The role of M_2-muscarinic receptors in mediating contraction of the pig urinary bladder *in vitro*

[1,2]Tomonori Yamanishi, [2]Christopher R. Chapple, [3]Kosaku Yasuda & *[,1]Russell Chess-Williams

[1]Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN; [2]Department of Urology, Royal Hallamshire Hospital, Sheffield and [3]Department of Urology, Dokkyo University, Koshigaya Hospital, Japan

1   In urinary bladder, M_2-muscarinic receptors predominate, but it is the smaller population of M_3-receptors which mediate detrusor contraction. This study examines the $M_2:M_3$ ratio and the role of M_2-receptors in contraction of pig urinary bladder.

2   Competition experiments with [³H]-QNB determined the ratio of $M_2:M_3$. In functional studies, affinity values (pK_B) for 4-DAMP, darifenacin and methoctramine were calculated. Similar experiments were performed on tissues following selective M_3-inactivation (incubation with 40 nM 4-DAMP mustard in the presence of 1 μM methoctramine to protect M_2-receptors), precontraction with 50 mM KCl and relaxation with isoprenaline (30 μM) or forskolin (1 μM).

3   In competition binding, displacement of [³H]-QNB by 4-DAMP, darifenacin and methoctramine best fitted a two-site model suggesting a predominant (70–80%) population of M_2-receptors.

4   On normal detrusor *in vitro*, 4-DAMP and methoctramine caused surmountable antagonism of responses to carbachol with pK_B values of 9.37 ± 0.07 and 6.05 ± 0.05 respectively. Darifenacin caused unsurmountable antagonism, the apparent pK_B value being 8.61 ± 0.10.

5   In tissues where the M_3-receptors had been inactivated and cyclic AMP levels elevated, 4-DAMP and darifenacin were less potent, with apparent pK_B values of 8.72 ± 0.08 and 6.74 ± 0.07. In contrast, methoctramine was more potent, the apparent pK_B value increasing significantly to 6.86 ± 0.06.

6   These data suggest that the pig bladder possesses a similar muscarinic receptor population to the human bladder and that the M_3-receptor subtype mediates contraction of the normal detrusor muscle. However an involvement of M_2-receptors in contraction can be observed following pharmacological manipulation of the receptor population.
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Introduction

The urinary bladder, like the majority of other smooth muscles from many species, exhibit heterogeneous populations of muscarinic receptors (Hegde *et al.*, 1997; Reddy *et al.*, 1995). A predominance of the M_2 muscarinic receptor subtype, with a minor population of M_3-receptors, has been reported for urinary bladder smooth muscle for several species. Immunoprecipitation data indicate that the proportion of muscarinic M_2 and M_3-receptors is 3:1 in bladders from humans, guinea-pigs and rabbits, and 9:1 in the rat bladder (Wang *et al.*, 1995). Radioligand binding studies also have revealed a population of 87% M_2-receptors and 13% M_3-receptors in rat urinary bladder (Monferini *et al.*, 1988). However, pharmacological characterization of muscarinic receptors mediating contraction of detrusor muscle in rat (Longhurst *et al.*, 1995), rabbit (Mutoh *et al.*, 1997; Choppin *et al.*, 1998) and guinea-pig bladder (Noronha-Blob *et al.*, 1989) suggest the singular involvement of M_3 receptors. These observations suggest that the M_3-receptor is the predominant muscarinic receptor subtype responsible for detrusor contraction to muscarinic agonists, and the M_2 receptors are not directly involved in contraction, thus a role

for the majority M_2 receptor in detrusor contraction is unclear.

The lack of discriminating pharmacological tools (particularly a selective M_2 receptor agonist) poses considerable difficulties in pharmacologically isolating a muscarinic receptor subtype (Eglen *et al.*, 1994). Although no direct contractile response to M_2 receptor activation can be demonstrated, an indirect influence on contraction *via* inhibition of cyclic AMP-mediated smooth muscle relaxation by β-adrenoceptors, forskolin, 5-hydroxytryptamine or vasoactive intestinal peptide has been reported (Griffin & Ehlert, 1992; Caulfield, 1993; Reddy *et al.*, 1995; Eglen *et al.*, 1996). M_2-receptors have also been suggested to be able to inhibit smooth muscle relaxation through decreasing the opening probability of K^+-channels (Caulfield, 1993; Kume & Kotlikoff, 1991).

