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RESEARCH PAPER

Dual modulation of urinary bladder activity and urine flow by prostanoid EP_3 receptors in the conscious rat

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Background and purpose: Cyclooxygenase inhibitors function to reduce levels of prostaglandin E_2 (PGE_2) and are broadly efficacious in models of bladder overactivity. We therefore investigated a regulation of urinary bladder function in conscious rats by modulation of the EP_3 receptor for PGE_2.
Experimental approach: The activity of the EP_3 receptor agonist GR63799X, and EP_3 receptor antagonists, CM9 and DG041, at recombinant EP_3 receptors was evaluated *in vitro*. *In vivo*, intraduodenal dosing during conscious, continuous-filling cystometry of spontaneously hypertensive rats was utilized to determine the urodynamic effect of EP_3 receptor modulation.
Key results: GR63799X dose-dependently (0.001–1 $mg \cdot kg^{-1}$) reduced bladder capacity, as indicated by a reduction in both the micturition interval and volume of urine per void. In contrast, CM9 (10 and 30 $mg \cdot kg^{-1}$) and DG041 (30 $mg \cdot kg^{-1}$) enhanced bladder capacity, as indicated by significantly longer micturition intervals and larger void volumes. CM9 and DG041 inhibited the responses to GR63799X supporting the *in vivo* activity of these pharmacological agents at the EP_3 receptor. In addition to its effect on bladder capacity, GR63799X increased endogenous urine production. Intra-arterial infusion of saline mimicked the enhancement of urine flow observed with GR63799X, and the response was inhibited by CM9.
Conclusions and implications: These data support the EP_3 receptor as a modulator of urinary bladder activity in the conscious rat, and in addition, indicate a role for EP_3 receptor activity in regulating urine flow.
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Keywords: EP3 receptor; cystometry; urinary bladder; urine flow; overactive bladder; SHR

Abbreviations: EP, receptor; EP-type, prostanoid receptor; FLIPR, fluorescence imaging plate reader; i.d., intraduodenal; KO, knockout; PGE_2, prostaglandin E_2; SHR, spontaneously hypertensive rat

Introduction

Cyclooxygenase inhibition has been shown to regulate urinary bladder function by enhancing the functional bladder capacity in a number of species under various conditions (Takagi-Matsumoto *et al.*, 2004; Angelico *et al.*, 2006; Wibberley *et al.*, 2006). Although cyclooxygenase inhibition decreases the production of many prostaglandins (PGs), these effects on bladder function are at least partially due to decreases in the levels of PGE_2 (Hu *et al.*, 2003). PGE_2 is implicated as a contributor to urinary bladder overactivity as evidenced by the ability of PGE_2 infused into the bladder to diminish bladder capacity (Maggi *et al.*, 1988a; Schüssler, 1990; Ishizuka *et al.*, 1995; McCafferty *et al.*, 2008), and by increases in PGE_2 production in bladder overactivity models (Park *et al.*, 1999; Hu *et al.*, 2003) as well as in overactive bladder patients (Kim *et al.*, 2005; 2006).

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PGE_2 is an agonist at EP, G protein-coupled, prostanoid receptors (EP_1–EP_4; nomenclature follows Alexander *et al.*, 2008) that mediate the physiological effects of PGE_2. Previously it has been suggested that the effects of PGE_2 on bladder function are mediated through EP_1 receptors; based on studies in knockout (KO) mice (Schröder *et al.*, 2004) and with EP_1 receptor antagonists (Maggi *et al.*, 1988b; Lee *et al.*, 2007). However, more recently it has been shown that EP_3 receptor KO mice have a diminished response to bladder infusion of PGE_2 and demonstrate an enhanced bladder capacity under basal conditions (McCafferty *et al.*, 2008). This suggests an important contribution for EP_3 receptors in the modulation of bladder function under physiological conditions, as well as under conditions of enhanced PGE_2 production evoking pathological bladder overactivity.

EP_3 receptors are expressed on dorsal root ganglion neurons (Oida *et al.*, 1995; Kozaki *et al.*, 2007; Wang *et al.*, 2007; Su *et al.*, 2008a) and have been shown to functionally sensitize nociceptor responses (Kumazawa *et al.*, 1993; 1996). Consistent with a neuronal role for EP_3 receptors in controlling urinary bladder function, bladder distension-induced afferent firing is dampened by EP_3 receptor antagonism (Su *et al.*, 2008a). Therefore, we have utilized spontaneously hypertensive rats (SHR), which present with afferent-based bladder overactivity (Persson *et al.*, 1998; Spitsbergen *et al.*, 1998; Patra *et al.*, 2007), to further evaluate EP_3 receptors as a target in the development of treatments for overactive bladder.

We have extended our previous *in vivo* findings in EP_3 receptor KO mice (McCafferty *et al.*, 2008) using pharmacological agonism and antagonism of the EP_3 receptor in conscious rats. We found that pharmacological activation and inhibition of the EP_3 receptor resulted in a reduction and enhancement of functional bladder capacity respectively. In addition, our cystometric studies also provide evidence supporting a role for EP_3 activity in urine production.

Methods

Cell-based assays of EP_3 receptor ligands

U2OS cells were thawed and diluted into DMEM/F12 (HAM'S) 1:1 with L-glutamine, 15 mM HEPES, phenol red, 10% fetal bovine serum and 1% penicillin-streptomycin solution. BacMam viruses encoding human (0.25%) or rat (0.15%) EP_{3c} (Namba *et al.*, 1993) and G_{qi5} were added to transduce U2OS cells plated into 384-well plates at 15 000 cells per well. Plates were then left at room temperature for 1 h followed by incubation at 37°C (5% CO_2) for 24 h. The following day Fluo4 (2 μM) and Brilliant Black (500 μM final concentration) calcium indicator dyes were loaded into the cells by incubation for 1 h at 37°C (5% CO_2) in Hank's buffered saline containing calcium, magnesium and probenecid (2.5 mM). Compounds were diluted into Hank's buffered saline containing calcium, magnesium and CHAPS (0.01%). A 20-point PGE_2 response curve was generated with half log concentrations of PGE_2, up to 2 μM. For each experiment an EC_{50} value was generated from a PGE_2 curve and was used to determine the EC_{80} challenge for the antagonist assay [calculated via EC_{80} = 4(EC_{50}/Hillslope)]. Antagonists were pretreated, and fluorescence measurements were made over 65 s after agonist addition by using a FLIPR system (Fluorescence imaging plate reader, Molecular Devices). IC_{50} values were utilized to calculate pK_i values for each compound (Cheng and Prusoff, 1973).

