Activation of Muscarinic Receptors in Rat Bladder Sensory Pathways Alters Reflex Bladder Activity

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Antimuscarinic drugs affect bladder sensory symptoms such as urgency and frequency, presumably by acting on muscarinic acetylcholine receptors (mAChRs) located in bladder sensory pathways including primary afferent nerves and urothelium. However, the expression and the function of these receptors are not well understood. This study investigated the role of mAChRs in bladder sensory pathways *in vivo* in urethane anesthetized rats. Intravesical administration of the mAChR agonist oxotremorine methiodide (OxoM) elicited concentration-dependent excitatory and inhibitory effects on the frequency of voiding. These effects were blocked by intravesical administration of the mAChR antagonist atropine methyl nitrate (5 μ M) and were absent in rats pretreated with capsaicin to desensitize C-fiber afferent nerves. Low concentrations of OxoM (5 μ M) decreased voiding frequency by $\sim$ 30%, an effect blunted by inhibiting nitric oxide (NO) synthesis with L-NAME (*N*_ω-nitro-L-arginine methyl ester hydrochloride; 5 mg/kg; i.v.). High concentrations of OxoM (40 μ M) increased voiding frequency by $\sim$ 45%, an effect blunted by blocking purinergic receptors with PPADS (0.1–1 mM; intravesically). mAChR agonists stimulated release of ATP from cultured urothelial cells. Intravenous administration of OxoM (0.01–5 μ g/kg) did not mimic the intravesical effects on voiding frequency. These results suggest that activation of mAChRs located near the luminal surface of the bladder affects voiding functions via mechanisms involving ATP and NO release presumably from the urothelium, that in turn could act on bladder C-fiber afferent nerves to alter their firing properties. These findings suggest that the urothelial-afferent nerve interactions can influence reflex voiding function.

Key words: urethane; antimuscarinics; urothelium; capsaicin; oxotremorine methiodide; afferent nerves

Introduction

Antimuscarinic agents are the main treatment for overactive bladder, a condition caused by aging, spinal cord injury (SCI), and other pathologies (Finney et al., 2006). According to traditional concepts, antimuscarinic drugs act by blocking postjunctional muscarinic acetylcholine receptors (mAChRs) in the bladder smooth muscle, thereby suppressing voiding contractions evoked by acetylcholine (ACh) released from parasympathetic efferent nerves (de Groat and Yoshimura, 2006; Finney et al., 2006). However, antimuscarinic drugs affect sensory bladder symptoms such as urgency, suggesting an action on bladder afferent pathways, which could involve a block of mAChRs in the urothelium and/or in the afferent nerves (Finney et al., 2006).

Bladder sensory pathways are potential targets for drugs used to treat various bladder dysfunctions because of their role in irritation symptoms (i.e., urgency, frequency) and in triggering reflex bladder activity (de Groat and Yoshimura, 2006; Birder and de Groat, 2007). Bladder afferent nerves originate in lumbosacral dorsal root ganglia and carry information regarding bladder full-

ness and pain. They consist of myelinated A δ -fibers and unmyelinated C-fibers, which terminate in the urothelium, suburothelial space and the muscle layers of the bladder (Gabella and Davis, 1998) and are activated by mechanical or chemical stimuli. Under normal conditions in conscious animals micturition is initiated by A δ -afferents. However, in pathological conditions and in anesthetized rats C-fibers are also involved (de Groat and Yoshimura, 2006).

The urothelium, a specialized epithelial tissue lining the urinary tract, is thought to play a role in bladder sensory mechanisms because it exhibits neuronal-like properties including the expression of various receptors (muscarinic, nicotinic, purinergic, transient receptor potential) and the ability to release various neurotransmitters (ACh, ATP, NO) that can influence the excitability of nearby afferent nerves (Birder and de Groat, 2007). M₁ to M₅ mAChR subtypes are expressed in the urothelium of different species (Giglio et al., 2005; Mansfield et al., 2005; Mukerji et al., 2006; Tyagi et al., 2006; Zarghooni et al., 2007; Kullmann et al., 2008). Activation of urothelial mAChRs releases a diffusible factor which inhibits carbachol induced smooth muscle contractions in pig (Hawthorn et al., 2000) or human (Chaiyaprasithi et al., 2003) bladder strips. In rat bladder tissue focal application of carbachol induces Ca²⁺ waves that originate in the urothelium and suburothelium and propagate into the muscle layer (Kanai et al., 2007).

Given the increasing evidence that the urothelium has neuronal-like properties that can affect sensory nerves and in

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turn influence bladder function, this study investigated the role of mAChRs located near the luminal surface of the bladder (i.e., urothelium and afferent nerves) in reflex voiding in rats by examining the effects of intravesical administration of a mAChR agonist and antagonist. The results indicate that activation of mAChR has both excitatory and inhibitory effects on voiding, which are mediated by C-fiber afferents and involve ATP and NO release, presumably from the urothelium.

Parts of this paper have been published previously in abstract form (de Groat et al., 2004).

Materials and Methods

Experimental animals. Experiments were performed in adult female rats (Harlan, Indianapolis, IN; 200–250 g) whose care and handling was in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee. One group of animals ($n = 11$ rats) was pretreated with capsaicin (125 mg/kg, s.c., dissolved in 10% ethanol, 10% Tween 80 and 80% physiological saline) to desensitize C-fiber afferent neurons. Capsaicin was administered under isoflurane anesthesia in three injections, divided in 25, 50, and 50 mg/kg doses over a 2 d period (at ~12 h intervals), and the experiments were performed 4 d after the last injection. Another group of rats ($n = 3$) was treated with the capsaicin vehicle. The efficiency of capsaicin treatment was evaluated in each rat just before the experiment using an eye wipe test (Cheng et al., 1993), in which a few drops of 100 μ M capsaicin were placed in the eye and the number of defensive forelimb movements was observed. No defensive forelimb movements were noted in capsaicin treated rats whereas >20 movements/2 min were noted in vehicle-treated rats. On visual inspection, the bladders from the capsaicin pretreated rats were larger than those of the untreated rats and had no signs of infection.

Continuous infusion cystometry. Rats were anesthetized with urethane (1.2 g/kg, s.c.; Sigma, St. Louis, MO). Under supplemental isoflurane anesthesia, the jugular vein was exposed and a polyethylene (PE-10) catheter was inserted for intravenous drug delivery. The urinary bladder was exposed via a midline abdominal incision and a flared PE-50 catheter was inserted through the bladder dome and secured with a silk tie. The catheter was connected via a three-way connector to a pump for saline infusion and to a pressure transducer for bladder pressure recording. Voiding responses were elicited by continuously infusing saline (0.9% NaCl) at a rate of 0.04 ml/min at room temperature (~22°C).

