Modulation of bladder function by prostanoid EP3 receptors in the central nervous system


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Su X, Leon LA, Wu CW, Morrow DM, Jaworski J-P, Hieble JP, Lashinger ES, Jin J, Edwards RM, Laping NJ. Modulation of bladder function by prostanoid EP3 receptors in the central nervous system. Am J Physiol Renal Physiol 295: F984–F994, 2008. First published July 16, 2008; doi:10.1152/ajprenal.90373.2008.—Prostanoid EP3 receptors in the central nervous system (CNS) may exert an excitatory effect on urinary bladder function via modulation of bladder afferent pathways. We have studied this action, using two EP3 antagonists, (2E)-3-[1-(2,4-dichlorophenyl)methyl]-5-fluoro-3-methyl-1H-indol-7-yl]-N-[4,5-dichloro-2-thienyl]sulfonfyl]-2-propanamide (DG041) and (2E)-N-[5-bromo-2-(methylxylo)phenyl]-sulfonfyl]-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide (CM9). DG041 and CM9 were proven to be selective EP3 antagonists with radioligand binding and functional fluorescent imaging plate reader (FLIPR) assays. Their effects on rhythm-induced bladder contraction and the visceromotor reflex (VMR) response to urinary bladder distension (UBD) were evaluated in female rats after intrathecal or intracerebroventricular administration. Both DG041 and CM9 showed a high affinity for EP3 receptors at subnanomolar concentrations without significant selectivity for any splice variants. At the human EP3C receptor, both inhibited calcium influx produced by the nonselective agonist PGE2. After intrathecal or intracerebroventricular administration both CM9 and DG041 dose-dependently reduced the frequency, but not the amplitude, of the bladder rhythmic contraction. With intrathecal administration DG041 and CM9 produced a long-lasting and robust inhibition on the VMR response to UBD, whereas with intracerebroventricular injection both compounds elicited only a transient reduction of the VMR response to bladder distension. These data support the concept that EP3 receptors are involved in bladder micturition at supraspinal and spinal centers and in bladder nociception at the spinal cord. A centrally acting EP3 receptor antagonist may be useful in the control of detrusor overactivity and/or pain associated with bladder disorders.

rhythmic contraction; nociception; bladder distension

SENSORY INNERVATION of the urinary bladder originates in hypogastric and pelvic nerve afferent fibers and is relayed to the spinal cord and supraspinal levels of the central nervous system (CNS). These nerves function both in normal micturition and in pathological states such as overactive bladder (OAB) and bladder pain. The literature suggests a complex regulatory role for prostanoids (PGs) in multiple aspects of urinary bladder physiology/pathophysiology. PGs are not stored in vesicles but are synthesized from arachidonic acid via the cyclooxygenase (COX-1 and COX-2) pathway in response to various physiologic/pathophysiologic stimuli. Prostaglandins (PGs) are not stored in vesicles but are synthesized from arachidonic acid via the cyclooxygenase (COX-1 and COX-2) pathway in response to various physiologic/pathophysiologic stimuli. PGs are released immediately after synthesis from urothelium and detrusor smooth muscle (29, 40) as well as from neurons and glial cells along bladder afferent pathways (28, 36). There are five naturally occurring PGs, PGD2, PGE2, PGF2, PGI2, and thromboxane A2; each has a distinct receptor class, named DP, EP, IP, and TP, respectively (6a). Actions of PGE2 are due to activation of EP receptor subtypes EP1, EP2, EP3, and EP4; these EP receptor subtypes are coupled to different signal transduction pathways (36).

PGE2 levels have been reported to be elevated in the urine of OAB patients (19, 20). Intravesical administration of PGE2 facilitates the micturition reflex (14, 28, 44); in contrast, inhibition of PG synthesis by COX inhibitors attenuates bladder hypersensitivity (2, 12, 18, 50). PGE2 contributes not only to bladder hypersensitivity but also to the processing of pain, by sensitizing the peripheral terminals of afferent nerves and neurons of the brain and spinal cord (21, 22, 34, 38, 42). In cutaneous pain models, activation of spinal EP1, EP2, and/or EP3 receptors caused allodynia and/or hyperalgesia (17, 31). The mechanisms involved in the modulation of nociception at the supraspinal level are different. EP receptor activation is involved in not only the progression (EP3 receptors) but also the suppression (EP1 receptors) of pain transmission (11, 37). There have only been a few reports on the role of EP receptors in visceral pain models. On the basis of studies in EP3 receptor-deficient mice, the EP3 receptor appears to mediate the enhancement of nociception by endotoxin (52).

