Role of Detrusor PDGFRα+ Cells in Cyclophosphamide-Induced Detrusor Overactivity

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Abstract

Cyclophosphamide (CYP)-induced cystitis is a rodent model that shares many features common to the cystitis occurring in patients, including detrusor overactivity (DO). Platelet-derived growth factor receptor alpha positive (PDGFRα+) cells regulate muscle excitability in murine bladders during filling. PDGFRα+ cells express small conductance \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) channels (predominantly SK3) that provide stabilization of membrane potential during filling. We hypothesized that down-regulation of the regulatory functions of PDGFRα+ cells and/or loss of PDGFRα+ cells generates the DO in CYP-treated mice. After CYP treatment, transcripts of \( Pdgfra \) and \( Kcnn3 \) were reduced, and PDGFRα and SK3 protein was also reduced in detrusor muscle extracts. The distribution of PDGFRα+ cells was also reduced. Inflammatory markers were increased in CYP-treated detrusor muscles. An SK channel agonist, CyPPA, increased outward current and hyperpolarization in PDGFRα+ cells. This response was significantly depressed in PDGFRα+ cells from CYP-treated bladders. \textit{Ex vivo} cystometry showed increased transient contractions in CYP-treated bladders, and the sensitivity of these bladders to apamin was reduced, reflecting the reduction in the SK conductance expressed by PDGFRα+ cells. In summary, PDGFRα+ cells were reduced and the SK3 conductance was downregulated in CYP-treated bladders. These changes are consistent with the development of DO after CYP treatment.
Introduction

Interstitial cystitis (IC) is characterized by suprapubic and/or bladder pain that is accompanied with an increase in urinary urgency, frequency and nocturia\(^1,2\). The pathology of IC does not follow that of other diseases/syndromes in the bladder, such as carcinoma, urinary tract infections or cystitis introduced by radiation or medication\(^3,4\). Although the pathogenesis of IC is still not fully understood, several factors have been suggested which include neuronal, urothelium, and myogenic causes with likely associated inflammation\(^4\).

Cyclophosphamide (CYP) causes cystitis in humans\(^5,6\). One of the commonly used models for cystitis in rodents is CYP-induced cystitis, usually induced by injections\(^7\). This model shares many features with cystitis occurring in human patients treated with CYP, as well as common features with bladder pain syndrome/interstitial cystitis (BPS/IC)\(^8-11\). CYP causes functional and histological changes in humans and rodents\(^12,13\), such as expression of receptors and signaling molecules in the urothelium/mucosa. CYP-induced changes include up-regulation of nitric oxide synthase\(^10,14,15\), increased urothelial muscarinic M5 receptors\(^16\), and mucosal permeability\(^15\). Furthermore, after CYP-injection, smaller micturition volumes have been noted\(^17-19\) that likely are due to upregulated afferent and efferent neural effects\(^16,20-22\). CYP can also induce detrusor overactivity (DO)\(^23\), but the functional changes in the detrusor smooth muscle responsible for DO have not been determined.

Recently, a unique population of interstitial cells were identified in the bladder. These cells are immunopositive for platelet-derived growth factor receptor alpha (PDGFR\(\alpha\)), and therefore are referred to by this chemical coding, i.e. PDGFR\(\alpha^+\) cells.
PDGFRα+ cells have been identified in murine, pig and human detrusor muscles\textsuperscript{24,25}. These cells are located on the edges of smooth muscle bundles and have multiple branches that couple to and form an electrical syncytium with smooth muscle cells (SMCs)\textsuperscript{24,26,27}. The function of PDGFRα+ cells in detrusor muscles has been investigated with molecular and electrophysiological techniques. PDGFRα+ cells displayed higher current density of small conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (SK) channels\textsuperscript{25,28}, as compared to detrusor SMCs. Activation of SK channels in PDGFRα+ cells provides a stabilizing influence on smooth muscle excitability. We hypothesized that loss-of-function in PDGFRα+ cells or SK channels could lead to DO in cystitis. This study describes investigation into the molecular and functional changes that occur in detrusor PDGFRα+ cells in CYP-injected bladder.
Results

Downregulation of PDGFR\(\alpha\) and SK channel in murine bladders from CYP-induced cystitis.

