The role of muscarinic receptor subtypes on carbachol-induced contraction of normal human detrusor and overactive detrusor associated with benign prostatic hyperplasia

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ABSTRACT
The aim of this study was to compare the effect of antimuscarinic antagonists on carbachol-induced contraction of normal human bladder and detrusor overactivity associated with benign prostatic hyperplasia (DO/BPH). Samples of human bladder muscle were obtained from patients undergoing total cystectomy for bladder cancer (normal bladder), and those undergoing retropubic prostatectomy for BPH. All of the patients with DO/BPH had detrusor overactivity according to urodynamic studies. Detrusor muscle strips were mounted in 10-ml organ baths containing Krebs solution, and concentration–response curves for carbachol were obtained in the presence of antimuscarinic antagonists (4-DAMP, methoctramine, pirenzepine, tolterodine, solifenacin, trosipium, propiverine, oxybutynin, and imidafenacin) or vehicle. All antagonists competitively antagonized concentration–response curves to carbachol with high affinities in normal bladder. The rank order of mean pA2 values was as follows: trosipium (10.1) > 4-DAMP (9.87), imidafenacin (9.3) > solifenacin (8.8) > tolterodine (8.6) > oxybutynin (8.3) > propiverine (7.7) > pirenzepine (7.4) > methoctramine (6.6). The effects of these antimuscarinic antagonists did not change when tested with DO/BPH detrusor, suggesting that each antimuscarinic antagonist has a similar effect in this condition. Schild plots showed a slope corresponding to unity, except for propiverine with DO/BPH detrusor. In conclusion, M3-receptors mainly mediate contractions in human bladder strips with normal state and DO/BPH.

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1. Introduction
For pharmacological treatment of overactive bladder (OAB), antimuscarinic agents have long been the drugs of choice. However, these agents can have unpleasant side effects, such as dry mouth, constipation, headache, blurred vision, and tachycardia, while the lower urinary tract symptoms of some patients are refractory (1–4). Worldwide, six antimuscarinic drugs are currently marketed for the treatment of OAB, which are oxybutynin, tolterodine, propiverine, trosipium, darifenacin, and solifenacin (2,5). Recently, imidafenacin and fesoterodine were also developed, and were reported to be effective for OAB (4,6–8).

The smooth muscle of the urinary bladder, like the majority of smooth muscle in many species, expresses a heterogeneous population of muscarinic receptors. Predominance of the M3 muscarinic receptor subtype, with a minor population of M2 receptors, has been reported in the bladder smooth muscle of several species. The proportion of muscarinic M2 and M3 receptors has been reported to be 70–80% and 20–30%, respectively, in the bladder smooth muscle of humans, guinea pigs, and rabbits (9–11). However, pharmacological characterization of the muscarinic receptor mediating detrusor muscle contraction in pigs and humans has suggested that only the M3 receptor is involved (9,11,12). This suggests that the M3 receptor is the predominant muscarinic receptor subtype responsible for detrusor muscle contraction in response to muscarinic agonists, while the M2 receptor is not directly involved (11,13).
However, it has been reported that M2 receptors participate in the contractile response of both rat and human bladder smooth muscle under pathologic conditions. M2 receptors have been reported to mediate detrusor contraction in rats with pelvic nerve denervation or spinal cord transection, and in patients with neurogenic bladder dysfunction (14–16). An increase of total muscarinic receptor density and M2 receptor density along with a reduction of M3 receptor density have been reported in obstructed, hypertrophic rat bladders (17). In contrast, the bladder contractile response to carbachol was reported to be mediated by the M3 receptor in rats with partial bladder outlet obstruction and in humans with idiopathic or neurogenic OAB (18,19). The present study was performed to investigate whether the antagonist effect of various antimuscarinic agents on carbachol-induced contraction differed between normal human bladder muscle and detrusor overactivity (DO) in patients with benign prostatic hyperplasia (BPH).

