Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT–PCR: changes in ageing

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1 We investigated muscarinic receptors in the detrusor and mucosa of the human bladder body. Radioligand-binding studies with [3H]QNB were conducted using specimens collected from patients (36–77 years) with normal bladder function, undergoing surgery. For RT–PCR, biopsies of normal bladder were obtained from patients (30–88 years) undergoing check cystoscopy.

2 Binding of [3H]QNB in detrusor (n = 20) was of high affinity (K_D 77.1 (55.2–99.0) pm) and capacity (B_max 181 ± 7 fmol mg protein^-1). Similar values were obtained in mucosa (n = 6) (K_D 100.5 (41.2–159.9) pm; B_max 145 ± 9 fmol mg protein^-1).

3 Competition-binding experiments in detrusor membranes with muscarinic receptor antagonists including trospium, darifenacin, 4-DAMP, methoctramine, AQ-RA 741, AF-DX 116 and pirenzepine indicated a receptor population of 71% M2, 22% M3 and 7% M1. In the mucosa, 75% of sites were M2 receptors, with 25% M3/M5.

4 Using RT–PCR, expression of M1, M2, M3 and M5 mRNA was demonstrated in both detrusor and mucosa.

5 The presence of a high density of mainly M2 muscarinic receptors in the mucosa appears to be a novel finding and raises the question of their physiological significance and the source of their endogenous ligand.

6 There was a negative correlation of receptor number (B_max) with age in detrusor muscle from male patients (P = 0.02). Quantitative competitive RT–PCR demonstrated a selective age-related decrease in mRNA for muscarinic M3 but not M2 receptors, in both male (P < 0.0001) and female (P = 0.019) detrusor. These findings correspond with reports of decreased detrusor contractility with ageing.

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Abbreviations: AF-DX 116, [2-(diethylamino)methyl]-1-piperidinyl[acycl][5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one; AQ-RA 741, (11-[[4-(diethylamino)butyl]-1-piperidinyl[acycl][5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine-methiodide; QCRT–PCR, quantitative competitive reverse transcription PCR; [3H]QNB, [3H]quinuclidinyl benzylate

Introduction

Five subtypes of G protein-coupled muscarinic receptors (M1–M5) have been cloned and pharmacologically characterised. In the urinary bladder, as in other smooth muscles, multiple muscarinic receptor subtypes have been identified (Eglen et al., 1996). Binding and immunoprecipitation studies in human detrusor (Nilvebrant et al., 1985; Wang et al., 1995; Goepel et al., 1998) have shown that the majority of muscarinic receptors present are of the M3 subtype. Although there is evidence that M2 receptors are of some functional importance (Matsui et al., 2002; Ehlerd, 2003), particularly in pathological states (Braverman & Ruggieri, 2003; Pontari et al., 2004), functional experiments in M3 knockout mice (Matsui et al., 2000) and human detrusor strips (Chess-Williams et al., 2001; Fetscher et al., 2002) have demonstrated that muscarinic M3 receptors are the main mediators of the contractile response.

Muscarinic receptor antagonists (anticholinergics) are the mainstay of treatment for the overactive bladder (Andersson & Yoshida, 2003), for example, in patients with frequency and urgency of micturition, with or without urge incontinence. Unfortunately, their lack of organ selectivity has resulted in many patients experiencing dry mouth and/or constipation. Recently, muscarinic receptor antagonists with greater subtype selectivity (darifenacin and methoctramine) or higher potency (tropium) have become available, but have not been used to more precisely define receptor subtypes in the human detrusor.

Historically, the urothelium has been considered a simple inert barrier. However, the urothelium is metabolically
active and some reviews of recent evidence suggest that the tissue acts as an important regulator of bladder contractility (Chess-Williams, 2002; Fry et al., 2004). Radioligand-binding studies with \(^{[3]H}\)quinuclidinyl benzylate (\(^{[3]H}\)QNB) in the pig urothelium have indicated that this tissue possesses a large number of muscarinic receptors (Hawthorn et al., 2000). Therefore, these urothelial receptors may represent a second site of action for the muscarinic receptor antagonists that are used to treat overactive bladder.

A recent large survey reported that 16.6% of European adults reported symptoms of overactive bladder, with the symptoms increasing consistently with advancing age (Milsom et al., 2001). Urodynamic tests have demonstrated an age-related reduction in bladder capacity, and an increased incidence of uninhibited contractions, decreased urinary flow rate and incomplete bladder emptying (Madersbacher et al., 1998). Despite these known age-related alterations in detrusor function, changes in muscarinic receptors with age have not been extensively studied in the human bladder.

The initial aims of this study were (1) to determine the affinity and density of muscarinic receptor proteins in both detrusor and mucosa (urothelium and lamina propria) of the human urinary bladder; (2) to use a range of muscarinic receptor antagonists to pharmacologically characterise the muscarinic receptor subtypes present in these regions; (3) to document the expression of muscarinic receptor subtype mRNA in detrusor and mucosa. During the course of this study, some age-related changes became apparent, and therefore we also examined (4) age-related changes in muscarinic receptor protein density (\(B_{\text{max}}\)) in male detrusor, and (5) age-related changes in expression of \(M_2\) and \(M_3\) receptor mRNA in the detrusor from both males and females.

