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Differential roles of M2 and M3 muscarinic receptor subtypes in modulation of bladder afferent activity in rats

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Abstract

Objectives—It has been proposed that the urothelium modulates the activity of bladder afferent pathways. However, the differential roles of muscarinic acetylcholine receptor (mAChR) subtypes in local bladder afferent activation remain unclear. We investigated the effects of various mAChR antagonists including selective M2 and M3 mAChR antagonists on bladder overactivity.

Methods—Cystometry was performed in urethane anesthetized female rats. We examined the effects of intravesical administration of antimuscarinic agents (non-selective mAChR antagonists; atropine sulfate, tolterodine tartrate and propiverine hydrochloride, M2-selective antagonists; dimethindene maleate and methoctramine hemihydrate, M3-selective antagonists; darifenacin hydrobromide and 4-DAMP) on bladder overactivity induced by oxotremorine-M (Oxo-M; non-selective mAChR agonist).

Results—Intravesical administration of Oxo-M (200 μ M) elicited bladder overactivity as evidenced by decreased intercontraction interval, bladder capacity and pressure threshold. These effects were blocked by intravesical administration of non-selective or M2-selective antagonists (30–60 μ M) whereas M3-selective antagonists (150 μ M) did not suppress the overactivity. When instilled intravesically by itself, none of antimuscarinic agents (non-selective, M2-selective or M3-selective antagonists) affected any cystometric parameters.

Conclusions—The M2 mAChR subtype plays an important role in the local cholinergic modulation of bladder afferent activity that contributes to bladder overactivity in normal rats. Therefore, it is expected that antimuscarinic agents that have antagonistic activity against M2 mAChR can be more beneficial for the treatment of patients with overactive bladder if enhanced ACh mechanisms are involved in pathogenesis of overactive bladder.

Keywords

rat; urinary bladder; afferent pathways; muscarinic receptor

Introduction

The most common treatment for overactive bladder is the use of antimuscarinic agents, which are considered to act on muscarinic acetylcholine receptors (mAChRs) in the detrusor that are stimulated by acetylcholine released from efferent (parasympathetic) nerves, thereby inhibiting

voiding contractions¹. However, antimuscarinic agents mainly relieve symptoms such as frequency and urgency during the storage phase, suggesting the effects on other sites including bladder afferent pathways.

Bladder afferent nerves originate from lumbosacral dorsal root ganglia and convey sensory information. They consist of A δ -fibers and C-fibers, which terminate in the urothelium, suburothelial layer and detrusor. The changes in afferent signaling from the bladder have been considered the important etiology of bladder overactivity. It has recently been reported that the urothelium expresses various receptors including muscarinic, nicotinic, purinergic and transient receptor potential, releases various neurotransmitters (such as ACh, ATP; adenosine 5'-triphosphate and NO; nitric oxide) in response to mechanical and/or chemical sensory stimuli, and thereby modulates activity of bladder afferent pathways².

mAChRs are present in the urothelium as well as in the detrusor of different species including humans^{3–6}. M2 and M3 mAChR subtypes are predominant, and M2 mAChR subtype outnumbers M3 mAChR subtype⁷. The M3 mAChR subtype in the detrusor is responsible for voiding contractions although the M2 mAChR subtype is more abundant⁸. Recent data showed that activation of mAChRs in bladder afferent pathways by intravesical administration of mAChR agonist alters reflex bladder contractions⁹. Accordingly, activation of mAChRs releases substances (ATP and NO) from the urothelium that modulate afferent nerves. Additionally, it is also shown that basal release of acetylcholine from the urothelium is increased by age and stretch¹⁰.

These evidences suggest that activation of mAChRs in the urothelial and/or suburothelial layers affects bladder afferent activity. However, the differential roles of M2 and M3 mAChR subtypes in local cholinergic modulation of bladder afferent activity remain unclear. We therefore investigated the effects of various mAChR antagonists including M2 and M3-selective mAChR antagonists on bladder overactivity induced by local mAChR stimulation.

