Key role for Kv11.1 (ether-a-go-go related gene) channels in rat bladder contractility.

Short Running title: ERG channels in rat bladder.

Vincenzo Barrese[1,2], Zena Wehbe[1], Alice Linden[1], Sarah McDowell[4], Elizabeth Forrester[1], Oleksander Povstyan[1], Karen D. McCloskey[4], Iain A Greenwood[1]*.

1. Vascular Biology Research Centre, Molecular & Clinical Sciences Research Institute, St George's, London, SW17 0RE.

2. Dept of Neuroscience, Reproductive Sciences and Dentistry, University of Naples Federico II, Naples, Italy

3. Institute of Medicine, University of Leeds, Leeds

4. Patrick G Johnston Centre for Cancer Research, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, BT9 7AE.

***Author for correspondence**

Corresponding Author Name: Iain Greenwood

- Address: Vascular Biology Research Centre, Institute of Molecular and Clinical Sciences, St George's University of London, London, UK
- Email: grenwood@sgul.ac.uk
- Phone number: 020 8672 9944- 2857

Email addresses:

VB: vincenzo.barrese@unina.it

ZW: m2109314@sgul.ac.uk

AL: m1503066@sgul.ac.uk

OP: o.povstyan@leeds.ac.uk

EF: m1802556@sgul.ac.uk

KM: k.mccloskey@qub.ac.uk

IAG: grenwood@sgul.ac.uk

Word Count: 4446

Acknowledgements

We would like to acknowledge the help with immunocytochemistry from Elizabeth Forrester, Alice Esseola and Sandra Ashton.

Conflict of Interest

The authors declare no conflict of interest.

Declaration of transparency and scientific rigour

We declare that our paper adheres to the principles for transparent reporting and scientific rigour of preclinic research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentations, as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Data Availability

The data generated within this paper is available upon reasonable request to the corresponding author.

Ethics Approval Statement

Animals used within the following investigation were handled in strict accordance with the Animal (Scientific Procedures) Act 1986.

Author contribution statement

VB performed molecular research. AL, KM, SM and ZW performed functional research. OP conducted electrophysiology research. ZW and IAG wrote the manuscript. IAG designed the research study. All authors contributed to the manuscript and approved the submitted version.

Funding

There are no funders for this research.

Abstract

Background and purpose: In addition, to their established role in cardiac myocytes and neurons, ion channels encoded by ether-a-go-go related genes (*kcnh*2) are functionally relevant in phasic smooth muscle. The aim of the study was to determine the expression and functional impact of Kv11.1 in rat urinary bladder smooth muscle.

Experimental approach: Quantitative polymerase chain reaction, immunocytochemistry, whole cell patch clamp, isometric tension recording.

Key Results: *kcnh*2 was expressed in rat bladder whereas *kcnh*6 and 3 expression was negligible. Immunofluorescence for Kv11.1 was detected in the membrane of isolated smooth muscle cells. Potassium currents with voltage-dependent characteristics consistent with Kv11.1 channels and sensitive to the specific blocker E4031 (1μM) were recorded from isolated detrusor smooth muscles. Disabling Kv11.1 activity with specific blockers (E4031 and dofetilide, 0.2- 20μM) augmented spontaneous contractions to a greater extent than BK_{Ca} channel blockers, enhanced carbachol-driven activity, increased nerve-stimulation mediated contractions and impaired β-adrenoceptor-mediated inhibitory responses.

Conclusion and implications: These data establish for the first time that Kv11.1 channels are key determinants of contractility in rat detrusor smooth muscle.

Key words: Detrusor smooth muscle, Ether-a-go-go related genes, *kcnh*2 gene, Kv11.1, BK channels

What is already known

- Kv11 channels are expressed in cardiac myocytes and are key modulators of normal cardiac electrical activity
- Kv11 channels reduce cellular excitability in smooth muscle cells.

What this study adds

- The Kv11.1 homologue is expressed in the rat detrusor
- Kv11.1 channels are involved in spontaneous, neurogenic and receptor-mediated contractions

Clinical significance

- Kv11.1 are key determinants of bladder contractility and may represent a more powerful brake on excitability than other Kv channels.
- Mutations in *kcnh*2 may be linked with bladder dysfunction
- Kv11.1 modulators could potentially restore normal contractility if developed with a cardiac-safe profile.

Introduction

The ether-a-go-go related gene (ERG) family comprises three members in humans, *ERG1,2,3* (*KCNH2, KCNH6* and *KCNH7*, HUGO gene nomenclature) that encode for tetrameric voltage-dependent potassium channels (Kv11.1-Kv11.3, respectively) (Gutman *et al.*, 2005). Kv11 channels, exhibit distinctive voltage-dependent kinetics due to a dominant C-type inactivation that is relieved quicker than channel deactivation by membrane hyperpolarisation (Spector et al., 1996; Hoshi & Armstrong, 2013; Dai & Zagotta, 2017).

KCNH2 is expressed predominantly in cardiac myocytes and congenital mutations in this gene result in gross perturbation of the normal cardiac electrical activity associated with hereditary long QT syndrome (London *et al.*, 1997; Vandenberg *et al.*, 2001). *KCNH6* and *KCNH7* expression is restricted mainly to neuronal cells, where the expression products contribute to the resting membrane conductance (Bauer & Schwarz, 2018). However, several studies have identified Kv11 channels as important dampeners of cellular excitability in several smooth muscles. *KCNH2* expression has been detected by q-PCR and immunocytochemistry in rodent stomach, portal vein and myometrium (Ohya *et al.*, 2002a; Ohya *et al.*, 2002b; Greenwood *et al.*, 2009). Moreover, currents with kinetics characteristic of Kv11 channels have been recorded in opossum oesophagus, mouse portal vein and non-pregnant mouse and human myometrium (Akbarali *et al.*, 1999; Ohya *et al.*, 2002b; Yeung & Greenwood, 2007; Greenwood *et al.*, 2009; Parkington *et al.*, 2014). In addition, selective Kv11 channel blockers like E4031 or dofetilide depolarise membrane potential and increase contractility in opossum oesophagus, rat stomach, mouse gall bladder, equine and human jejunum, mouse portal vein, bovine epididymis, mouse and human myometrium (Akbarali *et al.*, 1999; Ohya *et al.*, 2002a; Ohya *et al.*, 2002b; Farrelly *et al.*, 2003a; Lillich *et al.*, 2003; Parr *et al.*, 2003; Yeung & Greenwood, 2007; Mewe *et al.*, 2008; Greenwood *et al.*, 2009; Parkington *et al.*, 2014). Common to all these smooth muscles is the exhibition of spontaneous, phasic contractile behaviour associated with action potential discharge.

The detrusor smooth muscle of the urinary bladder also exhibits spontaneous contractions which, in tension recordings from bladder strips, manifest as low-amplitude contractions in contrast to much larger nerve-evoked or agonist-evoked (e.g. acetylcholine or ATP) contractions (Drake *et al.*, 2018). The genesis of the spontaneous contractions is a source of debate with both inherent myogenicity and urothelial-derived or mucosal mediators being implicated (Brading, 1997; Drake *et al.*, 2018). Their role is considered to provide a degree of tone and dynamic adjustment of optimal bladder shape during filling so that the bladder can be effectively emptied from any volume (Turner & Brading, 1997). During bladder filling, mechanisms that limit the amplitude of spontaneous contractions are essential for the bladder

to expand and act as a reservoir. Detrusor smooth muscle expresses a panel of potassium channels that act as brakes on contractility through repolarisation of the action potential and control of the resting membrane potential (Petkov, 2011, Thorneloe and Nelson, 2003). Of the myriad potassium channels, there is substantial evidence for the functional expression of BK (Petkov, 2014), Kv (Thorneloe and Nelson, 2003) and Kv7 channels (Anderson *et al.*, 2013, Provence *et al.*, 2018, Bientinesi *et al.*, 2017) and their contribution to spontaneous contractions . There is a paucity of work supporting expression of *ERG* in bladder and to the best of our knowledge, only one paper on ERG channel function where increased spontaneous contraction amplitude and corresponding decreased frequency in guinea-pig bladder strips by the ERG channel blocker, E4031 was reported (Imai *et al.*, 2001). The goal of the present study was to determine the molecular expression of *Kcnh* genes in rat detrusor smooth muscle and to ascertain the functional impact of ERG channels in detrusor contractility using various pharmacological selective modulators.

Materials and methods

Ex Vivo Bladder Preparation Techniques

Whole bladders were dissected from male Wistar rats (200-250 g) killed by cervical dislocation in accordance with the 1986 UK Animals Scientific Procedures Act (1986). Bladders were dissected free of fat and connective tissue then cut into transverse bands of 1 mm thickness from the mid-region of the bladder, whilst submerged in an ice-cold physiological saline solution (PSS) of the following composition (mmol-L-^{1}); 1.25 $CaCl_2$, 5 glucose, 25 $NaHCO_3$, 1.18 NaH_2PO_4, 1.17 $MgSO_4$, 4.5 KCl, 119 NaCl. The bands were then mounted on micro-pins within the tissue chambers of a myograph capable of isometric tension recording. Bands were set to an initial tension of 2 mN and reset to this level over a one hour equilibration period. Tissue viability was then assessed by a 60 mM KCl challenge. The effect of two structurally different, open channel blockers of Kv11.1, E4031 and dofetilide (Spector et al., 1996) was investigated using single concentrations per tissue. In an experimental series, carbachol was added cumulatively to all bladder bands. After washout of carbachol, tissue bands were incubated with one of the following agents: dimethyl sulphoxide (DMSO) (0.2%) as the vehicle control, or 2 µM E4031 and 20 µM E4031.