**Methods**

**Patients and specimens**

Collection of human bladder specimens was approved by the Human Ethics Committee of the University of New South Wales (HREC 03175). All patients displayed normal micturition frequency with no symptoms of urge incontinence or obstruction. Patients were characterised by their clinician as having no evidence of overactive bladder (frequency, nocturia with or without urge incontinence) nor features of outflow obstruction (poor stream, incomplete emptying). Previous pelvic radiotherapy or current bladder infection were exclusion criteria.

**Bladder segment collection** Whole-wall segments of macroscopically normal bladder (body) were collected from 33 patients (25 males, 8 females; age range 36–77 years) undergoing open bladder surgery (15 cystectomy for malignancy, 15 radical prostatectomy for malignancy, two colpoplasty and one ileal conduit). Bladder segments were placed immediately into ice-cold Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, NaHCO\(_3\) 25, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 2.5 and D-glucose 11.7), pre-gassed with carbogen (95% \(\text{O}_2\), 5% \(\text{CO}_2\)). Specimens were immediately transported to the laboratory and transferred to fresh, cold, pre-gassed solution. They were either refrigerated overnight before dissection or dissected immediately (Zeng et al., 1995).

The bladder segments were first separated into detrusor muscle and mucosa (containing urothelium and lamina propria) and then cut into portions of approximately 500 mg, which were frozen in liquid nitrogen and then stored at \(-70^\circ\text{C}\) until use in radioligand-binding experiments. All 33 detrusor specimens and eight (seven male and one female patients, age range 51–71 years) mucosal specimens were used for radioligand binding.

**Bladder biopsy collection** Bladder biopsies (3 × 4 mm\(^2\), 5–20 mg) were obtained from 73 patients (41 males, 32 females, age range 30–88 years) undergoing check cystoscopy for previous bladder cancer or asymptomatic haematuria. Cold-cup biopsies were taken from a site 2 cm lateral and cephalad from the left ureteric orifice. They were washed in saline, followed by immediate collection into ice-cold RNA Later (Ambion) in the operating theatre. After overnight storage at 4\(^\circ\text{C}\), biopsies were dissected into detrusor muscle and mucosa (containing urothelium and lamina propria) and stored at \(-80^\circ\text{C}\) until RNA was extracted (see below).

**Radioligand binding studies**

**Membrane preparation** Radioligand binding studies were carried out on bladder segments as described previously (Mansfield et al., 2003). Approximately 500 mg detrusor muscle or mucosa was finely minced in ice-cold sodium phosphate buffer (10 ml, 50 mM Na\(_2\)HPO\(_4\), pH 7.4) and homogenised with a Polytron (setting 5, for 3 × 10\(^s\)). The suspension was then centrifuged at 1000 \(\times\) g for 15 min. The pellet was discarded and the supernatant re-centrifuged at 40,000 \(\times\) g for 20 min. The final pellet was resuspended in 10 ml of 50 mM sodium phosphate buffer, pH 7.4.

**Kinetic, saturation and competition studies** These were carried out using the nonselective radioligand \(^{[3]H}\)QNB in a final volume of 0.5 ml of 50 mM phosphate buffer (pH 7.4) at 37\(^\circ\text{C}\). Nonspecific binding was defined in replicate tubes using 10 \(\mu\text{M}\) atropine. The incubation was initiated by addition of detrusor or mucosal membranes (2% wet weight final tissue concentration) to each tube. Binding of radioligand was <10% of total radioactivity in most experiments.

In preliminary studies, 200 \(\mu\text{M}\) \(^{[3]H}\)QNB was incubated with human detrusor muscle membranes at six time points for up to 3 h. Equilibrium appeared to be reached at approximately 1 h, and an incubation time of 2 h was chosen for subsequent experiments. In saturation experiments, eight concentrations of \(^{[3]H}\)QNB (15 \(\mu\text{M}\) to 2 \(\text{nm}\)) were incubated with detrusor or mucosal membranes for 2 h at 37\(^\circ\text{C}\). Protein content was determined by the Lowry method using BSA as a standard.

In competition studies, increasing concentrations of muscarinic receptor antagonists were incubated in 50 mM sodium phosphate buffer (pH 7.4) with detrusor or mucosal membranes and 200 \(\mu\text{M}\) \(^{[3]H}\)QNB for 2 h, before filtration and washing as above. For most compounds, 13 concentrations of competitor were used.