Material and Methods

Animals

Adult female Sprague-Dawley rats weighing 250 to 300g (Hilltop, Scottsdale, PA) were used. All experiments were conducted in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Drugs

In this study, we used oxotremorine methiodide (Oxo-M; Sigma, St. Louis, MO) as a non-selective mAChR agonist. Atropine sulphate (Sigma), propiverine hydrochloride (Taiho, Tokyo, Japan) and tolterodine tartrate (Kemprotec, Middlesbrough, UK) were used as non-selective mAChR antagonists. Dimethindene maleate (Tocris, Ellisville, MO) and methoctramine hemihydrate (Sigma) were used as M2-selective mAChR antagonists. Darifenacin hydrobromide (Kemprotec) and 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP; Tocris) were used as M3-selective mAChR antagonists. For intravesical administration, drugs except for 4-DAMP were dissolved in saline to a final concentration and applied. Because 2% DMSO solution had no effect on any cystometric parameters (data not shown), 4-DAMP was dissolved in 2% DMSO solution and administered intravesically.

Cystometry

Rats were anesthetized with urethane anesthesia (1.2 g/kg, subcutaneously; Sigma). A midline abdominal incision was made and a transvesical catheter with a fire-flared tip (PE-50) was inserted into the dome of the bladder and secured with a silk thread for bladder filling and

pressure recording. A three-way stopcock was connected to the transvesical catheter to monitor bladder pressure. During cystometry, intercontraction interval (ICI), pressure threshold (PT), maximum voiding pressure (MVP) and baseline pressure (BP) were measured by using a PowerLab unit and Chart software (AD Instruments, Colorado Springs, CO). Bladder capacity (BC) was calculated as the sum of voided volume and post-void residual urine (PVR). Voided volume was cumulatively collected into a urine cup placed on a microbalance. PVR was measured at the end of 2 to 3 micturition cycles before and after each drug administration. After constant voided volumes were collected, bladder infusion was stopped and PVR was measured by withdrawing intravesical fluid through the catheter by gravity and abdominal compression.

Experimental Protocols

Saline was continuously infused at a room temperature for at least 2 hours at a rate of 0.04 ml/min to record cystometrograms during a control period. After baseline cystometry was performed, vehicle (saline) or an antimuscarinic agent (30–60 μ M for non-selective and M2-selective mAChR antagonists or 150 μ M for M3-selective mAChR antagonists) was instilled intravesically for 30 min. Thereafter, 200 μ M Oxo-M was added to the infusate to examine whether intravesical application of Oxo-M with vehicle or an antimuscarinic agent induces bladder overactivity. We selected this dose of Oxo-M because 200 μ M Oxo-M elicited significant and consistent bladder overactivity as shown by a reduction in ICI without apparent effects on the detrusor function (MVP or BP) in our preliminary experiments.

Statistical Analysis

All data values are expressed as the mean $\pm$ standard error of the mean. $P < 0.05$ was considered significant. The overall comparisons among groups were performed using Prism software (GraphPad Software, San Diego, CA). For each cystometric parameter, two or three measurements were averaged. A paired t test was used to compare the cystometric parameters before and after drug administration.

Results

Intravesical administration of Oxo-M (200 μ M) produced bladder overactivity (Fig. 1A) as evidenced by decreased ICI, PT and BC (Table 1, 788.6 $\pm$ 74.1 to 469.0 $\pm$ 43.0 sec, 6.93 $\pm$ 0.40 to 5.75 $\pm$ 0.35 cmH₂O and 0.55 $\pm$ 0.04 to 0.36 $\pm$ 0.02 ml, respectively). MVP or BP was not altered during Oxo-M instillation (Table 2).