On the basis of affrent mechanisms, urgency and nociception seem to have different pathways because bladder overactivity and nociceptive behavior could be independently attenuated by intravesical administration of capsaicin (39). However, urgency and nociception do have many features in common with their sensitizations by similar agents, e.g., tachykinins, ATP, and other neuropeptides (1, 46).

In this study, we used specific antagonists to determine more precisely the role of central EP3 receptors in the modulation of bladder urgency and pain sensation. Two tool compounds, (2E)-3-[1-(2,4-dichlorophenyl)methyl]-5-fluoro-3-methyl-1H-indol-7-yl]-N-[4,5-dichloro-2-thienyl]sulfonfyl]-2-propanamide (DG041, Fig. 1A; Ref. 55) and (2E)-N-[5-bromo-2-(methylxylo)phenyl]sulfonfyl]-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide (CM9, Fig. 1B; compound 9 from Ref. 15), were evaluated for EP3 potency and selectivity with radioligand binding assays and functional studies on
PGE₂-induced calcium influx in hEP₃C U2OS cells. These compounds were then tested in anesthetized rat models by intrathecal (it) and intracerebroventricular (icv) routes. The micturition reflex sensitivity was tested in the bladder rhythmic contraction model. Pain sensitivity was tested by measuring visceromotor reflex (VMR) and cardiovascular (pressor) responses to noxious urinary bladder distension (UBD).

**MATERIALS AND METHODS**

**Binding studies.** U2OS (human osteosarcoma) cells (American Type Culture Collection, Manassas, VA) transected with the full-length cDNA of human (h)EP₃E, hEP₃C, rat (r)EP₃A, rEP₃C, or hDP receptors, Chinese hamster ovary (CHO) cells expressing the hEP₃A, hEP₁, hEP₂, and hTP receptors, and human embryonic kidney (HEK) cells expressing hEP₃E, hIP, and hFP receptors were used in this study. All cDNA clones were generated internally. However, binding studies for hIP and hFP receptors were done by Cerep (Redmond, WA).

Cell membranes were prepared according to the procedure of Nambi et al. (35). Briefly, the cells were grown in 245 mm tissue culture plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were washed with Dulbecco’s phosphate-buffered saline containing a protease inhibitor cocktail (Roche) and scraped in the same buffer. After centrifugation for 10 min to remove unbroken cells and nuclei, the supernatants were centrifuged at 40,000 x g, the cells were lysed by freezing in liquid nitrogen and thawing on ice, followed by homogenization in lysis buffer containing 20 mM Tris·HCl pH 7.5 and the protease inhibitor cocktail. After an initial centrifugation at 800 g for 10 min to remove unbroken cells and nuclei, the supernatants were centrifuged at 40,000 g for 1 h and the pellet was resuspended in 0.25 M sucrose, 50 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM EDTA, and protease inhibitor cocktail and stored in small aliquots at −70°C after freezing in liquid N₂. Protein was determined by the bicinchoninic acid (BCA) method with bovine serum albumin as the standard. The BCA protein assay kit was purchased from Pierce Biotechnology.

Binding assays using [³H]agonists (PerkinElmer) to cell membranes were performed at room temperature for 60 min in 1–6 μg of membrane protein, a total volume of 200 μl of 50 mM Tris pH 7.5, and 10 mM MgCl₂. Binding was initiated by the addition of increasing concentrations of indicated radioligand (Table 1) for saturation binding experiments in the absence (total binding) or presence (nonspecific binding) of 1 μM nonradiolabeled ligand. The incubation was stopped by dilution with cold buffer and filtering through Whatman GF/C glass fiber filters (Whatman International, Maidstone, UK) presoaked in water. The filters were washed five times (3 ml each time) with the same buffer and a Brandel (Gaithersburg, MD) cell harvester and counted with a liquid scintillation counter at an efficiency of 50%. Competition assays of the compounds were performed with 10 μM to 0.1 nM compounds (in DMSO) with 10-fold dilution. The final DMSO concentration in the assay tube was 1%. Saturation as well as competition binding experiments were performed in duplicate determinations, and each experiment was repeated two or three times.