Incubations were terminated by addition of 3 ml ice-cold 50 mM sodium phosphate buffer (pH 7.4). Membranes were filtered using a tissue harvester (Brandel Inc., Gaithersburg, U.S.A.) through GF/B filters (Whatman, Maidstone, U.K.)
pre-soaked in sodium phosphate buffer containing 0.5% polyethyleneimine (PEI) and 10 μM atropine. The filters were washed three times with 3 ml of ice-cold buffer and then placed into scintillation vials containing 2 ml scintillant (Beckman Ready Safe, Fullerton, U.S.A.). Vials were left overnight before measurement of radioactivity using liquid scintillation spectrometry (TriCarb Model 1900TR, Packard, Meriden, U.S.A.).

Data analysis For saturation studies, data from individual experiment were fitted with a one-site binding model using the nonlinear regression analysis program of GraphPad Prism (version 4.0, GraphPad Software Inc., San Diego, U.S.A.), to derive the binding parameters. The dissociation constant (K_D, in pm) is expressed as geometric mean (95% confidence limits) and the maximum number of binding sites present (B_max, in fmol mg protein^{-1}) is expressed as mean ± s.e.m.

For competition studies, all data were simultaneously analysed using the nonlinear regression analysis program of GraphPad Prism (version 3.0) and tested to determine if a one-site or two-site model was statistically preferred (F-test, P<0.05). Dissociation constants (K_i, values with 95% confidence limits) of competitors for [3H]QNB-binding sites were predicted from cDNA. M_1 PCR products were digested with Hinf (generated fragments of 225 and 113 bp); M_2 with HinfI (97, 111 and 150 bp); M_3 with PstI (57, 139 and 198 bp); M_4 with AvaI (302 and 186 bp); and M_5 with Ddel (200 and 95 bp). As the M_4 product was not detected in either detrusor or mucosa after first round PCR, nested-PCR reactions were performed with 1:10 and 1:100 dilutions of M_4 first round PCR product as templates. Nested primer pairs for M_4 receptor were used with TjiI polymerase: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 1 min and 70°C for 45 s, and a final elongation period at 70°C for 10 min.

In this study, we separated detrusor and mucosa and examined mRNA expression in each region independently. Expression of mRNA for calponin (smooth muscle marker) was used to check the dissections and, as expected, calponin mRNA expression was very high in detrusor muscle RNA, with very low or undetectable expression in the mucosa RNA (data not shown). The expression of calponin and β-actin gene transcripts was verified by RT–PCR. A PCR cycle of 25 amplifications was performed and extension time changed

| Table 1 | Primer sequence for RT–PCR of muscarinic receptor subtypes and β-actin |
|---|---|---|---|
| Code | Primer sequence (5‘→3’ ) | GenBank accession no. | Fragment size (bp) |
| M_1 | Sense: GCTCCCCCAATACAGTCAAGAG | NM_000738 | 338 |
| | Antisense: CAGCAGCAGCGGAAGGTTG | | |
| M_2 | Sense: GATGCGCCTGGGACACACAAC | NM_000739 | 358 |
| | Antisense: GCTGCTTAGTCATCTCACATC | | |
| M_3 | Sense: CGAGCAGATGGACACAAACG | NM_000740 | 390 |
| | Antisense: AGGTAAGTGGGCGCTGCTC | | |
| M_4 | Sense: TCAATAGTGTCGACGTCGCT | NM_000741 | 488 |
| | Antisense: AGACATAGCAGGCCGGGTGGTG | | |
| Nested M_4 | Sense: TCCAGATTTGGCAGCAAGCG | NM_000741 | 202 |
| | Antisense: AAGGCTAGCAGAAATGGCAAA | | |
| M_5 | Sense: GGACTATAGTGTCGACGTTG | NM_012125 | 295 |
| | Antisense: GGTAGACTGGGGCAGACACTTG | | |
| β-Actin | Sense: ACGGGGTCCACCACACACTGTC | NM_001101 | 659 |
| | Antisense: CTGAAGCTATTGGCGGTGAC | | |

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from 45 s to 2 min; otherwise the conditions were identical to RT-PCR for M2. PCR products were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide.

**Quantitative competitive RT–PCR** The expression of M2 and M3 receptor mRNA from the male and female detrusor was quantified using quantitative competitive RT–PCR (QCRT–PCR), where a known amount of competitor RNA is added to the target RNA sample and both the competitor and target RNA are amplified in the same reaction (Dash et al., 2000, Holsinger et al., 2000).

Competitor RNA (internally deleted standard RNA) was produced for M2 and M3 muscarinic receptors. M2 and M3 cDNA fragments (358 and 390 bp, respectively) were generated by RT–PCR from human bladder total RNA and then internally deleted 150 and 57 bp, using restriction enzymes HinfI and PstI, respectively. The modified cDNAs were subcloned into a pTargetT vector and used as templates to synthesise competitor RNA by T7 polymerase. The competitor RNA concentration was quantified by spectrophotometric absorbance at 260 nm.