When instilled intravesically by itself, none of the antimuscarinic agents (non-selective, M2-selective or M3-selective antagonist) changed any cystometric parameters (Fig. 1B, C, D and Table 1, 2). However, intravesical administration of Oxo-M concomitant with non-selective antagonists (atropine, propiverine or tolterodine; 30 μ M) did not produce bladder overactivity (Fig. 1B). M2-selective antagonists (dimethindene; 30 μ M or methoctramine; 60 μ M) also suppressed OxoM-induced bladder overactivity as well as non-selective antagonists (Fig. 1C). However, when Oxo-M was instilled with M3-selective antagonists (darifenacin or 4-DAMP; 30 μ M), ICI, PT and BC were significantly decreased (data not shown). Therefore, we used a high concentration (150 μ M) of M3-selective antagonists to confirm the difference in the effects of M2 and M3-selective antagonists. In spite of the high dose application of M3-selective antagonists, the inhibitory effect was not observed (Fig. 1D and 2), and ICI and BC were reduced (Table 1, darifenacin; 741.7 $\pm$ 39.9 to 473.5.0 $\pm$ 35.1 sec, 0.50 $\pm$ 0.03 to 0.35 $\pm$ 0.02 ml; 4-DAMP; 674.6 $\pm$ 72.8 to 413.1 $\pm$ 41.0 sec, 0.50 $\pm$ 0.06 to 0.26 $\pm$ 0.03 ml, respectively). In the darifenacin group, PT after adding Oxo-M was significantly lower than that of a control period or darifenacin alone (6.84 $\pm$ 0.32 to 5.36 $\pm$ 0.34 cmH₂O). In the 4-DAMP group, PT after adding

Oxo-M tended to be lower, but the difference was not significant. MVP or BP was not altered during instillation of Oxo-M with any antimuscarinic agents (Table 2).

Comment

The present study demonstrated that intravesical administration of non-selective or M2-selective muscarinic acetylcholine receptor (mAChR) antagonists, but not M3-selective antagonists, suppresses bladder overactivity induced by intravesical administration of oxotremorine-M (Oxo-M; non-selective mAChR agonist).

The sensation of bladder fullness is the first step in the micturition reflex. Afferent innervation of the bladder through the pelvic nerves includes myelinated (A δ) and unmyelinated (C) fibers. Most C-fibers end in the suburothelial layer immediately below the basal lamina of the bladder, and some nerve endings penetrate the urothelium¹¹. Previous studies suggest that urothelial cells can respond to chemical mediators or neurotransmitters released from nearby nerves, and that substances released from the urothelium can alter afferent neural transmission thresholds and excitability¹².

Recently, the role of mAChRs in the regulation of afferent pathways has attracted considerable interest. mAChRs are found on bladder urothelial cells in mice, rats, pigs and humans^{3–6}, suggesting that these receptors in the urothelium could participate actively in the control of bladder function. This is also supported by the evidence showing that basal release of acetylcholine from the urothelium is increased by age and stretch¹⁰.

In the present study, intercontraction interval was significantly reduced after intravesical infusion of Oxo-M. It is likely that Oxo-M activated local mAChRs in the bladder urothelial or suburothelial layers rather than in the smooth muscle because (a) Oxo-M was instilled intravesically and the effects of Oxo-M were observed immediately after instillation, (b) intravesical instillation of Oxo-M reduced intercontraction interval and pressure threshold without affecting maximum voiding pressure and baseline pressure which reflect detrusor activity and (c) Oxo-M has a quaternary structure and hydrophilic property, therefore it can not easily permeate the urothelium. Thus it is possible that locally expressed mAChRs in the urothelium and/or the suburothelial layer, where afferent nerve fibers are abundantly found, are involved in the emergence of bladder overactivity as basal release of acetylcholine from the urothelium is increased with bladder stretch as well as ageing¹⁰. A recent study also showed that expression of M2 and M3 mAChRs was increased in the urothelium of rats with partial bladder outlet obstruction (BOO), suggesting the possibility that urothelial mAChR responses to acetylcholine are enhanced in BOO-induced bladder overactivity¹³.

The mechanism by which local muscarinic receptors increase afferent activity to induce bladder overactivity is still unclear. One possible mechanism might be related with adenosine 5'-triphosphate (ATP) release from the urothelium. It has been shown that P2X receptors are expressed on bladder afferent nerves and that ATP released during bladder stretch or ATP administered intravesically activates afferent nerves, triggering the micturition reflex¹⁴. It has also been reported that P2X₃ knockout mice had bladder hyporeflexia¹⁵. These results indicate that P2X purinergic receptors are important in the control of bladder afferent activity. Kullmann et al.⁹ recently reported that intravesical administration of high dose Oxo-M (40 μ M or higher) induced bladder overactivity via release of ATP presumably from the urothelium whereas low dose Oxo-M (5 μ M) suppressed bladder activity via NO release in normal rats. Because the concentration (200 μ M) of Oxo-M used in this study is higher than 40 μ M, Oxo-M-induced bladder overactivity may involve activation of local mAChRs, which triggers ATP release from the urothelium and then stimulates P2X receptors on afferent nerves.