CMG data recording and analysis. Data were recorded for off-line analysis using Windaq data acquisition software (DATAQ Instruments, Akron, OH) and analysis was performed using Excel (Microsoft, Redmond, WA), Origin version 7 (OriginLab, Northampton, MA), and Prism 4 (GraphPad Software, San Diego, CA). The continuous infusion cystometry (CMG) parameters analyzed (see Fig. 1A) were intercontraction interval (ICI; defined as the time between two voiding episodes), amplitude of contractions (A; defined as the difference between bladder pressure at the peak of the contraction minus baseline bladder pressure), pressure threshold (PTh; defined as the bladder pressure necessary to evoke a voiding contraction), and baseline bladder pressure (BP; defined as the lowest bladder pressure just after voiding). Each of these parameters relates to a specific component of the reflex voiding (i.e., afferents, efferents, muscle). It is assumed that changes in ICI and PTh relate primarily to changes in the activity of the afferent nerves and/or changes in the afferent processing in the CNS (Yu and de Groat, 1999). Changes in BP reflect primarily changes in the bladder smooth muscle properties, whereas changes in contraction amplitude relate to changes in the activity of the efferent parasympathetic nerves and/or the contraction properties of the smooth muscle (Yu and de Groat, 1999). Control CMGs were performed for a period of 1.5–2 h before any drug application. Drugs used in this study were delivered intravesically or intravenously. For intravesical administration, drugs were dissolved in saline to a final concentration and applied for 30–60 min; washout periods lasted from 45–120 min or until a stable ICI was achieved. Antagonists were instilled for a period of 20–30 min before the agonist. For intravenous administration, drugs were dissolved in saline at desired dose and administered in small volumes (100–200 μ l) followed by 100 μ l of saline to flush the

catheter. Intravenous injection of saline (100–300 μ l) did not alter CMG parameters ($n = 2$ rats). For each parameter (ICI, A, BP, PTh), at least four measurements were averaged during the control period just before drug application. For intravesical drug administration measurements of each parameter were averaged for the entire period the drug was infused (30–60 min). For intravenous drug administration parameters were averaged for periods specified in the text. A change in a CMG parameter was considered a drug effect if the change was at least two times greater than the SD of that parameter in control conditions. Data are reported as percentage changes relative to control which was set to 100%.

Control experiments for the stability of CMG parameters were performed by infusing saline (at 0.04 ml/min) for 8–10 h. The ICI was stable during the course of the second to the eighth hour of recordings ($n = 7$ rats) (see Fig. 1B). Data included in this study were typically collected during hours 2–6 after the start of CMGs.

Drugs used in this study include (1) nonselective muscarinic receptor agonists, oxotremorine methiodide (OxoM; 1–160 μ M intravesical administration and 0.01–5 μ g/kg, i.v. administration) and oxotremorine sesquifumarate salt (OxoS; 10 μ M, *in vitro* testing; Sigma); (2) nonselective muscarinic receptor antagonist, atropine methyl nitrate (AMN; 1–100 μ M intravesical administration and 0.1–2 mg/kg i.v. administration; Sigma); (3) nonselective purinergic receptor antagonist 4-[4-formyl-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-2-pyridinyl] azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS; 0.1–1 mM intravesical administration; Sigma); and (4) nonselective inhibitor of nitric oxide (NO) synthase N_{ω} -nitro-L-arginine methyl ester hydrochloride (L-NAME; 5, 25 mg/kg, i.v. administration; Sigma).

Rat urothelial cell cultures were prepared as described previously (Birder et al., 1998). Female rats (200–250 g) were killed with 100% CO₂, the bladder was removed and placed in cold minimal essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with HEPES (2.5 g/l; Sigma) and containing penicillin/streptomycin/fungizone (PSF; 1%; Sigma). The bladder was cut open to expose the urothelium, and incubated in dispase (2.5 mg/ml, Worthington Biochemical, Lakewood, NJ) overnight at 4°C. Urothelial cells were gently scraped from the underlying tissue, placed in trypsin (0.25% w/v; Sigma) for 10–15 min at 37°C and dissociated by trituration. Cells were suspended in MEM containing 10% fetal bovine serum (FBS; Invitrogen) and centrifuged at 416 g for 10 min. The supernatant was removed and cells were suspended in keratinocyte media (Invitrogen) with 1% PSF, centrifuged again, and resuspended in fresh media. Cells were plated on collagen coated glass coverslips at densities of 50–125 $\times 10^4$ cells/ml. Media was added after 4 h of incubation at 37°C and changed every other day. Cells were used 48–96 h after dissociation.

ATP release from cultured urothelial cells was performed as described previously (Birder et al., 2002). Coverslips containing urothelial cells were superfused with oxygenated Krebs [containing (in mM) 4.8 KCl, 120 NaCl, 1.1 MgCl₂, 2.0 CaCl₂, 11 glucose, and 10 HEPES, pH 7.4; 25°C] at a flow rate of 1 ml/min. Drugs were bath applied via the perfusion system. Perfusate (100 μ l) was collected every 30 s before and after agonist stimulation and ATP levels were quantified using the luciferin-luciferase reagent (100 μ l; Adenosine Triphosphate Assay Kit, Sigma). Bioluminescence was measured using a luminometer (TD-20/20; Turner Biosystems, Sunnyvale, CA) whose detection limit was ~5 fmol ATP/sample. Data were normalized by comparison to the peak ATP release induced by the calcium ionophore, ionomycin (5 μ M; Sigma) used as a control at the end of each experiment. In these experiments, OxoS was used instead of OxoM because OxoM interfered with the assay. Pooled data are from a minimum of three cultures. Data analysis was performed using Excel (Microsoft) and Prism 4 (GraphPad Software).

Statistical analysis. Statistical significance was tested using paired, unpaired *t* test, and ANOVA between groups followed by Newman–Keuls multiple-comparison test, (significance set at $p < 0.05$) using Prism 4 (GraphPad Software). Data are presented throughout the text as mean $\pm$ SEM.