For QCRT–PCR, bladder total RNA (100 ng) was co-amplified with serial dilutions (30–0.03 pg) of competitor RNA, reverse transcribed with random hexamer (2 mM) and Amv reverse transcriptase, and the RT products were subsequently amplified by PCR with Tfl polymerase and a pair of M2 or M3 gene-specific primers (0.2 mM). The products were then separated by gel electrophoresis (2.5% agarose containing ethidium bromide) and quantified by densitometry (BioRad Gel Doc system).

**Data analysis** The amount of sample mRNA (M2 or M3 mRNA per 100 ng total RNA) was calculated from the competition equivalence point, determined by plotting the log relative intensity of DNA bands (total bladder RNA per competitor RNA) versus the log of the known concentration of competitor RNA.

The QCRT–PCR data for M2 and M3 receptor expression in detrusor muscle were then normalised for the expression of β-actin (cytoskeletal protein) in the same sample. Expression of β-actin did not vary with age (see Results) and was considered to be a suitable housekeeping gene for internal standardisation of target gene expression. Densitometry results from QCRT–PCR gels and age-related changes in muscarinic receptor expression were analysed using the linear regression analysis program of GraphPad Prism (version 3.0).

**Statistical tests**

Paired comparisons were carried out using the Wilcoxon ranked pairs test, and unpaired comparisons were carried out using the Mann–Whitney test.

**Materials**

[3H]QNB (specific activity; 37 Ci mmol\(^{-1}\)) was obtained from NEN (Boston, U.S.A.). Atropine, 4-DAMP, methoctramine and pirenzepine were obtained from Sigma (St Louis, U.S.A.). AQ-RA 741, and AF-DX 116 were gifts from Dr Karl Thomae GmbH (Biberach an der Riss, Germany). Darifenacin was a gift from Pfizer (Sandwich, U.K.). Trospium was supplied by Dr R. Pfleger GmbH (Bamberg, Germany). All other reagents were of analytical grade.

RNA extraction kits were obtained from Epicentre (Madison, U.S.A.). All other molecular reagents including Access RT–PCR kit, restriction enzymes, pTargetT vector, DNase treatment, RNasin and random hexamers were from Promega (Madison, U.S.A.).

**Results**

**Radioligand binding studies in detrusor muscle membranes**

**Saturation studies** When detrusor muscle membranes were incubated for 2 h in the presence of increasing concentrations of [3H]QNB, specific binding was saturable at approximately 500 pM (Figure 1a). Nonlinear regression analysis indicated that specific binding of [3H]QNB was consistent with binding to a single-site rather than to a multiple-site model (nH 0.905), with KD 77.1 (55.2–99.0) pM and Bmax 181 ± 7 fmol mg protein\(^{-1}\) (n = 20). The data for the male group (KD 84.2 [60.4–108.0] pM; Bmax 172 ± 11 fmol mg protein\(^{-1}\); n = 16) were not different from those for

![Figure 1](image-url) Results from one representative saturation-binding experiment (male, 63 years) with [3H]QNB. (a) Membranes prepared from the detrusor muscle. KD 75.9 pM; Bmax 186 fmol mg protein\(^{-1}\). (b) Membranes prepared from the mucosa. KD 50.7 pM; Bmax 151 fmol mg protein\(^{-1}\). Similar data were obtained from other specimens.
the small female group (K_D 57.9 [26.7–89.1], n = 4; B_max 192 ± 45 fmol mg protein⁻¹; n = 4).

**Competition studies** A number of muscarinic receptor antagonists were examined for their ability to compete with 200 pm [³H]QNB in detrusor muscle membranes (Table 2). The order of potency, based on IC₉₀ values, was atropine > trospium > AQ-RA 741 > 4-DAMP > methoctramine > darifenacin > AF-DX 116 > pirenzepine (Figure 2a, b). For some competitors, the competition curves displayed slope factors less than unity, and a two-site analysis resulted in significantly better resolution of some of these data sets (Table 2). The high-affinity component of binding of the muscarinic M₂ receptor-preferring antagonists methoctramine and AQ-RA 741 represented 71 and 65% of total binding sites, respectively. In contrast, the high-affinity components of the muscarinic M₁ receptor-preferring antagonists 4-DAMP and darifenacin represented 22 and 24% of total binding sites, respectively. Pirenzepine, which shows preference for M₁ receptors, also bound to more than one site: it showed high affinity for a minority of sites (8%) and very low affinity for the remainder.

The Kᵢ values obtained from all competition experiments (Table 2) were correlated with the Kᵢ values reported in the literature for the five cloned human muscarinic receptors (Table 3) by means of a log-log plot. A significant correlation (r² = 0.978, P < 0.0001) was found for the M₂ receptor subtype, whereas extremely poor correlations were obtained for M₁, M₃, M₄ and M₅ receptor subtypes (r² = 0.000, 0.004, 0.008, 0.000, respectively).