For isoprenaline experiments the level of spontaneous activity was enhanced by application of 20 mM KCl. Isoprenaline was applied cumulatively from (0.001 – 1 µM). After washout, tissue bands were pre-incubated with one of the following channel modulators: 20 µM E4031, 10 µM XE991 (Kv7 channel blocker),100 nM Iberiotoxin (BK_{Ca} blocker), or DMSO as vehicle control (0.2%) and isoprenaline was re-applied.

All drugs were obtained from HelloBio (Bristol, UK) except iberiotoxin (Alomone, Israel).

For electrical field stimulation (EFS) studies, transverse bands (1 mm thickness) were mounted in vertical baths attached to force transducers via wire hooks. EFS was delivered via silver/silver chloride electrodes placed at the top and bottom of the vertical organ baths using a Grass stimulator (Grass) and the following parameters, 0.3ms pulse width, 10s duration, 30-40V, 0.5 – 32 Hz frequency range. Organ baths were perfused with oxygenated PSS (solution as above) at 37^{o}C at a rate of 2-3ml/min; drugs were delivered via the perfusion system. Recordings were made via an AD/DA converter (National Instruments) and a personal computer running Chart software (University of Strathclyde, Dr J Dempster).

Contractions were measured in Clampfit (pClamp software, Axon Instruments) and data were collated in Excel (Microsoft Office) and analysed in GraphPad Prism software. EFS contraction amplitudes were normalised to a control contraction evoked by 60 mM K^+ Krebs solution for each experiment. Data are presented as mean ± standard error of the mean (SEM) and data sets were tested with two-way ANOVA and Bonferroni post-hoc tests with $p<0.05$ considered as significant.

Isometric tension data was recorded using LabChart software. The parameters that were then measured and analysed include, baseline tone, amplitude and frequency of individual phasic contractions and maximum change in contractility. Baseline tone is the lowest point of the trace from which phasic contractions develop. Maximum change in baseline contractility was measured for the highest crest amplitude and compared to the initial baseline tone. Amplitude was calculated as the average force (mN) of the peaks of the amplitudes two minutes prior to the addition of the next drug. Frequency was calculated as the average number of individual contractions occurring per minute.

Quantitative real-time polymerase chain reaction assay (qPCR)

cDNA was produced by reverse transcription of messenger RNA extracted from the intact bladder tissue samples. Quantitative real-time polymerase chain reaction (qPCR) was performed using specific primers for rat *Kcnh*2, *Kcnh*2-L, *Kcnh*6, and *Kcnh*7 **(Table 2),** and SYBR Green Master Mix from Primer design. Two isoforms are known for Kcnh2 (erg1) gene: a full-length isoform, encoding a protein composed by 1163 amino acids (previously termed erg1 long or erg1a), and a short one (erg1 short or erg1b), missing the first 5 exons of full length Erg1 and encoding a 821-amino acid protein with a different N-terminus with respect to Erg1a, encoded by an alternative exon 5 (Vandenberg et al., 2012).

Therefore, to evaluate the expression in rat bladder of the different isoforms, we used two sets of primers targeting the *Kcnh2* gene; these sequences were designed to amplify a common region of Kcnh2 found in the two transcripts variant (here defined as "*Kcnh2* primers"), and a specific sequence that can be found only in the longer variant of the same gene (here called "*Kcnh2-L* primers").

The reaction was carried out in a clean, designated environment to minimise risk of contamination. Primer and SYBR Green Mix were prepared and added into a 96-well plate, and the prepared cDNA template was added. Control wells containing no cDNA were used to detect the possible occurrence of contamination of the samples. Q-PCR reaction was performed using a CFX96 Real Time PCR machine (Biorad) according to the following protocol: 95ºC for 10 min (enzyme activation), then 40 cycles at 95ºC for 15 sec and 60ºC for 60 sec (denaturation and data collection). A melt curve to check for non-specific PCR products was performed at the end of the reaction. Results were then collected and analysed using BioRad CFX Manager Software. Data were normalized to the housekeeping gene Cytochrome C1 (CYC1) and expressed using the 2-ΔCt formula.

Table 2. Primers used for qPCR Experiments

Gene	***Kcnh2***	***Kcnh2-L***	***Kcnh6***	***Kcnh7***	***cyc1***
Primer Sequence *Forward*	5'-CCCCTCCATCAAGGACAAGT-3'	5'-CCTCGACACCATCATCCGCA-3'	5'GTGGATGTGGTCCCTGTGAA-3'	5'-GCCCGGGCTCAACCTGAAGA-3'	5'-TCGAAAACGCATGGGACTCA -3'
Primer Sequence Reverse	5'-TGAGCATGACACAGATGGAG-3'	5'-AGGAAATCGCAGGTGCAGGG-3'	5'-AGAGCCCAGGAAGCTGTGTG-3'	5'-TGGCCTGGATGTCCGTTGTC-3'	5'-TGACCACTTATGCCGCTTCA-3'
Amplicon size (base pairs)	128	156	141	169	85
GenBan/ NM number	2145-2272	369-533	328-468	3012-3180	300047

Electrophysiology

Single smooth muscle cells were isolated from whole bladder by enzymatic dispersal. Tissues were carefully cleaned of the mucosal layer (comprising urothelium, lamina propria, small blood vessels and connective tissues) and bathed for 10 minutes in nominally Ca^{2+}-free PSS. The vessels were then incubated at 37 ºC in Ca^{2+}-free PSS containing collagenase type IA (2

mg/ml) and protease type X (1 mg/ml) for 25-30 min followed by a 10 min wash in Ca^{2+}-free PSS at room temperature. Single cells were liberated by gentle mechanical agitation of tissues with a wide bore Pasteur pipette and the suspension was transferred to experimental chambers. All experiments were performed at room temperature using the whole cell patch-clamp technique. The solution used for recordings was of the following composition (mM): KCl 60, NaCl 66, $MgCl_2$ 1.2, $CaCl_2$ 0.1, D-glucose 12 and HEPES 10, pH was adjusted to 7.35 with NaOH. The solution was supplemented with paxilline (1 µM) and nicardipine (5 µM). Patch pipettes were fire-polished and had resistances of 4–8 MΩ when filled with the pipette solution containing (in mmol/L): 110 K gluconate, 30 KCl, 0.5 $MgCl_2$, 5 HEPES and 0.5 EGTA. The electrical signals were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA). Electrical signals were generated and digitized at 1 kHz using a Digidata 1322A hosted by a PC running pClamp 9.0 software (Axon Instruments, Sunnyvale, CA, USA). Data were analysed and plotted using pClamp and MicroCal Origin software. All data are presented as mean ± SEM. To isolate Kv11 channel currents we employed a protocol used previously (Ohya et al., 2002, Greenwood et al., 2009) that took advantage of the biophysical properties of the channel. Cells were held at 0 mV where Kv11.1 channels were in an inactive state and then stepped to hyperpolarized potentials from -120 mV to -20 mV every 5 s. Steps to hyperpolarized voltages removes the inactivation that is followed by channel closure (deactivation). This results in a distinctive 'hooked' appearance as both the recovery from inactivation and deactivation is faster at more hyperpolarized potentials (Smith et al., 1996; Ohya et al., 2002).

Immunocytochemistry

Bladder smooth muscle cells (SMC) were fixed with 3% paraformaldehyde solution at 22-24°C (RT) for 10 minutes, treated with 0.1 M glycine in PBS for 5 min and incubated in blocking solution (PBS containing 0.1% Triton X-100 and 1% bovine serum albumin) for 1h at RT. Subsequently, cells were incubated overnight at 4°C with goat polyclonal anti-HERG antibody (dilution 1:100) (catalogue number [Cat.#]: sc-377388) (Santa Cruz, USA), which had been validated previously using over-expression systems (Barrese et al.,2017), and mouse monoclonal anti-α-smooth muscle actin antibody (dilution 1:1,000) (Cat.#: A5228) (Sigma, Dorset, UK). Samples were washed with PBS and incubated for 1h with donkey anti-goat secondary antibody conjugated to Alexa Fluor 568 (Cat.#: A-11057) and donkey anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Cat.#: A32766) (dilution

1:100) (ThermoFisher, Paisley, UK). All antibodies were diluted in blocking solution. No primary antibody was used as a negative control. Coverslips were mounted on glass slides; images were acquired using a Nikon A1R confocal microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands).