Results

To determine whether *in vivo* activation of mAChRs located near the bladder lumen (i.e., urothelium and/or suburothelial afferent nerves) (Kullmann et al., 2008), affects voiding function, CMG

Figure 1. Inhibitory and excitatory effects of intravesical administration of OxoM. **A**, Typical CMG recording illustrating the parameters used for quantifying the effects of drugs on voiding. **B**, Variation of ICI during the course of 8 h of continuous saline infusion ($n = 7$ rats). **C**, Representative CMG recordings illustrating the inhibitory and excitatory effects of intravesical OxoM in the same animal. The duration of 5 and 40 μM OxoM application was ~ 35 and 45 min respectively, with a wash period of ~ 108 min between applications. **D**, Changes in ICI after intravesical administration of different concentrations of OxoM. $n = 9$ rats for 1 and 5 μM OxoM and $n = 9$ rats for 20, 40, and 80 μM OxoM. Because 40 and 80 μM OxoM produce similar effects, for the following experiments, data are pooled together. Asterisks indicate significant changes ($p < 0.05$) from control, tested with paired t test. **E**, Intravesical administration of low (white bars; 5 μM ; $n = 12$ rats) and high (black bars; 40 or 80 μM ; $n = 18$ rats) concentrations of OxoM do not significantly change A, PTh, and BP. Statistical significance was tested with paired t test for each parameter, $p > 0.05$. In **D** and **E**, dotted lines represent control set to 100%.

was performed in urethane anesthetized rats before and after intravesical administration of the mAChR agonist and antagonist, OxoM and AMN, respectively. These agents were selected because they have a reduced ability to pass through the urothelial barrier into the bladder wall because of their quaternary structure and their hydrophilic properties and therefore are less likely to activate mAChRs located in the bladder smooth muscle.

Inhibitory and excitatory effects of intravesical OxoM

Intravesical application of OxoM (1–80 μM) had both inhibitory and excitatory effects on voiding functions depending on the concentration (Fig. 1C,D). Low concentrations (1–5 μM) elicited predominantly inhibitory effects (i.e., decreasing voiding frequency), whereas high concentrations (>20 μM) elicited predominantly excitatory effects (i.e., increasing voiding frequency). Both effects could be elicited in the same animal (5 μM OxoM increased ICI by $37.5 \pm 16.0\%$, whereas 40 μM OxoM decreased

ICI by $38.6 \pm 6.4\%$; $n = 8$ rats; paired t test $p < 0.05$) (Fig. 1C). Small amplitude non-voiding contractions during bladder filling were also observed in the presence of OxoM at low and high concentrations (Fig. 1C) (data not quantified). To avoid prolonged instillation of OxoM into the bladder that may lead to penetration of OxoM into the bladder wall, concentration response curves were constructed in two groups of rats using two to three consecutive applications of increasing concentrations of OxoM, each lasting for ~ 30 –45 min. In one group of rats, low concentrations of OxoM, 1 and 5 μM , increased ICI by $26.9 \pm 9.9\%$ and by $32.1 \pm 9.6\%$, respectively, ($n = 9$ rats) (Fig. 1D) without affecting other CMG parameters. Therefore, for further experiments, 5 μM OxoM was used to elicit a consistent inhibitory effect and is referred to as “low” concentration. In another group of rats, high concentrations of OxoM, 20, 40 and 80 μM , were tested consecutively ($n = 9$ rats) (Fig. 1D). OxoM at 20 μM had mixed effects, producing inhibition or excitation. OxoM at 40 and 80 μM produced consistent excitation, decreasing the ICI by $38.2 \pm 3.7\%$ and $40.5 \pm 6.7\%$, respectively ($n = 9$ rats), without any significant changes in other CMG parameters. Because the effects of 40 and 80 μM OxoM on ICI were not different, the data obtained using 40 or 80 μM OxoM were combined and designated as responses elicited by “high” concentrations of OxoM.

Neither low (5 μM , $n = 12$ rats) nor high (40 or 80 μM , $n = 18$ rats) concentrations of OxoM had a significant effect on the amplitude of contractions, PTh, or BP (Fig. 1E, Table 1).

Effects of intravesical and intravenous administration of AMN

The possibility that luminal muscarinic receptors are tonically active and modulate

voiding function was tested by instilling AMN into the bladder. Intravesical administration of 1–100 μM AMN had no significant effect on ICI, PTh, and BP (Fig. 2A, Table 2). At 5 and 50 μM , AMN slightly but not significantly decreased the amplitude of contractions, whereas at 100 μM , AMN significantly decreased the amplitude of the contractions (Fig. 2A, Table 2). The effects were long lasting and only partially reversed after washout (amplitude reduced to: $58.5 \pm 8.7\%$ of control by AMN 100 μM and to $67.7 \pm 8.9\%$ of control after 57 ± 22 min of saline wash; $n = 2$ rats). In some rats, after observing the effect of intravesical administration of AMN (100 μM ; i.e., the decrease in the contraction amplitude) and while maintaining the intravesical infusion of AMN (100 μM), AMN was also administered intravenously (0.1–2 mg/kg, $n = 4$ rats) and voiding was monitored for another 30 min. Even at the lowest dose (0.1 mg/kg), AMN intravenously produced an immediate decrease in amplitude, ICI, and PTh, similar to previous reports (Hegde et al., 1997; Ishiura et al., 2001;

Takeda et al., 2002), lasting for ~5–10 min, followed by a partial recovery of the amplitude, ICI and PTh (Fig. 2*B,C*, Table 2). These data suggested that mAChRs located on the bladder sensory pathways are not tonically active. They also indicated that penetration of AMN through the urothelium is poor and that the sites of action of AMN are different depending on the route of administration. Because AMN does not readily cross the blood–brain barrier or the urothelial barrier, when delivered intravenously it most likely blocks mAChRs located in the smooth muscle, whereas when delivered intravesically it does not act directly on the smooth muscle but it blocks mAChRs located within or near the urothelium.

Inhibitory effects of intravesical administration of OxoM

Intravesical infusion of low concentrations of OxoM (1 and 5 μ M) decreased the frequency of voiding in 71% of the rats (12 of 17) (ICI increased to $134.3 \pm 5.3\%$ of control; $n = 12$ rats (Fig. 3*Ai,Bi,C*, Table 1). In four rats, OxoM (5 μ M) had an excitatory effect, reducing ICI to $58.4 \pm 9.7\%$ of control, whereas in one rat OxoM (1 and 5 μ M) had no effect (ICI was 92.4% and 93.9% of control, respectively); however, subsequent applications of OxoM (40 μ M) after a wash of >30 min in this rat produced excitation (decreasing ICI by ~50%). OxoM 5 μ M had no significant effect on other CMG parameters (A, PT, BP) (Fig. 1*E*). Continuous intravesical infusion of saline did not elicit a significant change in ICI (Fig. 3, compare *Bi*, *Bii*) ($n = 12$ rats for OxoM 5 μ M and $n = 7$ rats for saline; unpaired t test, $p < 0.05$), thus indicating that a spontaneous change in voiding was unlikely to account for the OxoM inhibitory effects. The inhibitory effects persisted for the period of OxoM infusion, in some cases >90 min, and were partially reversed after washout (from $129.2 \pm 18.2\%$ to $113.3 \pm 17.1\%$ of control after a washout time of ~80 min, $n = 5$ rats, paired t test $p > 0.05$) (Fig. 3*Aii*). However, the inhibition was not repeatable in the same animal; usually a second application of 5 μ M OxoM elicited an excitatory effect (ICI decreased to $66.2 \pm 3.6\%$ of control, $n = 5$ rats) (Fig. 3*Aii*). AMN (5 μ M) administered after the inhibitory effect of OxoM was established (~30 min) significantly reduced the inhibitory effects (from $128.1 \pm 4.0\%$ to $111.7 \pm 7.1\%$ of control; $n = 10$ rats; paired t test $p < 0.05$) (Fig. 3*Aiii,C*).