As two-site analysis is inadequate to resolve the relative proportions when more than two sites are present, a global fit of the data was undertaken for darifenacin, methoctramine, AQ-RA 741 and pirenzepine, with shared proportions of each of the three sites. The analysis gave the following percentages for the three putative muscarinic receptor subtypes: M₁ 7.2 ± 2.2%; M₂ 71.2 ± 6.3%; and M₃ 21.5 ± 5.9%. Correlation plots using log-log transformations of the appropriate Kᵢ values obtained from this analysis of the data for each of the four ligands versus Kᵢ values from the literature (Table 3) for M₁, M₂ and M₃ receptors gave significant correlations with each receptor subtype (M₁, r² = 0.99, P < 0.01; M₂, r² = 0.97, P < 0.05; M₃, r² = 0.99, P < 0.005).

**Radioligand binding studies in mucosal membranes**

**Saturation studies** In mucosal membranes, specific binding of [³H]QNB was saturable at approximately 500 pm (Figure 1b). Binding occurred to a single site (νₙ 0.982) with K_D 100.5 (41.2–159.9) pm and B_max 145 ± 9 fmol mg protein⁻¹ (n = 6). These studies were carried out in parallel with studies using detrusor membranes from the same patients (five males, one female); under these conditions, no significant differences between B_max or K_D values were seen between mucosa and detrusor (B_max, P = 0.33; K_D, P = 0.62; paired t-test, n = 6).

**Figure 2** Competition for specific binding of 200 pm [³H]QNB to human detrusor membranes by muscarinic receptor antagonists. Values are mean ± s.e.m. (n = 4–12).

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**Table 2** Dissociation constants (Kᵢ) for muscarinic receptor antagonists in human detrusor muscle membranes

<table>
<thead>
<tr>
<th>Drug</th>
<th>N</th>
<th>Hill slope*</th>
<th>Kᵢ (nM)*</th>
<th>Kᵢ, site 1</th>
<th>Kᵢ, site 2</th>
<th>% H⁺**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>4</td>
<td>0.82 ± 0.10</td>
<td>3.0 (2.1–4.3)</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropium</td>
<td>7</td>
<td>0.66 ± 0.06</td>
<td>4.9 (3.6–6.7)</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQ-RA 741</td>
<td>6</td>
<td>0.73 ± 0.03</td>
<td>12.3 (10.8–14.1)</td>
<td>4.5 (2.8–7.2)</td>
<td>97.5 (39.8–238)</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>4</td>
<td>0.63 ± 0.05</td>
<td>12.2 (9.2–16.4)</td>
<td>0.23 (0.05–1.19)</td>
<td>29.5 (18.5–47.1)</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>12</td>
<td>0.65 ± 0.03</td>
<td>38.6 (33.4–44.7)</td>
<td>13.7 (9.9–18.9)</td>
<td>752 (336–1680)</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>11</td>
<td>0.52 ± 0.05</td>
<td>42.0 (33.6–52.6)</td>
<td>0.23 (0.11–0.53)</td>
<td>146 (113–189)</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>6</td>
<td>0.75 ± 0.05</td>
<td>170 (140–208)</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8</td>
<td>0.78 ± 0.04</td>
<td>856 (734–998)</td>
<td>0.42 (0.06–3.10)</td>
<td>1134 (962–1340)</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Kᵢ values were calculated according to the equation Kᵢ = IC₉₀/(1 + 200/77.1).

*Data are expressed as mean ± s.e.m.

**Data are derived from simultaneous analysis of all competition curves and are expressed as geometric mean (95% confidence intervals).

**% H⁺ indicates % of high-affinity sites.**
Table 3 Reported $K_i$ values (nM) for muscarinic ligands in cell lines expressing human muscarinic receptor subtypes

<table>
<thead>
<tr>
<th>Compound</th>
<th>$M_1$</th>
<th>$M_2$</th>
<th>$M_3$</th>
<th>$M_4$</th>
<th>$M_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trosipium</td>
<td>0.8</td>
<td>0.6</td>
<td>0.5</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Atropine</td>
<td>0.3</td>
<td>0.8–1.3</td>
<td>0.2–0.3</td>
<td>0.1–1.2</td>
<td>0.2–0.8</td>
</tr>
<tr>
<td>AQ-RA 741</td>
<td>29–62</td>
<td>3.7–4.4</td>
<td>55–86</td>
<td>1.5–15</td>
<td>732–831</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>0.6–1.2</td>
<td>4–9</td>
<td>0.4–1.0</td>
<td>0.7–1.7</td>
<td>0.6–1.3</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>7–35</td>
<td>44–77</td>
<td>0.8–1.3</td>
<td>18–46</td>
<td>5–9</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>417–740</td>
<td>64–73</td>
<td>786–1290</td>
<td>211–545</td>
<td>5130</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>6–9</td>
<td>224–407</td>
<td>75–184</td>
<td>17–48</td>
<td>66–158</td>
</tr>
</tbody>
</table>

Values in bold indicate preferred receptor subtype.

Data from references cited in Mansfield et al. (2003).

aData from Madersbacher (2003).