Data analysis

Data was presented as mean ± SEM. For most graphical presentations, data was normalised and given as a percentage increase from resting conditions, or as a percentage difference from the control group. Data processing and presentation utilised Microsoft Excel and Prism GraphPad software. Statistical analysis was carried out with one-way and two-way ANOVAs, where statistically significant values *,**,***,****, represent $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$ respectively. The number of tissues utilised in an experiment is referred to as 'n', whereas the number of rats culled is denoted by 'N'.

Results

Kv11.1 transcript (Kcnh) is expressed in rat detrusor smooth muscle

Q-PCR using primers specific for all three *Kcnh* isoforms revealed robust expression of *Kcnh*2 but minimal expression of Kcnh6 and 7 in the intact rat detrusor (**Fig. 1A**). Of the two known variants of *Kcnh*2 (London *et al.*, 1997), the rat detrusor predominantly expressed the longer form **(Fig. 1A).** Immunofluorescence experiments using an anti-Kv11.1 antibody (Barrese et al., 2017) were undertaken to assess expression of *Kcnh*2 products in detrusor muscle. SMCs were identified by co-staining with α-smooth muscle actin. In these cells, anti-Kv11.1 immunofluorescence showed peripheral distribution, indicative of plasma membrane localisation of Kv11.1 channels **(Fig. 1B).**

Whole cell electrophysiology was performed to determine if functional Kv11.1 currents could be recorded from isolated detrusor SMC using a protocol utilised previously (Ohya *et al.*, 2002b; Greenwood *et al.*, 2009) that takes advantage of the distinctive properties of the Kv11.1 channel. When isolated detrusor SMC were hyperpolarized from a holding potential to -120 mV a large, slowly developing inward current was recorded. This current was the HCN-encoded mixed cation current known as the 'funny' current that is responsible for sino-atrial node pacing and that has been characterised in rat bladder and mouse portal vein previously (Green et al., 1990; Greenwood & Prestwich, 2002). The HCN channel current was preceded

by an inward current with a prominent 'hook' **(Fig. 2)** that decayed rapidly and was abrogated by the Kv11.1 specific blocker E4031 **(Fig. 2A-F)** (N=4, $p<0.05$). The kinetics of the development and subsequent decay of the E4031-sensitive current were voltage-dependent becoming slower at less negative potentials **(Fig. 2D).** These kinetics are consistent with the voltage-dependent properties of inactivation recovery and deactivation exhibited by Kv11.1 channels (Spector et al 1996; Ohya et al., 2002).

Kv11.1 blockers affect detrusor contractility

Transverse bands of bladder exhibited spontaneous phasic contractile activity that was sustained through the duration of typical recordings. Addition of two structurally different Kv11.1–specific blockers, E4031 and dofetilide (Yeung & Greenwood, 2007) augmented detrusor contractility considerably in a concentration-dependent manner **(Fig. 3).** In an intact bladder (in which the mucosa is retained), application of 2 µM E4031 or 2 µM dofetilide did not increase basal tone, whereas 20 µM E4031 caused a significant increase (+0.6 ± 0.1, N=5, $p<0.001$), as did 20 µM dofetilide (+0.56 ± 0.18, N=5, $P<.05$) **(Fig. 3B.i and 3B.ii)**. To confirm if neuronal input modulated the effect of E4031 on baseline tone, 1 µM of tetrodotoxin (TTX) was added prior to 20 µM E4031 to prevent neurotransmitter release. E4031 still increased baseline tone even in the presence of TTX (+0.14 ± 0.01, N=5) and this was not significantly different from its corresponding control **(Fig. 3B.iii)**.

Phasic contraction amplitude was significantly increased in the presence of 2 µM E4031 (566.7% ± 54.21%, N=5, $p<0.001$) and 20 µM E4031 (734.5% ± 106.8%, N=5, $p<0.0001$) in an intact detrusor **(Fig. 3C.i).** Similarly, 2 µM and 20 µM dofetilide also increased phasic contraction amplitude by 560.4% (± 131.7%, N=5, $p<0.05$) and 695.1% (± 110.1%, N=5, $p<0.01$), respectively **(Fig. 3C.ii).** This heightened contractile activity was unaltered over a 5-hour experimental period. E4031 still increased the amplitude of spontaneous contractions in the presence of TTX, (+355.2% ± 67.9, N=5) **(Fig. 3C.iii).** These data show that Kv11.1 channels have considerable impact upon spontaneous activity in the rat detrusor and that this effect is not likely mediated via neuronal stimulation.

Kv11.1 blockers and carbachol-mediated contractions

Application of the muscarinic receptor agonist, carbachol (1-10 µM) (CCh) evoked concentration-dependent contractions that were manifest as enhanced baseline superimposed by individual contractions (**Fig. 4 A.B**). All bladder strips were exposed to increasing concentrations of CCh followed by a wash out with PSS and this served as the internal control for each strip. The wash out was followed up another exposure to increasing

329 concentrations of CCh either with the prior incubation with 2 µM, 20 µM E4301 or vehicle
330 control. The subsequent increase in baseline tone, contractile amplitude or area under the
331 curve was measured as the change from its internal control (first exposure to CCh). Pre-
332 application of E4031 (2 µM) enhanced the effect of CCh on the amplitude of phasic
333 contractions and further enhancement was observed with 20 µM **(Fig. 4.C)** Neither
334 concentration of E4031 affected the force integral of the carbachol response measured as
335 area under curve during the first 3 minutes of CCh response **(Fig. 4D)**.

336

337 ***Kv11.1 blockers and nerve-mediated contractions***

338 Electrical field stimulation (EFS, 0.5 - 32 Hz) evoked frequency-dependent contractions (**Fig.**
339 **5A**), which are typically inhibited by the voltage-gated sodium channel blocker tetrodotoxin
340 (Campbell *et al.*, 2017). E4031 (20 µM) caused small but significant increases in EFS-
341 contraction amplitude across most frequencies (0.5, 1, 2, 4, 8, 16Hz; $p<0.05$, N=5) (**Fig. 5B.i**).
342 EFS-contraction amplitudes in each experiment were normalised to a control contraction
343 evoked by 60 mM K^+ Krebs solution, prior to calculations of means, SEM or statistical analysis.
344 Addition of TTX (1 µM) eliminated EFS contractions (30V, 30 Hz), and they were not restored
345 upon the addition of 20 µM E4301 **(Fig. 5B.ii)**.

346

347 ***Kv11.1 blockers on contractions of a denuded bladder***

348 The bladder was denuded in order to test the efficacy of E4301 on phasic and EFS
349 contractions in the absence of the mucosa **(Fig. 6A)**. There was still a significant increase in
350 baseline tone (+0.13 ± 0.01, N=5, $p<0.05$) **(Fig. 6B.i)** and phasic contraction amplitude,
351 relative to control (556.3% ± 63.9, N=5, $p<0.001$) in a denuded bladder **(Fig. 6B.ii)**. E4031 (20
352 µM) induced a significant increase in EFS-contraction amplitude across low frequencies (0.5,
353 2, 4 Hz; $p<0.05$, N=5), but had no effect at higher frequencies **(Fig. 6B.iii)**. EFS-contraction
354 amplitudes were normalised to a control contraction induced by 60mM K^+ Krebs solution
355 before calculating means and SEM.

356 ***The effect of Kv11.1 activators on EFS-evoked and spontaneous contractions***

357 The effects of two structurally dissimilar Kv11.1 activators PD118057 and NS1643 (Casis et
358 al., 2006, Zhou et al., 2005) were assessed on spontaneous and nerve-evoked contractions.
359 PD118057, significantly reduced spontaneous contractions to ~40% of basal levels (N=5,
360 $p<0.05$) **(Fig. 7).** NS1643 also reduced spontaneous contractions but this did not reach

significance (not shown). Both NS1643 and PD118057 had no significant effect on nerve-evoked contractions.

Comparison of BK and Kv11.1 blockers on spontaneous activity

BK channels are considered to have significant roles in bladder smooth muscle cell physiology (Meredith *et al.*, 2004; Petkov, 2014) and have been studied extensively in tissue myography and single-cell patch-clamp recordings (Cotton *et al.*, 1996; Huang *et al*., 1997; Herrera & Nelson, 2002). Here, we investigated the impact of blocking BK and Kv11.1 channels on bladder spontaneous contractions (**Fig. 8**). Application of the BK channel blocker, iberiotoxin (IbTX, 300 nM) increased the amplitude of spontaneous contractions amplitude by 156.6 ± 15.90 % (SEM, N=5) that was associated with a small (0.13 mN ± 0.04) increase in basal tone **(Fig. 8 B,C).** Subsequent addition of E4031 (20 μM) augmented the amplitude of spontaneous contractions (606.3 ± 76.42 %, N=5), similar to the increase induced by E4031 (20 μM) alone (~ 634% ± 82.89). Application of E4031 to tissues incubated in IbTX increased resting tension (0.50 ± 0.0.078 mN) to a level similar to that seen with E4031 (20 μM) alone (~0.5 ± 0.09) **(Fig 8 B,C).**

Isoprenaline-mediated relaxations are affected by Kv11.1 blockade

Stimulation of cAMP-linked β-adrenoceptors (predominantly β3) reduces detrusor smooth muscle contractility. As Kv11.1 channel activity is enhanced by cAMP-dependent protein kinase A (Chen *et al*., 2010) we speculated if Kv11 channels contributed functionally to β-adrenoceptor-mediated responses. Application of the general β-adrenoceptor agonist isoprenaline produced a concentration-dependent inhibition of spontaneous activity that had been augmented by the addition of 20 mM KCl **(Fig. 9A).** The presence of E4031 (2 μM) attenuated the inhibitory effect of isoprenaline markedly (**Fig. 9A)**. BK and Kv7 channels have also been implicated in relaxations produced by agonists of cAMP-linked receptors (eg Huang et al., 1997, Stott et al., 2018; Van der Horst et al., 2020). In contrast to the effect of E4031 on isoprenaline mediated responses, neither IBTX (100 nM) nor the Kv7 channel blocker XE991 (10 μM) altered the inhibitory effect of isoprenaline **(Fig. 9B)**. These data suggest that Kv11.1 channels provide a functional endpoint for β-adrenoceptor-related actions on bladder contractility.