2. Materials and methods

The procedures were approved by the local ethics committee of Dokkyo Medical University, and written informed consent was obtained from each patient before enrollment. Tissue samples (8 × 2 mm) were obtained from the dome of the bladder in patients without lower urinary tract symptoms undergoing total cystectomy for bladder cancer (normal bladder), and in patients undergoing retropubic prostatectomy for BPH whose bladders showed DO (DO/BPH). The serosa and urothelium were removed. A section of bladder dome tissue was dissected from an area free of cancer for use in the study. All of the patients with DO/BPH were proven to have DO and bladder outlet obstruction by video-urodynamic studies or ambulatory urodynamic studies before surgery. Muscle strips were mounted in 10 ml organ baths containing Krebs solution (in mM: NaCl 118.4, KCl 4.7, CaCl2 1.9, NaHCO3 24.9, MgSO4 1.15, KH2PO4 1.15, and glucose 11.7), which was maintained at 37 °C and aerated continuously with 95% O2 and 5% CO2. The strips were placed under a resting tension of 1 g (9.8 mN) and allowed to equilibrate for 60 min, during which time they were washed every 10 min and the resting tension was adjusted. The isometric tension generated by each muscle specimen was measured by a Power Lab data acquisition system (Analog Digital Instruments, Sydney, New South Wales, Australia).

Cumulative concentration–response curves (CRCs) for carbachol were obtained. Each muscle strip was washed for about 45 min until a stable resting tension was attained, followed by equilibration for 30 min with Krebs solution containing the appropriate concentration of antagonist or vehicle (time control). After incubation for 30 min, a second CRC for carbachol was obtained in the continued presence of the antagonist or vehicle. Antagonists were added at increasing concentrations after washing for 45 min. In this way, 4 CRCs for carbachol were obtained from each muscle strip, with three being obtained in the presence of increasing concentrations of a muscarinic antagonist ([4-DAMP (3, 10, 30 nM), methotramine (1, 3 and 10 μM), pirenzepine (1, 3 and 10 μM), tolterodine (3, 10, 30 nM), solifenacin (3, 10, 30 nM), trospium (3, 10, 30 nM), propiverine (10, 30, 100 nM), oxybutynin (3,10,30 nM), or imidafenacin (3,10,30 nM)] or in the presence of the vehicle. Control experiments were performed with addition of the vehicle instead of an antagonist and the data were used to correct for any tachyphylaxis or time-dependent changes of tissue sensitivity and responsiveness. The correction factors for EC50 and the maximum response between the first and second curves were both 1.0, those between the first and third curves were 1.5 and 0.85, respectively, and those between the first and fourth curves were 2.8 and 0.67, respectively. These correction factors were applied in all experiments (9,11–13,18).

2.1. Statistical analysis

The agonist potency and maximum response were expressed as the mean pEC50 ± SEM (−logarithm of the molar concentration of agonist resulting in 50% of the maximum response) and the mean maximum contraction ± SEM, respectively. The pA2 value for a muscarinic receptor antagonist (determined as the x-intercept on the Schild regression plot) was only measured when the Schild plot slope was unity. The dissociation constant (constant Ks value) for each antagonist was calculated from the following equation: $K_s = \text{agonist concentration (molar)} / (\text{concentration ratio} - 1)^{-1}$ where the concentration ratio is the ratio of EC50 values obtained in the presence and absence of the antagonist. Data are normalized for the maximal response in the first (control) curve, and are expressed as the mean ± SEM. The unpaired Student’s t-test was used for statistical comparison of normal bladder and DO/BPH bladder tissues.

2.2. Drugs and chemicals

Carbachol (carbamylcholine chloride), 4-diphenyl acetoxy- methyl piperidine methiodide (4-DAMP), methotramine, pirenzepine, oxybutynin, and propiverine were purchased from Sigma Chemical (St Louis, MO, USA). Tolterodine, trospium, solifenacin and imidafenacin were kind gifts from Pfizer Inc (New York, USA), Nihon Kemiifa Ltd. (Tokyo, Japan), Asteras Ltd. (Tokyo, Japan), and Kyorin Ltd. (Tokyo, Japan), respectively.

3. Results

Carbachol induced concentration-dependent contraction of normal bladder strips, with a mean pEC50 value and maximum response of 6.5 ± 0.1 and 9.6 ± 0.7 g, respectively. Carbachol also induced concentration-dependent contraction of DO/BPH bladder strips, with a mean pEC50 value and maximum response of 6.4 ± 0.1 and 9.8 ± 1.0 g, respectively. Both the mean pEC50 value and the maximum response did not differ significantly between the two tissues.

4-DAMP (a selective M3-muscarinic receptor antagonist) and methotramine (a selective M2-muscarinic receptor antagonist) produced parallel, rightward displacement of the CRCs to carbachol without affecting maximum responses and yielded mean (±SEM) pA2 values of 9.87 ± 0.09 and 6.64 ± 0.05, respectively in bladder strips from normal bladder. The pA2 values for 4-DAMP and methotramine decreased significantly (P = 0.0004 and P = 0.0347, respectively) in bladder strips from DO/BPH compared with the normal bladder (Table 1). Schild slopes for 4-DAMP and methotramine did not change significantly (Figs. 1 and 2).