Table 4 Dissociation constants ($K_i$) for muscarinic receptor antagonists in human mucosal membranes

<table>
<thead>
<tr>
<th>Drug</th>
<th>N</th>
<th>$Hill$ slope$^a$</th>
<th>$K_i$ (nM)$^b$</th>
<th>$K_i$, site 1</th>
<th>$K_i$, site 2</th>
<th>% $H^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ-RA 741</td>
<td>3</td>
<td>0.69±0.05</td>
<td>3.5 (2.8–4.3)</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoctramine</td>
<td>5</td>
<td>0.71±0.07</td>
<td>38.8 (28.9–52.3)</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darifenacin</td>
<td>5</td>
<td>0.67±0.05</td>
<td>144 (113–184)</td>
<td>1.9 (0.4–10.0)</td>
<td>325 (206–511)</td>
<td>22±4</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>5</td>
<td>0.87±0.06</td>
<td>1600 (1320–1940)</td>
<td></td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

$K_i$ values were calculated according to the equation $K_i = 1/C_{max}/(1 + [200]/100.5)$.

$^a$Data are expressed as mean ± s.e.m.

$^b$Data are derived from simultaneous analysis of all competition curves and are expressed as geometric mean $K_i$ (95% confidence intervals).

$^e$% $H_i$ indicates % of high-affinity sites.
and M₅ receptor mRNA was highly consistent, whereas that for M₄ was variable. In mucosa, on the other hand, mRNA for M₂ and M₃ receptors was expressed consistently, whereas mRNA for M₁ and M₁ receptors was detected only in some samples. The densitometric analysis demonstrated that band intensities of M₂ (n = 14 matched pairs, P < 0.001) and M₃ mRNA (n = 14 for detrusor and n = 10 for mucosa, P < 0.001) were greater in detrusor compared with mucosa. For both M₁ mRNA (n = 6) and M₅ mRNA (n = 5), no differences in expression were observed in detrusor compared with corresponding samples from the mucosa.

Neither detrusor nor mucosa showed any visible expression of M₄ receptor mRNA with RT–PCR, so nested-PCR reactions were performed to enhance the sensitivity. A weak band of M₁ receptor cDNA (202 bp) was then detected in all four detrusor and mucosa RNA samples, indicating the presence of very low levels of M₄ in human bladder.

Quantitative competitive RT–PCR studies in the male and female detrusor Following our observation of significant change in total muscarinic receptors in the male detrusor, we carried out QCRT–PCR studies in biopsy specimens of both male and female detrusor, to examine the expression of mRNA for the two major subtypes, M₂ and M₃ receptors. The amount of M₂ or M₃ mRNA in the total bladder RNA was calculated from the competition equivalence point, determined by plotting the log relative intensity of DNA bands (total bladder RNA relative to competitor RNA) versus the log of the known concentration of competitor RNA (Figure 6). The QCRT–PCR results were normalised to β-actin, as there was no change in β-actin expression with age (Figure 7a) in male (r² = 0.004, P = 0.69, n = 38) or female (r² = 0.128, P = 0.09, n = 23) detrusor.

There were no gender differences in the expression of M₂ or M₃ muscarinic receptor mRNA. The mean expression of M₂ muscarinic receptor mRNA was 0.37 ± 0.08 competitor RNA per 100 ng total RNA (male, n = 20) and 0.35 ± 0.08 competitor RNA per 100 ng total RNA (female, n = 16), whereas that of M₃ muscarinic receptor mRNA appeared higher, at 5.36 ± 1.16 competitor RNA per 100 ng total RNA (male, n = 33) and 6.23 ± 1.00 competitor RNA per 100 ng total RNA (female, n = 23).

The age-related expression of M₂ and M₃ muscarinic receptor mRNA is depicted in Figure 7b. c. The expression of M₂ muscarinic receptor mRNA remained constant with age (male: r² = 0.009, P = 0.69, n = 20 and female: r² = 0.019, P = 0.60, n = 16), while an age-related decrease in expression of M₃ receptor mRNA was demonstrated (male: r² = 0.41, P < 0.0001, n = 33, female: r² = 0.23, P = 0.019, n = 23) in detrusor.

Discussion

Detrusor muscle muscarinic receptors

Our binding study in detrusor muscle has used some new subtype-selective antagonists and more sophisticated
Radioligand-binding data analysis to confirm and extend previous findings that detrusor muscle contains not only M2 and M3 muscarinic receptor subtypes, but also M1 receptors. While the potent nonselective antagonists atropine and trospium showed only single-site binding, competition curves for the M1, M2 and M3 subtype-preferring antagonists could all be resolved into two-site binding (Table 2). However, global analysis is preferred when binding occurs to more than two sites. Global analysis of the data for the subtype preferring ligands, AQ-RA 741, darifenacin, methoctramine and pirenzepine showed the proportions of the three sites as 71:22:7 and the correlation plots of the appropriate $K_i$ values showed good agreement with the presence of M2, M3 and M1 receptors, respectively.