Conscious, continuous-filling cystometry

All animal care and experiments were approved by the institutional animal care and use committee (IACUC) of GlaxoSmithKline. Female SHR were purchased from Charles River Laboratories with urinary bladder and intraduodenal (i.d.) catheters surgically implanted by Charles River Surgical Services for saline infusion and compound dosing respectively. In some rats an additional femoral artery catheter was also implanted. After a 5 day minimum post-surgical recovery period, rats were placed in restraints (Braintree Scientific) located above a balance utilized to measure voided urine volumes. Urinary bladder catheters were extended with PE-50 tubing and connected to a pressure transducer and a saline infusion pump (Harvard Instruments) via a three-way connector. Bladder pressure was viewed and recorded via Chart software through a PowerLab data acquisition system (ADI Instruments). Bladders were continuously infused with room temperature saline at a rate of 100 μL·min^{-1} for the duration of studies. A 2 h infusion was used as an equilibration period allowing urodynamics to stabilize and the rats to become familiar with the restraints. After equilibration, an hour of control/basal urodynamics was obtained prior to i.d. compound dosing via the duodenal catheter. Urodynamics were continually assessed for a 2 h period post dosing. In antagonist pretreatment studies, pretreatment doses were administered 2 h prior to dosing with GR63799X. Dosing solutions of compounds were prepared in 10% Gelucire, sonicated and dosed intraduodenally (i.d.) as a suspension. In some rats saline was infused (10 μL·min^{-1}) into the femoral artery via the chronically implanted arterial catheter in order to enhance endogenous urine production.

Data analysis and statistics

Averages of cystometric parameters were obtained for 30 min time periods post dosing. These values were normalized to the control period and expressed as a percentage of control. Data were normalized to reduce numerical variability between groups and facilitate unpaired comparisons. Baseline/control micturition interval and void volume group values were also reported. In the cases where single time point data are shown the 90–120 min values were utilized for comparison, as this time point typically demonstrated the most stable and robust response over the treatment period. Interval was measured as the time between micturition events and void volume as the average volume of urine per micturition event. Peak micturition pressures were measured as the maximum pressure reached during urine voiding. Residual volumes were assessed as the difference between the volume infused (interval*100 μL·min^{-1}) and volume voided for a given cystometrogram. Residual volumes were then divided by the micturition interval providing a measure of endogenous urine flow.

Data were expressed as averages with associated standard errors, and statistics were performed by using GraphPad Prism

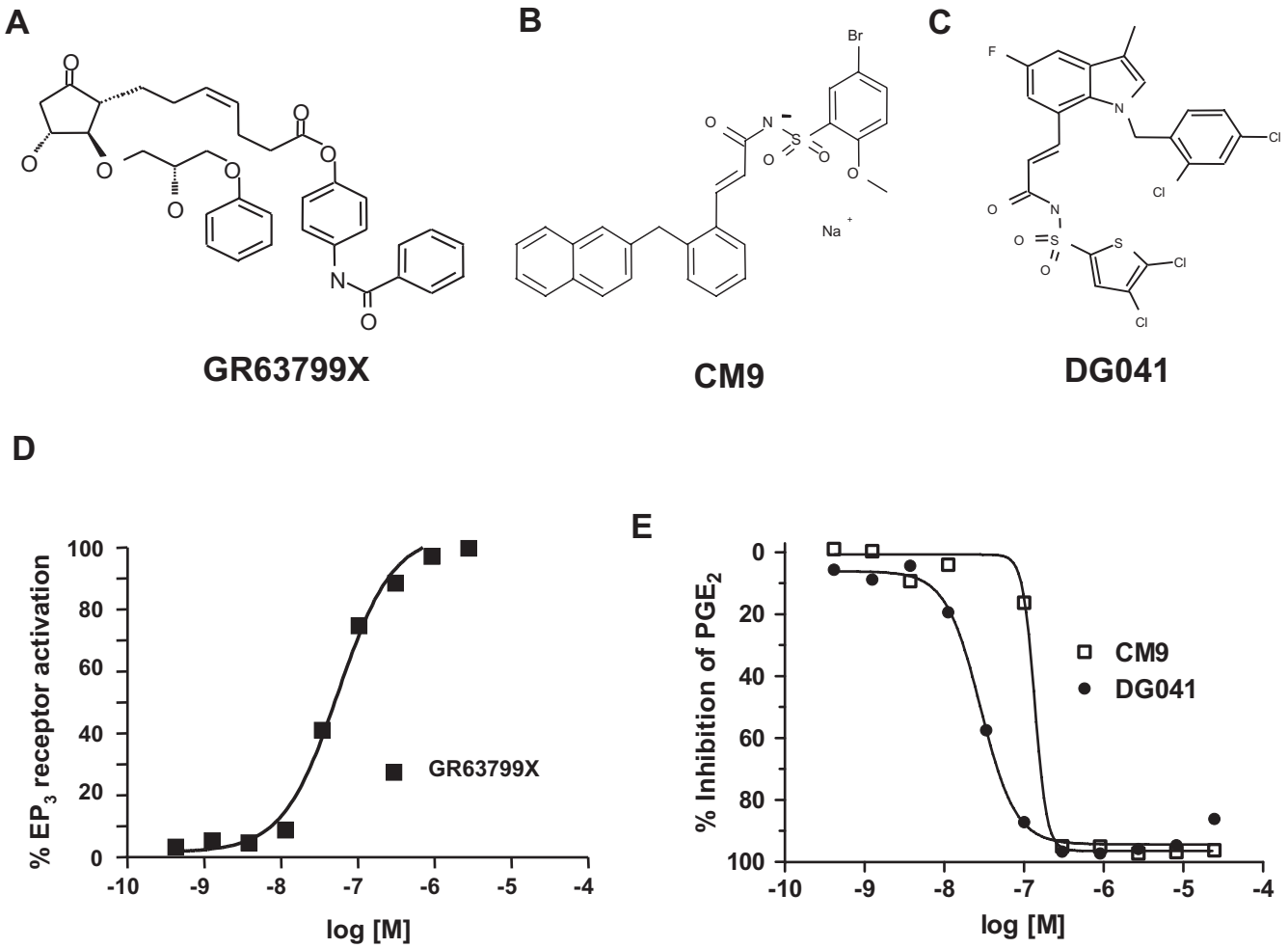

Figure 1 Effects of EP_3 receptor modulators. (A) GR63799X, EP_3 receptor agonist. (B) CM9, EP_3 receptor antagonist. (C) DG041, EP_3 receptor antagonist. (D) Representative dose–response to GR63799X on cells expressing human EP_3 receptors. Data are expressed as a percentage of the maximum PGE_2 response. (E) Representative dose–response curves to CM9 and DG041 demonstrating a complete inhibition of the PGE_2-evoked response at the rat EP_3 receptor.