Excitatory effects of intravesical administration of OxoM

High concentrations of OxoM (40 or 80 μ M) produced consistent excitation (i.e., increased voiding frequency) in 90% of the rats ($n = 18$ of 20), reducing the ICI to $57.8 \pm 4.1\%$ of control (Fig. 4*Ai*, Table 1). In one rat, OxoM 80 μ M had no effect (ICI was 98.6% of control) and in another rat OxoM had an inhibitory effect increasing ICI to 154% of control. Subsequent application of 160 μ M OxoM in this rat produced excitation, decreasing ICI

Table 1. Effects of intravesical administration of low (5 μ M; $n = 12$ rats) and high (40 or 80 μ M; $n = 18$ rats) concentrations of OxoM on CMG parameters

Parameters	Control	OxoM (5 μ M)	Control	OxoM (40 or 80 μ M)
ICI (s)	412.5 ± 53.2	$576.9 \pm 91.6^*$	491.3 ± 71.2	$285.2 \pm 42.2^*$
Amplitude (cmH ₂ O)	27.7 ± 0.8	26.9 ± 0.7	29.7 ± 1.2	30.7 ± 1.3
PTh (cmH ₂ O)	5.8 ± 0.6	7.4 ± 0.8	4.8 ± 0.6	4.9 ± 0.8
BP (cmH ₂ O)	4.3 ± 0.3	3.9 ± 0.4	4.1 ± 0.4	6.0 ± 1.2

*Statistically significant differences ($p < 0.05$) from control tested with paired t test.

Figure 2. Effects of intravesical and intravenous administration of AMN. **A**, Changes in CMG parameters after intravesical administration of 1, 5, 50, and 100 μ M AMN. Multiple concentrations were tested in each animal. Summary from $n = 3, 7, 4$, and 4 animals for 1, 5, 50, and 100 μ M AMN, respectively. **B**, Changes in CMG parameters after intravenous administration of AMN (0.1–2 mg/kg) in the presence of intravesical AMN (100 μ M). Control refers to parameters measured during intravesical AMN (100 μ M). Data are summarized during two time intervals: the first 10 min after intravenous delivery of AMN (white bars) and the remaining of 10–30 min (<10 min, gray bars; summary from $n = 4$ rats). **C**, Effects of intravenous administration of AMN (0.1 mg/kg) in the presence of 100 μ M intravesical AMN. In **A** and **B**, asterisks indicate significant changes ($p < 0.05$) from control, tested with paired t test, and dotted lines represent control set to 100%.

by 35%. The excitatory effects were reversible after washout, reproducible in the same animal ($n = 5$ rats) (Fig. 4*Aii*) and blocked by intravesical administration of AMN (5 μ M; $n = 5$ rats) (Fig. 4*Aiii*).

A plot of the time course of the changes in ICI before and after OxoM instillation revealed that the excitatory effects were time dependent, displaying two phases (Fig. 4*B*). The first phase consisted of a marked reduction in the ICI without any change in other parameters and was evident after the first or second voiding after OxoM application. The second phase consisted of a partial recovery (lengthening) of the ICI without detectable changes in other parameters in the majority of rats (78% of rats; 14 of 18) (Fig. 4*Bi*). In a small population of rats 22% (4 of 18) the second phase was accompanied by a slight, but not significant, increase in PTh (Fig. 4*Bii*) (to $153.9 \pm 34.5\%$ of control, $n = 4$, $p > 0.05$ paired t test), and in two of these rats also by an increase in baseline pressure (Fig. 4*Biii*) (to $272.7 \pm 23.1\%$ and to $158.5 \pm 5.3\%$ of control, respectively). The changes in ICI caused by high concentrations of OxoM were significantly different from changes in ICI caused by saline ($n = 18$ rats treated with OxoM

Table 2. Effects of intravesical and intravenous administration of AMN on CMG parameters

	Control	AMN
AMN 5 μ M ($n = 7$ rats)		
ICI (s)	536.6 $\pm$ 79.9	602.8 $\pm$ 76.8
Amplitude (cmH ₂ O)	31.2 $\pm$ 1.9	27.1 $\pm$ 1.7
PTH (cmH ₂ O)	7.2 $\pm$ 1.4	8.0 $\pm$ 1.9
BP (cmH ₂ O)	4.5 $\pm$ 0.5	4.5 $\pm$ 0.7
AMN 100 μ M ($n = 4$ rats)		
ICI (s)	411.1 $\pm$ 54.1	395.8 $\pm$ 54.9
Amplitude (cmH ₂ O)	32.8 $\pm$ 3.2	22.6 $\pm$ 2.4*
PTH (cmH ₂ O)	8.6 $\pm$ 2.1	8.4 $\pm$ 1.3
BP (cmH ₂ O)	5.0 $\pm$ 0.6	5.2 $\pm$ 0.7
Intravenous AMN (0.1–2 mg/kg) after intravesical AMN ($n = 4$ rats; control is 100 μ M intravesical AMN)		
		10 min/>10 min
ICI (s)	356.9 $\pm$ 69.8	185.0 $\pm$ 50.6*/225.1 $\pm$ 44.0
Amplitude (cmH ₂ O)	23.7 $\pm$ 3.1	14.1 $\pm$ 1.7*/16.7 $\pm$ 1.6
PTH (cmH ₂ O)	5.8 $\pm$ 1.5	2.8 $\pm$ 0.4/3.3 $\pm$ 0.92
BP (cmH ₂ O)	5.1 $\pm$ 0.7	6.1 $\pm$ 0.8/6.0 $\pm$ 0.8

For AMN intravenous, data were averaged in the first 10 min and in the next 10–30 min (>10 min) after delivery. Voiding was monitored in these rats for an average of 31.8 $\pm$ 7.5 min after intravenous delivery of AMN. Asterisks indicate statistically significant differences ($p < 0.05$) from control tested with paired t test.