Discussion

This study demonstrated that male rat bladders expressed both known variants of *Kcnh2* but not *Kcnh6* or *Kcnh7*. Immunocytochemistry established that the *Kcnh2* expression product, Kv11.1, was located predominantly at the cell membrane and patch clamp experiments demonstrated potassium currents with distinctive ‘hooked’ kinetics sensitive to the selective

Kv11.1 blocker, E4031, were present in bladder smooth muscle cells. In tension recordings, blockade of Kv11.1 by two structurally different agents, E4031 and dofetilide, enhanced contractile activity of the detrusor. In addition, Kv11.1 blockers augmented the contractile response of the muscarinic receptor agonist carbachol and attenuated the relaxant effect of the β-adrenoceptor agonist, isoprenaline.

Blockade of Kv11.1 channels with two structurally different agents enhanced the spontaneous contractile activity of rat bladders similar to preliminary data published by Imai et al (2001) in guinea pig bladder. These effects far exceeded those produced by blockers of BK_{Ca} and Kv7 channels (IBTX and XE991, respectively) and were manifest as an increase in the amplitude and duration of spontaneous events leading to a tonic increase in tension. These data are consistent with Kv11.1 channels contributing to the resting membrane potential and repolarisation of the action potential associated with spontaneous contractions (Hashitani & Brading, 2003). These pronounced effects of Kv11.1 blockers have also been observed in opossum oesophagus, mouse gall bladder, equine and human jejunum, bovine epididymis, mouse and human myometrium (Akbarali *et al.*, 1999; Farrelly *et al.*, 2003a; Lillich *et al.*, 2003; Parr *et al.*, 2003; Mewe *et al.*, 2008; Greenwood *et al.*, 2009; Parkington *et al.*, 2014). In many tissues, application of Kv11.1 blockers can convert mechanically quiescent smooth muscle to actively contracting tissues suggesting the contractile activity of the tissue may be dictated by the relative dominance of Kv11.1 channels. As this study demonstrated, the effects of Kv11.1 blockers on bladder contractility was mediated via detrusor smooth muscle cells, as even in the absence of a mucosa, phasic contraction amplitude was still significantly elevated. Moreover, inhibition of neuronal activation via TTX did not prevent the effect of E4031 on detrusor phasic contractility. However, this does not rule out that E4031 may also be affecting urothelial, interstitial cells or neuronal depolarization, and remains to be further investigated in future studies. Of note, Kv11.1 channels have been identified on neurons. In particular, ERG1 and the orthologs ERG2 and ERG3 have been identified in the brain and display regional specificity and they are involved in mediating the neuronal current (Sanchez-Conde, 2022).

Moreover, Kv11.1 protein was identified in the plasma membrane of α-smooth muscle actin-positive cells and E4031-sensitive currents with the distinctive voltage-dependent kinetics that are inherent to the channels were recorded from isolated smooth muscle cells. Kv11.1 currents have previously been recorded in smooth muscle cells isolated from opossum oesophagus, mouse portal vein and myometrium from mouse and human (Akbarali et al., 1999; Ohya et al., 2002b; Yeung & Greenwood, 2007, Greenwood et al., 2009, Parkington et

al. 2014). The voltage-dependent kinetics recorded in the present study were remarkably similar to those recorded in the other cell types. The combined data suggest that Kv11.1 is expressed by the detrusor smooth muscle cells and it contributes markedly to the regulation of smooth muscle contractility. There are two known rat isoforms of Kv11.1, previously known as erg1a and 1b. With respect to erg1a, erg1b lacks 373 amino acids and has a different N-terminus. The N-terminal region of erg1a forms the protein domain responsible for the characteristic deactivation kinetics of the channel, and the so-called “PAS domain”, that defines the ether-a-go-go subfamily of voltage gated potassium channels. Erg1a and 1b co-assemble in the endoplasmic reticulum to form a tetrameric channel, that mediates the “native” current in cardiac myocytes. Heteromeric erg1a/1b channels show faster activation, deactivation, and recovery from inactivation than channels formed by erg1a alone, a characteristic consistent with the lack of the N-terminal region responsible for the deactivation kinetics in erg1b. On the other hand, erg1b modulates channel trafficking and response to hormones and other endogenous agonists (Vanderberg et al, 2012). Whilst the molecular composition of the Kv11.1 channel in bladder remains to be define the relative lack of the shorter isoform suggests a heterotetramer is unlikely.

In addition to verifying the expression of *Kcnh2* and characterizing the role of Kv11.1, we also compared its effect to the large conductance voltage-activated and Ca^{2+} activated (BK) channel. The BK channel is activated by both voltage and Ca^{2+} and is well expressed in the detrusor (Petkov, 2011). Consistent with previous studies application of the BK channel blocker, IBTX, enhanced spontaneous activity; however, its impact was substantially smaller than that of the Kv11.1 inhibitors. Application of E4031 in the continued presence of iberiotoxin produced a striking enhancement of contractile activity reinforcing the view that Kv11.1 channels provide considerable capacity for suppressing bladder contractions and are major determinants of resting activity. It should be noted that detrusor smooth muscle cells also express a variety of other K+ channels, which collectively modulate and contribute to a resting membrane potential and repolarization. For example, SK channels, which are Ca^{2+} sensitive but voltage insensitive, are activated after the hyperpolarization phase and are present in much less abundance compared to BK channels. Pharmacological inhibition of these channels is associated with increased phasic contraction frequency (Herrera et al., 2003). Other channels which have been detected on the DSM include inward-rectifying ATP-sensitive K+ channels (K_{ATP}). These channels are activated at low intracellular concentrations of ATP, whereas they are inhibited at higher concentrations. Future studies will elaborate the interplay of Kv11.1 channels with other K channels expressed in the bladder.

E4031 also enhanced carbachol-induced contractility by ~25%. suggesting that Kv11.1 is not only important in regulating baseline tone and spontaneous activity, but also limits the amplitude of receptor-operated contractions. Our finding that EFS-mediated, neurogenic contractions could also be augmented by E4031, shows that Kv11.1 activity may provide a protective limit to bladder contraction amplitude during micturition. This would maintain metabolic homeostasis and also provide a further tuning mechanism for appropriate contraction amplitude. Such a mechanism might be expected to react to relaxation of bladder smooth muscle and this was tested in the present study using, isoprenaline, a non-specific β-adrenergic receptor agonist, that induced a concentration-dependent relaxation, almost abolishing spontaneous contractions. Notably, E4031 but not blockers of BK or Kv7 channels blunted the relaxation effect of isoprenaline. Activation of β-adrenergic receptors stimulates Kv11.1 channels via a cAMP-PKA mechanism (Kiehl, 2000; Vandenberg et al., 2012); furthermore, Kv11.1 protein contains numerous protein kinase A (PKA) phosphorylation sites within its intracellular domain, and, within cardiac myocytes, the interaction activates the channel enabling greater channel activation (Kiehl, 2000; Vandenberg et al., 2012). Thus, Kv11.1 may also have an important role in mediating β-adrenergic receptor bladder relaxation.

Dual actions of Kv11.1 blockers enhancing contraction (spontaneous, neurogenic and muscarinic-receptor operated) and impairing relaxation (β-adrenergic receptor operated) reveal the versatility of Kv11.1-mediated signalling in bladder physiology. While the complexity of mechanisms is not yet understood, Kv11.1 function is a promising area of study. Bladder dysfunction and lower urinary tract symptoms are common and represent many diverse pathophysiologies. Many targets have been identified and investigated for clinical translation, notably drugs targeting muscarinic receptors and β3-adrenergic receptor agonists are used clinically. BK channel activators were considered to be promising to reduce detrusor overactivity; however, the finding that BK is under-expressed in the neurogenic bladder impeded further development. Interestingly, gene therapies to restore BK channel expression in the neurogenic bladder have been in clinical trials and may bring benefit to patients. With the known key function of Kv11.1 channels in cardiac myocytes (Sanguinetti *et al.*, 1996), their importance is especially notable in individuals with congenital defects in Kv11.1 channels, where resulting cardiac arrhythmia may lead to sudden death. Mutations in *KCNH2* are responsible for approximately 25% of hereditary arrhythmias that present with QT interval prolongation (Sanguinetti *et al.*, 1996); it is not known whether this has consequences for bladder function All drugs are screened for activity against Kv11.1 to avoid adverse cardiac effects and this might suggest that Kv11.1 could not be progressed as a target to treat bladder dysfunction. Research directed at characterisation of bladder Kv11.1 protein structure, subtypes, pharmacological profile compared with cardiac channels may provide opportunity

to progress bladder-selective drugs. It is also important to discover whether existing bladder dysfunction treatments have actions on bladder Kv11.1 in addition to their known targets.