IC₅₀ values were determined by GraphPad analysis. The inhibition constant (Kᵢ) value was calculated with the equation Kᵢ = IC₅₀/(1 + L/Kₐ), where Kₐ is the dissociation constant and L is the concentration of radioligand (5). The Kᵢ values express the potency of DG041 and CM9 in competing for radioligand binding in cells expressing different PG receptors.

**Table 1. Effects of DG041 and CM9 on [³H]PGE₂ binding to prostaglandin receptors**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kᵢ, nM</th>
<th>Ligand (nM)</th>
<th>Ligand for Nonspecific Binding</th>
<th>DG041</th>
<th>CM9</th>
</tr>
</thead>
<tbody>
<tr>
<td>hEP₃A</td>
<td>1.9</td>
<td>[³H]PGE₂ (2)</td>
<td>PGE₂</td>
<td>0.08±0.01</td>
<td>0.21±0.17</td>
</tr>
<tr>
<td>hEP₃E</td>
<td>2.1</td>
<td>[³H]PGE₂ (2)</td>
<td>PGE₂</td>
<td>0.3</td>
<td>0.24</td>
</tr>
<tr>
<td>hEP₃C</td>
<td>2.1</td>
<td>[³H]PGE₂ (2)</td>
<td>PGE₂</td>
<td>0.12</td>
<td>N/A</td>
</tr>
<tr>
<td>rEP₃A</td>
<td>1.2</td>
<td>[³H]PGE₂ (2)</td>
<td>PGE₂</td>
<td>0.22±0.06</td>
<td>0.25±0.03</td>
</tr>
<tr>
<td>rEP₃C</td>
<td>1.1</td>
<td>[³H]PGE₂ (2)</td>
<td>PGE₂</td>
<td>0.32±0.04</td>
<td>0.78±0.17</td>
</tr>
<tr>
<td>hEP₃</td>
<td>8.9</td>
<td>[³H]PGE₂ (10)</td>
<td>PGE₂</td>
<td>1.000</td>
<td>&gt;8,400</td>
</tr>
<tr>
<td>hEP₃</td>
<td>20</td>
<td>[³H]PGE₂ (10)</td>
<td>PGE₂</td>
<td>9.200</td>
<td>&gt;9,200</td>
</tr>
<tr>
<td>hEP₃</td>
<td>0.8</td>
<td>[³H]PGE₂ (2)</td>
<td>PGE₂</td>
<td>500</td>
<td>&gt;3,200</td>
</tr>
<tr>
<td>hIP*</td>
<td>8</td>
<td>[³H]Iloprost (10)</td>
<td>Iloprost</td>
<td>5,300</td>
<td>5,300</td>
</tr>
<tr>
<td>hTP</td>
<td>2.4</td>
<td>[³H]SQ29845 (2)</td>
<td>SQ29845</td>
<td>600</td>
<td>574</td>
</tr>
<tr>
<td>hFP*</td>
<td>3.9</td>
<td>[³H]PGE₂ (2)</td>
<td>Cloprostenol</td>
<td>1,900</td>
<td>&gt;6,500</td>
</tr>
<tr>
<td>hDP</td>
<td>1.8</td>
<td>[³H]PGE₂ (2)</td>
<td>PGD₂</td>
<td>3.03</td>
<td>2,390</td>
</tr>
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</table>