Intravesical administration of mAChR antagonists regardless of their selectivity did not change any cystometric parameters in the present study, suggesting that antimuscarinic agents do not penetrate to the detrusor muscle layer enough to affect bladder contractility and that mAChRs are not involved in modulating bladder afferent activity in normal rats under urethane anesthesia. There are some reports showing that antimuscarinic agents have inhibitory effects on bladder afferent nerves. Yokoyama et al.¹⁶ demonstrated in conscious cystometry that intravesical administration of low dose tolterodine (0.2 nM) significantly increased bladder capacity without increasing residual volume in a rat model of bladder overactivity caused by occlusion of the middle cerebral artery. Iijima et al.¹⁷ recently reported that systemic administration of darifenacin reduced the bladder afferent spike number and sensitivity in urethane-anesthetized normal rats. Thus the efficacy of antimuscarinic agents on bladder afferent activity may be different depending on the animal models (normal vs. pathology), anesthetic conditions (awake vs. anesthetized) or the manner of drug administration (systemic vs. local).

Then, Oxo-M-induced bladder overactivity was prevented by concomitant application of non-selective or M2-selective mAChR antagonists, but not M3-selective antagonists. These results suggest that the M2 muscarinic receptor subtype plays a more important role than the M3 subtype in the local cholinergic modulation of bladder afferent activity which contributes to bladder overactivity. It has been considered that normal detrusor contraction is directly mediated by activation of the M3 mAChR subtype whereas the M2 subtype indirectly mediates detrusor contraction and the contribution of M2 subtype to detrusor contraction is less than that of the M3 subtype despite its dominant expression⁸. The present study implies that the M2 subtype additionally has excitatory effects on bladder afferent pathways. Some in vitro studies suggested the existence of excitatory mechanisms mediated by activation of the M2 mAChR subtype in other organs such as the respiratory and intestinal tracts^{18–20}. In addition, it has been shown that activation of M1, M2 and M3 mAChR subtypes releases ATP from cultured rat urothelial cells, possibly through increases in intracellular Ca^{2+} levels²¹. These studies support our in vivo results showing M2 receptor-mediated bladder overactivity after intravesical application of Oxo-M.

There are some limitations in the current study. First, it is unclear to which layer of bladder urothelium or suburothelium the drugs used in this study can penetrate. Differences in cell-layer distribution of mAChR subtypes in the urothelium have been reported in mouse and human^{3,22}, in which most of M1 mAChR receptors are distributed in basal cells of the urothelium and M2 subtype receptors are found only in umbrella cells while M3, M4 and M5 subtypes are distributed throughout the urothelium. Although similar results are not published in rats so far, if such cell layer-specific distribution exists in rats as well, the results may depend on the layer to which the drugs can penetrate. Thus, at present we can only conclude that intravesically applied drugs did not penetrate sufficiently to affect detrusor activity, and that activation of the M2 mAChR subtype in the urothelium and/or suburothelial layer is one possible cause to induce bladder overactivity. Second, we performed cystometry in urethane anesthetized rats. In the conscious condition, micturition is initiated by A δ -fibers whereas in anesthetized rats, C-fibers are also involved, and voiding efficiency and volume threshold are decreased¹. Therefore, it is not known whether similar results can be obtained in conscious and urethane anesthetized rats. Further studies are needed to clarify these points. Finally, since the concentration of M2 and M3-selective mAChR antagonists we used in this study is relatively high (30–150 μ M), non-selective receptor blockade might have occurred although minimal effects on the other subtypes have been reported²³. For example, methoctramine has also relatively high affinity for the M4 subtype as well as the M2 subtype whereas 4-DAMP has high affinity for the M5 subtype as well as the M3 subtype. Therefore, we used each two kinds of M2 and M3-selective mAChR antagonists to confirm the results.