Recently, an M_2-mediated contraction to muscarinic stimulation has been demonstrated following selective M_3-receptor inactivation and elevation of cyclic AMP levels in guinea-pig ileum (Thomas *et al.*, 1993; Reddy *et al.*, 1995) and in the rat bladder (Hegde *et al.*, 1997), an effect which manifests as a re-contraction.

Thus an M_2-mediated contraction can be demonstrated in the rat bladder where the $M_2:M_3$ ratio is approximately 9:1. Whether a role for M_2-receptors can be demonstrated in other species where the ratio is significantly lower e.g. human,

*Author for correspondence at: Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield, S10 2TN; E-mail: R.Chess@Sheffield.ac.uk

has yet to be investigated. The present study examines the $M_2:M_3$ ratio (radioligand binding experiments) and the role of M_2-receptors in mediating contraction (isolated tissue experiments) of the pig urinary bladder

Methods

In vitro *functional studies*

Female pig urinary bladder was collected from the abattoir and immediately placed in cold Krebs solution (4°C). Strips of tissue (10 × 3 mm) were cut from the bladder dome, and mucosa and serosa removed. The tissues were mounted in 30 ml organ baths containing Krebs solution which was maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. The tissues were subjected to a resting tension of 1 g and allowed to equilibrate for 60 min, during which time they were washed every 10 min and the resting tension was adjusted. Isometric tension generated by the tissue was measured from UF1 force transducers to PC *via* a Cambridge Electronic Design (CED) interface using CHART software.

Effects of muscarinic antagonists on concentration-response curve to carbachol in normal tissues

Cumulative concentration-response curves (CRCs) to carbachol were obtained. Tissues were then washed for about 45 min until a steady resting level of tension was attained, and were then equilibrated for 30 min with Krebs solution containing the appropriate concentration of the antagonist or vehicle (time control). After incubation for 30 min, a second CRC to carbachol was constructed in the continued presence of antagonist or vehicle. In this way, four CRCs to carbachol were obtained from the same strip, three in the presence of increasing concentrations of 4-DAMP (3, 10, 30 nM), darifenacin (3, 10 and 30 nM) and methoctramine (1, 3 and 10 μM) or in the presence of vehicle. Antagonists were added in increasing concentrations after washing for 45 min. Control experiments were performed with the addition of vehicle instead of antagonist and these were used to correct for any tachyphylaxis or time-dependent changes in tissue sensitivity and responsiveness. The correction factors of 1.0 – 2.0 for EC_{50} values (negligible on a logarithmic scale) and 1.0 – 0.8 for maximum responses were small, but were still applied in all experiments.

Effects of increasing cyclic AMP activity on concentration-response curves to carbachol in normal and M_3-inactivated tissues

After construction of the first CRCs to carbachol, tissues were washed with Krebs solution for 60 min. The tissues were then precontracted with KCl (50 mM). Once a stable contractile tone had been attained, the tissues were relaxed with isoprenaline (30 μM) to stimulate adenylate cyclase activity. Twenty minutes later, a second CRC to carbachol was obtained. Using separate tissues, selective M_3-inactivation was achieved (Eglen & Harris, 1993; Hegde *et al.*, 1997) after the first CRCs to carbachol by incubating with the alkylating agent 4-DAMP mustard (40 nM) for 60 min in the presence of methoctramine (1 μM) to protect M_2-receptors, the methoctramine being added 30 min before the 4-DAMP mustard. The drugs were then removed from the tissues by washing for 90 min with Krebs solution. The second curve to carbachol was obtained under conditions of stimulated adenylate cyclase activity i.e., isoprenaine-induced relaxation of KCl precontracted tissue as described below (Figure 1).

Effects of muscarinic antagonists on concentration-response curves to carbachol in M_3-inactivated tissues

After CRCs to carbachol had been obtained with selective M_3-inactivated tissue, the tissues were washed and incubated for 30 min with Krebs solution containing antagonists (4-DAMP 3, 10 and 30 nM, darifenacin 1, 3 and 10 μM or methoctramine 0.3, 1 and 3 μM), or vehicle. The tissues were then contracted with KCl (50 mM) and relaxed with isoprenaline (30 μM) or forskolin (1 μM) (for methoctramine) before CRCs to carbachol were again constructed. As with normal tissues, four CRCs to carbachol were obtained from the same strip, except for experiments with darifenacin in which CRCs to carbachol were obtained from separate strips from the same animal because the maximum contraction was significantly decreased by darifenacin.