Values are means ± SE. Kᵢ, dissociation constant; Kᵢ, inhibition constant; h, human; r, rat; N/A, not available. *Binding studies by Cerep (Redmond, WA).
Fluorescent imaging plate reader assay for functional evaluation of hEP3C receptor. Hanks’ balanced salt solution (1×, Sigma-Aldrich, St. Louis, MO) was supplemented with 20 mM HEPES (Sigma-Aldrich). Test compounds were diluted with 65 μL of Hanks’ solution with 20 mM HEPES and 0.01% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich) for a final concentration of 25 μM. The final concentration of EP3C receptor agonist PGF2α was 1 μM. Double transfection of U2OS cells was created with a vector carrying the full-length EP3 DNA (0.25% hEP3C BacMam). Parental U2OS cells were transfected with 0.25% Gqi5 virus. The cells were seeded into CellCoat 384-well black, clear bottom, poly-D-lysine-coated microplates (Greiner Bio-One, Frickenhausen, Germany) at a density of 15,000 cells per well and incubated for 24 h at 37°C and 5% CO2 to attain 80–100% confluency. The cell culture medium was removed, and cells were resuspended in 20 μL of dye loading buffer containing 2 μM fluo-4 AM (Molecular Devices, Sunnyvale, CA), 500 μM brilliant black (Molecular Devices), and 2.5 mM probenecid (Sigma-Aldrich) and incubated for 1 h at 37°C. Afterward, the plates were placed into a fluorometric imaging plate reader (FLIPR, Molecular Devices), and 10 μL of test compounds was added to the cell plates and incubated for 10 min at 37°C. After the incubation and baseline readouts, cells were stimulated with an EC80 of PGE2 and the fluorescence ([Ca2+]i) was captured via FLIPR for 65 s. [Ca2+]i responses per well were measured as the maximum [Ca2+]i relative fluorescence units (RFU) less the baseline RFU, and the statistical difference was plotted against the test compound dose-response concentrations for curve fitting with GraphPad Prism 4.0 (GraphPad Software). Negative log of the compound concentration giving 50% inhibition (pIC50) or negative log of the compound concentration giving 50% activation (pEC50) was calculated.

In vivo bladder rhythmic contraction. Female Sprague-Dawley rats weighing 200–300 g (n = 83) were anesthetized with 3% isoflurane during surgery. Anesthetized rats were placed on a hot water-circulating heating pad during the studies and were euthanized on completion of experimental procedures by an overdose of pentobarbital sodium (120 mg/kg iv; Vortech Pharmaceuticals, Dearborn, MI). The experimental protocol was approved by the Institutional Animal Care and Use Committee of GlaxoSmithKline Pharmaceuticals.

One jugular vein was cannulated with polyethylene tubing for intravenous administration of urethane. For intrathecal administration, an intrathecal catheter (CS-1, Recath) was inserted through the atlantooccipital membrane and passed caudally for 8.5 cm to ensure that the catheter tip was located just below the lumbosacral enlargement. For intracerebroventricular administration, rats with intracerebroventricular cannulation were ordered directly from Tacson and housed for 3–7 days before a study. Briefly, anesthetized rats were immobilized in a stereotaxic frame. A sterile guide cannula consisting of a length of 22-gauge stainless steel hypodermic tubing encased in plastic was stereotaxically placed, and the internalized tip was located in the lateral ventricle. Coordinates calculated in relation to bregma were AP (anterior/posterior) = −0.8 mm, ML (medial/lateral) = +1.2 mm (left side), and DV (dorsal/ventral) = −4.8 mm. The placement for intrathecal and intracerebroventricular cannulas was confirmed by showing the efficacy of local injection of morphine (100 μg/10 μl) in all experiments and by staining with malachite green dye (Alfa Aesar) in some experiments, after completion of studies. If these tests showed the intrathecal or intracerebroventricular cannula to be improperly placed, data from the experiment were excluded. A cannula (PE-50) was placed into the bladder via the urethra, and the urethra was ligated to ensure an isovolumetric bladder. On completion of surgical procedures, slow intravenous infusion of urethane (1.2 g/kg, ethyl carbonate; Sigma, St. Louis, MO) was given over 15 min. Saline bladder infusion procedures were begun 30 min after the final dose of urethane.