The presence of these three muscarinic receptor subtypes is supported by results of the RT–PCR studies. However, the relative expression of the different muscarinic receptor subtypes varies, so that the ratio of M2: M3 protein expression does not correlate with that of M2: M3 mRNA expression. While it is possible to correlate changes in receptor expression within a receptor subtype (M2 mRNA and M2 protein), it is inappropriate to compare changes across different receptor subtypes (M2 compared to M3). As well as different PCR conditions, differences could be due to variation in mRNA translation rates, which are governed by a variety of hormonal and cellular events, and/or mRNA stability. Fraser & Lee (1995) reported that M3 receptor mRNA expression was extremely stable compared to M1, but to our knowledge no one has reported on the stability of M2 receptor mRNA.

A novel and potentially important finding was the expression of M5 receptor mRNA in the human detrusor (and mucosa). Most reports of M5 receptors have concerned their

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Figure 6 Calculation of M2 receptor mRNA expression in human bladder detrusor RNA with quantitative competitive RT–PCR. (a) Typical gel image (65 years old, male, 2.5% agarose) showing band intensities for M2 RT–PCR products obtained from bladder RNA (upper band, 350 bp) and competitor RNA (lower band 250 bp). Competitor RNA is present in tubes 1–7 as follows: lanes 1 and 2, 10 pg; lane 3, 3 pg; lane 4, 1 pg; lane 5, 0.3 pg; lane 6, 0.1 pg; lane 7, 0.03 pg; lane 8, 0 pg. Bladder RNA (100 ng) is present in tubes 2–8. Lane 8 also shows a band for the $\beta$-actin internal control at 650 bp. Lane 9 is a 100 bp marker. (b) Quantitative analysis of gel shown in panel (a). At the point where the log ratio is zero, PCR band densities from the bladder RNA and competitor RNA are equal. The amount of the competitor RNA at this point represents the amount of M2 mRNA in the bladder RNA (here, 0.174 pg per 100 ng total RNA).

Figure 7 Age-related changes in mRNA expression in detrusor muscle, analysed using linear regression (males, solid line, females, dashed line). (a) $\beta$-Actin expression did not alter with age in male ($r^2 = 0.004, P = 0.69, n = 38$) or female patients ($r^2 = 0.128, P = 0.09, n = 23$). (b) M2 receptor mRNA expression remained constant with age in male ($r^2 = 0.009, P = 0.69, n = 20$) and female patients ($r^2 = 0.019, P = 0.60, n = 16$). (c) M3 receptor mRNA expression decreased with age in male ($r^2 = 0.41, P < 0.0001, n = 33$) and female patients ($r^2 = 0.23, P = 0.019, n = 23$). The level of muscarinic M2 and M3 receptor mRNA expression was determined using QCR–PCR. Data were normalised to expression of $\beta$-actin.
possible roles in the CNS and cerebral vasculature, although they have also been implicated in the eye, salivary gland and blood vessels (refer Eglen & Nahorski, 2000). The expression of M₃ receptor mRNA has been shown previously in the bladder trigone (Sigala et al., 2002) and in other human peripheral tissues such as oesophageal smooth muscle (Preiksaitis et al., 2000). The roles of the M₁ receptor in the bladder are at present unclear, but one speculative function might be in connection with blood vessels, which would be consistent with weak expression in both mucosa and detrusor. At present, there is no antagonist with high affinity that could have been used to define any M₁ receptors. Instead, we used AQ-RA 741 (which possesses a 10- to 200-fold lower affinity for M₁ receptors than for the other four subtypes, Table 3) and, although its competition curves could be resolved into two sites (Table 2), each had an affinity much higher than that reported for recombinant M₁ receptors (Table 3).

M₄ receptors were not detected in human detrusor (or mucosa) using either binding or RT–PCR. However, nested PCR revealed a very low level of expression of M₄ receptor mRNA. There is evidence from functional studies for prejunctional inhibitory M₄ receptors on cholinergic neurons in human bladder (Somogyi & De Groat, 1999; D’Agostino et al., 2000), which although of putative functional importance would not represent a significant proportion of total muscarinic receptors.

Mucosal muscarinic receptors

A novel result of our binding studies was the revelation of a substantial population of muscarinic receptors on the human urinary bladder mucosa, showing an affinity (Kᵦ) for [³H]-QNB and density (Bₘₐₓ) similar to that in the detrusor. The results are similar to those from a recent binding study with the same radioligand in the pig urinary bladder mucosa, where the Bₘₐₓ in the mucosa was 129 fmolmg protein⁻¹ compared with 81 fmolmg protein⁻¹ in the detrusor (Hawthorn et al., 2000).

The main muscarinic receptor subtype on the mucosa was the M₃ receptor. Binding studies using four subtype-selective antagonists suggested the presence of a majority of M₃ receptors and a minor population of darifenacin-prefering sites that might represent M₂ and/or M₃ receptors. Resolution of the inconclusive findings will rely on the future availability of a ligand with high subtype selectivity for the M₃ receptor. In the mucosal biopsies, RT–PCR demonstrated consistent strong expression of M₁ and M₃ receptor mRNA, but inconsistent expression of M₂ and M₅ receptor mRNA. The exact localisation of these mucosal receptors is unclear and not elucidated in our study, but they could be expressed on urothelium, myofibroblasts, blood vessels and/or nerves.