Radioligand binding studies

Tissues were homogenized at 0.2 g ml⁻¹ in 50 mM Tris-HCl buffer (pH 7.4), and filtered through muslin. Homogenates were centrifuged at 45,000 × g for 10 min at 4°C. The pellets were re-suspended in 5 ml of Tris-HCl buffer. Homogenates were again centrifuged and the pellets re-suspended in 5 ml of
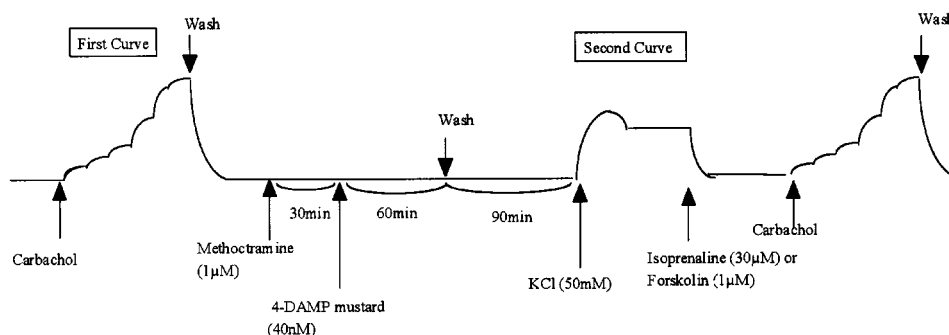

Figure 1 Schema of the procedures and time course for selective M_3-inactivation and cyclic AMP elevation in *in vitro* functional studies. Selective M_3-inactivation was achieved after the first CRCs to carbachol by incubating with 4-DAMP mustard (40 nM) for 60 min in the presence of methoctramine (1 μM) to protect M_2-receptors, the methoctramine being added 30 min before the 4-DAMP mustard. The drugs were then removed from the tissues by washing for 90 min with Krebs solution. The second curve to carbachol was obtained under conditions of stimulated adenylate cyclase activity i.e., pre-contraction with KCl (50 mM) and relaxation with isoprenaline (30 μM) or forskolin (1 μM) (for methoctramine).

Tris-HCl buffer. Membranes were used immediately in radioligand binding assays performed in duplicate.

Saturation experiments were conducted using seven concentrations (0.0625 to 4 nM) of 1-quinuclidinyl [phenyl-4-^{3}H] benzilate ([^{3}H]-QNB) (Goepel *et al.*, 1998). Binding was determined in a final volume of 0.25 ml and non-specific binding determined with 10 μM atropine. The assay tubes were incubated at 37°C for 60 min before filtration through a cell harvester (Brandel M30) and the radioactivity of filters determined by liquid scintillation spectrometry. Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Competition experiments with [^{3}H]-QNB were used to determine the ratio of M$_2$:M$_3$ receptors. Cell membranes were incubated (37°C) with 0.3 nM [^{3}H]-QNB and a range of concentrations of unlabelled antagonists (4-DAMP 0.1 nM – 1 μM, darifenacin 0.25 nM – 0.5 μM and methoctramine 25 nM – 0.25 mM).

Statistical analysis

In functional studies, agonist potencies and maximum responses were expressed as mean pEC$_{50}$ ± s.e.mean (−logarithm of the molar concentration of agonist resulting in 50% of the maximum response) and mean maximum contraction ± s.e.mean, respectively. Antagonist dissociation constants (K$_B$) were determined from the following equation: K$_B$ = antagonist concentration (molar) (dose ratio-1)$^{-1}$. PA$_2$ values for the muscarinic receptor antagonists were determined by Schild regression and taken as the x-intercept on the Schild plot (Arunlakshana & Schild, 1959).

Data are normalized to the maximal response generated by the first (control) curve, and are expressed as means ± s.e.mean. A non-paired Student's *t*-test was used for statistical analysis to compare normal and M$_3$-inactivated tissues. Radioligand binding data was analysed using Graphpad 'PRISM' software and data compared using paired Student's *t*-test.

Drugs and chemicals

1-Quinuclidinyl [phenyl-4-^{3}H] benzilate ([^{3}H]-QNB) (specific activity 42 Ci mmol^{-1}) was purchased from NEN Life Science Products, Inc. (Boston, MA, U.S.A.), and was stored at −20°C. Atropine, 4-DAMP and methoctramine were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Darifenacin was a gift kindly donated by Pfizer Ltd. (Sandwich, Kent, U.K.).

Results

Radioligand binding studies

Radioligand binding experiments were performed on paired tissues following incubation in gassed Krebs-bicarbonate

solution at 37°C (vehicle) or selective M$_3$-inactivation (see Methods), and the membranes prepared from these tissues.

