately transferred into ice-cold Krebs-Henseleit buffer (KHB; composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, Na HCO_3 25, HEPES 5, glucose 10, pH 7.4) and kept on ice prior to chopping. Each tissue was cross-chopped (300 μm x 300 μm) with a McIlwain tissue chopper. The resulting slices were transferred to 20 ml KHB, gassed with

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O_2/CO_2 (19:1) at 37^{o}C. Slices were shaken vigorously in a waterbath at 37^{o}C with 3-4 changes of KHB over a 30 min period.

Membrane preparation from guinea-pig tissues. Fresh or frozen tissue samples were finely chopped with scissors in 20 mM HEPES, 1 mM EDTA (pH 7.4) buffer at 4^{o}C and any excess fat decanted off. Tissue was then homogenized using a Polytron (14,000 r.p.m., 20 sec). Homogenates were centrifuged at 5000 x*g* (10 min, 4^{o}C) and the supernatant fraction pooled following passage through Miracloth (Calbiochem, San Diego, USA). The filtrate was then centrifuged at 40,000 x*g* (20 min, 4^{o}C). The resulting pellet was re-homogenized in buffer before a second high speed spin as before. This final pellet was re-suspended in 20 mM HEPES, 0.1 mM EDTA, pH7.4 buffer and aliquots frozen down at -80^{o}C at a concentration of 2-3 mg protein ml^{-1}.

CHO cell membrane preparation. Confluent monolayers of CHO cells were washed with HBS-EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4) and cells lifted from the flask by the addition of HBS-EDTA for approximately 15 min. A cell pellet was recovered by centrifugation at 1700 r.p.m. for 5 min. The cell pellet was homogenized, a membrane fraction recovered and stored exactly as described for the tissue membrane preparations described above.

Radioligand binding to membranes. Saturation binding was performed using a range of concentrations of [^{3}H]-NMS (0.03–6.0 nM; 81 Ci mmol^{-1}) in the absence and presence of atropine (10 μM) to define non-specific binding. Binding assays were performed in a final volume of 400 μl (assay buffer: 20 mM HEPES, pH 7.4) containing 25-100 μg membrane protein and were incubated for 120 min at 25^{o}C. Bound radioligand was separated from free by rapid vacuum filtration through Whatman GF/B filters on a 24 well Brandel cell harvester

and radioactivity quantified by liquid scintillation counting. Competition binding experiments were performed using a single concentration of [^{3}H]-NMS (0.3-0.5 nM) in the absence and presence of a range of antagonist concentrations. Bound radioligand was separated from free by rapid vacuum filtration through Whatman GF/B Unifilter Plates on a 96 well Packard Unifilter Harvester. All other conditions were as stated above for saturation binding assays.

Radioligand binding to intact cells. Intact cell binding assays were performed on cell monolayers on 24-well plates, in KHB. The medium was aspirated from each well and the cells washed 3 times with KHB at 37^{o}C. Where applicable, antagonists were pre-incubated for 30 min before the addition of [^{3}H]-NMS, in a total assay volume of 500 µl. Following 60 min incubation at 37^{o}C, cells were washed 3 times with warm, gassed KHB before solubilizing cells with NaOH (0.1 M) and subsequent liquid scintillation counting.

[^{3}H]-inositol phosphate accumulation in bladder slices. Following the 30 min washing period, bladder slices were transferred to a 50 cm^2 culture flask containing MEMα (10 ml), supplemented with 100 IU^{-1} penicillin and 100 µg ml^{-1} streptomycin. *Myo*-[^{3}H]-inositol (25 µCi; specific activity 88 Ci mmol^{-1}) was added to the flask and incubated for 24 h at 37^{o}C in CO_2/air (1:19). Following the labeling period, slices were transferred to KHB at 37^{o}C and incubated at 37^{o}C with vigorous shaking for 20 min. During this period the buffer was changed 3-4 times to ensure complete removal of medium. Gravity-packed slices (50 µl) were transferred to insert vials containing KHB. Vials were then gassed with O_2/CO_2 (19:1), capped and incubated at 37^{o}C. At this point, antagonists were added where appropriate. Lithium chloride (LiCl) (5 mM final concentration) was then added to each vial. Incubations were initiated, 15 min later, by the addition of carbachol (CCh), to give a final assay volume of 300 µl. Incubations were terminated after 30 min by the addition of ice-cold 1M

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trichloroacetic acid (TCA, 300 μl). Samples were left on ice to extract for 20 min. At the end of this period, vials were centrifuged for 15 min at 5000 r.p.m. 500 μl samples of the resulting supernatants were transferred to microfuge tubes containing 10 mM ethylenediaminetetra acetic acid (EDTA, 100 μl) and the [^{3}H]-IP_x fraction separated (Batty et al., 1997).

Ins(1,4,5)P_3 mass determination in cross-chopped submandibular gland. Gravity-packed submandibular gland slices (50 μl) were transferred to insert vials containing KHB. Vials were gassed with O_2/CO_2 (19:1) capped and incubated at 37^{o}C. At this point, antagonists were added where appropriate. Incubations were initiated, 15 min later, by the addition of CCh, to make a final assay volume of 300 μl. Incubations were terminated after 5 min by the addition of ice-cold 1M TCA (300 μl). Samples were left on ice to extract for 20 min. At the end of this period, vials were centrifuged for 15 min at 5000 r.p.m. A sample (500 μl) of the resulting supernatant was transferred to a microfuge tube containing 10 mM EDTA (100 μl). Samples were neutralized using 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1 v/v; 500μl) and assayed for Ins(1,4,5)P_3 mass content as described previously (Batty et al., 1997). Protein concentrations were determined according to the method of Lowry et al. (1951) and data corrected for these values.

Cyclic AMP accumulation in CHO cells. Following antagonist additions for 20 min CHO-m2 cell monolayers were incubated with methacholine (MCh, 1 μM) for 10 min prior to addition of forskolin (10 μM). Following further incubation for 10 min assays were stopped by aspiration and addition of ice-cold 0.5 M TCA (400 μl). Samples were neutralized as described above and cyclic AMP determined using the method of Brown et al. (1971).

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[^{3}H]-inositol phosphate accumulation in CHO cells. CHO-m3 cell monolayers were labelled with *Myo*-[^{3}H]-inositol for 24 h prior to each experiment. Following 30 min pre-incubation with antagonist, cells were incubated for 15 min with LiCl (10 mM) prior to addition of MCh (3 μM). Following further incubation for 10 min, assays were stopped by aspiration and addition of ice-cold 0.5 M TCA (400 μl). Samples were neutralized and the [^{3}H]-IP_x fraction separated (Batty et al., 1997).

Data and statistical analysis. Data are shown as mean ± standard error of mean (s.e.m.) for the indicated number of experiments. Saturation binding data were analyzed by non-linear regression using GraphPad Prism 3.0. The equilibrium binding dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were derived from the Langmuir equation $RL = R_tL/(K_D+L)$ where L is the concentration of free ligand, RL is the concentration of receptor-bound ligand at equilibrium and R_t is the total receptor concentration. Antagonist displacement curves were fitted to the "four parameter logistic equation" using GraphPad Prism 3.0 and the best fit between a variable Hill coefficient and a Hill coefficient fixed to unity was determined using an F-test. IC_{50} values, generated by these inhibition curves, were corrected to give binding constant (K_i) values for each test compound, using the Cheng-Prusoff equation (Craig, 1993). Sigmoidal concentration-response curves for carbachol and methacholine, as well as all antagonist inhibition curves, were fitted to the "four parameter logistic equation" using GraphPad Prism 3.0. IC_{50} values, generated by these inhibition curves, were corrected to give binding constant (K_B) values for each test compound, using the functional equivalent of the Cheng-Prusoff equation (Craig, 1993; Lazareno & Birdsall, 1993): $K_b = IC_{50}/[1 + (A/EC_{50})]$, where A = [agonist] and EC_{50} = agonist EC_{50} (derived from agonist concentration-response curve generated each day as part of the same experiment). The statistical significance of differences between data was determined using Student's *t*

test, with K_i and K_B values first being converted to the respective normally distributed negative logarithm (pK_i or pK_B).