Ideally, antimuscarinic therapy for overactive bladder should maintain the bladder contractility during normal voiding and have an acceptable tolerability. Existing antimuscarinic drugs for overactive bladder have high affinity for M3/M1 muscarinic receptors and consequently produce M3/M1-mediated adverse effects including dry mouth and constipation, resulting in poor patient compliance. Targeting overactive bladder with M2-selective antagonists may be an attractive alternative since it has the potential of inhibiting bladder overactivity without causing those adverse effects. However, the potential effects of M2-selective antagonists on heart rate need to be considered. M2 muscarinic receptors are expressed in high density in the heart and are responsible for slowing heart rate²⁴. Blockade of M2 receptors by antimuscarinic treatment for overactive bladder may lead to an imbalance between the sympathetic and parasympathetic influences on the heart, resulting in an increase in heart rate. Although M2-selective antagonists have never been clinically assessed in the target overactive bladder population and the effect on heart rate in this population is unknown at present, any treatment with a potential to increase heart rate could add unnecessary risk. Taken together, antimuscarinic agents with antagonistic activity against M2 mAChR could be effective for the treatment of overactive bladder, but antimuscarinics, which have higher bladder selectivity, is thought to be more beneficial.

Conclusions

The M2 muscarinic receptor subtype plays an important role in the local cholinergic modulation of bladder afferent activity that contributes to bladder overactivity in normal rats. Therefore, antimuscarinic agents which have antagonistic activity against M2 receptors could be more beneficial for the treatment of patients with overactive bladder if enhanced ACh mechanisms are involved in pathogenesis of overactive bladder.

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Figure 1.

Representative cystometograms. **A**, Intercontraction interval was decreased by intravesical administration of Oxo-M (200 μM). **B**, Bladder overactivity induced by Oxo-M was prevented when instilled with a non-selective antagonist (atropine 30 μM). **C**, Bladder overactivity induced by Oxo-M was also prevented by a M2-selective antagonist (dimethindene 30 μM). **D**, A M3-selective antagonist (darifenacin) did not inhibit the effect of Oxo-M in spite of its high concentration (150 μM). None of antimuscarinic agents alone affected any cystometric parameters (**B**, **C** and **D**).

Table 1

Effects of intravesical administration of Oxo-M with or without antimuscarinic agents on cystometric parameters (ICI, PT and BC). Data are shown as the mean $\pm$ standard error of the mean.