Binding of [^{3}H]-QNB was saturable and displaceable by atropine. Specific binding was 82.3 ± 1.8% of the total binding. Scatchard analysis of [^{3}H]-QNB binding (n = 8) demonstrated a single population of binding sites with a mean dissociation constant (K$_D$) of 0.30 ± 0.06 nM and a density of 89.3 ± 12.9 fmol mg protein^{-1} in normal tissues. After selective M$_3$-receptor inactivation the density of receptors was significantly reduced (P < 0.05) to 61.1 ± 10.1 fmol mg protein^{-1}, but the affinity of binding was unchanged (K$_D$ = 0.24 ± 0.04 nM).

Competition binding with muscarinic antagonists

Displacement experiments were performed using 4-DAMP (n = 9), methoctramine (n = 9) and darifenacin (n = 8). All experiments on normal tissue had low Hill slopes and fitted both one-site and two-site models. Correlation coefficients (r^2 values) were nearer unity with the two-site model and this model yielded a statistically significant better fit in four of the 4-DAMP, six of the methoctramine and one of the darifenacin experiments. The two-site model of competition data for these antagonists are summarized in Table 1.

After selective M$_3$-inactivation, 4-DAMP exhibited one-site binding in five of six experiments and the Hill slopes were significantly (P < 0.05) nearer unity (0.81 ± 0.04). Darifenacin exhibited one-site displacement with Hill slopes close to unity (1.06 ± 0.08) in all experiments (n = 6) and a mean pK$_i$ of 6.9, as did methoctramine (n = 5, Hill slopes of 0.77 ± 0.11 and mean pK$_i$ value of 7.9). Thus in no experiment, except one on M$_3$-inactivated tissue, was a two-site binding model statistically better fit than a one-site model.

Effects of muscarinic antagonists in normal tissues in functional *in vitro* studies

Carbachol produced concentration-dependent contraction of the pig urinary bladder with mean pEC$_{50}$ values and maximum responses of 5.86 ± 0.02 and 16.0 ± 2.02 g, respectively (n = 12). 4-DAMP and methoctramine produced parallel, rightward displacement of the CRCs to carbachol without affecting maximum responses and yielded mean (± s.e.mean) pK$_B$ values of 9.37 ± 0.07 and 6.05 ± 0.05, respectively. Schild slopes for 4-DAMP and methoctramine were not significantly different from unity (Figures 2 and 4, Table 2) Darifenacin, however, produced unsurmountable antagonism, maximum responses being significantly reduced at 30 nM (Figure 3, Table 2).

Effects of increasing cyclic AMP activity in normal and M$_3$-inactivated tissues

There was a slight, but significant decrease (P < 0.05) in pEC$_{50}$ values (5.61 ± 0.05) to carbachol in the presence of

Table 1 Competition data for [^{3}H]-QNB binding to normal pig detrusor membranes

	Tissue	pK$_i$-1 High affinity site	pK$_i$-2 Low affinity site	Hill slope	Fraction of high affinity sites*
4-DAMP	Normal (n = 9)	9.66 ± 0.28	7.26 ± 0.14	0.55 ± 0.09	0.31 ± 0.03
Darifenacin	Normal (n = 8)	9.35 ± 0.21	7.00 ± 0.14	0.66 ± 0.06	0.20 ± 0.04
Methoctramine	Normal (n = 9)	7.87 ± 0.19	6.0 ± 0.21	0.50 ± 0.09	0.76 ± 0.02

*Density of high affinity sites compared to low affinity sites, i.e. fraction of M$_3$-receptors for 4-DAMP and darifenacin and fraction of M$_2$-receptors for methoctramine.

Table 2 Affinity of antagonists determined using isolated pig detruscor muscle strips

Antagonist	Tissue	pK$_B$	pA$_2$	Schild slope
4-DAMP	Normal bladder (*n* = 12)	9.37 ± 0.07	9.6 ± 0.03	0.94 ± 0.12
	M3-inactivated bladder (*n* = 27)	8.72 ± 0.08**	8.7 ± 0.01	1.17 ± 0.09
Darifenacin	Normal bladder (*n* = 18)	8.61 ± 0.10	(unsurmountable)††	
	M3-inactivated bladder (*n* = 18)	6.74 ± 0.07****	(unsurmountable)††	
Methoctramine	Normal bladder (*n* = 18)	6.05 ± 0.05	6.12 ± 0.02	0.89 ± 0.15
	M3-inactivated bladder (*n* = 13) (KCl + Isoprenaline)	6.59 ± 0.11****		(Variable)
	M3-inactivated bladder (*n* = 24)† (KCl + Forskolin)	6.86 ± 0.06****	(unsurmountable)††	