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Results

Radioligand binding in intact CHO cell monolayers. The relative selectivity for M_2 and M_3 mACh receptor subtypes of atropine, darifenacin, oxybutynin and tolterodine was first determined at the level of receptor binding affinity, in CHO-m2 (M_2 B_{max} ≈1 pmol mg^{-1} protein) or CHO-m3 (M_3 B_{max} ≈1.5 pmol mg^{-1} protein) cells. Saturation binding analysis yielded affinity binding constant (K_D) values for [^{3}H]-NMS at CHO-m2 and CHO-m3 cells as 0.24 ± 0.07 and 0.37 ± 0.07 nM, respectively (data not shown). The affinity (pK_i) and associated Hill Slopes for atropine, darifenacin, oxybutynin and tolterodine for the M_2 and M_3 receptor subtypes are summarized, in Table 1. Darifenacin displayed marked selectivity (14.8 fold; $p<0.01$) for the M_3 over the M_2 mACh receptor. In contrast, oxybutynin was non-selective, while both atropine (5.1 fold; $p<0.05$) and tolterodine (6.6 fold; $p<0.05$) displayed modest, though significant M_2-selectivity (Table 1).

Functional affinity estimates in CHO cells. Fig. 1a illustrates the protocol adopted to examine the 'functional' affinity of mACh receptor antagonists at the M_2 receptor, showing a representative curve for the inhibition of forskolin-stimulated cAMP accumulation by MCh in CHO-m2 cells (EC_{50} = 0.27 ± 0.03 μM, n=7). This allowed the estimation of an approximate EC_{70} concentration (1 μM) (Fig. 1a) from which antagonist inhibition curves were constructed (Fig. 1b). pK_B values were derived from these curves as described earlier (see Methods) and are summarized in Table 2.

Fig. 2a shows a concentration-response curve for MCh-stimulated accumulation of [^{3}H]-IP_x (in the presence of Li^+) in CHO-m3 cells (EC_{50}, 1.26 ± 0.14 μM, n=6). An approximate EC_{70} concentration (3 μM) was estimated from this curve and used to construct antagonist inhibition curves (Fig. 2b). Affinity estimates were derived as described above and are

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summarized as pK_b values in Table 2.

Comparing the 'functional' affinity estimates at M_2 and M_3 mACh receptors, darifenacin was again found to be the most M_3-selective antagonist (32.4 fold; $p<0.01$), followed by oxybutynin which was modestly, but significantly M_3-selective (3.4 fold, $p<0.05$). Atropine (2.1 fold) and tolterodine (2.8 fold) were not significantly subtype selective in functional assays (Table 2).

Characterization of responses in guinea-pig tissue slices. In cross-chopped, *myo*-[^{3}H]-inositol-loaded guinea-pig bladder, carbachol (CCh) caused a time- and concentration-dependent accumulation of [^{3}H]-IP_x in the presence of LiCl (5 mM). Fig. 3a illustrates the time-course of the response to CCh (300 μM), which was linear over a 30 min time-course. The latter time-point was chosen for all subsequent experiments. Fig. 3b shows a concentration-response curve for the accumulation of [^{3}H]-IP_x; analysis of each curve yielded a mean EC_{50} value of 8.9 ± 1.8 μM (n=9).

Fig. 4a illustrates the time-course of the Ins(1,4,5)P_3 response to CCh (300 μM) in cross-chopped guinea-pig submandibular gland. The response increased linearly up to 60 s, after which a plateau was reached and maintained throughout the 10 min time-course investigated (Fig. 4a). Fig. 4b shows the concentration-dependent nature of Ins(1,4,5)P_3 accumulation in response to 5 min CCh stimulation. The EC_{50} value calculated for this response was 25.3 ± 4.8 μM (n=9). Since differences in agonist potency are accounted for within the functional equivalent of the Cheng-Prusoff relationship, comparison of pharmacological values derived from the two tissue responses is valid, despite the slight differences in carbachol potency.

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Functional affinities of mACh receptor antagonists for inhibition of agonist-stimulated phosphoinositide turnover in guinea-pig tissues. Fig. 5 shows inhibition curves for each of the test compounds, expressed as a percentage of the maximal response (i.e. that evoked by 50 μM CCh in the absence of antagonist) in both cross-chopped guinea-pig bladder and submandibular gland. Oxybutynin (9.3 fold; $p<0.01$), darifenacin (7.9 fold; $p<0.05$) and tolterodine (7.4 fold; $p<0.05$) each displayed significant selectivity for inhibition of the functional response in the bladder over that seen in the submandibular gland. In contrast, atropine displayed similar affinities for inhibition of phosphoinositide responses seen in each tissue (Fig. 5a; Table 2). This provides further support for the validity of the comparison of pharmacological values derived from the two different tissue responses.

Determination of mACh receptor subtypes present in guinea-pig bladder and submandibular gland. Saturation binding analysis with [^{3}H]-NMS established the total mACh receptor populations in bladder and submandibular gland as 335 ± 23 and 120 ± 22 fmol mg^{-1} protein, respectively (data not shown).

In order to determine the mACh receptor subtypes comprising these populations, a range of subtype-selective mACh receptor antagonists were utilized in competition radioligand binding assays against [^{3}H]-NMS. The compounds tested were as follows: atropine (non-selective), pirenzepine (M_1-selective), 4-DAMP (M_1/M_3-selective), methoctramine (M_2-selective), darifenacin (M_3-selective) and MT-7 (a highly M_1-selective toxin; see Adem and Karlsson, 1997). Fig. 6 illustrates the resulting inhibition curves generated for the six compounds against membranes prepared from guinea-pig bladder (Fig. 6a) or submandibular gland (Fig. 6b). Affinity binding constant (pK_i) values were derived from these curves and are summarized, along with the corresponding slope factors, in Table 3.

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The binding profile in membranes prepared from bladder tissue was consistent with a mixed M_2/M_3 mACh receptor population. Thus, the displacement curve for the M_2-selective antagonist was best fitted by 2-site analysis. The high affinity site, accounting for approx. 84% of the total mACh receptor population, was consistent with the reported affinity of methoctramine at the M_2 receptor (Eglen et al., 1996), while its affinity at the remaining 16% of bladder mACh receptors was consistent with the reported affinity of methoctramine at the M_3 receptor (Eglen et al., 1996). The low affinity of pirenzepine and the absence of any displacement by the highly M_1-selective toxin MT-7 was inconsistent with a detectable M_1 mACh receptor population in the guinea pig bladder (Table 3).