version 4.02. Student's unpaired *t*-tests or a one-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* analysis was utilized. Time course data were analysed by two-way repeated measures ANOVA followed by a Bonferroni *post hoc* analysis to compare equivalent time points where applicable.

Materials

The EP_3 receptor agonist GR63799X and EP_3 receptor antagonists (CM9 and DG041) were synthesized in house at GlaxoSmithKline Pharmaceuticals (King of Prussia, PA, USA).

Results

In vitro *potency of EP_3 receptor ligands*

The EP_3 receptor agonist GR63799X (Bunce *et al.*, 1991; Figure 1A) demonstrated an ability to stimulate Ca^{2+} entry into U2OS cells transduced with full-length human EP_3 receptors demonstrating a pEC_{50} of 7.2 ± 0.2 (n = 6; Figure 1D). EP_3 receptor antagonists CM9 (Juteau *et al.*, 2001; Figure 1B) and DG041 (Zegar *et al.*, 2007; Figure 1C) were confirmed as active at EP_3 receptors. CM9 inhibited PGE_2-induced Ca^{2+} entry in cells with either human or rat EP_3 receptors (Figure 1E; Table 1). DG041 also dose-dependently inhibited Ca^{2+} influx initiated by activation of human and rat EP_3 receptors (Figure 1E; Table 1). For both CM9 and DG041, the potency at the rat receptor appeared to be somewhat reduced when compared with the equivalent human EP_3 receptor.

Table 1 Potencies of EP_3 receptor antagonists in the fluorescence imaging plate reader assay

		Human EP_3	*Rat EP_3*
CM9	pIC_{50}	7.03 ± 0.04 (n = 101)	6.58 ± 0.04 (n = 73)
	fpK_i	7.77 ± 0.04	7.12 ± 0.04
DG041	pIC_{50}	7.66 ± 0.04 (n = 101)	7.04 ± 0.03(n = 70)
	fpK_i	8.41 ± 0.04	7.57 ± 0.03

pIC_{50} values were measured in response to an EC_{80} challenge of the EP_3 receptor agonist PGE_2.

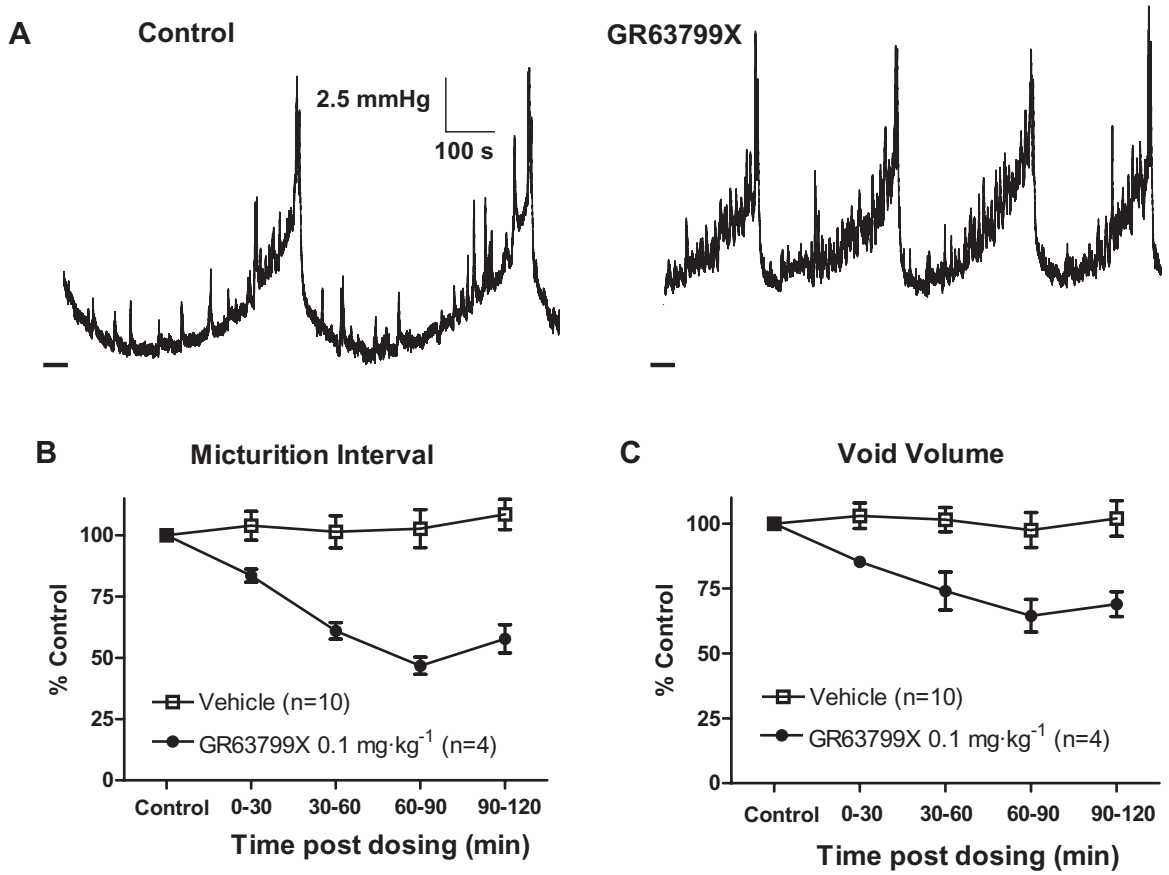

Figure 2 Effect of GR63799X on rat urodynamics. (A) Bladder pressure recordings before and after administration of GR63799X (0.1 mg·kg^{-1}, intraduodenal). Dashed line at the lower left of traces is 0 mmHg. (B and C) Time course of the response to GR63799X and vehicle, demonstrating a reduction in micturition interval ($P = 0.002$ vs. vehicle, two-way repeated measures ANOVA) and average void volume ($P = 0.01$ vs. vehicle) respectively.