M$_3$-inactivation was achieved by incubating tissues with 4-DAMP mustard (40 nM) for 60 min in the presence of methoctramine (1 μM) to protect the M$_2$-receptor population. †Forskolin was used to increase cyclic AMP level. ††Antagonists did not act as competitive but reduced maximum responses to carbachol. **$P < 0.01$, ****$P < 0.0001$.

isoprenaline and KCl compared with those (6.06 ± 0.01) in the absence of these agents (*n* = 6). Maximum responses in the presence of isoprenaline and KCl (9.7 ± 0.26 g) were also significantly ($P < 0.0001$) decreased by 40% compared with those in the absence of isoprenaline and KCl (23.7 ± 0.15 g). In tissues where the M$_3$-receptors had been inactivated, carbachol produced contractile responses in KCl-precon-tracted/isoprenaline-relaxed tissues (pEC$_{50}$ = 5.23 ± 0.14, *n* = 12). Maximum responses after M$_3$ inactivation were 6.06 ± 0.41 g, which was 33.7% of control values (15.8 ± 5.32 g).

Effects of muscarinic antagonists in M$_3$-inactivated tissues

In tissues where the M$_3$-receptors had been inactivated and cyclic AMP levels elevated (isoprenaline plus KCl), 4-DAMP produced antagonism of the contractile responses to carbachol, but was less potent, the pK$_B$ value being 8.72 ± 0.08, significantly ($P < 0.01$) lower than in the normal tissues. Maximum responses were unaffected by the antagonist and the Schild slope was 1.17 ± 0.09 (Figure 2, Table 2). Darifenacin showed unsurmountable antagonism of the responses to carbachol, and was also less potent, the apparent pK$_B$ value being 6.74 ± 0.07, significantly ($P < 0.0001$) lower than in the normal tissues (Figure 3, Table 2). In contrast, methoctramine was more potent after M$_3$-inactivation (KCl and isoprenaline), the apparent pK$_B$ value increasing significantly ($P < 0.0001$) to 6.59 ± 0.11. Maximum responses were unaffected by the antagonist but the Schild slope for methoctramine was variable (0.90 ± 0.58). In M$_3$-inactivated tissues where cyclic AMP levels were increased by forskolin (1 μM), the apparent pK$_B$ value for methoctramine again was increased significantly ($P < 0.0001$) to 6.86 ± 0.06. The Schild slope was less variable and was similar to unity (0.90 ± 0.12), but maximum responses were reduced by all concentrations of methoctramine ($P < 0.0001$) (Figure 4, Table 2).

Discussion

Previous studies indicate that the contractile response of detrusor muscle to muscarinic agonists is wholly mediated *via* the M$_3$-receptor subtype in all species so far examined (Claufield 1993; Eglen *et al.*, 1994; Longhurst *et al.*, 1995). This is surprising since immunoprecipitation studies suggest that in most species the density of M$_2$ receptors exceeds that of the M$_3$ population by a ratio of 3 : 1 and in the rat by a ratio of 9 : 1 (Wang *et al.*, 1995). This conclusion is supported
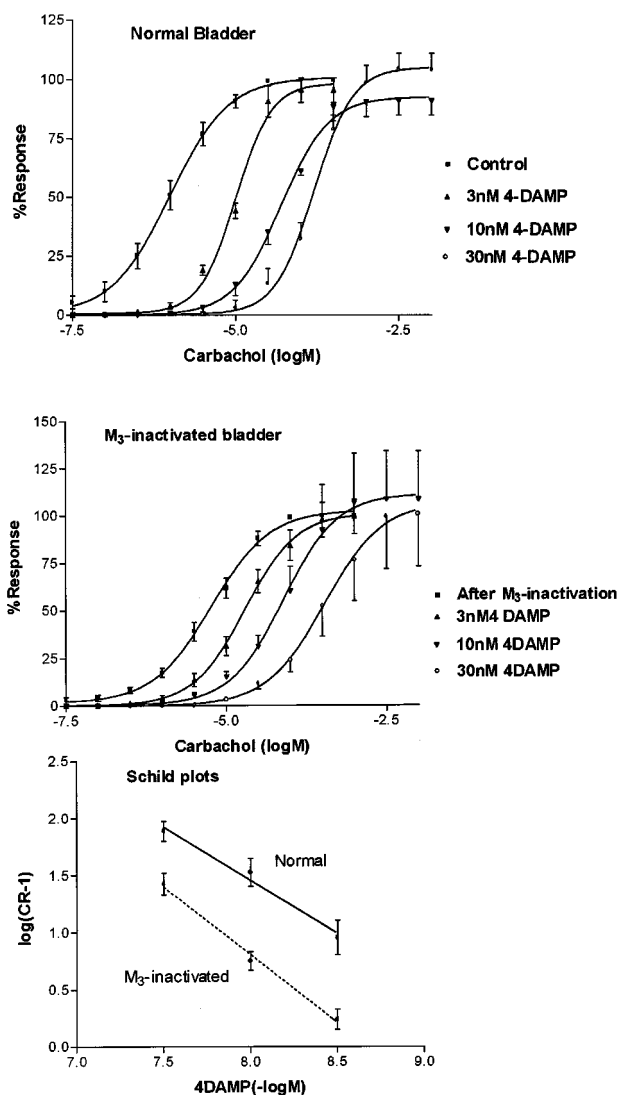