We compared the transcriptional expression of \(Pdgfra\) and \(Kcnn1-4\) in detrusor muscles from CYP-treated and saline-treated mice. \(Pdgfra\) and \(Kcnn3\) (SK3) were significantly downregulated, but neither SK1-2 (\(Kcnn1\) and \(Kcnn2\)) nor IK (\(Kcnn4\)) were changed significantly after CYP-treatment \((n=4, \text{Fig. 1A and B})\). Detrusor muscles from CYP-treated mice displayed a significant increase in \(Il6\) and \(Tnf\) (inflammatory markers) \((n=4, \text{Fig. 1C})\) suggesting the onset of bladder inflammation after CYP treatment. Levels of transcripts do not necessarily translate linearly into protein expression. Therefore, we also employed immunohistochemistry and Wes analysis to confirm parallel changes in protein expression. Immunohistochemistry revealed PDGFR\(\alpha\) immune-positive cells were down-regulated in CYP-treated detrusor compared with age-matched saline-injected controls \((\text{Fig. 2A& B})\). Wes analysis showed in a more quantitative way that PDGFR\(\alpha\) and SK3 were significantly downregulated in CYP-treated detrusor muscles, as compared to controls \((n=4, \text{Fig. 3})\). These data are consistent with transcriptional data, and suggested the possibility that down-regulation of \(Pdgfra\) and/or \(Kcnn3\) transcripts could be involved in generation of DO.

The effect of SK channel activator on the generation of outward currents and membrane potentials in detrusor PDGFR\(\alpha^{+}\) cells from CYP-treated bladders.

We tested the effect of a SK channel activator \((\text{CyPPA, 10 \& M})\) on freshly dispersed detrusor PDGFR\(\alpha^{+}\) cells. The cells were dialyzed with K\(^{+}\)-rich solution (see
Methods). CyPPA hyperpolarized control detrusor PDGFRα+ cells from -31.4 ± 4.6 mV to -61.6 ± 2.9 mV under current clamp (I=0, red dot line, n= 5, Fig. 4A). In the same cells, CyPPA activated outward current at a holding potential of -40 mV in voltage-clamp (V-C) mode (Fig. 4A, blue dotted line). CyPPA-activated current amplitude averaged 39.3 ± 7.1 pA. Figure 4B shows currents evoked by ramp depolarization before (Fig. 4Ba, black trace) and in the presence of CyPPA (Fig. 4Bb, red trace). The resting membrane potentials (RMP) of CYP-treated detrusor PDGFRα+ cells were depolarized to -16 ± 1.4 mV (n=5, P<0.01, as compared to untreated control PDGFRα+ cells).

CyPPA induced less hyperpolarization in CYP-treated detrusor PDGFRα+ cells (∆mV 9.1 ± 2.0), as compared to untreated control PDGFRα+ cells (n=5, P<0.01, Fig. 4C) under current-clamp mode (I=0) and generated smaller outward currents under V-C mode (5.2 ± 0.9 pA; n= 5, P<0.01, Fig 4D) at -40 mV. These data suggest that SK current density was significantly decreased in PDGFRα+ cells isolated from CYP-treated bladders, as compared with current density in control PDGFRα+ cells isolated from non-treated bladders.

The effect of apamin on CYP-treated bladders using ex vivo preparation.