Urodynamic response to EP$_3$ receptor activation

In conscious, continuous-filling cystometry in the SHR, GR63799X (0.1 mg·kg^{-1}) given i.d. increased the urination frequency as compared with control (Figure 2A). This was indicated by a time-dependent decrease in the micturition interval (Figure 2B) and was also reflected by a decrease in void volume, after treatment with GR63799X (Figure 2C). Vehicle was without effect, and the GR63799X response was significantly different than vehicle, for both micturition interval and void volume. Control micturition intervals for the vehicle- ($n = 10$) and GR63799X ($n = 4$)-treated groups were 12 ± 2 and 13 ± 1 min, and control void volumes were 1.2 ± 0.2 and 1.2 ± 0.1 mL respectively. The response to GR63799X was dose-dependent with an IC$_{50}$ of 0.01 mg·kg^{-1} i.d., as measured by the micturition interval (Figure 3). Control interval values for the GR63799X treatment groups were the following: 0.001 mg·kg^{-1}, 11 ± 2 min ($n = 8$); 0.003 mg·kg^{-1}, 12 ± 1 min ($n = 7$); 0.01 mg·kg^{-1}, 12 ± 1 min ($n = 7$); 0.1 mg·kg^{-1} 13 ± 1 min ($n = 4$); 0.3 mg·kg^{-1}, 9 ± 1 min ($n = 3$); and 1 mg·kg^{-1} 12 ± 2 min ($n = 4$). GR63799X had no significant effect on peak micturition pressure at the doses evaluated (percentage control: vehicle 104 ± 4%, 0.001 mg·kg^{-1} 83 ± 7%, 0.003 mg·kg^{-1} 108 ± 7%, 0.01 mg·kg^{-1} 104 ± 9%, 0.1 mg·kg^{-1} 96 ± 8%, 0.3 mg·kg^{-1} 97 ± 6% and 1 mg·kg^{-1} 106 ± 7%).

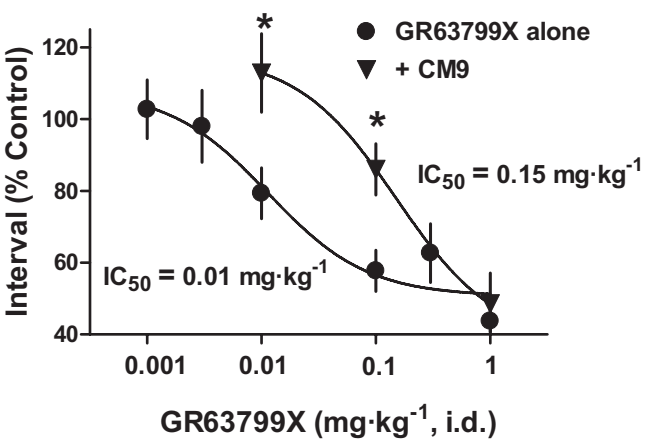

Figure 3 EP$_3$ receptor antagonist CM9 antagonized the reduction in micturition interval, induced by GR63799X. Dose–response curves of the effect of GR63799X on micturition interval, in the presence (+CM9) and absence (GR63799X alone) of the EP$_3$ receptor antagonist CM9 [30 mg·kg^{-1}, intraduodenal (i.d.)]. Each data point represents a separate group of 3–8 rats (*$P < 0.05$ vs. GR63799X alone same GR63799X dose, unpaired *t*-test).