Figure 2 Concentration-response curve to carbachol in normal (upper panel) and M$_3$-inactivated (middle panel) pig detrusor muscle strips in the absence and presence of 4-DAMP (3 – 30 nM). Data are normalized to the maximal response generated during the first (control) curve. Lower panel shows Schild slopes for normal and M$_3$-inactivated bladder tissues.

in radioligand binding studies where the ratio of M$_2$: M$_3$ receptors has been shown to be about 7 : 1 in the rat (Monferini *et al.*, 1988). A previous study of the pig and human bladder concluded that only one subtype exists in both these species and it was identified as being of the M$_2$-
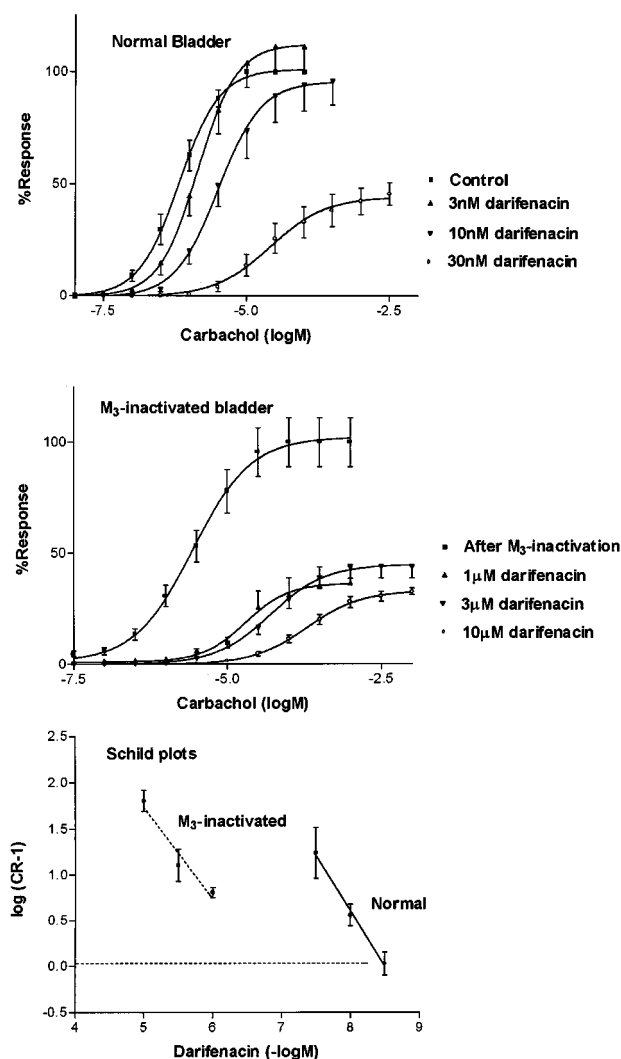
Figure 3 Upper panel: concentration-response curve to carbachol in normal pig detrusor muscles in the absence and presence of darifenacin (3–30 nM). Middle panel: concentration-response curve to carbachol in M$_3$-inactivated pig detrusor muscles in the absence and presence of darifenacin (1 μ – 10 μM). Data are normalized to the maximal response generated during the first (control) curve. Lower panel shows Schild slopes for normal and M$_3$-inactivated bladder tissues.
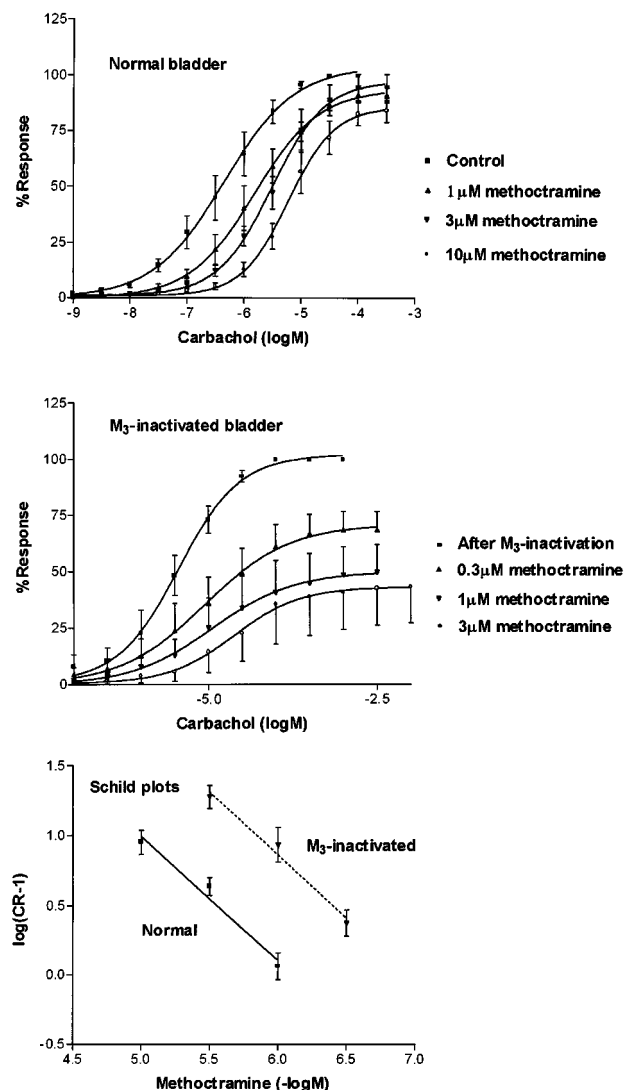
Figure 4 Upper panel: concentration-response curve to carbachol in normal pig detrusor muscles in the absence and presence of methoctramine (1 – 10 μM). Middle panel: concentration-response curve to carbachol in M$_3$-inactivated pig detrusor muscles in the absence and presence of methoctramine (0.3 – 3 μM). Data are normalized to the maximal response generated during the first (control) curve. Lower panel shows Schild slopes of normal and M$_3$-inactivated bladder tissues.