	saline	antimuscarinic	Oxo-M+antimuscarinic	n=7
ICI (intercontraction interval, second)				
control	788.6 $\pm$ 74.1	N/A	469.0 $\pm$ 43.0*	
atropine	859.3 $\pm$ 81.5	804.6 $\pm$ 62.4	833.2 $\pm$ 51.0	
propiverine	784.9 $\pm$ 116.3	680.6 $\pm$ 101.7	719.2 $\pm$ 97.1	
tolterodine	729.0 $\pm$ 72.8	695.3 $\pm$ 60.1	720.0 $\pm$ 60.1	
dimethindene	855.6 $\pm$ 119.5	734.9 $\pm$ 72.8	854.1 $\pm$ 110.6	
methoctramine	741.3 $\pm$ 55.7	720.2 $\pm$ 52.2	660.8 $\pm$ 99.5	
darifenacin	741.7 $\pm$ 39.9	815.4 $\pm$ 49.8	473.5 $\pm$ 35.1*	
4-DAMP	674.6 $\pm$ 72.8	719.4 $\pm$ 65.0	413.1 $\pm$ 41.0*	
PT (pressure threshold, cmH₂O)				
control	6.93 $\pm$ 0.40	N/A	5.75 $\pm$ 0.35*	
atropine	6.74 $\pm$ 0.80	5.59 $\pm$ 0.55	8.14 $\pm$ 1.11	
propiverine	8.79 $\pm$ 0.86	7.41 $\pm$ 0.43	8.48 $\pm$ 0.97	
tolterodine	6.60 $\pm$ 0.61	5.61 $\pm$ 0.62	5.86 $\pm$ 0.60	
dimethindene	7.41 $\pm$ 0.62	6.18 $\pm$ 0.76	6.83 $\pm$ 0.71	
methoctramine	4.26 $\pm$ 0.48	4.17 $\pm$ 0.38	5.64 $\pm$ 0.85	
darifenacin	6.84 $\pm$ 0.32	6.71 $\pm$ 0.34	5.36 $\pm$ 0.34*	
4-DAMP	6.51 $\pm$ 0.72	6.30 $\pm$ 0.56	5.82 $\pm$ 0.35	
BC (bladder capacity, ml)				
control	0.55 $\pm$ 0.04	N/A	0.36 $\pm$ 0.02*	
atropine	0.61 $\pm$ 0.06	0.60 $\pm$ 0.05	0.52 $\pm$ 0.04	
propiverine	0.46 $\pm$ 0.06	0.47 $\pm$ 0.06	0.45 $\pm$ 0.05	
tolterodine	0.49 $\pm$ 0.05	0.48 $\pm$ 0.03	0.51 $\pm$ 0.04	
dimethindene	0.67 $\pm$ 0.08	0.48 $\pm$ 0.05	0.69 $\pm$ 0.08	
methoctramine	0.46 $\pm$ 0.04	0.53 $\pm$ 0.03	0.45 $\pm$ 0.06	
darifenacin	0.50 $\pm$ 0.03	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02*	
4-DAMP	0.50 $\pm$ 0.06	0.52 $\pm$ 0.04	0.26 $\pm$ 0.03*	

* P<0.05 vs saline and antimuscarinic alone.

Table 2

Effects of intravesical administration of Oxo-M with or without antimuscarinic agents on cystometric parameters (MVP and BP). Data are shown as the mean $\pm$ standard error of the mean.

	saline	antimuscarinic	Oxo-M+antimuscarinic	n=7
MVP (maximum voiding pressure, cmH₂O)				
control	31.3 $\pm$ 1.2	N/A	32.5 $\pm$ 1.3	
atropine	29.2 $\pm$ 1.6	27.7 $\pm$ 1.3	27.5 $\pm$ 1.5	
propiverine	34.3 $\pm$ 1.5	32.7 $\pm$ 1.4	34.2 $\pm$ 0.9	
tolterodine	32.3 $\pm$ 1.9	30.4 $\pm$ 1.4	29.9 $\pm$ 1.6	
dimethindene	34.9 $\pm$ 2.9	32.7 $\pm$ 1.7	31.6 $\pm$ 2.2	
methoctramine	31.5 $\pm$ 1.9	29.6 $\pm$ 1.6	30.7 $\pm$ 2.3	
darifenacin	30.6 $\pm$ 1.6	30.2 $\pm$ 2.1	32.0 $\pm$ 1.7	
4-DAMP	30.3 $\pm$ 1.2	30.8 $\pm$ 1.3	33.8 $\pm$ 1.5	
BP (baseline pressure, cmH₂O)				
control	1.56 $\pm$ 0.25	N/A	2.31 $\pm$ 0.36	
atropine	1.51 $\pm$ 0.26	1.32 $\pm$ 0.20	1.75 $\pm$ 0.28	
propiverine	2.74 $\pm$ 0.70	2.42 $\pm$ 0.45	2.71 $\pm$ 0.45	
tolterodine	1.74 $\pm$ 0.33	1.66 $\pm$ 0.37	1.64 $\pm$ 0.29	
dimethindene	1.03 $\pm$ 0.14	1.03 $\pm$ 0.11	0.89 $\pm$ 0.16	
methoctramine	0.83 $\pm$ 0.35	0.50 $\pm$ 0.11	0.62 $\pm$ 0.10	
darifenacin	1.72 $\pm$ 0.09	1.51 $\pm$ 0.16	1.90 $\pm$ 0.17	
4-DAMP	2.04 $\pm$ 0.53	1.71 $\pm$ 0.48	2.36 $\pm$ 0.51	