Figure 3. Inhibitory effects of intravesical administration of OxoM. **Ai**, Low concentrations of OxoM (5 μ M) increased the ICI (summary from $n = 12$ rats). **Aii**, The inhibitory effects on ICI (white bars) were partially reversible after ~ 80 min wash (gray bars), and a second application produced excitation (black bar; summary from $n = 5$ rats). **Aiii**, The effects on ICI (white bars) were significantly reduced by intravesical administration of AMN (5 μ M; black bars; summary from $n = 10$ rats; example in **C**). Asterisks indicate significant changes ($p < 0.05$) from control, tested with paired t test. Dotted lines represent control set to 100%. s, Significant difference; ns, no significant difference (tested with paired t test between the groups indicated). **Bi**, Time course of the inhibitory effects of OxoM ($n = 12$ rats). ICI number (#) 0 indicates when OxoM was applied. A negative ICI # refers to ICIs before drug application and a positive ICI # refers to ICIs after drug application. The thick line represents mean and dotted lines represent confidence interval (5, 95%). **Bii**, Similar data from control experiments in which saline was instilled instead of OxoM ($n = 7$ rats). **C**, Inhibitory effect of OxoM (5 μ M) on ICI, which is reversed by intravesical administration of AMN (5 μ M).

40–80 μ M and $n = 7$ rats treated with saline; unpaired t test, $p < 0.05$) (compare Figs. 3Bii, 4C).

Effects of intravenous administration of OxoM

To demonstrate that the effects of intravesical OxoM application were caused by activation of mAChRs located in urothelium and/or afferent nerves rather than activation of receptors located

in the smooth muscle, OxoM was administered intravenously. Systemic effects of OxoM intravenously including increased salivation were observed starting at very low doses (effects not quantified). Because OxoM does not readily cross the blood–brain barrier, when delivered intravenously it likely activates receptors in the periphery. In preliminary experiments, dose–response curves for intravenous OxoM (0.01–5 μ g/kg, i.v., $n = 5$ rats) (Fig. 5A) were constructed by administering increasing doses every 30–45 min. Doses that produced significant effects (4–5 μ g/kg) on CMG parameters were chosen for further testing. The effects of intravenous OxoM on CMG parameters were short lasting, returning to control within 10–30 min (depending on the dose). For this reason data were summarized for a period of 10–20 min immediately after drug application. In contrast to intravesical administration, intravenous administration of OxoM had no significant effect on the ICI and PTH (Fig. 5C, white bars) ($n = 5$ rats paired t test $p > 0.05$), but produced an acute increase in the intravesical pressure (IVP; Δ IVP = 10.4 $\pm$ 2.0 cmH₂O after OxoM 4 μ g/kg, i.v.; $n = 5$ rats; paired t test $p < 0.05$) (Fig. 5A, Bi) and a significant increase in A and BP (Fig. 5C, white bars) (paired t test $p < 0.05$). In experiments with multiple OxoM applications, the effects of OxoM were not prevented by intravesical administration of AMN (5 μ M; 50–60 min before administration of the next dose of OxoM) (Fig. 5A, Bii, C, black bars) (Δ IVP = 13.3 $\pm$ 2.9 cmH₂O after OxoM 5 μ g/kg, i.v.; $n = 5$ rats; paired t test $p > 0.05$). These results indicate that the effects on CMG parameters of intravesical and i.v. OxoM were different, consistent with activation of mAChRs located at different sites in the bladder.

Capsaicin pretreatment prevents the effects of intravesical OxoM administration

As intravesical OxoM produced changes in voiding frequency, likely reflecting an action on afferent nerves (Lecci et al., 2001), we next investigated what types of afferent nerves (C or A δ type) mediate these effects. Previous studies showed that C-fiber afferents play a significant role in voiding in rats, especially under urethane anesthesia (Cheng et al., 1993). To assess whether the effects of intravesical OxoM involve activation of C-fiber afferents, rats were pretreated with capsaicin 4 d before assessing CMG parameters to desensitize the C-fibers. Similar to previous findings (Yu and de Groat, 1999), these rats had longer ICIs and higher PTH than untreated or vehicle-treated rats (Fig. 6A, B). In vehicle-treated rats ($n = 3$ rats), which had CMG parameters (ICI, PTH, BP, A) comparable

Figure 4. Excitatory effects of intravesical administration of OxoM. **Ai**, High concentrations of OxoM (40, 80 μ M) reduced ICI (summary from $n = 18$ rats). **Aii**, The effects of OxoM (40, 80 μ M) on ICI (white bars) were reversible after washout (gray bars) and repeatable (black bar); summary from $n = 5$ rats. **Aiii**, The effects of OxoM (80 μ M) were blocked by intravesical administration of AMN (5 μ M; summary from $n = 5$ rats). Dotted lines represent control set to 100%. Asterisks indicate significant changes ($p < 0.05$) from control, tested with paired t test. s, Significant differences tested with paired t test between the groups indicated in figure. **B**, Time dependence of the excitatory effects of intravesical OxoM. Examples from three rats. **Bi** illustrates the most common pattern (found in 78% of rats): initially the ICI becomes shorter (phase 1), then ICI lengthens (phase 2), but it is still shorter than control, without any changes in other CMG parameters. Numbers on top of voiding contractions represent the number of the ICI before (negative values) and after (positive values) OxoM application, which are used for quantification in **C**. **Bii** illustrates an example in which PTh increases slightly during phase 2. **Biii** illustrates an example in which BP increases during phase 2. Dotted lines indicate baseline pressure before OxoM application. Arrows in each panel indicate the time when OxoM infusion was started. **C**, Quantification of the time dependence of the excitatory effects of OxoM (40, 80 μ M; summary from $n = 18$ animals). ICI number (#) indicates when OxoM was applied. The line shows average of control data and dotted line represents linear fitting of the data during OxoM application.

with those of untreated rats, OxoM (40 μ M) had similar excitatory effects; thus, data from vehicle-treated and untreated rats were combined and compared with data from capsaicin pretreated rats.

In the capsaicin pretreated rats intravesical administration of OxoM (5–160 μ M) did not significantly change the voiding frequency, A and BP, but increased significantly PTh (Fig. 6C,D). This increase was associated with a change in the slope of the filling curve (Fig. 6C), suggesting that OxoM may have reached the muscarinic receptors in the detrusor muscle. This may have occurred as a result of an increased permeability of the urothelium. It is known that the absorption of intravesical fluids is increased in over-distended bladders (Sugaya et al., 1997) and bladders of capsaicin pretreated rats are over-distended (Santicioli et al., 1985), therefore making it possible for drugs to penetrate the urothelial barrier during infusion. However, there was no increase in BP as expected from a direct action of OxoM on the muscle (Fig. 6C) (OxoM, i.v.), therefore the amount of OxoM that may have reached the bladder smooth muscle was likely very small. Administration of OxoM intravenously (0.01–2 μ g/kg) elicited effects similar to those observed in untreated rats (i.e., acute increase in intravesical pressure without affecting the voiding frequency; Δ IVP = 8.8 ± 1.2 cmH₂O; $n = 3$ rats; paired t test $p < 0.05$) (Fig. 6E) and these effects were not prevented by intravesical administration of AMN (5 μ M; ~50 min preincubation; Δ IVP = 8.3 ± 2.0 cmH₂O; $n = 3$ rats; paired t test $p < 0.05$) (Fig. 6E).