receptor subtype (Goepel *et al.*, 1998). This highlights the problems found when using drugs without a great selectivity between the various subtypes of muscarinic receptor. In the present study some of the most selective agents available were used, and we were able to demonstrate that, as found in other species, it is a minor population of M$_3$-receptors which mediates contraction of the pig detrusor muscle to carbachol. The M$_3$ selective agents 4-DAMP and darifenacin had relatively high affinities for the detrusor muscarinic receptor, the values of 9.4 and 8.6 being similar to those reported for these antagonists at the M$_3$-receptor, whilst the M$_2$-selective antagonist methoctramine had a relatively low apparent affinity, which again was similar to values reported for this antagonist at M$_3$-receptors (Hegde *et al.*, 1997).

As found by other laboratories working in this area (Goepel *et al.*, 1998), demonstrating the M$_3$-receptor population in radioligand binding assays was difficult, although we had more selective antagonists available. In competition experiments, displacement of [^{3}H]-QNB was usually best fitted by a two-site model for the antagonists, but there was only a statistically better fit with the two-site

model in four of the 4-DAMP experiments, one of the darifenacin and six of the methoctramine experiments. Hill slopes for all three antagonists were low, which supports the concept of two-site binding and this was further supported in tissues following M$_3$-alkylation, where only one binding site (M$_2$) could be observed. The radioligand binding data therefore demonstrated a minority M$_3$-receptor population of about 25% in the pig bladder, and this ratio is similar to that reported previously for the human bladder (Wang *et al.*, 1995; Yamaguchi *et al.*, 1996). For this reason we investigated the receptor subtype mediating functional responses in this species.

