Blocking $\alpha 2\delta-1$ Subunit Reduces Bladder Hypersensitivity and Inflammation in a Cystitis Mouse Model by Decreasing NF-kB Pathway Activation

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Bladder pain is frequently associated with bladder inflammation, as in conditions like interstitial cystitis (IC), for which current analgesic therapies have limited efficacy. The antinociceptive effect of alpha-2-delta (α2δ) ligands on inflammation-associated visceral pain like that experienced in cystitis has been poorly investigated. To investigate the effect of pregabalin (PGB), an α2δ ligand, we evaluated its impact on mechanical hyperalgesia in a mouse model of cystitis induced by cyclophosphamide (CYP). We further studied its effect on inflammation and NF-kB pathway activation. Acute cystitis was induced by intraperitoneal injection of 150 mg kg\(^{-1}\) of CYP in C57Bl/6J male mice. PGB was subcutaneously injected (30 mg kg\(^{-1}\)) 3 h after CYP injection. The effect of PGB on CYP-induced mechanical referred hyperalgesia (abdominal Von Frey test), inflammation (organ weight, cytokine production, α2δ subunit level, NF-kB pathway activation) were assessed 1 h after its injection. In parallel, its effect on cytokine production, α2δ subunit level and NF-kB pathway activation was assessed in vitro on peritoneal exudate cells (PECs) stimulated with LPS. PGB treatment decreased mechanical referred hyperalgesia. Interestingly, it had an anti-inflammatory effect in the cystitis model by reducing pro-inflammatory cytokines. Acute cystitis was induced by intraperitoneal injection of 150 mg kg\(^{-1}\) of CYP in C57Bl/6J male mice. PGB was subcutaneously injected (30 mg kg\(^{-1}\)) 3 h after CYP injection. The effect of PGB on CYP-induced mechanical referred hyperalgesia (abdominal Von Frey test), inflammation (organ weight, cytokine production, α2δ subunit level, NF-kB pathway activation) were assessed 1 h after its injection. In parallel, its effect on cytokine production, α2δ subunit level and NF-kB pathway activation was assessed in vitro on peritoneal exudate cells (PECs) stimulated with LPS. PGB treatment decreased mechanical referred hyperalgesia. Interestingly, it had an anti-inflammatory effect in the cystitis model by reducing pro-inflammatory cytokine production. PGB also inhibited NF-kB pathway activation in the cystitis model and in macrophages stimulated with LPS, in which it blocked the increase in intracellular calcium. This study shows the efficacy of PGB in hypersensitivity and inflammation associated with cystitis. It is therefore of great interest in assessing the benefit of α2δ ligands in patients suffering from cystitis.

Keywords: cystitis mouse model, α2δ-1 ligands treatment, pain, inflammation, NF-kB pathway
INTRODUCTION

Bladder pain is frequently associated with bladder inflammation, as in interstitial cystitis (IC) (Ogawa et al., 2015). The mechanisms include an increase in mucosal bladder permeability (Buffington and Woodworth, 1997), leading to sensitization of bladder afferent pathways and inflammation (Yoshimura et al., 2002), and sensitization of peripheral and/or central pain pathways (Ogawa et al., 2015). At the periphery, inflammatory processes could be involved. Increased levels of pro-inflammatory cytokines (Erickson et al., 2002) and a decrease in those of the anti-inflammatory IL-4 cytokine (Ueda et al., 2000) have been observed in IC patients. The efficacy of anti-nerve growth factor (NGF) therapy in humans (Evans et al., 2000; Sasaki et al., 2001). However, some preclinical studies failed to find any effect of GBP in rodent cystitis models (Rudick et al., 2000; Sasaki et al., 2001). In addition, previous studies have demonstrated potent and selective binding of PGB to α2δ-1 and α2δ-2 subunits (Li et al., 2011). These molecules are now proposed in first line with antidepressant drugs for treatment of neuropathic pain (Johannessen Landmark, 2008). Their beneficial effect has been observed in cystitis patients (Hansen, 2000; Sasaki et al., 2001). However, some preclinical studies failed to find any effect of GBP in rodent cystitis models (Rudick et al., 2009). Our study aimed to investigate the potential beneficial effects of PGB, an α2δ ligand, on a mouse model of cystitis induced by cyclophosphamide (CYP). It has been reported that α2δ ligands reduce the activation of the nuclear factor kB (NF-kB) in neuroblastoma and glioma cells (Park et al., 2008). In light of these findings, we further investigated the mechanism of action of α2δ ligands by studying their interaction with NF-kB pathway activation.