We also examined the pressure-volume relationships of excised bladders in ex vivo preparations to investigate changes in functional expression of SK channel in control and CYP-treated bladders. Ex vivo pressure-volume measurements exclude extrinsic neural regulation during filling, so this technique highlights regulation via myogenic mechanisms. In in vivo cystometry, voiding contractions start around 20 cmH₂O in murine bladder²⁹. Thus, we analyzed the pressure amplitude and frequency
up to 15 cmH\textsubscript{2}O (see Fig. 5A \& B) to associate contractile activity (i.e. transient contractions or TCs) to the non-voiding contractions (NVCs) observed in \textit{in vivo} cystometry. The passive pressure underlying was normalized to 0 cmH\textsubscript{2}O (see Fig. 5Aa \\& Bb). In control bladders, infusion of KRB solution (15 μl/min) induced a small increase in intravesical amplitude (1.3 ± 0.3 cmH\textsubscript{2}O) with the frequency of TCs equal to 17 ± 2 events at intravesical pressures up to 15 cmH\textsubscript{2}O. Addition of apamin (300 nM) into the bath increased TC frequency to 35 ± 3 events (P<0.001, Fig. 5A-C) and the amplitude of TCs to 3.8 ± 0.4 cmH\textsubscript{2}O (n=6, P<0.01, Fig. 5A, B & D). The SK channel agonist, SKA-31, showed no significant effect on control bladders (data not shown). CYP-treated bladders displayed increased amplitude of TCs with high frequency before apamin treatment (Fig. 6A-C). SKA-31 and apamin treatment did not significantly affect the amplitude or frequency of TCs in CYP-treated bladders (n=6, Fig. 6B & C).
Discussion

This study investigated the mechanisms of DO in CYP-induced cystitis. We found the downregulation of gene transcripts and protein levels of PDGFRα and SK3 in CYP-treated detrusor muscles. The current density from SK channels evoked by a SK channel activator, CyPPA, was depressed in PDGFRα⁺ cells isolated from CYP-treated detrusor muscles suggesting that gene and protein expression levels have an important functional effect on bladder function. In ex vivo experiments, an SK channel antagonist, apamin, increased the amplitude and frequency of TCs during filling in control bladders. However, the effects of apamin were reduced significantly in the CYP-treated bladders. Similarly the effects of an SK agonist were also minimal in CYP-treated bladders. These data suggest that loss of PDGFRα⁺ cells and the major functional conductance provided by SK channels in these cells in CYP-treated bladders can lead to increased TCs and induction of DO.

Chemical cystitis is one of the adverse effects observed after administration of CYP chemotherapy in humans. CYP also induces cystitis in mice and rats. Therefore, CYP treatments of rodents has been used widely as an experimental model of IC/BPS. Single CYP intraperitoneal injection leads to urinary bladder inflammation, visceral pain and DO. However, the mechanisms of DO induced by CYP have not been clarified by previous studies. In the current study we tested the hypothesis that functional causes of DO after CYP treatment could be related to loss-of-function of bladder regulation provided by PDGFRα⁺ cells.

Previous studies have shown that as the bladder fills, volume increases and the walls are stretched, but intravesical pressure remains low during much of the period of filling. This accommodation occurs even though there is a natural tendency for
detrusor SMCs to contract in response to stretch\textsuperscript{34,35}. During bladder filling, NVCs are detected in cystometric records from various species\textsuperscript{36-39}. NVCs appear to correspond to localized contractions that are also observed in ex vivo bladder preparations and have been termed ‘spontaneous phasic contractions’, ‘micromotions’ or ‘TCs’\textsuperscript{40-44}. TCs increase as bladder filling proceeds\textsuperscript{40}. Activation of non-selective cation channels expressed in detrusor SMCs\textsuperscript{34,35} generates TCs which initiate sensory inputs via activation of afferent nerves\textsuperscript{40} that propagate to the central nervous system during bladder filling\textsuperscript{45-48}. However, under physiological condition, development of TCs can be restrained due to activation of SK channels in detrusor muscles\textsuperscript{25}. For instance, SK channel activators reduce and SK channel blocker increase detrusor muscle contractions\textsuperscript{49-53}. Furthermore, $Kcnn3$ knockout mice show an increase in NVCs in in\textit{vivo} cystometry and TCs in ex\textit{vivo} bladders\textsuperscript{40,54}. Previous reports demonstrate that $Kcnn3$ are highly expressed in detrusor PDGFR$\alpha^+$ cells\textsuperscript{25,28,55}. Since the current density of SK channels is very low in SMCs compared to detrusor PDGFR$\alpha^+$ cells\textsuperscript{25}, it seems clear that PDGFR$\alpha^+$ cells serve an important stabilizing role in regulating contractile activity of SMCs during bladder filling. Therefore, disruption of PDGFR$\alpha^+$ cells and/or downregulation of SK channels and intracellular signaling pathways regulating SK channel activity could lead to DO.