In submandibular gland membranes, pirenzepine displayed low affinity and MT-7 caused no significant displacement of [^{3}H]-NMS binding (Fig. 6b; Table 3). Affinities of the other compounds were consistent with a homogeneous population of M_3 mACh receptors; thus, these data indicate that no significant population of M_1 mACh receptors is expressed in guinea-pig submandibular gland. Consistent with the radioligand binding data, MT-7 (100 nM) had no significant effect on the Ins(1,4,5)P_3 response to CCh (1 mM) in cross-chopped submandibular gland (data not shown).

To confirm that MT-7 does displace [^{3}H]-NMS binding at the (guinea-pig) M_1 receptor, competition binding assays were performed using membranes derived from CHO-m1 cells and membranes prepared from guinea-pig cerebral cortex. Fig. 7 shows that MT-7 binds with high affinity to both human and guinea-pig M_1 mACh receptors, displacing the specific [^{3}H]-NMS binding component by 94 ± 1% and 46 ± 3% in CHO-m1 and guinea-pig cortex membranes, respectively. MT-7 displayed high affinity for the human M_1 mACh receptor

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expressed in CHO cells (pK_i, 9.48 ± 0.01; n=3) and a similar affinity for the 46% of mACh receptors that bound MT-7 with high-affinity in guinea-pig cortex (pK_i, 9.86 ± 0.04; n=3).

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Discussion

Muscarinic receptor antagonists are a mainstay in the pharmacological management of overactive bladder (OAB) (Wynedaele, 2001). However, a lack of receptor subtype selectivity can result in a significant side-effect profile (e.g. dry mouth), which is associated with a high degree of patient non-compliance (Wallis and Napier, 1999). This has prompted a search for anti-muscarinic compounds with greater selectivity. The observation that some, but not all, mACh receptor antagonists display *in vivo* functional selectivity for bladder versus salivary gland (see Introduction) represents a significant step towards more selective OAB therapies.

The main aim of this study was to investigate the potential for mACh receptor antagonists to exhibit tissue-dependent 'functional selectivity' at the level of second messenger generation. Initially, the binding and functional affinities of the four compounds chosen for investigation were determined in CHO cells stably expressing either the human M_2 or M_3 mACh receptor. Darifenacin was shown to be M_3-selective, both at the level of receptor binding (15 fold) and inhibition of agonist-induced second messenger responses (32 fold), in agreement with earlier studies (Eglen et al., 1996; Wallis and Napier, 1999). Oxybutynin was found to be non-selective in competition radioligand binding assays and only weakly (3-4 fold) M_3-selective in functional assays. This is in contrast to previous reports, which found oxybutynin to be approximately 10 fold M_3-over-M_2 selective in radioligand binding studies in membrane homogenates (Nilvebrant et al., 1997). Atropine and tolterodine were weakly M_2-selective in both binding and functional assays. This differs somewhat from other work that reports these compounds to be non-selective (Nilvebrant et al., 1997; Caulfield and Birdsall, 1998). These small discrepancies may be explained by differences in binding affinity and selectivity of mACh receptor antagonists between membrane preparations,

which are widely used to characterize ligand binding profiles, and intact cell assays that were employed in this study (Nelson et al., 2002).

Using indices of mACh receptor-stimulated phospholipase C activity we have been able to show that darifenacin, oxybutynin and tolterodine each display higher affinity for the inhibition of responses in urinary bladder slices compared to submandibular gland slices. Data presented here therefore support the notion that certain mACh receptor antagonists show differential inter-tissue effects on cellular responses reputedly mediated by the same mACh receptor subtype in the same species. We have demonstrated that, in the case of darifenacin, oxybutynin and tolterodine, this tissue-specific selectivity is manifested *in vitro* at the level of inhibition of agonist-induced second messenger turnover, in line with earlier reports of the *in vivo* selectivity of these compounds (Nilvebrant et al., 1997; Wallis and Napier, 1999; Gupta et al., 2002).

Our finding that oxybutynin displays selectivity (9.3 fold) for bladder versus salivary gland is at odds with previous reports in the literature. Newgreen and Naylor (1996) found that oxybutynin was non-selective *in vitro* in the guinea-pig (by comparing inhibition of bladder contractions and ^{86}Rb efflux from submandibular glands). Ikeda et al. (2002) also found that oxybutynin inhibited bladder contraction and salivation with similar potency *in vivo*, in anaesthetized rat. In contrast, Nilvebrant et al. (1997) found oxybutynin to exhibit selectivity for inhibition of salivation over bladder contraction in anaesthetized cat. Oxybutynin is subject to metabolism *in vivo*, producing both active and inactive metabolites (Waldeck et al., 1997), and has also been reported to have significant 'non-muscarinic' effects, such as blockade of Ca^{2+} channels (Wada et al., 1995). Thus, the selectivity we observe *in vitro* may be reduced or lost *in vivo*. In contrast, the bladder selectivity demonstrated by darifenacin and tolterodine is in agreement with previous studies. Thus, Gupta et al. (2002) reported that

darifenacin displays a 10 fold selectivity for inhibition of pelvic nerve-stimulated bladder contractions versus salivary secretion in dog, while tolterodine was 4-5 fold selective in the same study (Gupta et al., 2002). It should also be emphasized that in the vast majority of studies, including this one, atropine fails to discriminate bladder and salivary gland responses. This cogently argues against an affinity 'frame-shift' between preparations and strongly supports the conclusion that observed tissue selectivities are particular to a subset of mACh receptor antagonists.

While it is generally accepted that acetylcholine-induced phosphoinositide hydrolysis and contraction are mediated almost exclusively by the M_3 receptor in bladder (Harriss et al., 1995; Chess-Williams et al., 2001), some controversy remains over the potential involvement of 'non-M_3' mACh receptors in cholinergic responses of salivary glands (Laniyonu et al., 1990; Watson et al., 1996). We therefore sought clarification of whether the presence of 'non-M_3' (in particular M_1) muscarinic receptors could be involved in the phosphoinositide response in submandibular gland, using competition radioligand binding assays with a range of the most selective muscarinic ligands available. It was found that while the binding profile was consistent with a mixed M_2 and M_3 receptor population in bladder, affinities derived for submandibular gland membranes were indicative of a homogeneous population of M_3 receptors. The striking absence of any significant displacement of [^{3}H]-NMS binding by the highly M_1-selective toxin MT-7 indicates that the guinea-pig submandibular gland does not express a significant population of M_1 receptor. This was confirmed by the lack of effect of MT-7 (at 100 nM) on carbachol-stimulated phosphoinositide responses in submandibular gland slices (data not shown). In contrast, MT-7 did cause significant inhibition of specific [^{3}H]-NMS binding to membranes prepared from CHO-m1 cells and guinea-pig cortex, confirming that this toxin binds to both human and guinea-pig M_1 mACh receptors with high affinity.

While our findings are in agreement with some other studies (Laniyonu et al., 1990; Moriya et al., 1999) it is likely that the make up of the mACh receptor population in submandibular gland varies from species to species. Thus, a recent study, utilizing similar methods and range of muscarinic antagonists to those used here, reported a significant M_1 mACh receptor sub-population in canine submandibular gland (Clarke et al., 2003). It should also be noted that controversy regarding 'non-M_3' mACh receptor expression in submandibular gland is not limited to the potential involvement of the M_1 subtype and there is some evidence for the presence of M_5 mACh receptors (e.g. Meloy et al, 2001). Recent studies of transgenic mice lacking the individual mACh receptor subtypes implicate the M_5 receptor in slow salivary secretion, as mice lacking the M_5 receptor gene showed reduced salivation in response to pilocarpine only at longer time (15-60 min) after injection (Yeomans et al., 2001). In contrast, Bymaster et al (2003) reported that salivation measured at 15 min post-injection was not altered in M_5 knockout mice. Additionally, we found that the binding affinities of a range of the most M_3/M_5-selective agents available (darifenacin, *p*-FHHSiD and AF DX 384) in submandibular gland membranes did not differ significantly from affinity estimates in CHO-m3 membranes (data not shown). Our data are therefore consistent with the expression of a homogeneous M_3 mACh receptor population in the guinea-pig submandibular gland.