MATERIALS AND METHODS

Acute Cystitis Induction

All experiments were performed according to the ethical guidelines set out in the Guide for the Care and Use of Laboratory Animals and with approval of the “Comité d’Ethique pour l’Expérimentation Animale Auvergne” (C2E2A), the local ethics committee (Reference number: EU0116-5330). All experiments were performed on C57Bl/6 male mice weighing 20–24 g (JANVIER LABS, Le Genest Saint Isle, France). Animals were given access to food and water ad libitum and housed with a 12 h light-dark cycle. Acute cystitis was induced by intraperitoneal injection of 150 mg kg$^{-1}$ of CYP. Control mice received saline injection.

Pregabalin Treatment

Pregabalin ((S)-(++)-3-(aminomethyl)-5-methylhexanoic acid; Dochem lot PRE20110601) was dissolved in 0.9% saline. 3 h after CYP injection, mice were subcutaneously injected with PGB (30 mg kg$^{-1}$) or saline. Tests were performed 1 h later.

Mechanical Referred Hyperalgesia Testing

Mechanical cutaneous abdominal sensitivity was assessed with von Frey filaments (Biosed, Vitrolles, France) before the animals were injected and 4 h after CYP injection. The filaments were applied to the lower abdominal area close to the urinary bladder and the median 50% threshold (T50) was determined by the up-and-down method (Chaplan et al., 1994). Briefly, this method is based on the use of the 3.22 filament size (0.16 g), which corresponds to the intermediate size of the filament range. The filament is applied perpendicular to the lower abdominal area close to the urinary bladder, exerting sufficient force to flex it for a period of 5 s. Two answers were possible: (i) animal reacts (abdominal contraction), this response is marked “X” and the test continues with the smaller filament on the range (2.83/0.07 g); (ii) animal does not respond, this response is marked “O” and the test continues with the smaller filament on the range (2.83/0.07 g). This scheme continues until the objectivized response of the animal changes compared to the first reaction observed with the 3.22 filament size. From that moment, four filaments are applied (always according to the same method) and the test is finished. The pattern thus obtained corresponds to a score available in the appendix of the Chaplan publication (Chaplan et al., 1994). Finally, thanks to the Dixon formula (Dixon, 1980), it possible to calculate the median 50% threshold (T50): (10^Xf / (Xf + kX)) / 10000, with Xf, size of the last applied filament, k, score and δ, average difference between stimuli.

Bladder Culture

Following euthanasia, the bladder was removed, cut open longitudinally, washed in PBS and cultured in RPMI1640 medium containing penicillin and streptomycin. After 24 h incubation at 37°C with 5% CO2, supernatants were centrifuged at 4°C and used for assaying cytokines by ELISA.

Enzyme-Linked Immunosorbent Assay

All ELISA kits are DuoSet kits from R&D Systems, and assays were performed according to the manufacturer’s protocol.
Cells (PECs) were loaded with 2 µM of Fura-2-acetoxyethyl ester (Fura-2/AM, Life Technologies), 0.5% BSA in the recording saline solution. After 1 h, cells were stimulated for 2 min with LPS (1 µg/mL). When indicated, cells were also pre-incubated with PGB (11.3 µM) for 15 min. Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)] with the ratiometric fluorescent probe Fura-2 in PECs. The MetaFluor Imaging System (Molecular Devices) was used for fluorescence acquisition and analysis of individual cells. Pairs of images were acquired every 2 s. A single PEC was considered as a responder if the F340/F380 ratio for a single PEC increased by 0.05.

**Statistical Analysis**

All data were expressed as mean ± SEM and analyzed with GraphPad Prism5 software. Differences in T50 and the effects of PGB on ex vivo parameters were analyzed by a 1-way ANOVA (Treatment) followed by Tukey post hoc test for multiple comparisons. The effects of PGB on IkBa, phospho-p65 and α28-1 expression in PECs culture were analyzed by a 2-way ANOVA (Model, Treatment) followed by Bonferroni post hoc test for multiple comparisons. For calcium imaging experiments, statistical differences were elicited by a Mann–Whitney U-test. A p-value less than 0.05 was considered statistically significant.