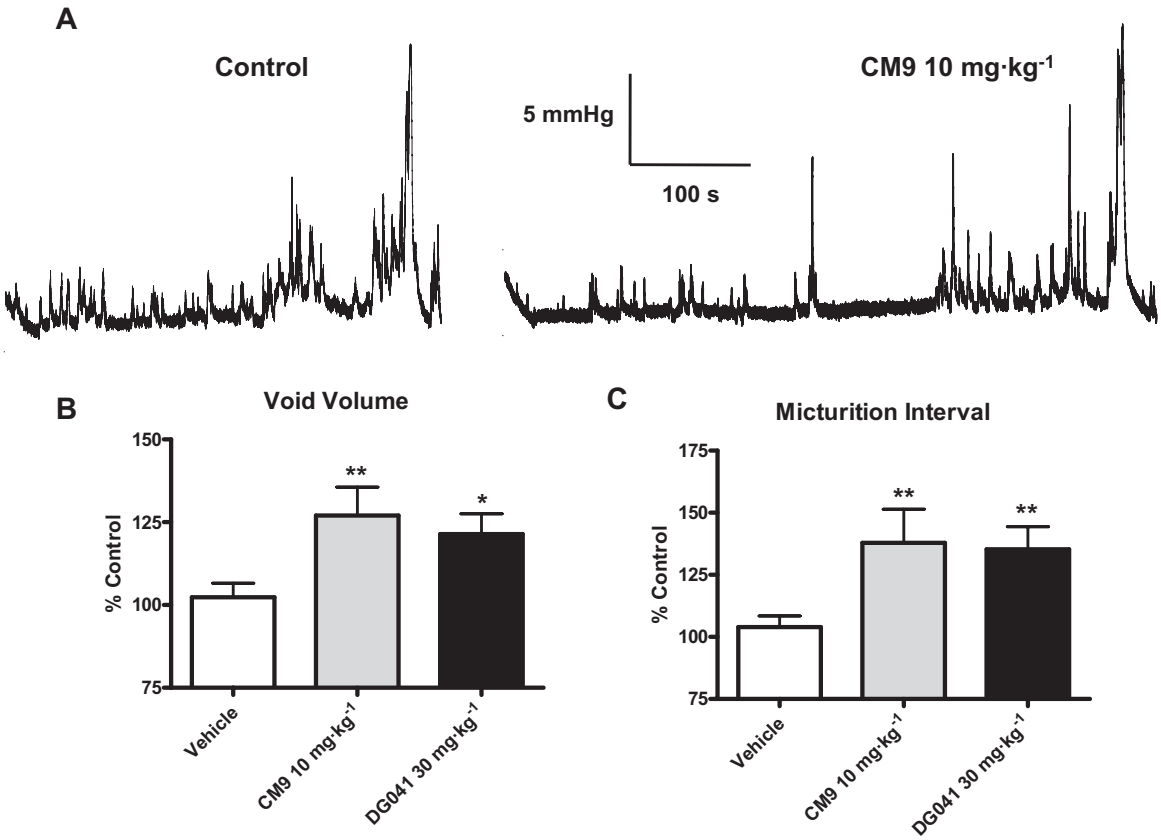

Figure 4 EP_3 receptor antagonists CM9 and DG041 increased bladder capacity. (A) Representative recording of the effect of 10 mg·kg^{-1} CM9 on cystometric urodynamics. CM9 and DG041 increased void volume (B) and micturition interval (C), compared with vehicle treatment. Data are expressed as a percentage of control for each group. Vehicle (n = 18), CM9 (10 mg·kg^{-1}, n = 8) and DG041 (30 mg·kg^{-1}, n = 8). (*P < 0.05, **P < 0.01, vs. vehicle, unpaired t-test).

Urodynamic response to EP_3 receptor antagonism

EP_3 receptor antagonism with the chemically distinct antagonists CM9 (10 mg·kg^{-1}, i.d.) and DG041 (30 mg·kg^{-1}, i.d.) enhanced the bladder capacity, as indicated by significant increases in both void volume and micturition interval when compared with vehicle treatment (Figure 4B and C respectively). In this study control void volumes (mL) and intervals (min) were the following: vehicle 0.9 ± 0.1, 9 ± 1 (n = 18); CM9 0.7 ± 0.1, 7 ± 1 (n = 8); and DG041 0.66 ± 0.08, 6.1 ± 0.7 (n = 8) respectively. A representative cystometric pressure recording under control conditions and after CM9 treatment at 10 mg·kg^{-1} is shown in Figure 4A. As was observed with EP_3 receptor activation (Figure 2), peak micturition pressure was not affected by EP_3 receptor antagonism with either CM9 or DG041 (Figure 4A; percentage control: vehicle 99 ± 4%, DG041 83 ± 7% and CM9 113 ± 10%).

EP_3 receptor antagonists inhibit the response to GR63799X

The ability of GR63799X to reduce the micturition interval and void volume was antagonized by pretreatment with CM9 (30 mg·kg^{-1}, i.d.). Assaying micturition interval, CM9 pretreatment evoked a clear rightward shift in the GR63799X dose–response curve, shifting the IC_{50} value for GR63799X from 0.01 mg·kg^{-1} in the absence of CM9 to 0.15 mg·kg^{-1} in the presence of CM9 (Figure 3). The reductions in void volume and micturition interval induced by GR63799X (0.1 mg·kg^{-1}, i.d.) were also antagonized by pretreatment with DG041 (3 and 30 mg·kg^{-1}, i.d.) and by CM9 at 3 mg·kg^{-1}, i.d. (Figure 5). Control void volume (mL) and interval (min) values prior to GR63799X (0.1 mg·kg^{-1}) challenge (after respective pretreatments) were the following: vehicle 1.1 ± 0.1, 12 ± 1; DG041 (3 mg·kg^{-1}) 0.72 ± 0.07, 6.8 ± 0.6; DG041 (30 mg·kg^{-1}) 1.2 ± 0.2, 10 ± 2; and CM9 (3 mg·kg^{-1}) 0.74 ± 0.09, 6 ± 1. Consistent with the effects of DG041 and CM9 on the GR63799X response being EP_3 receptor-mediated, and not via an alternative competing mechanism, vehicle challenge (instead of GR63799X) of CM9 (30 mg·kg^{-1}, i.d.)-pretreated rats was without effect (Figure 5; baseline values CM9 alone 30 mg·kg^{-1} 0.9 ± 0.1 mL, 8.8 ± 0.9 min). As was observed with GR63799X (Figure 2), or antagonist treatment alone (Figure 4), in the GR63799X/antagonist combination studies no significant alteration in peak micturition pressure was detected with GR63799X (0.1 mg·kg^{-1}) challenge after the various pretreatments (peak pressure as percentage control: vehicle 104 ± 6% (n = 10), DG041 (3 mg·kg^{-1}) 104 ± 76% (n = 4), DG041 (30 mg·kg^{-1}) 109 ± 12% (n = 4), CM9 (3 mg·kg^{-1}) 95 ± 9% (n = 4) and CM9 (30 mg·kg^{-1}) alone 97 ± 4% (n = 4)).

Activation of EP_3 receptors modulates urine production

As described above GR63799X evoked a significant reduction both in the micturition interval and average voided urine volume (Figure 2). However, in GR63799X-treated rats, the