So if agonist-stimulated phosphoinositide turnover in both bladder and submandibular gland is mediated via activation of M_3 mACh receptors, the question remains as to why some, but not all mACh receptor antagonists inhibit these responses with different affinities? In the absence of any evidence for genetic heterogeneity of the M_3 receptor, the influence of the cellular environment on M_3 receptor pharmacology must be considered. In this context it could be speculated that GPCRs express 'phenotypic' profiles specific to the host cell

environment, such that the same gene can exhibit distinct pharmacologies when expressed in different cells (Kenakin, 2003).

Interactions with other integral membrane proteins expressed by the cell can influence receptor phenotype, as demonstrated by the influence of receptor activity-modifying proteins (RAMPs) upon GPCR pharmacology. Co-expression of RAMP3 with the calcitonin receptor has been shown not only to influence agonist potency ranking orders, but also antagonist affinity estimates (Armour et al., 1999). Numerous recent studies have also highlighted the potential for GPCRs to form dimer or higher order oligomer assemblies (see Angers et al., 2002). Such receptor-receptor interactions, especially between non-identical monomers (i.e. heterodimerization), can generate novel properties in the resulting complex (e.g. Jordan and Devi, 1999). Evidence has been provided for dimerization of mACh receptor subtypes (Wreggett and Wells, 1995) and it is therefore tempting to speculate that heterodimerization can occur between M_2 and M_3 mACh receptors to influence the pharmacology in a tissue-dependent manner.

In conclusion, the main finding of this study is that tolterodine, darifenacin and oxybutynin each display selectivity for the inhibition of phosphoinositide turnover in guinea-pig urinary bladder versus submandibular salivary gland in the same species. In contrast, atropine is non-selective between these two tissues. We have also shown that the lower functional affinities of tolterodine, darifenacin and oxybutynin in the submandibular gland are unlikely to be due to the involvement of an M_1 mACh receptor population in the functional response of this tissue. Our data therefore support the notion that certain mACh receptor antagonists are able to display a functional selectivity for the inhibition of responses in bladder versus salivary gland. The molecular mechanism(s) underlying this phenomenon remains to be resolved.

Acknowledgements

We gratefully acknowledge the financial support of Pfizer Global Research and Novartis Pharma. We thank Rajendra Mistry for excellent technical assistance with the CHO cell second messenger experiments. We also thank Dr. Nick Clarke (Pfizer, Sandwich, UK) for his helpful discussions and advice regarding the radioligand binding work in native tissues.

References

Adem A and Karlsson E (1997) Muscarinic receptor subtype selective toxins. *Life Sci* **60:**1069-1076.

Andersson KE (1993) Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol Rev* **45:**253-308.

Angers S, Salahpoou A and Bouvier M (2002) Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu Rev Pharmacol Toxicol* **42:**409-435.

Armour SL, Foord S, Kenakin T and Chen WJ (1999) Pharmacological characterization of receptor-activity-modifying proteins (RAMPs) and the human calcitonin receptor. *J Pharmacol Toxicol Methods* **42**:217-224.

Batty IH, Carter AN, Challiss RAJ and Hawthorne JN (1997) Receptor-linked phosphoinositide metabolism. In Neurochemistry – A Practical Guide (2nd Ed., Turner AJ and Bachelard), pp. 229-268, IRL Press, Oxford.

Brown BL, Albano JD, Ekins RP, Sgherzi AM and Tampion W (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem J* **121:**561-562.

Bymaster FP, Carter PA, Yamada M, Gomeza J, Wess J, Hamilton SE, Nathanson NM, McKinzie DL and Felder CC (2003) Role of specific muscarinic receptor subtypes in cholinergic parasympathomimetic responses, in vivo phosphoinositide hydrolysis, and pilocarpine-induced seizure activity. *Eur J Neurosci* **17**:1403-1410.

Caulfield MP and Birdsall NJM (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50:**279-290.

Chess-Williams R, Chapple CR, Yamanishi T, Yasuda K and Sellers DJ (2001) The minor population of M_3-receptors mediate contraction of human detrusor muscle in vitro. *J Auton Pharmacol* **21:**243-248.

Clarke NP, Smith K, Napier CM and Gupta P (2003) Characterisation of muscarinic receptor expression in dog submandibular gland. *Br J Pharmacol* **138**, 123P.

Craig DA (1993) The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharmacol Sci* **14:**89-91.

Eglen RM, Hegde SS and Watson N (1996) Muscarinic receptor subtypes and smooth muscle function. *Pharmacol Rev* **48:**531-565.

Eglen RM, Michel AD, Montgomery WW, Kunysz EA, Machado CA and Whiting RL (1990) The interaction of parafluorohexahydrosiladiphenidol at muscarinic receptors in vitro. *Br J Pharmacol* **99:**637-642.

Ehlert FJ (2003) Contractile role of M2 and M3 muscarinic receptors in gastrointestinal, airway and urinary bladder smooth muscle. *Life Sci* **74**:355-366.

Gillberg PG, Sundquist S and Nilvebrant L (1998) Comparison of the in vitro and in vivo profiles of tolterodine with those of subtype-selective muscarinic receptor antagonists. *Eur J Pharmacol* **349:**285-292.

Gupta P, Anderson CWP, Carter AJ, Casey JH and Newgreen DT (2002) *In vivo* bladder selectivity of darifenacin, a new M_3 antimuscarinic agent, in the anaesthetised dog. *Eur Urol Suppl* **1:**515.

Harriss DR, Marsh KA, Birmingham AT and Hill SJ (1995) Expression of muscarinic M_3-receptors coupled to inositol phospholipid hydrolysis in human detrusor cultured smooth muscle cells. *J Urol* **154:**1241-1245.

Ikeda K, Kobayashi S, Suzuki M, Miyata K, Takeuchi M, Yamada T and Honda K (2002) M_3 receptor antagonism by the novel antimuscarinic agent solifenacin in the urinary bladder and salivary gland. *Naunyn-Schmiedeberg's Arch Pharmacol* **366:**97-103.

Jordan BA and Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* **399:**697-700.

Kenakin T (2003) Predicting therapeutic value in the lead optimization phase of drug discovery. *Nat Rev Drug Discov* **2**:429-438.

Laniyonu A, Sliwinski-Lis E and Fleming N (1990) Muscarinic M_3 receptors are coupled to two signal transduction pathways in rat submandibular cells. *Eur J Pharmacol* **188:**171-174.

Lazareno S and Birdsall NJ (1993) Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng-Prusoff equations. *Br J Pharmacol* **109**:1110-1119.

Longhurst PA, Leggett RE and Briscoe JA (1995) Characterization of the functional muscarinic receptors in the rat urinary bladder. *Br J Pharmacol* **116:**2279-2285.

Lowry OH, Rosebrough NJ, Farr AC and Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193:**265-275.