**RESULTS**

**Effect of Pregabalin on Cutaneous-Referred Bladder Hypersensitivity in Acute Cyclophosphamide-Induced Cystitis**

Before CYP injection, the von Frey test showed no difference between the different groups (saline: 0.37 ± 0.14 g, PGB: 0.34 ± 0.08 g, CYP: 0.48 ± 0.16 g, CYP/PGB: 0.38 ± 0.10 g, data not shown). In the group receiving adjuvant and saline injection...
**FIGURE 2** | Effect of PGB (30 mg kg\(^{-1}\), s.c.) on spleen (A) and bladder (B) weight, mucosal thickness (C,D) and bladder MPO activity (E) in cyclophosphamide (CYP)-induced cystitis in mice. The values are expressed as a mean ±S.E.M. and compared by a 1-way ANOVA (Treatment) followed by Tukey post hoc test for multiple comparisons. For panels A, B, E, the result represented \( n = 8 \) animals / group and for panel C, 4 measured per section and 3 sections per animals were analyzed. \( {}^* p < 0.05, {}^{**} p < 0.01, {}^{***} p < 0.001 \) vs. control group.
(control group), T50 scores (0.43 ± 0.16 g) were comparable to those obtained before animals were injected. Cyclophosphamide treatment induced a significant decrease in the von Frey scores (0.006 ± 0.001 g, p < 0.05 vs. control group). Subcutaneous acute PGB administration led to a marked significantly increase in the T50 score in healthy mice (1.29 ± 0.11 g, p < 0.001 vs. control group) and in CYP-treated animals (1.14 ± 0.12 g, p < 0.001 vs. control group) (Figure 1).

Effect of Pregabalin on Inflammatory Parameters in Acute Cyclophosphamide-Induced Cystitis
Cyclophosphamide induced a significant increase in the weight of the spleen (3.24 ± 0.17 g/100 g body weight, p < 0.05 vs. control group) and bladder (1.42 ± 0.05 g/100 g body weight, p < 0.01 vs. control group). This increase was not reproduced when animals were treated with PGB (spleen weight: 2.85 ± 0.04 g/100 g body weight; bladder weight: 1.13 ± 0.06 g/100 g body weight, ns, vs. control group) (Figures 2A,B).

The cyclophosphamide treatment induced a significant increase in mucosal thickness of the bladder (124.50 ± 6.21 μM, p < 0.001, vs. control group). In contrast, PGB acute treatment prevented bladder thickening (83.55 ± 3.60 μM, ns, vs. control group) (Figure 2C). The deleterious impact of CYP on bladder structure and the beneficial effect of PGB treatment were confirmed in a morpho-anatomical observation (Figure 2D).

Cyclophosphamide treatment induced an increase in bladder MPO activity (14.76 ± 3.49 U/g of bladder, p < 0.01, vs. control group). In contrast, PGB-treated animals had normal levels of MPO activity (3.85 ± 0.64 U/g of bladder, ns, vs. control group) (Figure 2E).

Local (bladder) and systemic (plasma) markers of inflammation (IL-6, KC, TNFa cytokines) presented the same profile with an increased level in animals receiving CYP and treated with saline, and normal levels in animals receiving CYP and treated with PGB (Table 1).

Effect of Pregabalin on Membrane Addressing α2δ-1 Subunit
Cyclophosphamide induced a significant increase in membrane expression of α2δ-1 subunit in the bladder of the CYP-treated animals (575.50 ± 141.80, p < 0.05, vs. control group) (Figure 3A). The membrane expression of α2δ-1 subunit in animals receiving CYP and treated with PGB was similar to that in control animals (131.80 ± 58.44, ns, vs. control group) (Figure 3A). No differences were observed between the groups in α2δ-1 subunit cytoplasmic expression (Figure 3B) and in total α2δ-1 subunit expression (Figure 3C).

Effect of Pregabalin on NF-kb Pathway Activation in Bladder
Cyclophosphamide induced a strong and significant decrease in IkBα expression in the bladder of saline-treated animals (20.63 ± 12.45, p < 0.05, vs. control group) but not in that of PGB-treated mice (74.29 ± 25.05, ns, vs. control group) (Figure 3D). Concomitantly, PGB treatment blocked the significant increase in phospho-p65 expression in the bladder of mice receiving cyclophosphamide (234.20 ± 39.07, ns, vs. control group) (Figure 3E). Since a lot of other signaling pathways are involved in inflammation, we have checked the phospho-ERK1/2 pathway. Similarly, as for the phospho-p65, cyclophosphamide induced an increase in phospho-ERK1/2 expression in the bladder of saline-treated animals, and a PGB treatment did not change the level of phospho-ERK1/2 expression in cyclophosphamide-treated mice (Figure 3F).

Effect of Pregabalin on LPS-Induced α2δ-1 Expression, NF-kb Pathway Activation, Cytokine Production and Intracellular Calcium Increase on Peritoneal Exudate Cell Culture
In PEC culture, LPS treatment induced a significant increase in α2δ-1 expression blocked by PGB treatment (Figure 4A).

LPS induced a decrease in IkBα expression (Figure 4B) and an increase in phospho-p65 expression (Figure 4C) in LPS-stimulated PECs blocked by PGB treatment.