In the normal pig isolated detrusor strips, Schild slopes for the antagonists were close to unity, suggesting that only one receptor subtype was mediating the contractile responses to carbachol in these tissues. Other tissues have also been identified which have a high M$_2$-receptor density, but where responses *in vitro* appear to be mediated solely *via* the M$_3$-subtype (Eglen *et al.*, 1994). It has been suggested that the conditions *in vitro* are not favourable to the demonstration of

an M_2-response which involves the inhibition of adenylate cyclase. Indeed in guinea-pig ileum, responses normally mediated *via* M_3-receptor can involve M_2-activity following M_3 inactivation and elevation of cyclic AMP levels, the response then being regarded as a re-contraction or reversal of relaxation (Thomas *et al.*, 1993; Reddy *et al.*, 1995). This has also been shown in the rat bladder, but in this species the M_2-receptor population is large (Hegde *et al.*, 1997; Braverman & Ruggieri, 1999) and the $M_2 : M_3$ ratio is far greater than found in other species. The present study investigated whether an M_2-mediated response could be demonstrated in the pig bladder where the $M_2 : M_3$ ratio is lower than that found in the rat. The $M_2 : M_3$ ratio of $3 : 1$ obtained in the present study is significantly lower than that found in the rat, but is similar to the value recorded for the human bladder (Wang *et al.*, 1995; Yamaguchi *et al.*, 1996).

Using a protocol previously used on the rat bladder to inactivate M_3-receptors and raise cyclic AMP levels, the apparent affinities of the three antagonists were significantly altered and suggested that the M_2-receptor was responsible for mediating responses under these conditions. The affinity of the M_3-selective agents 4-DAMP and darifenacin were reduced, while that for the M_2-selective methoctramine was increased, compared to control tissues. Affinity estimates for the antagonists under these conditions were similar to those reported in the literature for M_2-mediated responses (Hegde *et al.*, 1997) and Schild plots had slopes similar to unity for 4-DAMP and darifenacin. The Schild plots for methoctramine were variable, but when cyclic AMP levels were elevated using forskolin instead of isoprenaline, a similar affinity estimate was obtained and the Schild plot slope was more consistent and close to unity. The reason for the more reliable results obtained using forskolin are not known but presumably may result from a larger elevation of cyclic AMP levels (Griffin & Ehlert, 1992). In contrast to the normal tissues, the Schild plots suggested that responses after M_3-inactivation and cyclic AMP elevation were mediated solely *via* M_2-muscarinic receptors. Radioligand binding experiments supported this finding. Incubation of tissues with 4-DAMP mustard in the presence of methoctramine to protect M_2-receptors resulted in a 30% decrease in total muscarinic receptor density, but competition curves had Hill slopes close to unity for all three antagonists, and no experiment could be fitted to a two-site model of binding, suggesting that the M_3-receptor population had been almost completely removed.

These findings may have relevance in both physiological and pathological states. Hegde *et al.* (1997) have demonstrated a contribution by M_2-receptors to bladder contraction in the rat *in vivo* and M_2-muscarinic receptor density has been shown to be selectively elevated following denervation of the bladder, albeit again in the rat (Braverman *et al.*, 1998). It has been suggested that M_2-receptors may mediate the dominant parasympathetic control over smooth muscle tone under conditions of high sympathetic activity or where M_3-receptors are dysfunctional (Eglen *et al.*, 1994). Beta-adrenoceptors predominate over alpha-adrenoceptors in the urinary bladder body, where their tonic stimulation is thought to facilitate the storage phase of micturition by relaxing the detrusor smooth muscle (Levin *et al.*, 1988). The cholinergic activity is inhibited during this filling phase. On the contrary, during the voiding phase, sympathetic nerve activity is inhibited and acetylcholine is released (deGroat, 1993). Activation of M_2 receptors during the voiding phase may oppose inhibitory sympathetic activation of beta-adrenoceptors (Nilvebrant *et al.*, 1997), resulting in more efficient bladder emptying or initiation of voiding.

In summary, pig detrusor muscle possesses a population of muscarinic receptor in which the M_2 is the major subtype (75%), but where M_3-receptors mediate all the contractile responses in isolated muscle strip *in vitro*. However, under altered pharmacological conditions M_2-receptors can mediate contractile (re-contraction) responses to muscarinic agonists.

Since the pig has been shown to have a $M_2 : M_3$ ratio similar to that reported for human, the data suggest that less selective muscarinic agents with regard to M_2/M_3-selectivity might be more effective than M_3-selective antagonists in treating detrusor over-activity.

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