Malone-Lee J, Shaffu B, Anand C and Powell C (2001) Tolterodine: superior tolerability than and comparable efficacy to oxybutynin in individuals 50 years old or older with overactive bladder: a randomized controlled trial. *J Urol* **165:**1452-1456.

Meloy TD, Daniels DV, Hegde SS, Eglen RM and Ford AP (2001) Functional characterization of rat submaxillary gland muscarinic receptors using microphysiometry. *Br J Pharmacol* **132:**1606-1614.

Moriya H, Takagi Y, Nakanishi T, Hayashi M, Tani T and Hirotsu I (1999) Affinity profiles of various muscarinic antagonists for cloned human muscarinic acetylcholine receptor

(mAChR) subtypes and mAChRs in rat heart and submandibular gland. *Life Sci* **64:**2351-2358.

Nelson CP, Gupta P, Napier CM and Challiss RAJ (2002) Affinity estimates for darifenacin, tolterodine and oxybutynin at M_2 and M_3 muscarinic receptors in membrane and intact Chinese hamster ovary cell preparations. *Br J Pharmacol* **135:**341P.

Newgreen DT and Naylor AM (1996) Comparison of the functional muscarinic receptor selectivity of darifenacin with tolterodine and oxybutynin. *Br J Pharmacol* **117:**107P.

Nilvebrant L, Andersson KE, Gillberg PG, Stahl M and Sparf B (1997) Tolterodine - a new bladder-selective antimuscarinic agent. *Eur J Pharmacol* **327:**195-207.

Wada Y, Yoshida M, Kitani K, Kikukawa H., Ichinose A., Takahashi W, Gotoh S, Inadome A, Machida J and Ueda S (1995) Comparison of the effects of various anticholinergic drugs on human isolated urinary bladder. *Arch Int Pharmacodyn Ther* **330:**76-89.

Waldeck K, Larsson B and Andersson KE (1997) Comparison of oxybutynin and its active metabolite, N-desethyl-oxybutynin, in the human detrusor and parotid gland. *J Urol* **157**:1093-1097.

Wallis RM and Napier CM (1999) Muscarinic antagonists in development for disorders of smooth muscle function. *Life Sci* **64:**395-401.

Wang P, Luthin GR and Ruggieri MR (1995) Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. *J Pharmacol Exp Ther* **273:**959-966.

Watson EL, Abel PW, Dijulio D, Zeng W, Makoid M, Jacobson KL, Potter LT and Dowd FJ (1996) Identification of muscarinic receptor subtypes in mouse parotid gland. *Am J Physiol* **271:**C905-C913.

Watson N, Reddy H, Stefanich E and Eglen RM (1995) Characterization of the interaction of zamifenacin at muscarinic receptors in vitro. *Eur J Pharmacol* **285:**135-142.

Wreggett KA and Wells JW (1995) Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. *J Biol Chem* **270:**22488-2299.

Wyndaele JJ (2001) The overactive bladder. *BJU Int* **88:**135-140.

Yamaguchi O, Shishido K, Tamura K, Ogawa T, Fujimura T and Ohtsuka M (1996) Evaluation of mRNAs encoding muscarinic receptor subtypes in human detrusor muscle. *J Urol* **156:**1208-1213.

Yeomans J, Forster G and Blaha C (2001) M5 muscarinic receptors are needed for slow activation of dopamine neurons and for rewarding brain stimulation. *Life Sci* **68**:2449-2456.

JPET Fast Forward. Published on May 12, 2004 as DOI: 10.1124/jpet.104.067140
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This work was financially supported by Pfizer Global Research and Novartis Pharma.

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JPET Fast Forward. Published on May 12, 2004 as DOI: 10.1124/jpet.104.067140
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FIGURE LEGENDS

Fig. 1. (a) Representative curve illustrating the inhibition of forskolin (10 μM)--stimulated cyclic AMP accumulation by MCh. Arrow indicates MCh concentration (1 μM) selected for generation of antagonist-inhibition curves. **(b)** Effect of mACh receptor antagonists on 1 μM MCh-induced inhibition of 10 μM forskolin-stimulated cyclic AMP production in CHO-m2 cells. Results are expressed as mean percent forskolin (10 μM) -stimulated cyclic AMP accumulation ± s.e.m., n ≥3.

Fig. 2. (a) Representative curve illustrating the stimulation of [^{3}H]-IP_x accumulation in response to MCh in CHO-m3 cells. Arrow indicates MCh concentration (3 μM) selected for generation of antagonist-inhibition curves. **(b)** Effect of mACh receptor antagonists on [^{3}H]-IP_x accumulation in response to 3 μM methacholine in CHO-m3 cells. Results are expressed as mean percent maximal stimulation ± s.e.m., n ≥ 3.

Fig. 3. (a) Time-course of [^{3}H]-IP_x response to CCh (1 mM) in guinea-pig bladder slices. **(b)** Concentration-response curve of total [^{3}H]-IP_x accumulation to CCh in guinea-pig bladder slices. Data are expressed as percent maximum response. Results are expressed as mean ± s.e.m , n ≥ 3.

Fig. 4. (a) Time-course of Ins(1,4,5)P_3 response to CCh (300 μM) in guinea-pig submandibular gland slices. **(b)** Concentration-response curve of Ins(1,4,5)P_3 response to CCh in guinea-pig submandibular gland slices. Data are expressed as percent maximum response. Results are expressed as mean ± s.e.m , n ≥ 3.

Fig. 5. Inhibition of CCh (50 μM)-mediated functional responses in guinea-pig bladder (open symbols) and submandibular gland (closed symbols) slices by atropine **(a)**, darifenacin **(b)**, oxybutynin **(c)** and tolterodine **(d)**. Data are expressed as percent maximum response (in the absence of antagonist). Results are expressed as mean ± s.e.m, n ≥ 3.

Fig. 6. Inhibition of specific [^{3}H]-NMS binding to guinea-pig bladder **(a)**, and submandibular gland **(b)** membranes by mACh receptor antagonists. Data are expressed as mean percent control specific binding ± s.e.m., n=4.

Fig. 7. Inhibition of specific [^{3}H]-NMS binding to CHO-m1 **(a)**, and guinea-pig cerebral cortex **(b)** membranes by atropine, pirenzepine and MT-7. Data are expressed as mean percent control specific binding ± s.e.m., n ≥ 3.

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Table 1. Binding affinity constant (pK_i) and Hill slope estimates for mACh receptor antagonists at intact CHO-m2 and CHO-m3 cells.

	CHO-m2		CHO-m3	
	pK_i	Hill slope	pK_i	Hill slope
atropine	9.13 (0.10)	0.91 (0.06)	8.42 (0.10)	1.02 (0.05)
darifenacin	6.97 (0.07)	0.98 (0.07)	8.14 (0.11)	0.95 (0.02)
oxybutynin	7.21 (0.09)	0.99 (0.01)	7.12 (0.16)	1.13 (0.03)
tolterodine	8.49 (0.12)	0.92 (0.03)	7.67 (0.10)	1.00 (0.04)

Values are expressed as mean (S.E.M.) pK_i and Hill slope values from $n \geq 3$ experiments.

Table 2. Functional affinity (pK_b) estimates for mACh receptor antagonists at CHO cells recombinantly expressing human m2 and m3 mACh receptors, and guinea-pig bladder and submandibular salivary gland slices.