The increase in p65 subunit was located in the nucleus of the cells, as shown by detection of the p65 subunit by nucleus marker (DAPI). This localization was not observed following PGB treatment (Figure 4D).

**Table 1.** Effect of pregabalin (30 mg kg\(^{-1}\), s.c) on IL-6, KC and TNFa concentration in bladder and plasmatic level in cyclophosphamide (CYP)-induced cystitis mice model.

| Cytokines | Saline/Saline (n = 8) | Saline/PGB (n = 8) | Cyp/Saline (n = 8) | CPY/PGB (n = 8) |
|-----------|----------------------|-------------------|-------------------|---------------|
| Bladder   |                      |                   |                   |               |
| pg/ml/mg of bladder | 181, 3 ± 11, 5 | 190, 4 ± 23, 1 | 300, 5 ± 40, 2** | 166, 2 ± 181, 1 |
| KC        | 772, 6 ± 98, 1       | 743, 9 ± 91, 8    | 1454, 1 ± 215, 2** | 210, 6 ± 37, 4* |
| TNFa      | 135, 6 ± 7, 5        | 146, 4 ± 16, 5    | 244, 9 ± 30, 3*** | 117, 5 ± 13, 6 |
| Plasmatic |                      |                   |                   |               |
| pg/ml     | 1473, 8 ± 10, 7     | 1627, 6 ± 67, 4   | 2942, 9 ± 78, 4*** | 1461, 5 ± 58, 9 |
| TNFa      | 453, 1 ± 65, 7      | 289, 1 ± 31, 8    | 839, 9 ± 105, 4**  | 491, 7 ± 76, 9 |

The values are expressed as a mean ± S.E.M. and compared by a 1-way ANOVA (Treatment) followed by Tukey post hoc test for multiple comparisons. N = 8/group. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control group.
FIGURE 3 | Effect of PGB (30 mg kg\(^{-1}\), s.c.) on membrane addressing \(\alpha_2\delta-1\) subunit and on NF-\(\kappa\)B pathway activation in cyclophosphamide (CYP)-induced cystitis in mice. The expression of \(\alpha_2\delta-1\) subunit was evaluated by western blot on cytoplasmic membrane (A), cytoplasm (B), and total cell (C) level. The NF-\(\kappa\)B pathway activation was evaluated by the semi quantification of \(\kappa B\alpha\) (D) and phospho-p65 (E). The phospho-ERK1/2 pathway activation was also evaluated by the semi quantification ERK1/2 and phosphor-ERK1/2 and the ratio between these two forms was calculated (F). The values are expressed as a mean ± S.E.M and compared by a 1-way ANOVA (Treatment) followed by Tukey post hoc test for multiple comparisons. \(N = 8\)/group. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) vs. control group.
FIGURE 4 | Effect of PGB (11.3 µM) on α2δ-1 subunit (A) IkBα (B), and phospho-p65 (C) expression in resident peritoneal exudate cells (PECs) stimulated with LPS (100 ng ml⁻¹). The expression was measured 10, 20, 30, 45, 60, and 90 min after LPS with or without PGB treatment by western blot analyses (n = 4/condition). Intracellular localization of p65 in LPS-stimulated (100 ng ml⁻¹) PEC treated or not with PGB (11.3 µM) was visualized by immunohistochemistry. Arrowheads indicate cells which are magnified in side panels (scale bar: 100 µm). (D) Effect of PGB on IL-6 level in resident PECs stimulated with LPS 24 h after these treatments.
The increased production of IL-6 cytokine by PECs stimulated with LPS was significantly reduced after PGB treatment (Figure 4E) as was KC cytokine production (data not shown).

As αδ-1 ligands inhibit activation of VGCCs, we decided to further investigate the cellular mechanisms of PGB by measuring intracellular \([Ca^{2+}]\) in cultivated PECs with a Fura-2 probe. In three independent experiments, stimulation with LPS induced an increase in intracellular \(Ca^{2+}\) concentrations in a total of 126 out of 225 PECs (53.3 ± 9.3%). Pre-incubation with PGB significantly reduced the number of PECs having an intracellular \([Ca^{2+}]\) rise in response to LPS to 105 out of 377 (21.2 ± 3.6%, \(p < 0.05\) vs. LPS group) (Figure 4F).

**DISCUSSION**

Alpha 2 delta ligands, developed as anticonvulsants and used to treat neuropathic pain, exerted a potent anti-hypersensitive and anti-inflammatory effect in a murine cystitis model. We clearly show that CYP treatment induced an increase in bladder sensitivity and inflammation that is blocked by PGB treatment. The marked decrease in cytokine overexpression induced by PGB could be due to reduced activation of the NF-κB pathway.