The inhibitory effects of intravesical OxoM involve NO

Previous studies have shown that NO can be released from the urothelium and nerves (Birder et al., 1998) and can affect voiding frequency (Pandita et al., 2000). NO donors can also affect bladder afferent neuron excitability by modulating Ca²⁺ channels (Yoshimura et al., 2001) and when administered intravesically NO donors can suppress bladder hyperactivity induced by cystitis (Ozawa et al., 1999). To test the involvement of NO in the observed OxoM effects, the NOS inhibitor L-NAME (Masuda et al., 2007) was administered before OxoM. L-NAME alone at 5 mg/kg and 25 mg/kg (i.v.) increased the ICI, PTh, A, and BP in a dose-dependent manner ($n = 9$, 6 rats in each group respectively) (Fig. 7A). These effects lasted for >3–4 h and might be related to a block of NO mediated urethral smooth muscle relaxation and an increase in the urethral outlet resistance during micturition (Bennett et al., 1995; Masuda et al., 2007). Because 5 mg/kg L-NAME had fewer effects on CMG parameters (i.e., only the amplitude increased significantly from control; paired t test $p < 0.05$; $n = 9$ rats), this dose was chosen for further studies. OxoM was instilled intravesically ~45–60 min after L-NAME, when ICI was stable. Intravesical instillation of low concentrations of OxoM (5 μ M) slightly, but not significantly, increased the ICI in 78% of rats (7 of 9; ICI increased to $111.4 \pm 7.3\%$ of control, $n = 7$ rats; paired t test $p > 0.05$) (Fig. 7B,C) with no changes in other CMG parameters. This inhibitory effect was significantly less than the inhibitory effect of OxoM in the untreated rats (where ICI increased to $134.3 \pm 5.3\%$ of control; $n = 12$ rats; unpaired t test $p < 0.05$) (compare with Fig. 3Bi). In the remaining 22% of rats (2 of 9) low concentrations of OxoM produced an excitatory effect, reducing the ICI to $66.3 \pm 2.0\%$ of control without any changes in other CMG parameters. Instillation of high concentrations of OxoM (40 μ M) after L-NAME administration produced consistent excitation reducing the ICI to $72.8 \pm 7.4\%$ of control ($n = 6$ rats; paired t test $p < 0.05$) (compare Figs. 4C, 7D,E) without any other changes in CMG parameters. L-NAME

Figure 5. Effects of intravenous administration of OxoM on CMG parameters. **A**, Effects of increasing doses of intravenous OxoM on CMG parameters. The highest dose (5 µg/kg) was administered in the presence of intravesical AMN (5 µM for ~50 min before intravenous administration of OxoM). Note that the effects of intravenous OxoM were not blocked by intravesical administration of AMN (5 µM). **Bi** and **Bii** show enlargements of the dotted areas in **A** illustrating an acute increase in the IVP after administration of OxoM (black circle). IVP was measured before (IVP₁) and after (IVP₂) intravenous OxoM delivery at the time points indicated by arrows. **C**, Quantification of the changes in CMG parameters produced by intravenous administration of OxoM in the absence (white bars) and in the presence (black bars) of intravesical AMN (5 µM; summary from $n = 5$ rats). Asterisks indicate statistically significant differences (paired t test $p < 0.05$) relative to control.

did not affect the time-dependency of the excitatory effects of OxoM (i.e., the two phases of the excitation) (Fig. 7D).

The excitatory effects of OxoM are mediated in part by ATP

Previous studies have shown that bladder distention triggers ATP release from the urothelium (Ferguson et al., 1997; Kumar et al., 2004), which can then act on afferent nerves to increase their excitability (Cockayne et al., 2000; Vlaskovska et al., 2001; Burnstock, 2006). To test whether the excitatory effects of OxoM on ICI were mediated via purinergic mechanisms, OxoM (40–80 µM) was administered in the presence of the nonselective purinergic antagonist PPADS (0.1, 0.2, and 1 mM, intravesically, in concentrations shown to significantly reduce the afferent nerve activity during bladder distension) (Vlaskovska et al., 2001). Regardless of the PPADS concentration, the excitatory effect of OxoM was attenuated (OxoM reduced ICI to $61.8 \pm 16.8\%$ of control in the absence of PPADS and to $75.2 \pm 17.0\%$ of control in the presence of PPADS, $n = 13$ rats; paired t test $p < 0.05$) (Fig. 8A, B). There was variation from rat to rat in the size of the effect (Fig. 8A), which was not correlated with the concentration of PPADS, suggesting that even the lower concentrations of PPADS were effective at blocking purinergic receptors. The two phases of the excitatory effect still occurred in the presence of PPADS (Fig. 8B, C) and PPADS (0.1–1 mM) alone did not significantly alter CMG parameters ($n = 13$ rats; paired t test $p > 0.05$).

Muscarinic agonists release ATP from cultured urothelial cells

To determine whether activation of urothelial mAChRs results in ATP release, ATP was measured in perfusate of cultured urothelial cells. In these experiments OxoS was used because OxoM interfered with the ATP assay. The calcium ionophore ionomycin (5 µM) was applied at the end of each experiment to elicit a maximal ATP release that was used to normalize data between coverslips with varying number of cells (Birder et al., 2003). OxoS (10 µM, applied for 60–120 s) evoked release of ATP that lasted

for 1–6 min and was repeatable in the same preparation ($n = 5$ coverslips) (Fig. 8D) (first and second OxoS responses were $14.1 \pm 7.1\%$ and $11.0 \pm 3.6\%$ of the ionomycin response, respectively). Preincubation with AMN (20 µM) reduced OxoS-evoked ATP release by $60.9 \pm 9.7\%$ ($n = 5$ coverslips) (Fig. 8E). AMN alone did not have an effect on ATP levels (basal ATP levels, 6.6 ± 0.7 fmol/100 µl ATP, and in 20 µM AMN, 8.7 ± 0.9 fmol/100 µl; $n = 5$ coverslips; paired t test $p > 0.05$).