Pirenzepine (a selective M1-muscarinic receptor antagonist) also produced parallel, rightward displacement of the CRCs to carbachol without affecting maximum responses and yielded mean (±SEM) pA2 values of 7.38 ± 0.09 and Schild slopes not significantly different from unity. The pA2 values and Schild slopes for pirenzepine did not change significantly in bladder strips from DO/BPH (Table 1).

Tolterodine, trospium and propiverine (nonselective antimuscarinic antagonists), and oxybutynin, solifenacin and imidafenacin (M1- and M3-selective antimuscarinic antagonists) also caused parallel, rightward displacement of the CRCs for carbachol without affecting the maximum response for strips of normal and DO/BPH bladder, respectively. The mean (±SEM) pA2 value and Schild slope for the effect of each muscarinic antagonist on the CRCs for carbachol are summarized in Table 1. The pA2 values of these muscarinic antagonists did not differ significantly between the normal bladder and DO/BPH bladder. In addition, the slope of
4. Discussion

Detrusor overactivity (DO) has been reported to be the cause of overactive bladder symptoms. It has been reported that DO is noted in 40–60% of patients with BPH (20). Bladder outlet obstruction in BPH can be a factor contributing to DO, possibly through cholinergic denervation of the detrusor and hypersensitivity of muscarinic receptors to acetylcholine, although the prevalence of OAB is similar in men and women across the age groups (2). Anti-muscarinic agents are the most widely used treatment for OAB, and it has been reported to be safe and effective for overactive bladder in men with BPH (21,22). The affinity (pA2) value of tolterodine against CRC to carbachol in our study in the human bladder from normal and DO/BPH appeared to be similar to the previous reports in the normal human bladder (23).

Solifenacin and imidafenacin are antimuscarinic drugs that are widely used for the treatment of OAB with selectivity for M3 and M1 muscarinic receptor subtypes (3,4,24), and the affinity values for normal human bladder in the previous report were also similar to our result for bladder muscle strips from normal states and DO/BPH (7,25). Propiverine has a nonselective antimuscarinic action and is also a calcium antagonist; it has been widely used in Japan and Europe (26), and the affinity values for normal human bladder in the previous report were also similar to our result for bladder muscle strips from normal states and DO/BPH (7,25). However, the slope for Schild plot was less than unity in propiverine with DO/BPH muscle strips (0.49 ± 0.14). The reason may be that propiverine also shows activity as a calcium antagonist, so both its antimuscarinic and calcium antagonist effects may have operated in DO/BPO.

The present study showed that all of the muscarinic antagonists tested, including clinical available antimuscarinic agents, the Schild plot corresponded to unity for all antagonists and did not change significantly, except for propiverine with DO/BPH muscle strips. The Schild slope for propiverine were different from unity in tissues from DO/BPH patients, and decreased significantly compared to that of normal bladder (P = 0.0476). The rank order of pA2 value of muscarinic antagonists in the normal bladder was, tropium (10.1) > 4-DAMP (9.9) > imidafenacin (9.3) > solifenacin (8.8) > tolterodine (8.6) > oxybutynin (8.3) > propiverine (7.7) > pirenzepine (7.4) > methoctramine (6.6). That in DO/BPH was, tropium (10.1) > imidafenacin (9.2) > 4-DAMP (9.0) > solifenacin (8.7) = tolterodine (8.7) > oxybutynin (8.4) > propiverine (7.6) > pirenzepine (7.3) > methoctramine (6.4).

The affinity values of 4-DAMP and methoctramine decreased significantly in pathologic states in DO/BPH. However the affinity value of pirenzepine and the rank order of these muscarinic antagonists did not change in this pathologic condition indicating that M3-receptor still predominantly mediated contraction in this pathological condition. The antagonist activity may not be the same between in the normal and in the pathologic state. We cannot explain the reasons, but one of the reasons may be that the urothelium was removed in the present study because the presence of urothelium may influence contraction of the detrusor. The purpose of this study was to investigate whether the antagonist affinity of bladder smooth muscle changed between the normal and the pathological states. Thus we have removed the urothelium to study solely the affinity of muscarinic receptor subtypes for the bladder smooth muscle. The affinity of these muscarinic antagonists may have changed when the urothelium was intact. The further study remains to be necessary to investigate the interaction of urothelium and detrusor.