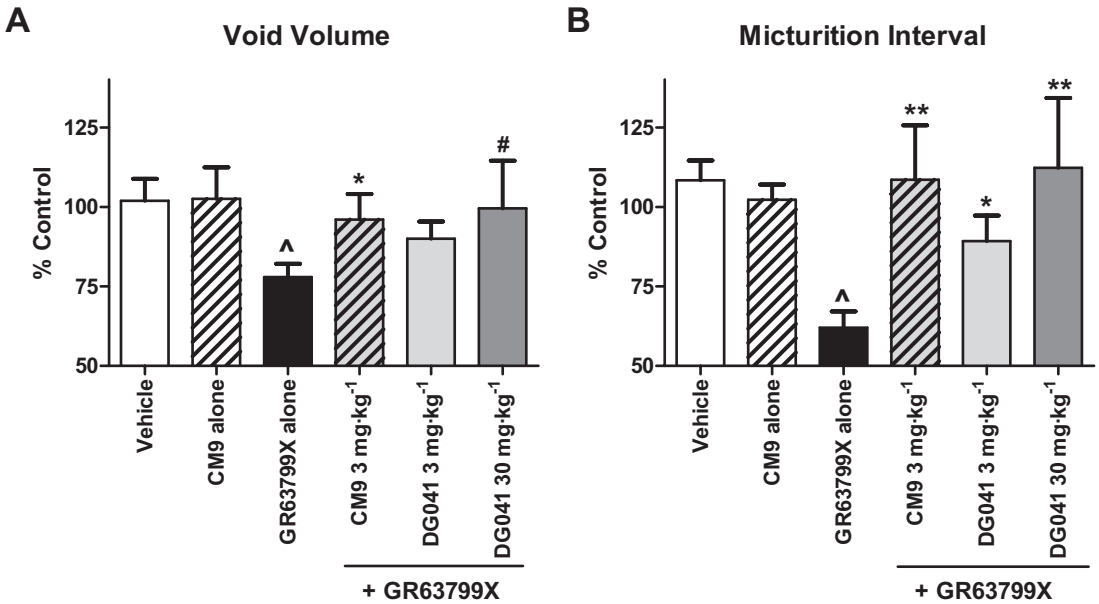

Figure 5 EP_3 receptor antagonists CM9 and DG041 antagonized the cystometric responses to EP_3 receptor activation with GR63799X. GR63799X [0.1 mg·kg^{-1}, intraduodenal (i.d.)] evokes a reduction in void volume (A) and micturition interval (B) that was antagonized by pretreatment with CM9 (3 mg·kg^{-1}, i.d.) or DG041 (3 and 30 mg·kg^{-1}, i.d.). CM9 pretreatment followed by vehicle instead of GR63799X challenge was without effect (CM9 alone, 30 mg·kg^{-1}). Vehicle and GR63799X alone $n = 10$ per group, and CM9 alone (30 mg·kg^{-1}), CM9 (3 mg·kg^{-1}), DG041 (3 mg·kg^{-1}) and DG041 (30 mg·kg^{-1}) $n = 4$ per group. ($^{\wedge}P = 0.008$ void volume vs. vehicle, $^{\wedge}P < 0.0001$ micturition interval vs. vehicle, unpaired *t*-test; $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{\#}P = 0.07$ vs. GR63799X alone, unpaired *t*-test).

reduction in micturition interval was significantly greater than that observed for the void volume (Figure 6A; $P = 0.01$, two-way repeated measures ANOVA). These 10 rats had an average control interval of 12 ± 1 min and void volume of 1.1 ± 0.1 mL at baseline. Comparing the volumes of urine voided to those infused into the bladder, via the bladder catheter during cystometrigrams, we noted that in the presence of GR63799X, the rats were voiding more volume than was infused into their bladders. This was reflected by the increase of urine flow in the presence of GR63799X, compared with vehicle treatment (Figure 6B). Vehicle treatment alone had no effect on urine flow. This suggests that in addition to reducing functional bladder capacity as indicated by the reduction in the average volume per void (Figure 2C), GR63799X (i.d.) also increased endogenous urine production.

Under control conditions, in these cystometric studies, endogenous urine flow is undetectable (Figure 6B control time points and vehicle group; Figure 6C control). In order to enhance endogenous urine production during cystometry rats were intra-arterially (IA) infused with saline (10 μL·min^{-1}), in addition to the typical continuous-filling of the bladder lumen (100 μL·min^{-1}). This IA infusion increased the vascular blood volume and thereby significantly enhanced endogenous urine flow (Figure 6C). The increase in urine flow observed with IA infusion of saline was comparable to that observed with GR63799X treatment (compare Figures 6B and C). Administration of the EP_3 receptor antagonist CM9 (30 mg·kg^{-1}) to IA saline infused rats nearly abolished the induced urine flow (Figure 6C). This suggests that EP_3 receptor antagonism could decrease endogenous urine production and was consistent with our observation that EP_3 receptor activation increased urine flow.

Discussion

Here we have demonstrated that EP_3 receptor modulation alters both functional urinary bladder capacity and endogenous urine flow in conscious SHR. EP_3 receptor activation induced bladder overactivity by decreasing the functional bladder capacity (void volume), and EP_3 receptor antagonism, with two chemically distinct antagonists, was shown to increase functional bladder capacity. In addition, in the same rats, activation of EP_3 receptors evoked diuresis, and EP_3 receptor antagonism induced an antidiuretic effect. Therefore, in addition to a role for EP_3 receptors in regulating bladder activity it appears that the EP_3 receptor has a role in regulating urine production.

We conclude that the observed effects on bladder activity and urine flow involve two mechanisms of action, and that the enhancement of urine flow was not the sole underlying cause of bladder overactivity induced by EP_3 receptor activation. As a result of an enhanced urine production alone, under these study conditions it would be expected that the volume of urine per micturition (void volume) would remain unaffected and a decrease in the interval would occur, or alternatively the void volume could increase, as observed in dogs and rabbits in response to diuresis (Levin *et al.*, 1995; McCafferty *et al.*, 2009). Because the void volume demonstrated a significant decrease in response to EP_3 receptor activation, this indicates that a kidney-independent, bladder-based, effect is likely. A bladder-based effect is further supported by a response to GR63779X delivered locally to the bladder lumen of mice (McCafferty *et al.*, 2008). Bladder infusion of GR63799X into mice did not evoke a detectable increase in urine production; probably as a result of the local