The urethral cannula was connected via a T connector to a low-volume transducer (ADInstrument MLT0380D, Colorado Springs, CO) and to a 20-ml syringe with a perfusion pump. The signal was amplified through a DC amplifier (ADInstrument ML119). For the rhythmic bladder contraction studies, saline at room temperature was infused into the bladder at a rate of 50 μl/min until the first spontaneous contraction (≥10 mmHg) was observed. The infusion rate was then lowered to 10 μl/min and continued until rhythmic bladder contraction (3–5 contractions) was established; the saline infusion was then terminated. The vehicle or test compound was administered after a 15-min control period. After administration, bladder contractions were recorded for 20–30 min. The frequency/interval and amplitude of the bladder contractions were recorded. The mean control value was calculated from the average of values obtained during the last 5 min before dosing. After dosing, mean responses were determined for each 5-min interval (24).

In vivo VMR and pressure responses to urinary bladder distention. Female Sprague-Dawley rats (n = 60) were anesthetized initially with 3% isoflurane. Cannulas for intrathecal or intracerebroventricular administration and for bladder distention were implanted as described above. To measure blood pressure, the right carotid artery was catheterized with PE-50 tubing. The arterial catheter was linked to a low-volume transducer (ADInstrument MLT0380D), and signal was amplified through a DC amplifier (ADInstrument ML119). The bladder catheter was linked to a bladder distention control device. The bladder was distended with saline by regulating air inflow into a Mariott bottle from a valve interface distension control device (University of Iowa Bioengineering, B482C-1; Ref. 47). Two needle electrodes were sutured into the oblique abdominal musculature just above the inguinal ligament. Abdominal contractions were quantified.
by action potentials of electromyographic activity. Action potentials were initially amplified through a low-noise AC differential amplifier (ADInstrument EC4-400) and processed with the AD data acquisition program (PowerLab 16/30, ML880). Raw action potentials of myoelectric activities, bladder pressure, and blood pressure were displayed online continuously. All data were analyzed off-line with the AD PowerLab program (Chart 8).

After completion of the surgical preparation, the isoflurane concentration was reduced until flexion reflex response could be evoked by pinch of the foot without spontaneous escape behaviors (1% isoflurane). For UBD, all rats received a series of at least six phasic bladder distensions at 60 mmHg for 30 s at 3-min intervals to evaluate response stability to repeated bladder distension. Test compound or vehicle was administered only after four consistent responses had been elicited and given 2 min before the onset of distension.

The electromyographic activity was integrated and calculated as the area under the curve. The VMR response to the stimulus was defined as the increase in electromyographic activity during UBD from the baseline activity before each response. Pressor response was quantified as the peak change in mean arterial pressure during UBD compared with the average level during a baseline period immediately before UBD. After compound administration, response was expressed as a percentage of the mean control response (the average of the 4 UBD responses before compound administration).

Data analysis. All data are expressed as means ± SE. Results were analyzed with Student’s t-test or analysis of variance (ANOVA) with repeated measures by Prism 4 (GraphPad Software, San Diego, CA). A value of <0.05 was considered statistically significant.

Compounds. DG041 (mol wt 592.326) and CM9 (mol wt 558.43) were synthesized from the Department of Medicinal Chemistry, Glaxo-SmithKline. Compounds were dissolved in 10% DMSO in 34% (2-hydroxypropyl)-β-cyclodextrin and injected in 10 μl after 20-μl (it) or 5-μl (icv) saline flush.

RESULTS

Determination of antagonist activities of DG041 and CM9 at EP3 receptors. The nonselective agonist PGE2 and the selective agonist sulprostone bound with high affinity to membranes prepared from U2OS cells expressing the C splice variant of the human EP3 receptor (hEP3C) (Fig. 2A). Both DG041 and CM9 had high affinities to hEP3C as well as other splice variants of human and rat EP3 receptors; no significant differences in affinity (Ki) were observed (Table 1). Similar experiments showed both compounds to have a high degree of selectivity versus other PG receptors (IP, TP, FP, and DP receptors), except that DG041 showed some affinity to DP receptor. The ratio of binding affinity for EP3 to DP was ~10- to 40-fold (Table 1).