Tolterodine is a nonselective antimuscarinic agent that is widely used for the treatment of OAB, and it has been reported to be safe and effective for overactive bladder in men with BPH (21,22). The affinity (pA2) value of tolterodine against CRC to carbachol in our study in the human bladder from normal and DO/BPH appeared to be similar to the previous reports in the normal human bladder (23).

Table 1


<table>
<thead>
<tr>
<th>Antagonist</th>
<th>4-DAMP</th>
<th>Methoctramine</th>
<th>Pirenzepine</th>
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</thead>
<tbody>
<tr>
<td>Normal bladder</td>
<td>N = 9</td>
<td>N = 6</td>
<td>N = 4</td>
</tr>
<tr>
<td>pA2 ± SEM</td>
<td>9.87 ± 0.09</td>
<td>6.64 ± 0.05</td>
<td>7.38 ± 0.09</td>
</tr>
<tr>
<td>Schild slope</td>
<td>1.09 ± 0.37</td>
<td>0.69 ± 0.01</td>
<td>0.77 ± 0.17</td>
</tr>
<tr>
<td>DO/BPH</td>
<td>N = 5</td>
<td>N = 5</td>
<td>N = 5</td>
</tr>
<tr>
<td>pA2 ± SEM</td>
<td>9.01 ± 0.12</td>
<td>6.36 ± 0.09</td>
<td>7.33 ± 0.11</td>
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<tr>
<td>Schild slope</td>
<td>0.93 ± 0.15</td>
<td>0.76 ± 0.18</td>
<td>0.89 ± 0.42</td>
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</table>

<table>
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<tr>
<th>Antagonist</th>
<th>Tolerodine</th>
<th>Tropium</th>
<th>Propiverine</th>
<th>Oxybutynin</th>
<th>Solifenacin</th>
<th>Imidafenacin</th>
</tr>
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<tbody>
<tr>
<td>Normal bladder</td>
<td>N = 6</td>
<td>N = 5</td>
<td>N = 6</td>
<td>N = 4</td>
<td>N = 8</td>
<td>N = 6</td>
</tr>
<tr>
<td>pA2 ± SEM</td>
<td>8.63 ± 0.17</td>
<td>10.12 ± 0.11</td>
<td>7.74 ± 0.20</td>
<td>8.33 ± 0.16</td>
<td>8.80 ± 0.10</td>
<td>9.29 ± 0.26</td>
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<tr>
<td>Schild slope</td>
<td>1.29 ± 0.01</td>
<td>0.91 ± 0.29</td>
<td>1.06 ± 0.20</td>
<td>1.01 ± 0.12</td>
<td>1.06 ± 0.20</td>
<td>0.93 ± 0.12</td>
</tr>
<tr>
<td>DO/BPH</td>
<td>N = 5</td>
<td>N = 7</td>
<td>N = 5</td>
<td>N = 4</td>
<td>N = 5</td>
<td>N = 6</td>
</tr>
<tr>
<td>pA2 ± SEM</td>
<td>8.74 ± 0.16</td>
<td>10.14 ± 0.64</td>
<td>7.60 ± 0.11</td>
<td>8.41 ± 0.12</td>
<td>8.73 ± 0.09</td>
<td>9.24 ± 0.09</td>
</tr>
<tr>
<td>Schild slope</td>
<td>1.23 ± 0.06</td>
<td>1.04 ± 0.44</td>
<td>0.49 ± 0.14</td>
<td>1.16 ± 0.11</td>
<td>0.98 ± 0.04</td>
<td>1.22 ± 0.17</td>
</tr>
</tbody>
</table>

a The Schild slope was different from unity in tissues from DO/BPH patients, and decreased significantly compared to that of normal bladder (P = 0.0476). Apparent pKB values were calculated because of non-competitive antagonism.

b Detrusor overactivity associated with benign prostatic hyperplasia.