In conclusion, the findings of the study reveal functional expression of *kcnh*2 transcript as Kv11.1 ion channels in rat bladder smooth muscle. Pharmacological modulators reveal that Kv11.1 are key determinants of bladder contractility and may represent a more powerful brake on excitability than BK or other Kv channels. The findings show participation of Kv11.1 in spontaneous, neurogenic and receptor-mediated contractions, suggesting a central role in bladder physiology. It is not yet known whether *kcnh*2 mutations are linked with bladder dysfunction; or whether Kv11.1 modulators could restore normal contractility if developed with a cardiac-safe profile; we highlight this as an important area for further research.

References

Akbarali HI, Thatte H, He XD, Giles WR & Goyal RK. (1999). Role of HERG-like K(+) currents in opossum esophageal circular smooth muscle. *Am J Physiol* **277,** C1284-1290.

Anderson UA, Carson C, Johnston L, Joshi S, Gurney AM & McCloskey KD. (2013). Functional expression of KCNQ (Kv7) channels in guinea pig bladder smooth muscle and their contribution to spontaneous activity. *Br J Pharmacol* **169,** 1290-1304.

Bauer CK & Schwarz JR. (2018). Ether-à-go-go K. *J Physiol* **596,** 769-783.

Brading AF. (1997). A myogenic basis for the overactive bladder. *Urology* **50,** 57-67; discussion 68-73.

Campbell PC, McDonnell B, Monaghan KP, Baysting L, Little O & McCloskey KD. (2017). Mucosal modulation of contractility in bladder strips from normal and overactive rat models and the effect of botulinum toxin A on overactive bladder strips. *Neurourology and urodynamics* **36,** 1052-1060.

Casis O, Olesen SP, Sanguinetti MC. Mechanism of action of a novel human ether-a-go-go-related gene channel activator. *Mol Pharmacol.* 2006;**69**:658–665.

Cotton KD, Hollywood MA, Thornbury KD & McHale NG. (1996). Effect of purinergic blockers on outward current in isolated smooth muscle cells of the sheep bladder. *Am J Physiol* **270,** C969-973.

Dai G & Zagotta WN. (2017). Molecular mechanism of voltage-dependent potentiation of *kcnh* potassium channels. *Elife* **6**.

Drake MJ, Fry CH, Hashitani H, Kirschner-Hermanns R, Rahnama'i MS, Speich JE, Tomoe H, Kanai AJ & McCloskey KD. (2018). What are the origins and relevance of spontaneous bladder contractions? ICI-RS 2017. *Neurourology and urodynamics* **37,** S13-S19.

Farrelly AM, Ro S, Callaghan BP, Khoyi MA, Fleming N, Horowitz B, Sanders KM & Keef KD. (2003a). Expression and function of *kcnh*2 (HERG) in the human jejunum. *Am J Physiol Gastrointest Liver Physiol* **284,** G883-895.

Farrelly AM, Ro S, Callaghan BP, Khoyi MA, Fleming N, Horowitz B, Sanders KM & Keef KD. (2003b). Expression and function of *kcnh*2 (HERG) in the human jejunum. *American journal of physiologyGastrointestinal and liver physiology* **284,** G883-895.

Greenwood IA, Yeung SY, Tribe RM & Ohya S. (2009). Loss of functional K+ channels encoded by ether-à-go-go-related genes in mouse myometrium prior to labour onset. *J Physiol* **587,** 2313-2326.

Gutman GA, Chandy KG, Grissmer S, Lazdunski M, McKinnon D, Pardo LA, Robertson GA, Rudy B, Sanguinetti MC, Stuhmer W & Wang X. (2005). International Union of

Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. *Pharmacol Rev* **57,** 473-508.

Hashitani H & Brading AF. (2003). Ionic basis for the regulation of spontaneous excitation in detrusor smooth muscle cells of the guinea-pig urinary bladder. *Br J Pharmacol* **140,** 159-169.

Herrera GM & Nelson MT. (2002). Differential regulation of SK and BK channels by Ca(2+) signals from Ca(2+) channels and ryanodine receptors in guinea-pig urinary bladder myocytes. *J Physiol* **541,** 483-492.

Herrera, G. M., Pozo, M. J., Zvara, P., Petkov, G. V., Bond, C. T., Adelman, J. P., & Nelson, M. T. (2003). Urinary bladder instability induced by selective suppression of the murine small conductance calcium-activated potassium (SK3) channel. *The Journal of physiology*, *551*(Pt 3), 893–903. https://doi.org/10.1113/jphysiol.2003.045914

Hoshi T & Armstrong CM. (2013). C-type inactivation of voltage-gated K+ channels: pore constriction or dilation? *J Gen Physiol* **141,** 151-160.

Imai T, Okamoto T, Yamamoto Y, Tanaka H, Koike K, Shigenobu K & Tanaka Y. (2001). Effects of different types of K+ channel modulators on the spontaneous myogenic contraction of guinea-pig urinary bladder smooth muscle. *Acta Physiologica Scandinavica* **173,** 323-333.

Kiehl,Johann. (2000). Regulation of the cardiac repolarizing HERG potassium channel by Protein Kinase A. *Trends in Cardiovascular Medicine*, **10,** 205-209.

Lillich JD, Rakestraw PC, Roussel AJ, Finley MR, Ganta S & Freeman LC. (2003). Expression of the ether-a-go-go (ERG) potassium channel in smooth muscle of the equine gastrointestinal tract and influence on activity of jejunal smooth muscle. *Am J Vet Res* **64,** 267-272.

London B, Trudeau MC, Newton KP, Beyer AK, Copeland NG, Gilbert DJ, Jenkins NA, Satler CA & Robertson GA. (1997). Two isoforms of the mouse ether-a-go-go-related gene coassemble to form channels with properties similar to the rapidly activating component of the cardiac delayed rectifier K+ current. *Circ Res* **81,** 870-878.

Malysz J & Petkov GV. (2020). Urinary bladder smooth muscle ion channels: expression, function, and regulation in health and disease. *Am J Physiol Renal Physiol* **319,** F257-F283.

Meredith AL, Thorneloe KS, Werner ME, Nelson MT & Aldrich RW. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J Biol Chem* **279,** 36746-36752.

Mewe M, Wulfsen I, Schuster AM, Middendorff R, Glassmeier G, Schwarz JR & Bauer CK. (2008). Erg K+ channels modulate contractile activity in the bovine epididymal duct. *Am J Physiol Regul Integr Comp Physiol* **294,** R895-904.

Ohya S, Asakura K, Muraki K, Watanabe M & Imaizumi Y. (2002a). Molecular and functional characterization of ERG, KCNQ, and KCNE subtypes in rat stomach smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **282,** G277-287.

Ohya S, Horowitz B & Greenwood IA. (2002b). Functional and molecular identification of ERG channels in murine portal vein myocytes. *Am J Physiol Cell Physiol* **283,** C866-877.

Ohya S, Horowitz B & Greenwood IA. (2002c). Functional and molecular identification of ERG channels in murine portal vein myocytes. *American journal of physiologyCell physiology* **283,** C866-877.

Parkington HC, Stevenson J, Tonta MA, Paul J, Butler T, Maiti K, Chan EC, Sheehan PM, Brennecke SP, Coleman HA & Smith R. (2014). Diminished hERG K+ channel activity facilitates strong human labour contractions but is dysregulated in obese women. *Nat Commun* **5,** 4108.

Parr E, Pozo MJ, Horowitz B, Nelson MT & Mawe GM. (2003). ERG K+ channels modulate the electrical and contractile activities of gallbladder smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **284,** G392-398.

Petkov, G. V., Heppner, T. J., Bonev, A. D., Herrera, G. M., & Nelson, M. T. (2001). Low levels of K(ATP) channel activation decrease excitability and contractility of urinary bladder. *American journal of physiology. Regulatory, integrative and comparative physiology*, *280*(5), R1427–R1433. https://doi.org/10.1152/ajpregu.2001.280.5.R1427

Petkov G. V. (2011). Role of potassium ion channels in detrusor smooth muscle function and dysfunction. *Nature reviews. Urology*, *9*(1), 30–40. https://doi.org/10.1038/nrurol.2011.194

Petkov GV. (2014). Central role of the BK channel in urinary bladder smooth muscle physiology and pathophysiology. *American journal of physiologyRegulatory, integrative and comparative physiology* **307,** R571-584.