With a FLIPR assay, PGE2 was shown to induce Ca2+ influx in U2OS cells expressing the hEP3C receptor. The pEC50 for this response was 8.52 ± 0.02 (8.48–8.55). DG041 and CM9 produced a concentration-related inhibition of the PGE2 response (Fig. 2B). Serum significantly reduced the antagonist potency (pIC50) of DG041 [8.07 ± 0.02 (8.02–8.12) to 7.82 ± 0.02 (7.78–7.88); P < 0.0001] and CM9 [7.25 ± 0.03 (7.19–7.30) to 6.03 ± 0.04 (5.95–6.11); P < 0.0001, 2-way ANOVA]. Confirming the radioligand binding results, a functional study using the FLIPR assay showed DG041 to have a
pIC$_{50}$ of 7.67 ± 0.12 ($n = 4$) as an antagonist of PGD$_2$-induced Ca$^{2+}$ influx in cells expressing the DP receptor. DG041 had no agonist activity at the DP receptor (pEC$_{50}$ < 4.3, $n = 3$).

**Effect of intrathecal or intracerebroventricular administration of DG041 and CM9 on micturition reflex.** Intrathecal administration of vehicle [10% DMSO in 34% (2-hydroxypropyl)-β-cyclodextrin] did not produce a significant change in either frequency or amplitude of volume-induced rhythmic bladder contractions. Administration of DG041 or CM9 inhibited the frequency of bladder contraction, sometimes completely shutting down contractions. In the latter case, contractions resumed after a short period of disappearance (<20 min). Unless contractions were shut down, the contraction amplitude was not reduced by either antagonist. When contractions disappeared, the contraction amplitude was not analyzed in summarized graphs. Examples of the effect of DG041 and CM9 are shown in Fig. 3. Figure 4 summarizes the effect of DG041 and CM9 on bladder rhythmic contraction following intrathecal administration. Analysis of their effect on the frequency of the rhythmic contraction did not show a significant difference in the potency of DG041 and CM9. The minimal effective dose was 30 nmol for either compound.

Administrations of DG041 and CM9 via the intracerebroventricular route produced a similar pattern of inhibition on the bladder rhythmic contraction, reducing the frequency but not the amplitude of the contraction. The minimal effective doses for DG041 and CM9 were 30 nmol and 300 nmol, respectively. Examples of experimental records are shown in Fig. 5 and summarized data in Fig. 6.

**Effect of intrathecal or intracerebroventricular administration of DG041 and CM9 on nociceptive responses to bladder distension.** As we have shown previously (47, 48), VMR and pressor responses were reproducible with repeated noxious UBds. Vehicle [10% DMSO in 34% (2-hydroxypropyl)-β-cyclodextrin it or icv] did not produce significant changes in VMR or pressor responses to UBD.

Intrathecal administration of DG041 at 30 nmol and CM9 at 300 nmol produced a long-lasting inhibition of the VMR responses to noxious UBD (Fig. 7). A two-way repeated-measures ANOVA test followed by a post hoc analysis showed that the regression of the VMR response produced by either DG041 or CM9 was statistically significant ($P < 0.05$, post hoc analysis). Both antagonists tended, in some experiments, to attenuate the pressor response to UBD; statistical analysis showed this effect to be nonsignificant (Fig. 8).

At maximal intrathecal doses, DG041 (100 nmol) and CM9 (300 nmol) produced a sustained inhibition of the VMR response. In contrast, intracerebroventricular administration of the same doses of DG041 and CM9 produced only a transient inhibition of the VMR response to UBD. Figure 9 depicts typical examples of such actions on both VMR and pressor responses to noxious UBD by intracerebroventricular administration of vehicle, DG041 (100 nmol), and CM9 (300 nmol). Figure 10 summarizes the effect of intracerebroventricular DG041 and CM9 on VMR and pressor responses to noxious UBD.