c The pA2 values for 4-DAMP and methoctramine decreased significantly (P = 0.0004 and P = 0.0347, respectively) in bladder strips from DO/BPH compared with those from the normal bladder.
(tolterodine, solifenacin, propiverine, trospium, oxybutynin and imidafenacin) competitively antagonized carbachol responses with high affinity in normal human detrusor. The rank order of their pA₂ value was as follows: trospium (10.1) > 4-DAMP (9.9) > imidafenacin (9.3) > solifenacin (8.8) > tolterodine (8.6) > oxybutynin (8.3) > propiverine (7.7) > pirenzepine (7.4) > methoctramine (6.6). The pA₂ values did not differ significantly between the normal state and detrusor overactivity (DO/BPH). The pA₂ values for these antimuscarinic agents and their rank order seemed to be similar to those in previous reports. Chess-Williams et al. (9), and Sellers et al. (12), reported that the antagonistic affinity of various muscarinic antagonists was best correlated with the affinity for cloned M₃ muscarinic receptor subtype. These observations suggest that the M₃ receptor is the predominant muscarinic receptor subtype mediating detrusor contraction in response to muscarinic agonists, while the M₂ receptor is not directly involved in contraction of the normal bladder (1,2,9,12). Reports about the role of the M₂ receptor in animal models of detrusor overactivity have been conflicting. Braverman and Ruggieri (17) found a significant increase of M₂ receptor mRNA transcript and protein levels in hypertrophic urinary bladders after major pelvic ganglion electrocautery and partial bladder outlet obstruction. However, the M₃ receptor protein level was reported to be decreased (although M₃-receptor transcription was increased) in hypertrophic bladders after bladder outlet obstruction. There was a shift in the affinity of muscarinic antagonists towards M₂-mediated contraction in addition to muscarinic supersensitivity in rat models of neurogenic bladder (denervation and spinal cord injury) (14,15). On the contrary, Krivecksky et al. (19), found no change in the affinity of muscarinic antagonists between the normal bladder and partial bladder outlet obstruction in rats. In humans, Pontari et al. (16), reported that the M₂ receptor played a role in the contraction of overactive detrusor muscle in patients with neurogenic bladder. However, Stevens et al. (18), reported no change in the affinity of muscarinic antagonists between normal and overactive (idiopathic and neurogenic) detrusor muscle in humans.

Limitation of this study is that it is difficult to get human detrusor tissues, similar to other studies (7,9e13). As a control, we collected bladder tissues from patients undergoing total cystectomy for bladder cancer. We selected patients without lower urinary tract symptoms, but we could not exclude patients with DO because we could not perform urodynamic study in these patients for ethical reasons. It was more difficult and limited to obtain human bladder tissues from patients undergoing retropubic prostatectomy because transurethral resection of prostate is the mainstay of surgery for BPH.
In the present study, carbachol evoked concentration-dependent contraction with a similar potency (pEC\textsubscript{50}) and maximal effect in strips of both normal and DO/BPO detrusor muscle. This result was different from that reported by Stevens et al. (18), who found an increase of sensitivity to muscarinic agonists (pEC\textsubscript{50}), highlighting a change of receptor-mediated contraction in idiopathic and neurogenic DO compared with the normal detrusor. The reasons for the discrepancy are unknown, but may be related to differences of the underlying disease (idiopathic or neurogenic OAB vs. BPH).

The above results suggest that antimuscarinic agents are effective for DO/BPO, and that the M\textsubscript{3} receptor mainly mediates detrusor contraction in this condition.

Although the role of M\textsubscript{2} receptor could not be demonstrated in this present study, it may have effects when M\textsubscript{3} receptor was inactivated (11,13). M\textsubscript{2} receptor in the urothelium may also have a role in activating afferent c-fibers in the pathological conditions (27). The role of M\textsubscript{2} receptor should be elucidated in the future studies.

5. Conclusions

4-DAMP, a selective M\textsubscript{3}-receptor antagonist, oxybutynin, solifenacin and imidafenacin, selective M\textsubscript{1}- and M\textsubscript{3}-receptor antagonists, and trospium, tolterodine and propiverine, non-selective muscarinic receptor antagonists antagonized CRCs to carbachol with high affinities. Methoctramine, a selective M\textsubscript{2}-receptor antagonist, and pirenzepine, a selective M\textsubscript{1}-receptor antagonist antagonized carbachol-induced contractions with relatively low affinities. These results suggest that M\textsubscript{3}-receptors mainly mediate contractions in normal human bladder. The rank order of the affinity values of these antimuscarinic antagonists did not change when tested with DO/BPH bladder, suggesting that each antimuscarinic antagonist has a similar effect in this condition. Schild plots showed a slope corresponding to unity, except for propiverine with DO/BPH detrusor. In conclusion, M\textsubscript{3}-receptors mainly mediate contractions in human bladder strips with normal state and DO/BPH.

Conflicts of interest

The authors have no conflict of interest.

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