In the present study we found that $Kcnn3$ and $Pdgfra$ transcripts were downregulated in CYP-treated detrusor muscles. Western analysis confirmed that both PDGFR$\alpha$ and SK3 proteins were reduced in CYP-treated detrusor muscles. Immunohistochemistry also showed a reduction in the density of PDGFR$\alpha^+$ cells in CYP-injected detrusor muscle. At present we do not know what factors, activated by
CYP, might be responsible for loss of PDGFRα+ cells and downregulation of SK channels in detrusor muscles, however the upregulation of inflammatory factors, such as *Tnfa* and *Il6*, suggest that an inflammatory mechanism may be involved.

Functional studies to evaluate the state of the SK conductance in detrusor PDGFRα+ cells before and after CYP treatment were performed using the patch clamp technique. Our recordings confirmed the presence of an SK conductance in control (untreated) PDGFRα+ cells, and the availability of this conductance was decreased in PDGFRα+ cells isolated from CYP-treated bladders. As above, CYP treatment led to reduced PDGFRα+ cell density, and the patch clamp experiments showed that there was concomitant reduction in the SK conductance normally prominent in these cells. Since SK channels provide stabilization of membrane potential and excitability of SMCs during bladder filling, reducing the availability of the SK conductance would lead to increased generation of TCs. This hypothesis was confirmed using *ex vivo* bladder preparations of mice treated with CYP. We found an increase in TCs as the bladders were filled and less sensitivity of apamin in comparison to the control mice.

In conclusion, we found that CYP treatments induced DO was caused by reduced detrusor PDGFRα+ cells and reduction in the prominent SK conductance expressed by these cells that is utilized to regulate SMC excitability during bladder filling. These experiments provide a novel understanding of detrusor PDGFRα+ cells and how defects in these cells can contribute to the development of abnormal bladder activity.
Methods

Preparation of tissue

Male C57BL/6J and Pdgfra<sup>tm11(EGFP)Sor</sup>/J (PDGFRα/eGFP) mice were purchased from Jackson Laboratory, Bar Harbor, ME. The mice were maintained and experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All methods are reported in accordance with ARRIVE guidelines. Animal protocols were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee. Mice were housed in a pathogen-free barrier facility on a 12-h light/dark cycle with free access to water and food (Prolab 5P76 Isopro 3000; 5.4% fat by weight). All mice were males and used at 8-10 weeks of age for all experiments (purchased from Jackson Laboratory, Bar Harbor, ME, USA). Mice were sacrificed with isoflurane inhalation (AErrane; Baxter, Deerfield, IL, USA) followed by cervical dislocation. The abdomens were opened and bladders were removed, then placed in Krebs-Ringer Bicarbonate (KRB) buffer solution (see below). The bladders were opened and the urothelium and detrusor layer were isolated by sharp dissection<sup>25</sup>.

Induction of CYP-induced cystitis

Murine CYP-induced cystitis was established according to previously described protocols<sup>23,32,56</sup>. To induce acute cystitis, mice were injected with 1.2 mg of CYP per 100 μl of saline solution and control mice were injected only with saline solution. Both mice were sacrificed on day 7 after CYP treatment.

RNA isolation, reverse-transcription PCR and quantitative PCR
Total RNA was isolated from the detrusor smooth muscle tissue using Direct-zol RNA miniPrep Kit (Zymo Research, Irvine, CA, USA), and first-strand cDNA was synthesized using qScriptTM cDNA SuperMix (Quanta, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Endpoint PCR was performed with specific primers (Table 1) using Go-Taq Green Master Mix (Promega Corp., Madison, WI, USA). Products of the end-point PCR were run on a 2% agarose gel and visualized by ethidium bromide. Next, the standard curve method of Quantitative PCR (qPCR) was performed as previously described in Bookout et al. 2005 with the same primers as PCR. The fast SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA) on the Quantstudio 3 Real Time PCR System (Applied Biosystems) was also employed. In summary, mean values of duplicate samples for the diluted cDNA had regression analysis performed on them and this was used to generate standard curves. This resulted in transcriptional quantification of each gene, log transformation of corresponding raw data was taken and transcription expression was given relative to the endogenous glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