![Fig. 4. Time course for the effect of vehicle, DG041 (A and B), and CM9 (C and D) on the frequency (A and C) and the amplitude (B and D) of volume-induced contractions after intrathecal administration. Responses are represented as % of control, where the baseline response before administration is defined as 100%. Significance of differences between test and control values was determined by ANOVA test.]
DISCUSSION

In the present study we demonstrated that DG041 and CM9 were suitable pharmacological tools for evaluation of the functional roles of central EP3 receptors in the control of the micturition reflex and bladder nociceptive responses. The EP3 receptor antagonists produced a strong and sustained inhibition of the bladder rhythmic contraction after either intrathecal or intracerebroventricular administration but a significant inhibition of the VMR responses to bladder distension only by the intrathecal route. This suggests a different site of action for EP3 receptor-mediated modulation of different responses to activation of afferent fibers from the urinary bladder. Therefore, urgency and pain could be independently modulated.

Both DG041 and CM9 are potent EP3 antagonists (affinity at subnanomolar concentrations). The in vitro efficacies by binding assay and FLIPR assay were consistent with the original reports (3, 15, 55). Several differences in the PG receptor selectivity profiles of the two EP3 antagonists were noted in the in vitro assays. 1) CM9 is more selective for EP3 receptors versus other PG receptors, and DG041 is only ~10- to 40-fold selective for EP3 versus DP receptors. 2) DG041 seems slightly more potent than CM9. 3) Binding to serum proteins could significantly decrease the in vivo potency of both compounds, especially CM9. Similar findings have been reported by Belley et al. (Ref. 3, e.g., compound 7c).

DG041 may act as a mixed EP3/DP receptor antagonist, but its DP antagonist activity does not seem important in the readout of our in vivo studies since DG041 and CM9 showed similar activity. Indeed, DP receptors have a very discrete tissue distribution, highly localized in the CNS and intestine (4, 53, 54). If the DP antagonist activity of DG041 did contribute, a reduced inhibitory effect might be expected with this antagonist because of the opposite roles of PGD2 and PGE2 in the CNS (8, 32).

DG041 and CM9 were injected locally into the cerebrospinal fluid. Thus effects of the compounds in this experiment can be attributed to actions on EP3 receptors located either pre- or postsynaptically on CNS neurons. The spinal fluid proteins may significantly change the potency of the compounds, especially CM9. Therefore, it is difficult to predict the absolute concentration of the antagonists at CNS receptors and to correlate in vitro receptor affinity with in vivo potency.

DG041 and CM9, applied intrathecally or intracerebroventricularly, attenuated the contraction frequency of the rhythmic contraction, which often resulted in the disappearance of voiding contractions. The disappearance of contractions in the isovolumetric rhythmic contraction assay is reported for compounds targeting the bladder afferent pathway (7, 10, 25, 49, 51). The inhibition of the rhythmic bladder contractions often corresponds to an increased micturition threshold and increased bladder capacity in cystometric studies in conscious rats (25). Blockade of CNS EP3 receptors by DG041 and CM9 may affect the parasympathetic motor outflow from sacral regions of the spinal cord. Nerve fibers arise from cell bodies in the sacral parasympathetic nucleus, traverse the ventral roots, and, via the pelvic nerve, end in parasympathetic bladder...
ganglia (6b). Indeed, activation of prejunctional EP3 receptors appears to inhibit acetylcholine release from parasympathetic nerves (6,45). The contraction amplitude is dependent on the efferent arm of the micturition reflex, whereas the frequency is related to the afferent arm of the reflex and to the integrity of the micturition center (26,27). In this study, both DG041 and CM9 failed to change the amplitude of the bladder rhythmic contraction, indicating that the efferent/detrusor functions were not influenced by EP3 blockade.

When tested on nociceptive responses to bladder distension via intrathecal administration, DG041 and CM9 were more potent inhibitors of the VMR vis-à-vis the pressor response to UBD (Fig. 7). A similar profile was observed with morphine and with mexiletine, a sodium channel blocker (47), and with a Cav2.2 channel blocker (48). An explanation for this potency difference is not clear. The threshold bladder pressure for inducing the VMR response is higher than that for the pressor response, and VMR is considered to be a more reliable readout for the nociceptive response of the bladder (24,47). Recent studies suggested that EP1 receptor activation can stimulate spinal nitric oxide formation in the maintenance of neuropathic pain (30,33). Nitric oxide is also involved in the facilitation of the micturition reflex when nociceptive bladder afferents are activated by noxious chemical irritation of the bladder (1,16). This is consistent with a role of spinal EP1 receptors in control of bladder afferent function.