Discussion

Intravesical instillation of the muscarinic agonist OxoM elicited inhibitory and excitatory effects on voiding frequency that were abolished by intravesical instillation of the muscarinic antagonist AMN or by pretreatment with capsaicin, indicating that they were dependent on C-afferent fiber activation. The inhibitory effects involved NO because L-NAME, a NOS synthesis inhibitor, blunted the inhibition. The excitatory effects were reduced by a purinergic receptor antagonist PPADS; and OxoS released ATP from cultured urothelial cells, suggesting that the excita-

tion is partially mediated through ATP likely released from urothelium. I.v. administration of OxoM had different effects on bladder reflex activity and these effects were not blocked by AMN administered intravesically. These results suggest that *in vivo* activation of mAChRs located in bladder sensory pathways alters voiding frequency via multiple mechanisms likely involving urothelial derived factors that in turn alter the excitability of C-afferent nerves.

Site of action of intravesical OxoM

mAChRs are expressed throughout the bladder: in urothelium, myofibroblasts, afferent and efferent nerves and smooth muscle (Abrams et al., 2006). For several reasons, it is likely that the effects of OxoM administered intravesically were caused by activation of mAChRs located in the urothelium or just beneath the urothelium (afferent nerves, myofibroblasts), rather than a direct action on the bladder smooth muscle. First, OxoM and AMN have limited ability to cross the urothelial barrier because of their quaternary structure and hydrophilic properties. Our experiments indicated that this is indeed the case because (1) both OxoM and AMN delivered intravesically had different effects than when delivered intravenously (Figs. 2–5), and (2) AMN intravesically blocked the intravesical effects of OxoM, but did not alter the intravenous effects of OxoM (Figs. 3–5). Furthermore, to reach the smooth muscle, OxoM would have to pass through the lamina propria, a tissue rich in blood vessels and therefore likely to be taken up in the bloodstream to produce systemic effects such as increased salivation. However, systemic effects were absent after intravesical OxoM, but were observed after intravenous injection of OxoM even at low doses (0.1 µg/kg) that had minimal effects on bladder activity. Second, intravesical OxoM altered the frequency of voiding without significantly changing the amplitude of contractions or basal bladder pressure (Figs. 1E, 3, 4). This is similar to the effects of agents such as capsaicin, known to act on afferent nerves (Lecci et al.,

Figure 6. Effects of intravesical and intravenous administration of OxoM in capsaicin pretreated rats. **A**, Comparison of CMG parameters between untreated and capsaicin pretreated rats, before OxoM application (summary from $n = 21$ untreated rats and $n = 14$ capsaicin pretreated rats). Asterisks indicate significant changes ($p < 0.05$) from control, tested with paired t test. **B**, CMG recordings from an untreated rat (**Bi**) and a capsaicin pretreated rat (**Bii**), illustrating the differences in ICI. Traces are on the same time scale for comparison. **C**, Effects of intravesical OxoM in a capsaicin pretreated rat. The arrow points to PTh. **D**, Intravesical administration of OxoM at increasing concentrations did not affect ICI, A, or BP, but increased PTh (summary from $n = 5, 3, 9, 7$, and 3, animals for OxoM 5, 20, 40, 80, and 160 μM , respectively). Dotted line represents control set to 100%. Asterisks indicate significant changes ($p < 0.05$) from control, tested with paired t test. **E**, Effects of intravenous OxoM in a capsaicin pretreated rat in the absence and in the presence of intravesical AMN (5 μM for >50 min before i.v. administration of OxoM). Note that the effects of intravenous OxoM were not blocked by intravesical administration of AMN.

2001), indicating an action of OxoM on the afferent nerves. In contrast, OxoM delivered intravenously produced a marked increase in intravesical pressure and contraction amplitude with little effect on voiding frequency (Fig. 5), suggesting an action on the smooth muscle mAChRs. Third, systemic capsaicin pretreatment to desensitize C-fibers, abolished the intravesical effects of OxoM, but did not affect the intravenous effects (Fig. 6). Fourth, even low intravesical concentrations of OxoM (1 and 5 μM), that presumably only activated mAChRs on the luminal surface of the urothelium, elicited changes in the ICI that were blocked by intravesical administration of AMN (5 μM) (Fig. 3). Fifth, the effects of intravesical OxoM were rapid, occurring shortly after infusion (Fig. 4). This coupled with limited permeability of OxoM strongly argue that the effects were not caused by a direct action on the muscle. Sixth, OxoM did not significantly affect the amplitude of the contractions, suggesting that OxoM did not penetrate the urothelium to reach the parasympathetic efferent nerves. These nerves contain presynaptic M_1 and M_2/M_4 mAChRs that modulate transmitter release (Somogyi and de Groat, 1992; Somogyi et al., 1994; Braverman et al., 1998; Zhou et al., 2002). These data provide strong evidence that intravesical OxoM activates mAChRs located in the bladder sensory pathways including urothelium and afferent nerves. The expression of mAChRs in the sensory pathways was shown with RT-PCR or immunohistochemistry (Giglio et al., 2005; Mansfield et al., 2005; Mukerji et al., 2006; Tyagi et al., 2006; Zarghooni et al., 2007), but

this is the first demonstration that *in vivo* activation of these receptors elicits complex effects on bladder function.

Inhibitory mechanisms

Intravesical administration of low concentrations (1, 5 μM) of OxoM had a predominant inhibitory effect decreasing the voiding frequency without significantly changing other CMG parameters (Figs. 1E, 3). These experiments were performed in anesthetized rats where C-fiber afferents are involved in micturition (Cheng et al., 1993), and the effects of OxoM were absent in capsaicin pretreated rats (Fig. 6), suggesting that mAChR activation affects C-fiber activity. NO contributed to the inhibitory effects because suppressing NO synthesis with L-NAME blunted the inhibition without altering the excitation (Fig. 7). The urothelium could be a source of NO because it releases NO in response to stimulation of various receptors (i.e., TRPV1 or β -adrenoreceptor) (Birder et al., 1998, 2002), although no study has shown NO release in response to mAChR stimulation. Because the bladder smooth muscle has low sensitivity to NO (Fujiwara et al., 2000), the site of action of NO is likely the afferent neurons (Yoshimura et al., 2001) and/or the interstitial cells (Lagou et al., 2006). In dissociated bladder afferent neurons, NO donors modulate N-type Ca^{2+} channels (Yoshimura et al., 2001) and in cultured C-type DRG neurons NO donors block fast TTX-sensitive as well as slow and persistent TTX-resistant Na^+ cur-