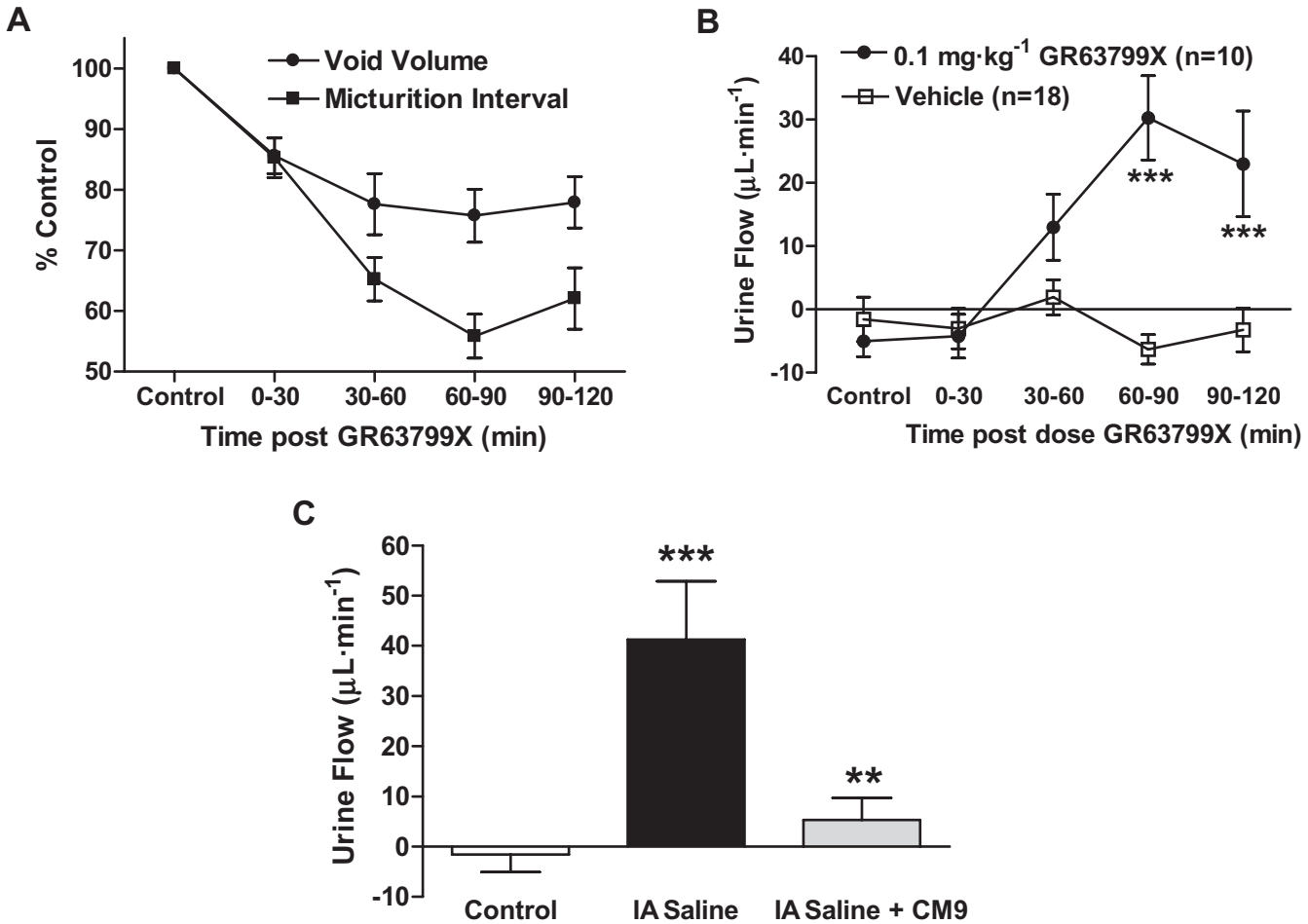

Figure 6 EP_3 receptor modulation altered urine production as assessed during continuous-filling cystometry. (A) Comparison of the average normalized response on void volume and micturition interval in response to GR63799X treatment ($n = 10$). The reduction in micturition interval was significantly greater than that in void volume ($P = 0.01$, two-way repeated measures ANOVA). (B) GR63799X [0.1 mg·kg^{-1}, intraduodenal (i.d.)] increased endogenous urine flow, compared with vehicle treatment ($P = 0.006$, two-way repeated measures ANOVA, ***$P < 0.001$ Bonferroni *post hoc* vs. vehicle). (C) Intra-arterial infusion of saline (IA saline) increases urine production, as indicated by the increase of urine flow. CM9 (30 mg·kg^{-1}, i.d.) antagonized this response ($P < 0.0001$ one-way ANOVA, **$P < 0.01$ vs. IA saline, ***$P < 0.001$ vs. control).

delivery of the compound to the bladder. However, dosing GR63799X i.d. provides access to the urinary bladder and to the renal and cardiovascular systems, and has revealed the additional diuretic consequence of systemic EP_3 receptor activation shown here. Consistent with a dual functional contribution (bladder activity and urine flow) to the effect of GR63799X on micturition interval, GR63799X demonstrated a significantly larger reduction on the interval than on the void volume. The interval reduction due to GR63799X represents the additive effect of activating EP_3 receptors on bladder function and on urine production, whereas the smaller response on the void volume presumably represents the effect on bladder function alone.

Previously we have shown that inhibiting EP_3 receptor function by genetic ablation of the EP_3 receptor gene enhanced conscious bladder capacity under basal conditions (McCafferty *et al.*, 2008). Our pharmacological data presented here utilizing conscious rats demonstrated an enhancement of bladder capacity by pharmacological inhibition of the EP_3 receptor with chemically distinct antagonists. These findings in conscious rats are consistent with the phenotype displayed by EP_3 receptor knockout mice. EP_3 receptor-mediated modulation of bladder activity has been proposed to occur via an afferent mechanism (McCafferty *et al.*, 2008; Su *et al.*, 2008a). Therefore, SHR were chosen for the current study as they have been reported to display afferent-based urinary bladder hyperactivity (Persson *et al.*, 1998; Spitsbergen *et al.*, 1998). However, no differences in the expression or function of EP_3 receptors in the SHR have been observed compared with normal rats (Su *et al.*, 2008a), suggesting that the EP_3 receptor-mediated responses reported here may well be observed in other rat strains. This is supported by equivalent EP_3 receptor-mediated responses observed in anaesthetized bladder models performed in several strains (Su *et al.*, 2008a).