Sanchez-Conde, F. G., Jimenez-Vazquez, E. N., Auerbach, D. S., & Jones, D. K. (2022). The ERG1 K^+ Channel and Its Role in Neuronal Health and Disease. *Frontiers in molecular neuroscience*, *15*, 890368. https://doi.org/10.3389/fnmol.2022.890368

Sanguinetti MC, Curran ME, Spector PS & Keating MT. (1996). Spectrum of HERG K+-channel dysfunction in an inherited cardiac arrhythmia. *Proc Natl Acad Sci U S A* **93,** 2208-2212.

Smith PL, Baukrowitz T, Yellen G (1996). The inward rectification mechanism of the HERG cardiac potassium channel. Nature.;379(6568):833-6

660 Spector PS, Curran ME, Keating MT, Sanguinetti MC (1996). Class III antiarrhythmic drugs
661 block HERG, a human cardiac delayed rectifier K+ channel. Open-channel block by
662 methanesulfonanilides. Circ Res.;78(3):499-503

663
664 Svalo J, Sheykhzade M, Nordling J, Matras C & Bouchelouche P. (2015). Functional and
665 molecular evidence for Kv7 channel subtypes in human detrusor from patients with
666 and without bladder outflow obstruction. *PLoS One* **10,** e0117350.

667
668 Turner WH & Brading AF. (1997). Smooth muscle of the bladder in the normal and the
669 diseased state: pathophysiology, diagnosis and treatment. *Pharmacology &*
670 *therapeutics* **75,** 77-110.

671
672 Vandenberg JI, Walker BD & Campbell TJ. (2001). HERG K+ channels: friend and foe. *Trends*
673 *Pharmacol Sci* **22,** 240-246.

674
675 Yeung SY & Greenwood IA. (2007). Pharmacological and biophysical isolation of K+ currents
676 encoded by ether-à-go-go-related genes in murine hepatic portal vein smooth muscle
677 cells. *Am J Physiol Cell Physiol* **292,** C468-476.

678

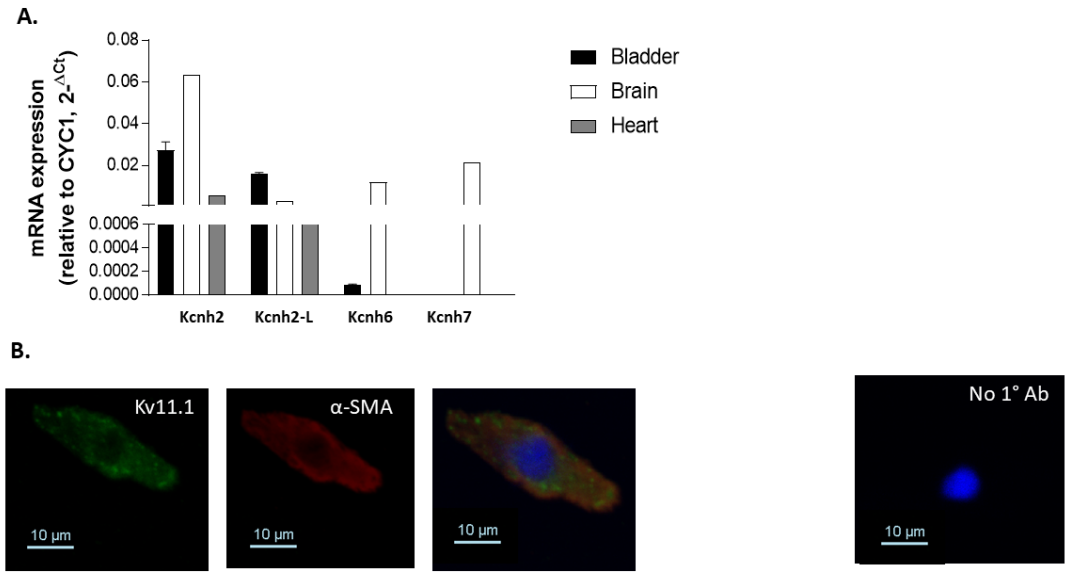
679 Zhou J, Augelli-Szafran CE, Bradley JA, Chen X, Koci BJ, Volberg WA, Sun Z, Cordes JS.
680 Novel potent human *ether-a-go-go*-related gene (hERG) potassium channel
681 enhancers and their *in vitro* antiarrhythmic activity. *Mol Pharmacol.* 2005;**68**:876–884.

682

683

684

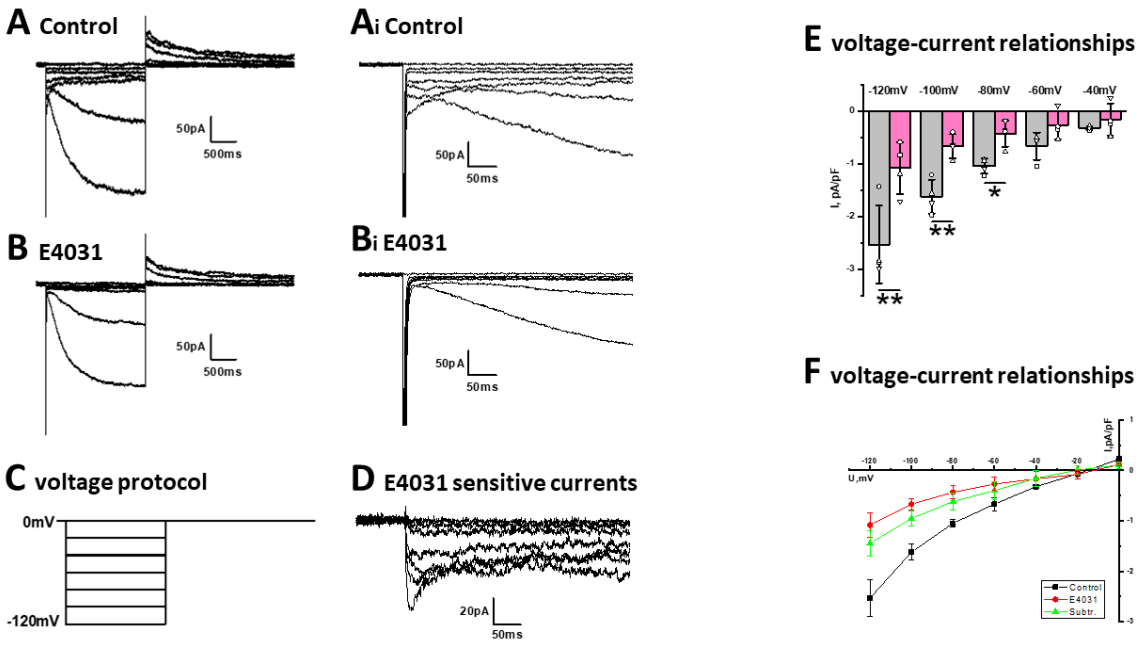
685 Legends.



686

687 **Figure 1. Expression of Kcnh2 channels in rat detrusor.** (A) Quantitative PCR experiments
688 showing the expression of total Kcnh2, long Kcnh2 isoform (Kcnq2-L), Kcnh6 and Kcnh7
689 mRNAs in rat detrusor, normalized to the housekeeping gene Cyc1. The rat brain and heart
690 were used as positive controls. Data are expressed as mean ± s.e.m. N=5. (B). Fluorescent
691 images of smooth muscle cells isolated from rat detrusor and labelled with K_v11.1 antibody
692 (left column) and α-smooth muscle actin (α-SMA, middle column). Cell where primary
693 antibodies were omitted (incubated with secondary antibodies only) are also shown (right
694 panel). Each image is representative of 5 separate dispersals. Scale bar= 10 µm.

695

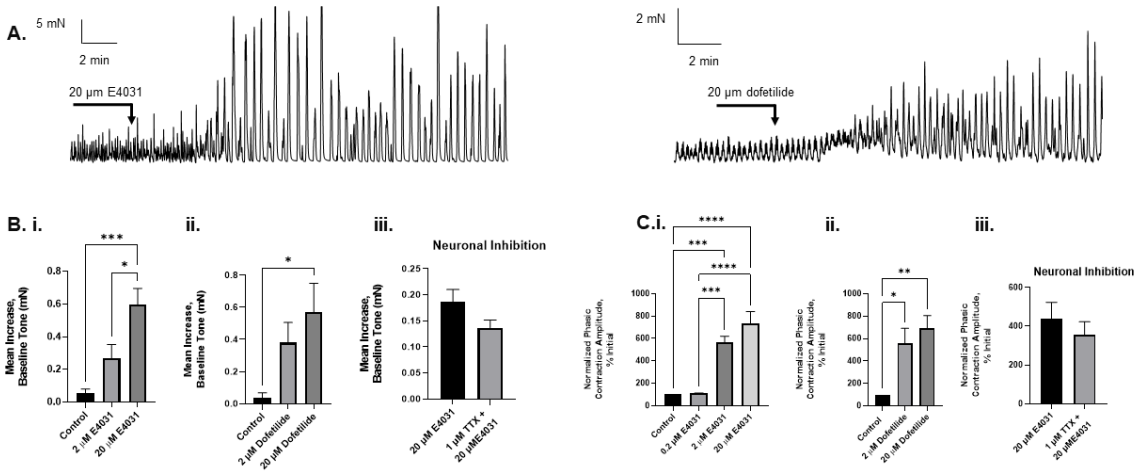


696

697 **Figure 2. Whole cell electrophysiology of isolated detrusor smooth muscle cells.** Panel
698 A and B show currents evoked by the protocol shown in panel C in the absence (A) and
699 presence (B) of E4031 (2 µM). The right hand panels (Ai and Bi) show a zoom of the initial
700 200 ms of the test step to highlight the E4031-sensitive current. D shows a representative set
701 of E4031-sensitive currents. Panels E and F show the mean current amplitude at different test
702 potentials in the absence and presence of E4031. Data taken from 8-12 cells from 4 animals
703 **(N=4, *p<0.05, ** p<0.01, one-way ANOVA, error bars represent SEM).**