Cyclophosphamide-induced cystitis is a widely used model to assess bladder inflammation and related pain (Lantéri-Minet et al., 1995). Using this model, Boudes et al. (2011) performed von Frey stimulation and, as in our study, found increased sensitivity in the lower abdominal area. We first showed that PGB treatment greatly increases von Frey scores in CYP-treated animal, evidence that this anticonvulsant drug has an antinociceptive effect. To our knowledge, this is the first time that preclinical data show a potential benefit of using αδ ligands in an experimental IC model. One previous preclinical study failed to find any effect of GBP in rat or mice cystitis models (Rudick et al., 2009). However, these preclinical results were surprising according the reports of its efficacy in treatment of IC (Hansen, 2000; Sasaki et al., 2001). Another clinical study reported the beneficial effects of a mixed treatment using GBP, amitryptiline and non-steroidal anti-inflammatory agents on bladder pain syndrome (Kwon et al., 2013). The antinociceptive effect of αδ ligands is not surprising given that these drugs are frequently used in the treatment of chronic pain, mainly neuropathic (Verma et al., 2014) but also in fibromyalgia (Traynor et al., 2011). There is some evidence of their efficacy in the treatment of visceral pain, notably in IBS patients (Gale and Houghton, 2011). Their effect does not seem to involve a GABAergic mechanism but could result from their blockage of VGCC activation. In fact, αδ ligands bind to the αδ subunit exclusively expressed by high-voltage gated channels (Cheng and Chio, 2006). Of the four isoforms, PGB seems to exhibit greater affinity for αδ subunits expressed in the peripheral and central nervous systems (Cheng and Chio, 2006). PGB has a better affinity for these units than GBP, another αδ ligand (Taylor, 2009) several findings suggest an involvement of these subunits in the context of visceral pain at a peripheral level in intestinal primary afferent fibers (Needham et al., 2010), or at a central level (Liao et al., 2010). Blockage of αδ subunits at both peripheral and central levels could explain their antinociceptive effect in our cystitis model. However, another peripheral mechanism could be involved. Our study clearly showed that PGB is able to decrease several signs of bladder inflammation, a property that, to our knowledge, has never been mentioned in any of the preclinical models of inflammatory pain in which αδ ligands were tested (Hurley et al., 2002).

The key mediator of pro-inflammatory mediator production and inflammation triggering is NF-kB (p65) (Hayden et al., 2006). Given that GBP or PGB binding on αδ subunits is able to inhibit this factor in neuroblastoma and glioma cells (Park et al., 2008), the anti-inflammatory effect of PGB could be due to the blockade of NF-kB pathway activation. In the CYP model or in an in vitro model of PEC culture acute administration of PGB can prevent IkBα (main negative regulator of NF-kB pathway) degradation, and p65 phosphorylation (the mark of nuclear translocation of this transcription factor). A decrease in NF-kB pathway activation could result from a decrease in intracellular calcium \([Ca^{2+}]_i\) induced by blockade of αδ subunits. An increase in \([Ca^{2+}]_i\) is important for the activation of intracellular function as transcriptional control (Mandeville and Maxfield, 1996) to promote NF-kB activation and subsequently the expression of genes involved in inflammatory responses (Martin et al., 2006). While the major \(Ca^{2+}\) entry pathway in non-excitable cells involves store-operated calcium channels, recent works also suggest that functional VGCCs are expressed in cells such as dendritic cells and macrophages (Gupta et al., 2009). Here, we propose that PGB induces a blockage in αδ-1 subunit membrane expression thereby reducing the number of PECs that respond to LPS by increasing \([Ca^{2+}]_i\).

**CONCLUSION**

To conclude, our study shows that αδ ligands can reduce bladder hypersensitivity in a cystitis model and therefore is of potential benefit in patients with bladder pain. Our findings also suggest that αδ ligands possess anti-inflammatory properties that contribute to their beneficial effect. We showed that
the mechanism of this anti-inflammatory effect involves a decrease in αδ-1 membrane expression, in [Ca²⁺]i and in NFKB pathway activation. Clinical studies in patients with bladder pain syndrome are needed to confirm these preclinical data.

**AUTHOR CONTRIBUTIONS**

LB was involved in protocol and project development, collected and analyzed the data, and wrote the manuscript. SM collected and analyzed the data. MM, YA, and BS were involved in protocol and project development. AL, LU, and FR collected the data. FC and DA were involved in protocol/project development, the analysis of data, and wrote the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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