In contrast to the equivalent effect of intrathecal or intracerebroventricular administration of an EP3 antagonist on the micturition reflex, less inhibition of the VMR response was observed when DG041 and CM9 were administered via the intracerebroventricular route. After intracerebroventricular administration, compounds would be expected to diffuse in the cerebrospinal fluid and have access to all supraspinal sites. Since this pattern was observed with two structurally dissimilar antagonists, it is unlikely that variations in the accessibility of neuronal structures to the exogenously applied compounds accounts for the potency difference between the two in vivo assays. Rather, our results suggest that there is a difference between the neuronal pathways involved in the modulation of micturition and nociception by EP3 receptors. Thus the inhibitory effects of EP3 receptor antagonists on bladder nociception may be mediated primarily by blockade of spinal EP3 receptors.

This study and our previous report (49) detail in vivo efficacy of EP3 receptor antagonists in preclinical models of the bladder rhythmic contraction and responses to noxious UBD. The diverse downstream actions of endogenous PGE2 are mediated by other receptor subtypes, too. Studies utilizing a pharmacological blockade of EP1 receptors with tool antagonists have demonstrated therapeutic potentials for OAB (13,23,43), acid-induced esophageal hypersensitivity, and visceral pain associated with irritable bowel syndrome (Ref. 41; see review in Ref. 9).

In summary, we have evaluated the effect of two selective EP3 antagonists, administered directly at different levels of the CNS, on the micturition reflex and responses to nociceptive bladder distension. We have demonstrated that EP3 receptors are involved in bladder micturition at supraspinal and spinal centers and in bladder nociception at the spinal

Fig. 6. Time course for the effect of vehicle, DG041 (A and B), and CM9 (C and D) on the frequency (A and C) and the amplitude (B and D) of volume-induced contractions after intracerebroventricular administration. Responses are represented as % of control, where the baseline response before administration is defined as 100%. Significance of differences between test and control values was determined by ANOVA test.
Fig. 7. Typical experimental recording of visceromotor reflex (VMR, mV; top) and pressor responses (mmHg; bottom) of intrathecal administration (arrow) of vehicle (A), DG041 (100 nmol; B), and CM9 (300 nmol; C). Location of the intrathecal cannula was confirmed by showing the efficacy of intrathecal administration of morphine (100 µg, arrowhead) 30 min after dosing (//).

Fig. 8. Effect of intrathecal administration of DG041 (A and B) and CM9 (C and D) on VMR (A and C) and pressor (B and D) responses to bladder distension in the anesthetized rat. Responses are represented as % of control, where the baseline response before administration of drugs is defined as 100%. Significance of differences between test and control values was determined by ANOVA test.
Fig. 9. Typical experimental recording of VMR (mV, top) and pressor responses (mmHg, bottom) of intracerebroventricular administration (arrow) of vehicle (A), DG041 (100 nmol; B), and CM9 (300 nmol; C). Location of the intracerebroventricular cannula was confirmed by showing the efficacy of intracerebroventricular administration of morphine (100 μg, arrowhead) 30 min after dosing (\(//\)).

Fig. 10. Effect of intracerebroventricular administration of DG041 (A and B) and CM9 (C and D) on VMR (A and C) and pressor (B and D) responses to bladder distension in the anesthetized rat. Responses are represented as % of control, where the baseline response before administration of drugs is defined as 100%. Significance of difference between test and control values was determined by unpaired Student’s t-test. \(*P < 0.05\).
cord. This is the only study that has examined the CNS effects of PGs on bladder activity or bladder reflex pathways. To further elucidate the functional roles of EP3 receptors in micturition and in the response to bladder pain, an excitatory effect by EP3 receptor agonists via local delivery in the CNS requires further investigation. Our present data provide novel insights into the differential roles of PGE2 in bladder function at the CNS and provide additional guidance for the development of EP antagonists for the treatment of overactive bladder or bladder pain.

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