Figure 7. Inhibitory effects of intravesical administration of OxoM involve NO. **Ai**, Summary of the effects of intravenous administration of 5 mg/kg L-NAME (white bars; $n = 9$ rats) and 25 mg/kg L-NAME (black bars; $n = 6$ rats) on CMG parameters. Asterisks represent significant changes from control (paired t test between control and L-NAME $p < 0.05$). Significant (s ; unpaired t test $p < 0.05$) or nonsignificant (ns ; unpaired t test $p > 0.05$) differences in between 5 mg/kg L-NAME and 25 mg/kg L-NAME are shown. **Aii**, Effects of 5 mg/kg (i.v.) L-NAME on CMG parameters. **Aiii**, Effects of 25 mg/kg (i.v.) L-NAME on CMG parameters. Arrows indicate the time when L-NAME (i.v.) was injected. **B**, Time course of the effects of intravesical administration of low concentrations of OxoM (5 μ M) in rats treated with L-NAME ($n = 7$ rats). Negative ICI values represent the ICI number (#) before OxoM application and positive ICI values represent ICI # after OxoM application. ICI # 0 was taken when OxoM was applied. **C**, The effects of intravesical administration of low concentrations of OxoM (5 μ M) on CMG parameters after L-NAME, illustrating the absence of OxoM-induced inhibition. **D**, Time dependence of the effects of high concentrations of OxoM (40 μ M) in rats treated with L-NAME ($n = 6$ rats). **E**, The effects of 40 μ M OxoM on CMG parameters after L-NAME illustrating the presence of OxoM-induced excitation.

rents (Renganathan et al., 2002), thereby affecting neuronal excitability.

Excitatory mechanisms

Intravesical administration of OxoM at concentrations $>20 \mu$ M produced a predominant excitatory effect on ICI without signif-

icantly changing other CMG parameters (Figs. 1*E*, 4), consistent with previous findings using carbachol (Kim et al., 2005). These effects were dependent on C-fiber activation as they were abolished in capsaicin pretreated animals (Fig. 6). One mechanism contributing to these effects is ATP release from the urothelium, followed by activation of purinergic receptors on afferent nerves. This is supported by our data demonstrating that (1) cultured urothelial cells release ATP after stimulation with OxoS (Fig. 8*D*, *E*) and (2) blocking purinergic receptors *in vivo* with PPADS reduced the excitatory effects of OxoM (Fig. 8*A–C*). Other studies have shown that P2X_{2/3} are expressed on bladder afferent nerves and that ATP released during bladder stretch or ATP administered intravesically can activate afferent nerves, triggering the micturition reflex (Ferguson et al., 1997; Cockayne et al., 2000; Vlaskovska et al., 2001; Pandita and Andersson, 2002; Kumar et al., 2004; Nishiguchi et al., 2005; Burnstock, 2006; Ruggieri, 2006). P2X and P2Y receptors are also expressed in urothelial cells (Birder et al., 2004) and myofibroblasts (Wu et al., 2004; Sui et al., 2006) and their activation may trigger ATP release, that can further stimulate the afferent nerves (Birder and de Groat, 2007).

The excitatory effects displayed two phases, an initial large decrease in the ICI followed by a smaller decrease in the ICI (Fig. 4). This could be interpreted as initial excitation followed by inhibition or two excitatory pathways one of which rapidly desensitizes. Blocking NO synthesis (Fig. 7*D*, *E*) or blocking purinergic receptors (Fig. 8*A–C*) did not affect the two-phase response, suggesting different mechanisms. One possibility involves activation of distinct mAChR subtypes located at different sites: urothelium and afferent nerves.

Sensory role of urothelial and afferent nerve mAChRs and pathological implications

The role of urothelium in bladder sensory mechanisms has recently attracted considerable attention. Historically this tissue has been viewed as a passive physical barrier preventing the leak of urine constituents into the bladder wall. Previous research indicating that urothelial cells express various receptors and release neurotransmitters in response to mechanical and chemical stimuli that could influence the excitability of the nerves suggests that the urothelium may play an active role in modulating bladder functions (Birder and de Groat, 2007). Our data provide evidence for such a role by demonstrating that activation of mAChRs located near the luminal surface of the bladder can modulate bladder reflexes via mechanisms involving NO and ATP, likely released from the urothelium and acting on C-fiber afferents to alter their excitability.

Intravesical AMN had no effect on voiding frequency (Fig. 2), suggesting that under normal physiological conditions urothelial and/or suburothelial mAChRs are not tonically active. However, OxoM-induced increases in voiding frequency resemble the pathological condition of overactive bladder (OAB), suggesting that alteration of mAChRs in sensory pathways might contribute to some of the OAB symptoms. In the urothelium, alteration of mAChRs might lead to increased ATP release. Indeed, in bladders that are overactive as a result of inflammation (Smith et al., 2005) or SCI (Salas et al., 2007) and in urothelial cells from patients or cats suffering from interstitial cystitis (Birder et al., 2003; Sun and Chai, 2006), ATP release is upregulated. In the afferent system, increased excitability of primary sensory neurons occurring after SCI or cystitis is considered a “fingerprint” of bladder hyperactivity (de Groat and Yoshimura, 2006). mAChRs might be involved in these changes as preliminary studies indicate that mus-

carinic agonists can alter the firing properties of bladder afferent neurons (Negoiita et al., 2004). Further investigation of the underlying muscarinic modulatory mechanisms, specifically whether a particular mAChR subtype (M_1 – M_5) or a mAChR subtype located at a particular site (urothelium or nerves) is involved, might help to identify new targets for the treatment of overactive bladder.

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Figure 8. Mechanisms of the excitatory effects of intravesical administration of OxoM. **A**, The excitatory effect of OxoM (40–80 μ M) in the absence and in the presence of PPADS (0.1–1 mM) are illustrated for all rats ($n = 13$). Each empty round symbols represent data from a single rat and lines connect data from the same rat. Filled round symbols represent the average data from all rats before (left) and after (right) PPADS. Asterisk indicates significant differences ($p = 0.038$) between the effect of OxoM on ICI before and after PPADS. **B**, Time course of the excitatory effects of OxoM (40–80 μ M) in the presence of PPADS (0.1–1 mM; intravesical) ($n = 13$ rats). Negative ICI values represent the ICI number (#) before OxoM application and positive ICI values represent ICI # after OxoM application. ICI # 0 was taken when OxoM was applied. **C**, Effects of OxoM in the absence (**i**) and in the presence (**ii**) of PPADS. **D**, In cultured urothelial cells, two consecutive applications of OxoS (10 μ M) evoked ATP release of similar magnitude (summary from $n = 5$ coverslips). Baseline indicates baseline ATP levels and Ionomycin indicates ATP release in response to the calcium ionophore ionomycin (5 μ M). **E**, OxoS-evoked ATP release was greatly reduced by AMN (20 μ M; summary from $n = 5$ coverslips). In **D** and **E**, asterisks indicate significant differences tested with ANOVA between groups followed by Newman–Keuls *post hoc* test.

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