The contribution of the urinary mucosa to bladder function is now the subject of intense research. Urothelial cells express vanilloid receptors (Birder et al., 2002) and muscarinic receptors (Hawthorn et al., 2000) and can respond to stretch and other stimuli by release of a number of agents including Ach (Yoshida et al., 2004), ATP (Ferguson et al., 1997; Fry et al., 2004) and other mediators (Birder et al., 2002), which may stimulate suburothelial nerves directly or via myofibroblasts (Fry et al., 2004). Recently, studies in pig and human bladder strips have suggested that activation of urothelial muscarinic receptors, by carbachol, leads to the release of an inhibitory factor that can regulate detrusor contractility (Templeman et al., 2002; Chaiyaprasithi et al., 2003). The role of muscarinic receptors in the human mucosa has not been extensively investigated but recent speculation has centred on possible afferent functions. It is now accepted that muscarinic antagonists act mainly during bladder filling to increase bladder capacity and decrease urge (Andersson & Yoshida, 2003), actions not necessarily related to inhibition of detrusor contraction. Therefore, the urothelial muscarinic receptors, present in unexpectedly high density, may represent another site of action for the muscarinic receptor antagonists. The source of the endogenous ligand for these receptors may not be neuronal Ach but may be Ach of urothelial origin (for discussion, refer Yoshida et al., 2004).

Age-related changes in muscarinic receptor density, expression and function

Several studies have examined changes in muscarinic receptors with age in rabbit (Latifpour et al., 1990) and rat (Ordway et al., 1986; Pagala et al., 2001) bladders, but results are variable, complex and region-dependent, and binding data do not necessarily correlate with functional data. In humans, previous urodynamic studies have shown that bladder capacity and urinary flow rate decline with age (Elbadawi et al., 1998, Madersbacher et al., 1998), while detrusor overactivity (involuntary detrusor contractions) becomes more common in the elderly. In the human detrusor, Yoshida et al. (2001) have demonstrated an age-related reduction in atropine-sensitive contractile responses elicited by electrical field stimulation and an age-related reduction in Ach release whose source appears to be predominantly urothelial (Yoshida et al., 2004). A large clinical study found a diminished efficacy of the muscarinic antagonist tolterodine in elderly patients (Michel et al., 2002). With the exception of an early study with [³H]NMS reporting no age-related differences in Bₘₐₓ (Lepor et al., 1989), minimal information is available in the literature concerning the effect of age on the density and distribution of total or individual muscarinic receptor subtypes in human detrusor.

Our results using the technique of radioligand binding indicate a decrease in total muscarinic receptor numbers (Bₘₐₓ) with age in male detrusor muscle (Figure 4b). These findings in male detrusor are unlikely to be due to obstruction due to prostatic hypertrophy, since such patients were excluded from our study. Furthermore, obstruction in the rat bladder leads to an increase in the number of total and M₃ muscarinic receptors (Braverman & Ruggieri, 2003). This binding component of our study was unable to demonstrate which receptor subtype(s) was decreased with age. However, using quantitative competitive RT–PCR, we were able to show a decrease in the level of expression for M₃ receptor mRNA with age in both male and female subjects, but no corresponding change for M₁ receptor mRNA over a wide age range (30–88 years).

It would be simplistic and probably inaccurate to ascribe the decrease in total muscarinic receptor protein (Bₘₐₓ) to the decrease in M₃ receptor mRNA. The expressions of mRNA and protein do not always follow a simple correlation since many mechanisms may be involved in post-transcriptional control. While the expression of gene-specific mRNA can be accurately determined, reliable measurements of associated...
protein can be problematic. To date, there are no highly subtype-selective radioligands, antagonists, and antibodies commercially available for the muscarinic receptors. Until a reliable protein quantitative technique is developed for individual receptor subtypes, determination of a change in expression of mRNA is the only mainstream technique available to indicate corresponding protein changes in ageing or disease.

Conclusions

Our data from normal bladder show the existence of multiple muscarinic receptor subtypes in the human detrusor and mucosa. In the detrusor, evidence from both radioligand-binding and molecular studies is consistent with the presence of a majority of M2 and lesser populations of M3 and M1 receptors. Molecular studies also indicated a small population of M3 receptors. In the mucosa, both techniques revealed a significant population of M3 receptors. Minor populations of M2, M3, and M1 receptors were suggested by the molecular studies.

The age-related decline in total muscarinic receptor protein, and specifically in M1 receptor mRNA expression, may partly account for the previous urodynamic findings of reduced contractility/flow rate and incomplete emptying in the elderly, particularly in males. However, the relationship between the decrease in muscarinic receptor numbers with age and the development of conditions of bladder overactivity remains unclear.

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