704

705



706

707 **Figure 3. Effect of Kv11.1 channel inhibitors on spontaneous contractile activity.** (A)
708 Representative trace of basal tone and phasic contraction amplitudes after addition of 20 µm
709 of the Kv11.1 inhibitors, E4031 (left) and Dofetilide (right). (B) Maximum increase in baseline
710 tone at various concentrations of Kv11.1 inhibitors E4031 (i) and dofetilide (ii), and in the
711 presence of neuronal inhibition with TTX (iii) (N=5). (C) Percent increase in the amplitude of
712 phasic contractions as compared to control, in the presence of Kv11.1 inhibitors, E4031 (i)
713 and dofetilide (ii), and in the presence of neuronal inhibition (iii) (N=5). (*p<0.05, **p<0.01,
714 *** p<0.001, **** p<0.0001, one-Way ANOVA, error bars represent SEM).

715

716

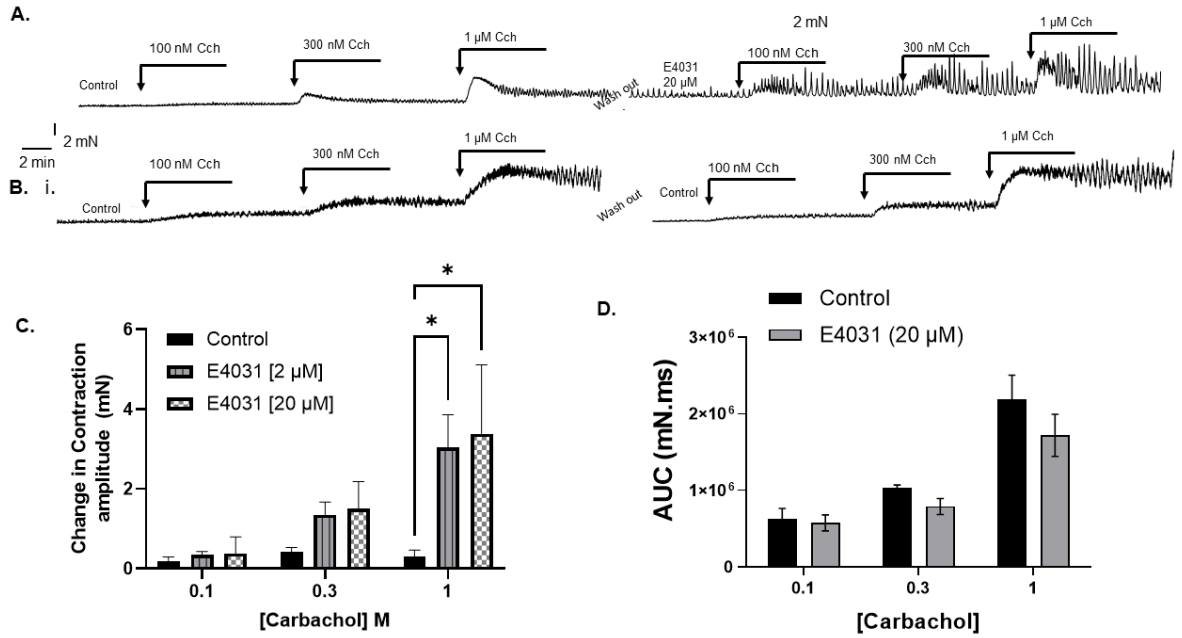
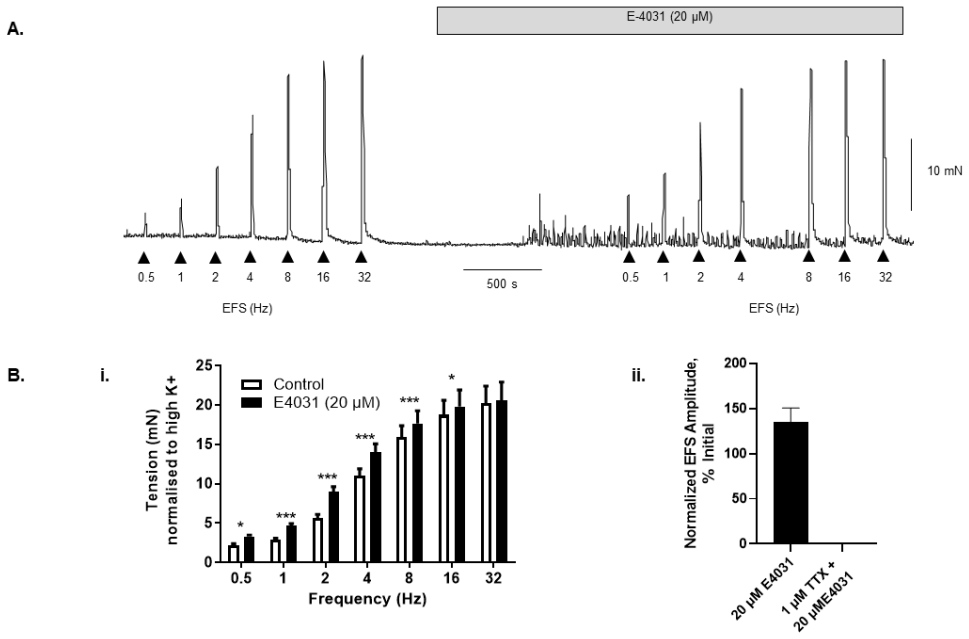


Figure 4. Assessing amplitude of phasic contractions in response to carbachol in the presence and absence of potassium channel inhibitors. (A) Representative traces of contraction amplitudes in response to carbachol before and after the addition of the Kv11.1 inhibitor, E4031 (20 µM) within the same tissue (internal control). (B) Representative traces of contraction amplitudes in response to carbachol before and and after the addition of vehicle control (DMSO). (C) The change in mean amplitude of contractions taken over a 2-minute period when the effect of Cch reaches plateau and prior to the addition of the next concentration. The greatest change in amplitude, compared to control was observed in the presence of E4031 with 1 µm CCh. **(N=5), *p<0.05, error bars represent SEM; statistical analysis using 2-WAY ANOVA, with Bonferroni multiple comparison test.** (D) Area under the curve analysis carried out at the maximum change in baseline tone after the addition of each concentration of Cch, in the presence of 20 µM E4201 **(N=5; 2-way ANOVA; error bars represent SEM).**

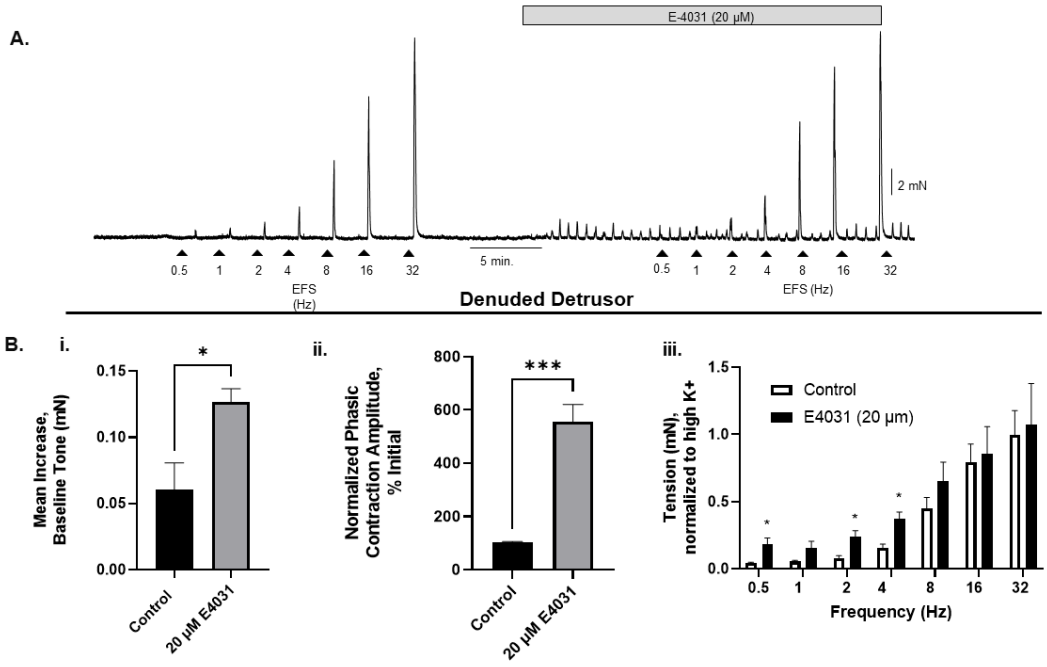


734

735 **Figure 5. Effect of E-4031 on nerve-evoked contractions**. (A) Electrical field stimulation
736 (EFS) across the frequency range 0.5, 1, 2, 4, 8, 16, 32 Hz evoked frequency-dependent
737 contractions. Following treatment with E4031 (20 µM), the stimulation protocol was repeated.
738 Note the enhancement of spontaneous contractions in the presence of E4031. (B.i) Summary
739 data from experimental series (N=5), normalised to high K+ (60mM) contraction. (B.ii) Mean
740 data showing effect of E4031 on EFS (30 Hz, 30V) with and without prior incubation with TTX
741 (t-test). ***p<0.05, **p<0.01, ****p<0.0001; error bars represent SEM.** (2-way ANOVA with
742 Bonferroni post-hoc test).