The responses to activation of EP_3 receptors with GR63799X on void volume and micturition interval were inhibited by EP_3 receptor antagonism with the two chemically distinct EP_3 receptor antagonists, CM9 and DG041, suggesting that the reported effects of GR63799X are indeed mediated via the EP_3 receptor. The selectivity of GR63799X

for EP_3 receptor is further supported by the loss of GR63799X-induced bladder overactivity in EP_3 receptor KO mice (McCafferty *et al.*, 2008), along with previously published binding studies (Bunce *et al.*, 1991; Kiriyama *et al.*, 1997). Using a FLIPR-based calcium influx assay we demonstrated the ability of CM9 and DG041 to antagonize the rat EP_3 receptor and confirmed activity at the human receptor (Su *et al.*, 2008b). In general DG041 was more potent than CM9, and antagonism at the human EP_3 receptor was more potent than at the rat receptor for both compounds under our conditions. The selectivity of CM9 and DG041 for the EP_3 receptor is supported by binding assays to other members of the EP receptor family (EP_1, EP_2 and EP_4) and other PG receptors (IP, TP and FP; Coleman *et al.*, 1994; Su *et al.*, 2008b). DG041 demonstrates a low nanomolar affinity for the PGD_2 (DP) receptor (Su *et al.*, 2008b). This is not the case for the chemically distinct antagonist, CM9, which we have used to replicate the effects of DG041, and therefore DG041 acting via the DP receptor is unlikely to explain the functional DG041 responses reported here in the conscious rat. Furthermore, the ability of CM9 and DG041 to antagonize the response to GR63799X *in vivo* strongly suggests that these compounds are active at the EP_3 receptor at these doses in the rat. The possibility that CM9 was working via an independent competing mechanism in these experiments was ruled out by the lack of response (increase in void volume and interval) in CM9-pretreated rats, challenged with vehicle in the place of GR63799X. Taken together, our *in vivo* and *in vitro* selectivity data support the functional effects observed here with CM9 and DG041 as being mediated by EP_3 receptors.

The mRNA for EP_3 receptors has been specifically detected in L6/S1 dorsal root ganglia that innervate the rat urinary bladder, and this expression was comparable to that detected in the intact rat bladder (Su *et al.*, 2008a). Expression of EP_3 receptors in intact bladders may represent their expression on nerve terminals located within the bladder wall. Alternatively, or in addition to a neuronal expression, EP_3 receptors may be expressed in the smooth muscle, urothelium and/or other cell types present within the intact bladder. A functional role for the bladder-based EP_3 receptor expression is supported by the ability of GR63799X delivered to the bladder to selectively induce bladder overactivity in EP_3 receptor wild-type mice (McCafferty *et al.*, 2008), while the functional importance of an afferent-based expression is supported by the finding that inhibition of peripheral EP_3 receptors decreased bladder-induced afferent firing (Su *et al.*, 2008a). In addition, efficacy in anaesthetized, bladder models has also been described via central delivery of EP_3 receptor antagonists suggesting a modulatory role for EP_3 receptors in the central control of the micturition reflex (Su *et al.*, 2008b). The effects on conscious rat bladder urodynamics described here with i.d. administration of compounds were likely to have been mediated via modulation of peripheral EP_3 receptors as DG041 does not penetrate the blood–brain barrier (Su *et al.*, 2008a). Expression of EP_3 receptors on afferent nerves is likely to play an important functional role in the regulation of urodynamics. We cannot, however, eliminate a contribution from EP_3 receptors expressed in other cell types, in the modulation of bladder function.

EP_1 receptors have been implicated in urinary bladder function based on studies utilizing EP_1 receptor KO mice (Schröder *et al.*, 2004) and EP_1 receptor antagonists (Maggi *et al.*, 1988b; Lee *et al.*, 2007). In these studies PGE_2-induced bladder overactivity was inhibited by EP_1 receptor KO or antagonist administration. Although the EP_3 receptor KO yielded a reduced sensitivity to infusion of PGE_2 into the bladder, the response was not completely prevented (McCafferty *et al.*, 2008). Therefore, under pathological conditions of bladder overactivity when PGE_2 levels are increased, as occurs in preclinical models and patients (Park *et al.*, 1999; Hu *et al.*, 2003; Kim *et al.*, 2005; 2006), EP_1 and EP_3 receptors may both play a role in PGE_2-mediated overactivity. The data provided here in conscious rats using pharmacological modulators further supports an involvement of EP_3, in addition to EP_1 receptors, in regulating conscious urodynamics.

PGE_2 is produced by various cell types within the kidney, regulates blood flow and the permeability of various segments of the nephron, including the collecting ducts (Hao and Breyer, 2008). In this study EP_3 receptor activation with GR63799X increased urine flow indicating that EP_3 receptor activity favours the production of urine, while EP_3 receptor antagonism with CM9 inhibited urine flow elicited by arterial saline infusion indicating that EP_3 receptors play a functional role during volume overload. These results are consistent with studies in EP_3 receptor KO mice suggesting that EP_3 receptor activity functions to regulate urine osmolality (Fleming *et al.*, 1998). EP_3 receptors are highly expressed in the renal medulla and cortical collecting duct (Breyer *et al.*, 1993; Sugimoto *et al.*, 1994; Takeuchi *et al.*, 1994a,b; Taniguchi *et al.*, 1994) where PGE_2 has been shown to inhibit water re-absorption by reducing epithelial cAMP levels (Hébert *et al.*, 1991; Hao and Breyer, 2008). Furthermore, EP_3 receptors are coupled to G_i proteins that reduce cAMP and thus promote diuresis as exemplified by its ability to inhibit the antidiuretic response of vasopressin (Grantham and Burg, 1966; Takeuchi *et al.*, 1994a,b; Breyer *et al.*, 1996; Nielsen *et al.*, 1999).

In addition, the changes in urine flow, dependent on EP_3 receptors, described here could result from altered renal blood flow (Edwards, 1985; Tang *et al.*, 2000; Audoly *et al.*, 2001), and/or changes in mean arterial pressure in response to the EP_3 receptor modulators (Audoly *et al.*, 1999; Zhang *et al.*, 2000; van Rodijnen *et al.*, 2007). Volume overload by saline infusion may increase EP_3 receptor activity in the renal/cardiovascular system and contribute to the induced urine flow. While it is likely that EP_3 receptor-mediated modulation of renal output involves an alteration in tubular permeability, haemodynamic changes or other undescribed EP_3 receptor-mediated responses, may also contribute.

In conclusion, we have demonstrated in conscious rats that activation of EP_3 receptors has a dual functional consequence inducing bladder overactivity and diuresis, and that antagonism of EP_3 receptors has the expected, opposing response of reducing bladder activity and urine output. A development of EP_3 receptor antagonists as a therapy for bladder overactivity would require a particular consideration of the renal consequences of EP_3 receptor modulation.

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Conflict of interest

No conflicts of interest. All the authors are employees of GlaxoSmithKline Pharmaceuticals.

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