**Immunohistochemistry**

Whole bladder was fixed in paraformaldehyde [4% w/v in 0.1 M phosphate buffer solution (PBS) for 60 min at 4°C] and washed with PBS. Fixed tissue was passed through sucrose gradient (up to 30%). Tissues were bisected and snap frozen on liquid nitrogen in Tissue Tek OCT compound (Sakura Finetek, USA). Ten micron cryosections were cut on a cryostat (Leica CM3050) and placed on to Vectabond (Vector Labs, USA) coated slides. Tissues were washed 5 times and incubated in BSA (1%) for 1 h at room
temperature containing Triton X-100 (0.3% in PBS) to reduce non-specific antibody binding. Tissue sections were incubated overnight in primary anti PDGFRα antibody (R&D Systems, 1:100 dilution) diluted in 0.5% Triton X-100 at 4°C. Excess primary antibody was washed in PBS and were then incubated Alexa Fluor 488 (Invitrogen, Grand Island, NY, USA) secondary antibody diluted 1:1000 in PBS for 1 hr. Excess secondary antibody was washed in PBS and mounted with Aqua mount mounting media (Lerner Laboratories, Pittsburgh, PA, USA). Sections on glass slides and cover slipped were imaged with the Olympus FV1000 (Olympus America Inc., Center Valley, PA, USA) and Carl Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging, LLC, Thornwood, NY, USA). Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA) was used to arrange the images taken.

Wes Simple Western automated capillary electrophoresis and immunodetection

Tissue samples were prepared by homogenizing at 4°C in 0.3ml radioimmune precipitation assay (RIPA) buffer (1X/ type) with added protease inhibitor tablet (Thermo-Fisher mini tablets EDTA free) with a Bullet Blender (5 mins, speed 5, 1 stainless steel bead per detrusor muscle). The homogenate was centrifuged at 4°C, 3000 x g for 10 minutes, to remove cell debris. The supernatant was aliquoted and stored at -80°C. Other tissue samples were subjected to differential centrifugation to obtain a plasma membrane-enriched fraction for PDGFRα and SK3 detection. In this case, the tissues were homogenized in ice cold lysis buffer (mM; 50 Tris HCl pH 8.0, 60 beta-glycerophosphate, 100 NaF, 2 EGTA, 25 Na-pyrophosphate, 1 DTT, and protease inhibitor tablet). Each tissue was homogenized in 0.3 ml lysis buffer, centrifuged at 16,000 x g at 4°C for 10 min, and the supernatants centrifuged at 100,000 x g for 1 hour.
at 4°C. The 100,000x g pellet was resuspended into 0.3ml of lysis buffer, and stored at -80°C. Protein concentrations of the supernatants were determined by the Bradford assay using bovine γ-globulin as the standard. Automated western blotting (Wes Simple Western, ProteinSimple, Santa Clara, CA) was utilized to measure PDGFRα and SK3 protein levels. Simple Western analysis was performed according to the ProteinSimple user manual. The primary antibodies and total protein lysate concentrations for each protein were determined by initial titrations of lysate amounts and antibody dilutions. Final concentrations of bladder lysates were 1.0mg/ml and 0.3 mg/ml for SK3 and PDGFRα respectively. The antibody for SK3 (catalog #sc-28621, Santa Cruz Biotechnologies, CA, USA) was used at a 1:100 dilution. The antibody for PDGFRα (catalog #sc-338, Santa Cruz Biotechnologies, CA, USA) was used at a 1:100 dilution. The boiled samples, biotinylated protein ladder, blocking buffer, primary antibodies, secondary antibodies, chemiluminescent substrate, and wash buffer were loaded into the plate (Wes 12-230 kDa Pre-filled Plates with Split Buffer, ProteinSimple). The plate was then loaded onto the automatic size-based Simple Western system for protein separation, antibody incubation and imaging using the Wes default parameters. Image reconstruction of the detected proteins was generated by Compass software (ProteinSimple). The protein signals were quantified from the electropherogram of the area under the chemiluminescent intensity peak obtained by Compass software.