	CHO-m2	CHO-m3	Bladder	Submandibular	Selectivity (Bladder v Submandibular)
atropine	9.37 (0.08)	9.04 (0.08)	8.91 (0.08)	9.05 (0.02)	0.7
darifenacin	7.30 (0.17)	8.81 (0.14)	9.31 (0.21)	8.41 (0.29)	7.9
oxybutynin	7.21 (0.11)	7.74 (0.10)	8.24 (0.19)	7.27 (0.04)	9.3
tolterodine	8.59 (0.09)	8.15 (0.11)	8.91 (0.22)	8.04 (0.11)	7.4

Values are expressed as mean pK_b (s.e.m.) values from $n \geq 3$ experiments.

JPET Fast Forward. Published on May 12, 2004 as DOI: 10.1124/jpet.104.067140
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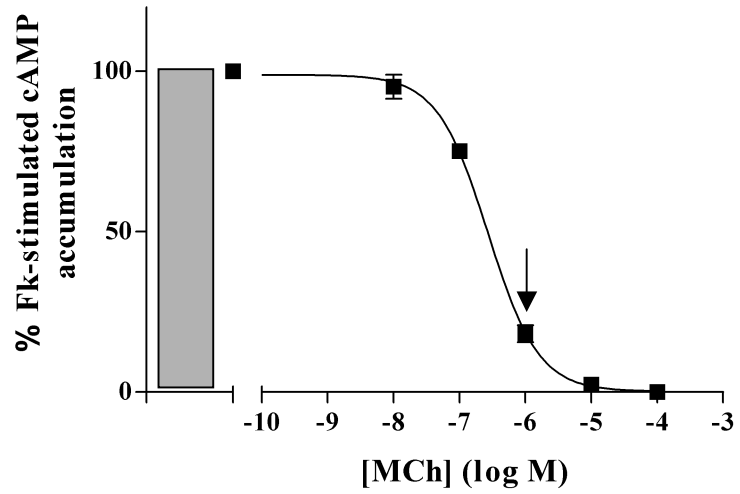
Table 3. Binding affinity constant (pK_i) and Hill slope estimates for mACh receptor antagonists at guinea-pig bladder and submandibular salivary gland membranes.

	Bladder		Submandibular gland	
	pK_i	Hill slope	pK_i	Hill slope
atropine	9.26 (0.09)	1.02 (0.05)	9.23 (0.09)	1.16 (0.07)
pirenzepine	6.84 (0.03)	1.05 (0.03)	6.92 (0.09)	0.94 (0.07)
4-DAMP	8.79 (0.05)	1.04 (0.04)	9.20 (0.04)	1.23 (0.02)
methoctramine	8.58 (0.09) 6.76 (0.12)	N/A (2-site fit)	6.57 (0.01)	1.71 (0.07)
darifenacin	7.99 (0.03)	0.92 (0.03)	8.54 (0.05)	1.27 (0.04)

Values are expressed as mean pK_i and Hill slope (s.e.m.) values from $n \geq 3$ experiments.

Figure 1

(a)



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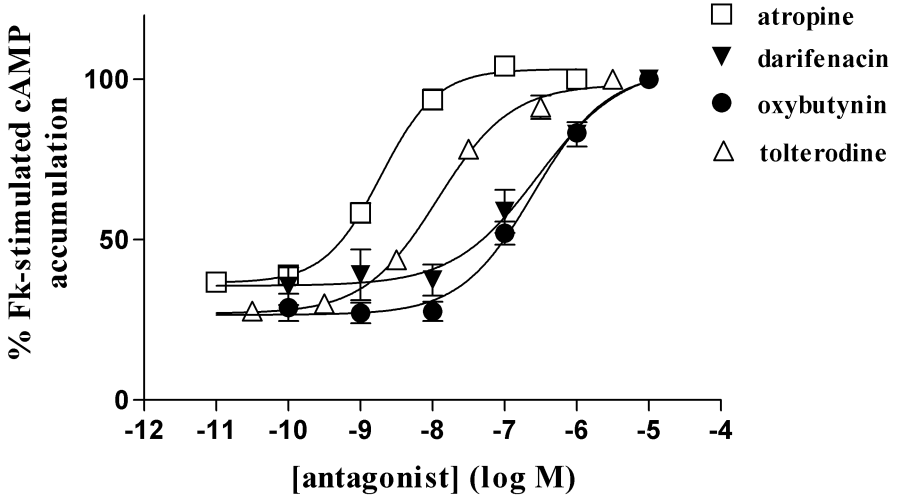


Figure 2

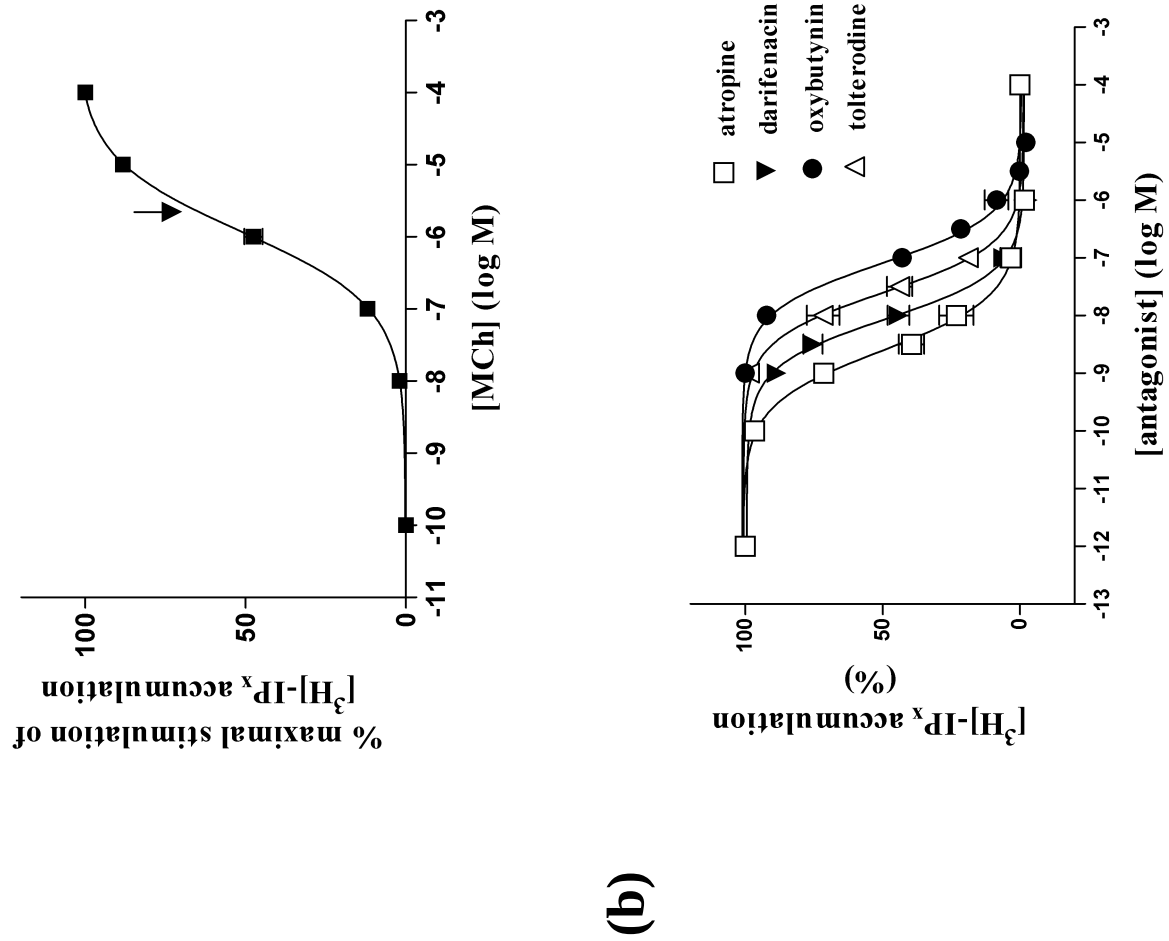
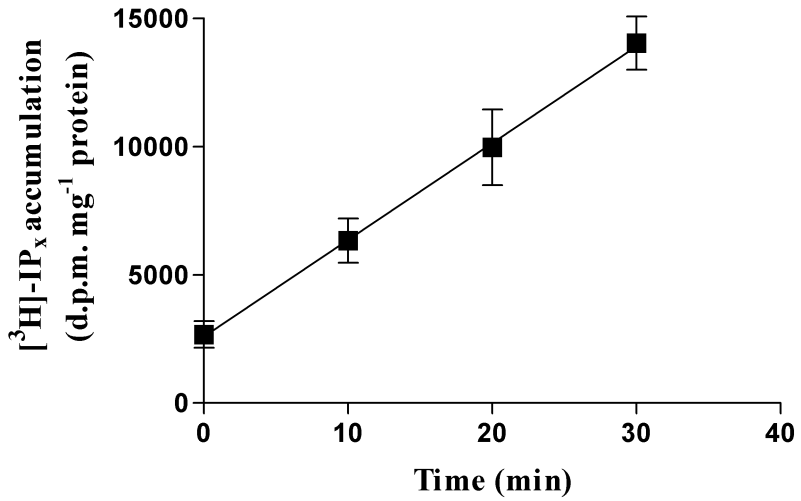