743

744



745

746 **Figure 6. Effect of E-4031 on contractile activity of a denuded bladder**. (A) Electric field
747 stimulation (EFS) of a denuded bladder across a range of frequencies (0.5, 1, 2, 4, 8, 16, 32
748 Hz). The protocol was conducted before and after incubation with E4031 (20 μM). (B.i) Mean
749 data indicating increase in baseline tone in the absence (control) and presence of E4301. (B.ii)
750 Average data of increase in phasic contractile amplitude after the addition of E4301 or in the
751 presence of the vehicle (control) (t-test). (B.iii) Summary data of EFS-induced contractions
752 (normalised to K+ (60 mM). *p<0.05, ***p<0.0001, N=5, error bars represent SEM).

753

754

755

756

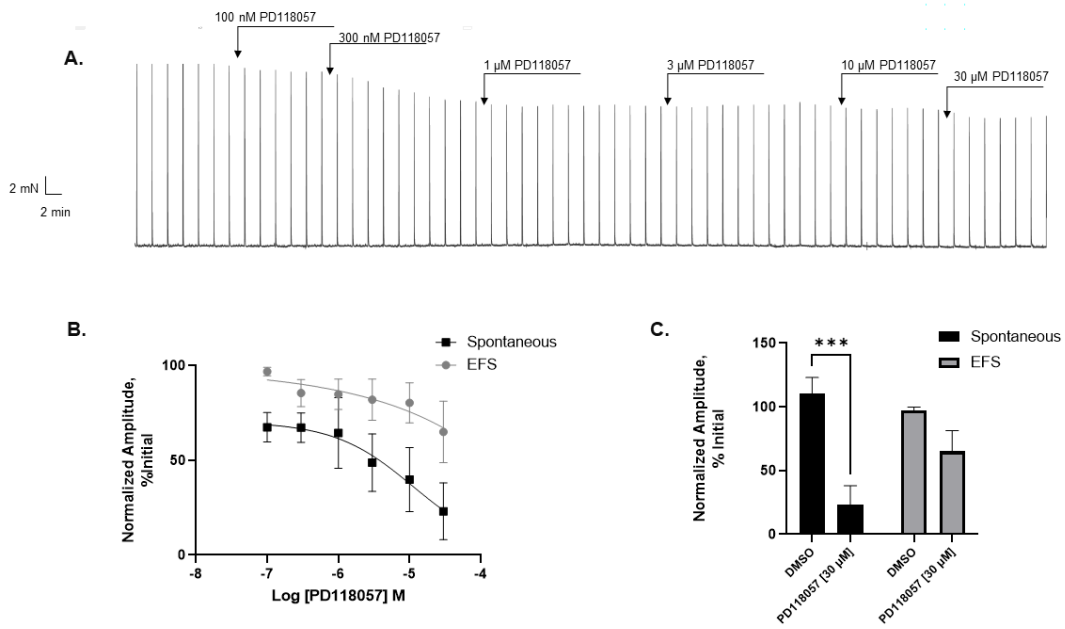


Figure. 7 Effect of the Kv11.1 activator, PD118057 on the amplitude of spontaneous and EFS (electric-field stimulated) contractions (20 Hz). (A) Representative trace of cumulative concentration of PD118057. (B) Normalized amplitudes of spontaneous and EFS contractions at increasing concentrations of PD118057. (C) Normalized amplitudes of spontaneous and EFS contractions in response to PD118057, relative to vehicle. N=5, Error bars represent SEM, ***p<0.001, Statistical analysis using two-way ANOVA.

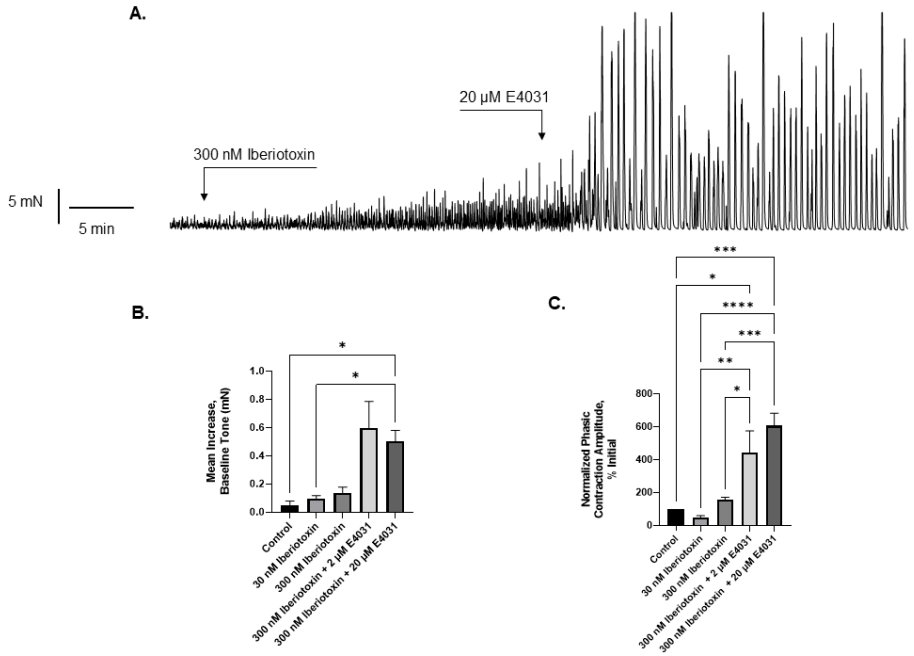
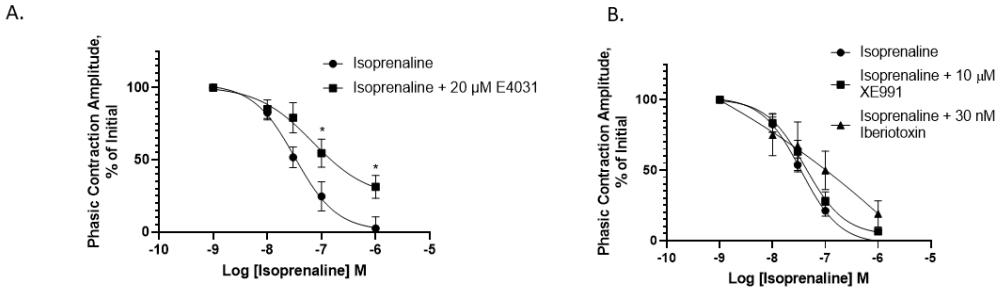


Figure 8. Characterization of baseline tone and phasic contraction amplitude in the presence of BK channel inhibitors in the presence and absence of E4031. (A) Representative trace of basal tone and phasic contraction amplitudes after addition of 20 µm of the Kv11.1 inhibitor, following preincubation with 300 nM of the BK inhibitor iberiotoxin. (B) Maximum increase in baseline tone in the presence of iberiotoxin with and without E4031 (N=5). (C) Percent increase in the amplitude of phasic contractions as compared to control, in the presence of the Kv11.1 inhibitor, E4031 with and without prior incubation with iberiotoxin (N=5). **p<0.01, *** p<0.001, **** p<0.0001, error bars represent SEM. Statistical testing using One-Way ANOVA. (N=5, One-way ANOVA, **p<0.01, error bars represent SEM).



779

780 **Figure 9. The effect of isoprenaline in the presence of potassium channel inhibitors.** (A)
781 Decrease in phasic contraction amplitude of detrusor muscle strips upon treatment with
782 Isoprenaline. The relaxation effect of Isoprenaline is significantly counteracted upon pre-
783 treatment with E4031 (B) Pre-incubation with either XE991 or Iberiotoxin did not significantly
784 alter the adrenergic effect of Isoprenaline. (N=5), *p<0.05; error bars represent SEM; statistical
785 analysis using 2-WAY ANOVA, with Bonferroni multiple comparison test.