_Electrophysiological recordings_

Whole cell currents and membrane potentials were recorded using whole cell voltage- and current-clamp techniques. Cells were placed in a 0.5 ml chamber mounted
on an inverted microscope (Nikon, Japan). PDGFRα⁺ cells, isolated from Pdgfra<sup>tm11(EGFP)Sor</sup>/J mice, were identified by the fluorescence of eGFP in nuclei. Pipette tip resistances were: 4–6 MΩ for PDGFRα⁺ cells. An Axopatch 200B amplifier with a CV-4 headstage (Molecular Devices, Sunnyvale, CA, USA) was used. All data were analysed using pCLAMP software (Axon Instruments, USA) and Graphpad Prism (v. 3.0, Graphpad Software Inc., SanDiego, CA, USA). All recordings were made at room temperature of ~21°C.

**Ex Vivo Preparation**

This technique is used to establish the relationship between storage volume and pressure. The bladders were extracted and the urethras ligated, close to the vesico-ureteric junctions. A catheter was placed in the urethral opening to record pressure and filling. Intravesical pressure was recorded in reference to atmospheric pressure. An amplifier was connected to a transducer that has been filled with water. A syringe filled with KRB solution was connected to a pump. The infusion rate of the KRB solution was 15-25 µL/min by automatic infusion and this was kept at 37°C. The filling of the bladder with KRB solution was stopped when the pressure reaches 45-50 cmH₂O. This is to avoid distending the bladder which can cause permanent tissue damage. Recordings obtained were analyzed by Clampfit (Molecular device) which had the baseline adjusted to examine the amplitude and frequency of pressures during filling (see Fig. 5).

**Solutions and Chemicals**

Whole-cell configuration was achieved in Ca²⁺-containing physiological saline bath solution (mm): NaCl 135, KCl 5, MgCl₂ 1.2, CaCl₂ 2, glucose 10, Heps 10, pH 7.4
with Tris-base. The pipette solution contained (mm): KCl 135, CaCl$_2$ 0.012, MgATP 3, Na$_2$GTP 0.1, creatine phosphate disodium 2.5, EGTA 0.1, glucose 10, Hepes 10, pH 7.2 with Tris-base. The KRB buffer solution contained (mM): NaCl 120, KCl 5, CaCl$_2$ 2, MgCl$_2$ 1 NaHCO$_3$ 25, D-glucose 5.5. All drugs and reagents including apamin and CyPPA (N-cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine) were purchased from Sigma.

**Statistical Analyses**

All Patch clamp data were expressed as means ± SEM. All statistical analyses were performed using Graphpad Prism. Student's paired or non-paired $t$ test were used to compare groups of data and differences were considered to be significant at $P < 0.05$. Data analysis for the westerns was performed using Compass software (ProteinSimple, San Jose, CA, USA), and expressed as intensity area/ug of protein. Lane view images of the western blots were saved as JPEGs, opened with Adobe Photoshop, converted to TIFFs; and saved after adjusting the image resolution with the Auto Res function.
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Authors’ contributions: HL, KMS and SDK designed research. HL, BHK, LEP, HJW and BAP performed patch, immunohistochemistry, molecular, ex vivo and Wes experiments and analysis, respectively. HL, SDK and KMS edited, reviewed the MS. All authors approved the final version of the manuscript.