Figure 3

(a)



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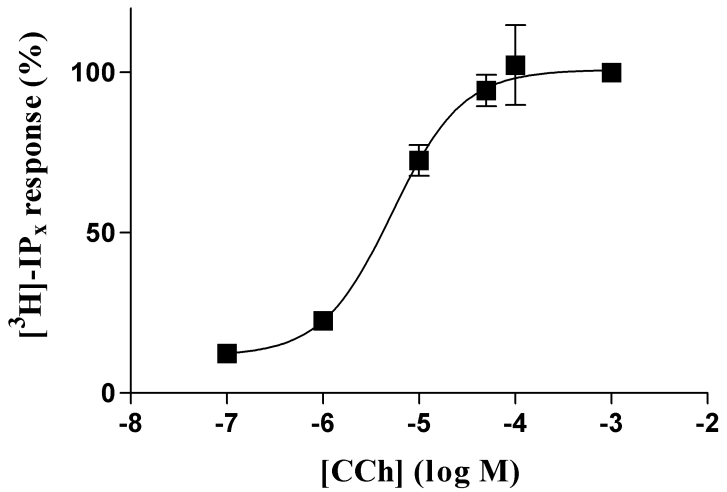

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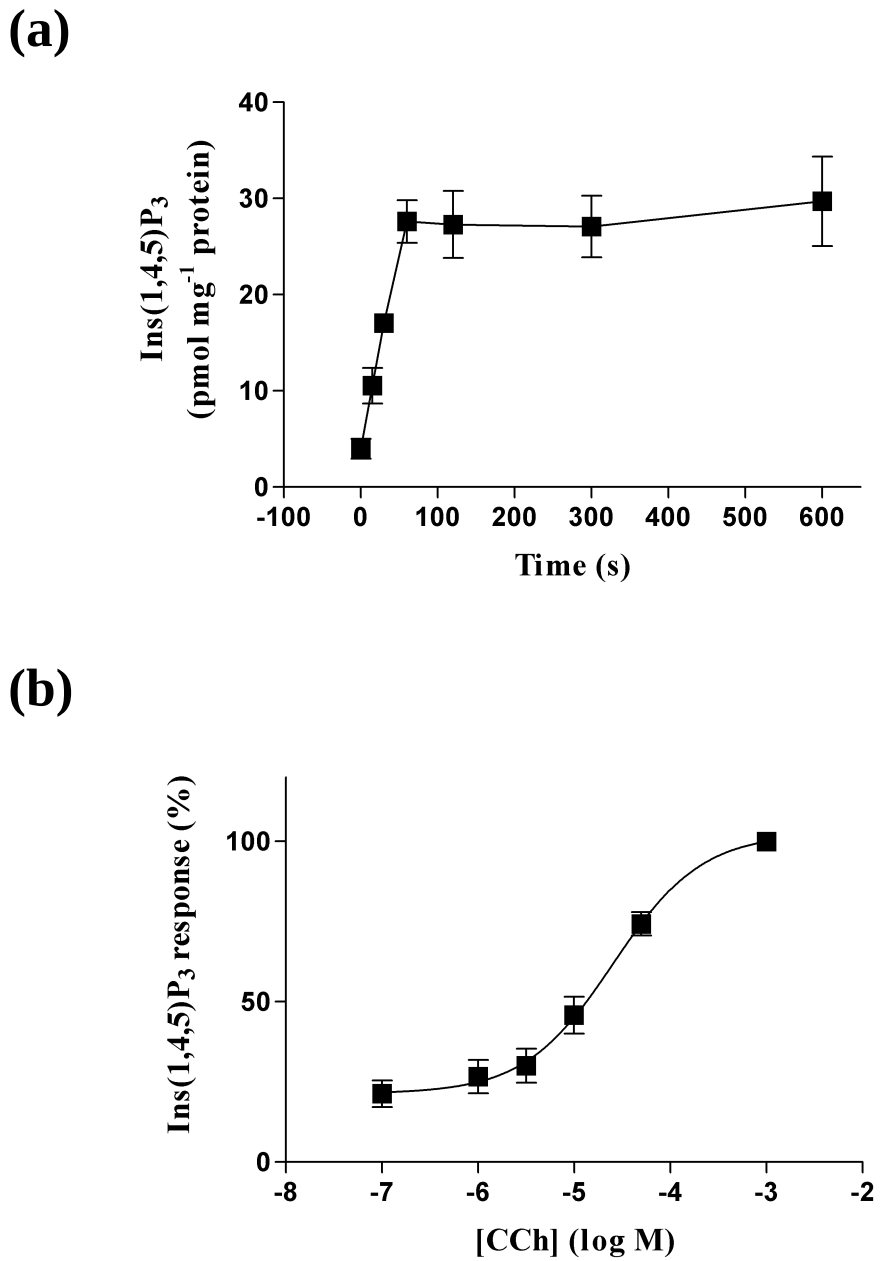