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Figure 1. Quantitative analysis of transcripts in control and CYP-treated detrusor muscles. A. Transcripts for Pdgfra in control and CYP-treated detrusor muscle. B. Transcripts for Kcnn in control and CYP-treated detrusor muscle. C. Transcripts for Tnf and Il6 in control and CYP-treated detrusor muscle. Expression of all transcripts was normalized to Gapdh.
Figure 2. Immunohistochemistry of PDGFRα in murine detrusor from control and CYP-treated bladders. A. Whole mount detrusor with PDGFRα staining (green) from saline-treated control mouse. B. Immunoreactivity with PDGFRα (green) antibody was decreased in CYP-treated bladder detrusor compared with control (A). L and D denote lamina propria and detrusor muscle, respectively.
Figure 3. Expression levels of PDGFRα and SK3 in control and CYP-treated detrusor muscles. **A & C.** Representative gels of PDGFRα (A) and SK3 (C) expression in control and CYP-treated detrusor muscle. **B & D.** Quantification analysis of expression level of PDGFRα (B) and SK3 (D) in control and CYP-treated detrusor muscle. ** denotes P< 0.005, *** denotes P< 0.001.
Figure 4. The Effect of SK channel activator on membrane currents and potential in PDGFRα+ cells from control and CYP-treated mice. A, in detrusor PDGFRα+ cells from control bladders, CyPPA (10 µM) induced membrane hyperpolarization under current clamp (I = 0, red dot line). In the same cell, CyPPA activated outward current (current above blue dotted line) at a holding potential of −40 mV under voltage-clamp mode (V-C). B, current responses to ramp-depolarizations from A before (a) and during (b) CyPPA. Inset denotes voltage-protocol. C, in detrusor PDGFRα+ cells from CYP-treated bladders, CyPPA(10 µM) induced membrane hyperpolarization under current clamp (I = 0, red dot line). In the same cell, CyPPA activated outward current (current above blue dotted line) at a holding potential of −40 mV under voltage-clamp mode (V-C). D, current responses to ramp-depolarizations from C before (a) and during (b) CyPPA. Inset denotes voltage-protocol.
Figure 5. The Effect of SK channel blocker on spontaneous activity using the ex vivo volume-pressure relationship in control bladders. A & B. Ex vivo compliance curves for control (A) and after apamin application (B) from control bladders up to 15 cmH₂O (see blue dotted line). Expanded time scales with adjustment of baseline under control (Aa) and apamin (Bb) from panel A and B respectively. Blue dots were detected by threshold (0.2 cmH₂O) and used for frequency analysis. C & D. Summarized frequency (D) and amplitude (E) under control and after apamin application (n=6). ** and *** denote P<0.01 and P<0.001, respectively.
Figure 6. The Effect of SK channel activator and blocker on TCs in the *ex vivo* volume-preparation from CYP-treated bladders. **A.** *Ex vivo* compliance curves for control, SKA-31 and apamin application from CYP-treated bladders. **B & C.** Summarized frequency (**B**) and amplitude (**C**) under control, SKA31 and apamin application (n=6) from CYP-treated bladders.
Table 1: Primer sequences used for qPCR.

| Gene Name | Primer Sequences | Accession Number |
|-----------|------------------|------------------|
| Gapdh     | F-GCCGATGCCCCCATGTTTGTGA<br>R-GGGTGGCAGTGATGGCATGGAC | NM_008084.3 |
| Pdgfra    | F-ATGACAGGAGGGAGGGCTTCAACG<br>R-CCGCACAGGTCACCACGATCGTTT | NM_011058.2 |
| Tnf       | F-CTGAACTTCCGGGGTGATCGG<br>R-GGCTTGTCACTCGAATTTTGAGA | NM_013693.3 |
| Il6       | F-TCCAGTTGCCCTCTTGGGAC<br>R-GTACTCCAGAAGACCAGAGG | NM_031168.2 |
| Kcnn1     | F-TGTGTGTGTGTTCTCTCAGCG*<br>R-ACACACCTTCCCACAGTAG | NM_032397.2 |
| Kcnn2     | F-TTCTAACAACCTGGCGCTCT<br>R-CCAGCTTGTAGCCGATGTTC | NM_080465.2 |
| Kcnn3     | F-CTGCTGGTGTCAGCATCTCTCTG<br>R-GTCCCCCATAGCCAATGGAAAGGAAC | NM_080466.2 |
| Kcnn4     | F-AAGATGCTGCGCCGATCCACA<br>R-TCTTCTCCAGGGCAGGCTGGA | NM_008433.4 |