Figure 5

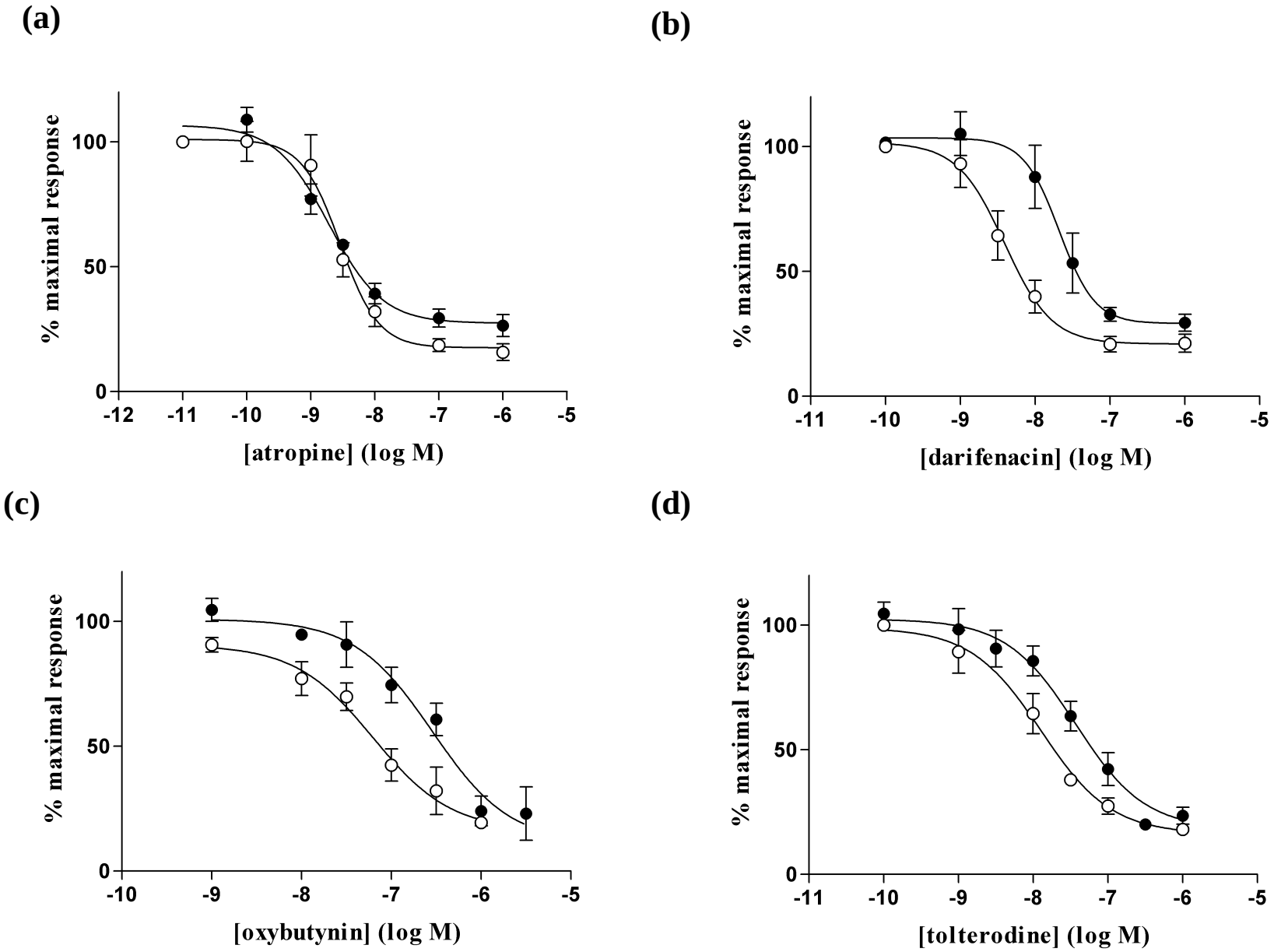
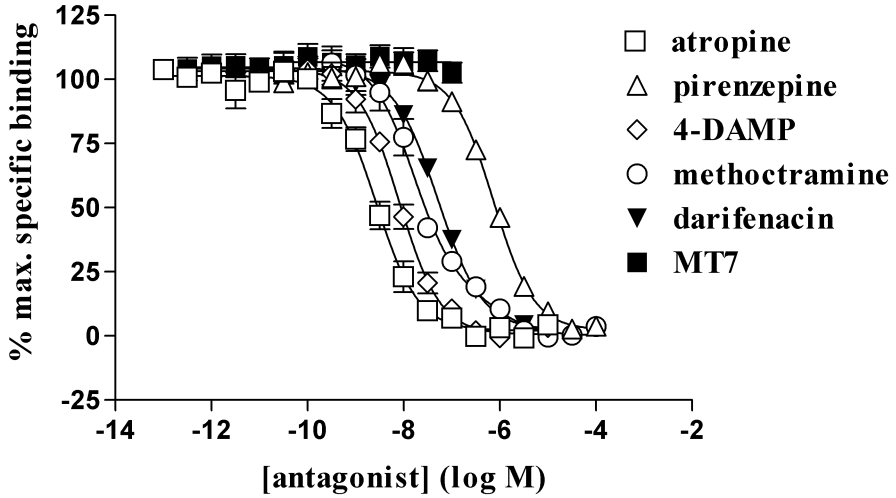


Figure 6

(a)



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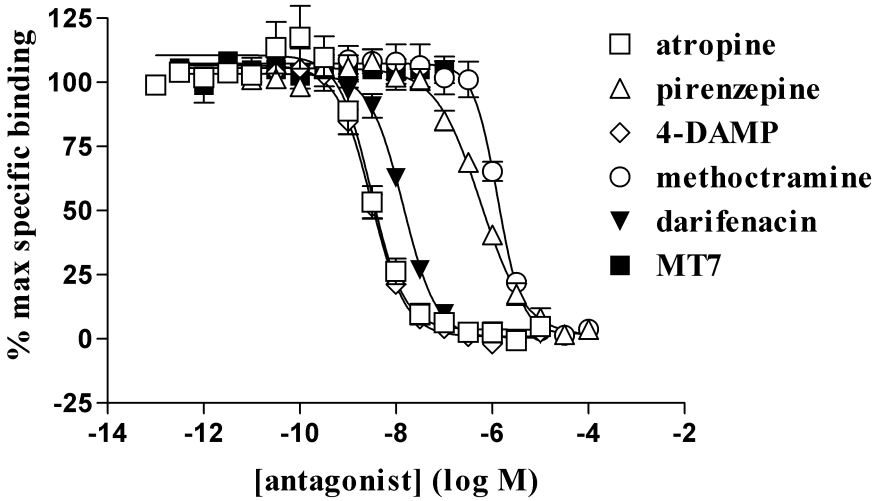
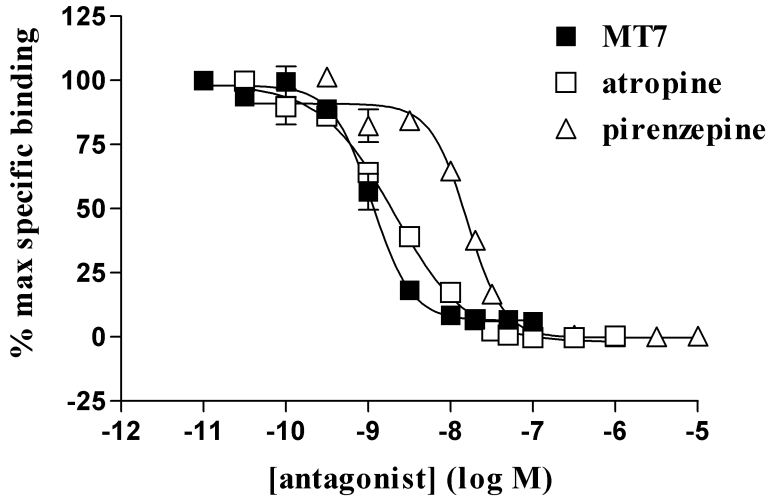


Figure 7

(a)



(b)