Constitutively active HCN Channels Constrain Detrusor Excitability and Modulate Evoked Contractions of Human Bladder

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Abstract

BACKGROUND AND PURPOSE: Although expression of Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in bladder is reported, their functional role remains unsettled. Here, we immunolocalized the expression of HCN1 and HCN4 subtype in human bladder and investigated their functional significance. EXPERIMENTAL APPROACH: Bladder procured from ten human organ donors were dissected into mucosa (containing urothelium and submucosa) and detrusor for double immunofluorescence of HCN with markers and isometric tension recordings. Mucosa intact and denuded detrusor strips were stretched to a basal tension of 10 mN for eliciting either tetrodotoxin (TTX) resistant spontaneous contractions or TTX sensitive electrical field stimulated (EFS) evoked contractions or carbachol evoked contractions before and after the addition of HCN blocker, ZD7288 or activator, Lamotrigine. KEY RESULTS: Double immunofluorescence revealed prominent immunolocalization of HCN1 and HCN 4 subtype with calcitonin gene related peptide (CGRP), choline acetyl transferase and gap junction proteins in mucosa and detrusor. Removal of mucosa significantly enhanced the basal tension and the spontaneous contractions upon cumulative addition of ZD7288 but not with Lamotrigine ($p<0.05$). ZD7288[10nM] did not influence the carbachol response, but in presence of Neostigmine [1μM], significantly enhanced the atropine and TTX sensitive EFS contractions of mucosa intact strips. CONCLUSION & IMPLICATIONS: Overall, HCN channels immunolocalized in mucosa, smooth muscle, gap junctions and nerve fibers exert a tonic constraint on detrusor excitability, enable spatio-temporal integration of evoked contraction and constrain the release of neurotransmitters, respectively. In contrast to the pacemaker role in other organs, findings argue for a non-pacemaking role of HCN channels in bladder.

Introduction

Micturition is described by a prolonged phase of urine storage that usually last hours and a voluntary voiding phase typically lasting a few seconds. Voluntary voiding involves bladder contraction evoked by stimulation of muscarinic receptors on detrusor smooth muscle (DSM) by acetylcholine and the storage phase of overactive bladder (OAB) patients is characterized by uninhibited detrusor contractions as seen during urodynamics of OAB patients (Cullingsworth et al., 2019). The storage symptoms of OAB patients are typically managed by diminishing the strength of uninhibited detrusor contractions(Kashyap et al., 2015) by two main classes of drugs namely, β3 receptor agonists(Gillespie et al., 2015), and muscarinic receptor antagonists (Frazier et al., 2008) .

Importantly, the mechanism of action for β3 receptor agonists and muscarinic receptor antagonists entails modulation of a second messenger called cyclic adenosine monophosphate (cAMP)(Kretschmannova et al., 2012) with multiple downstream signaling effects. One of those effects is shifting the voltage dependence of hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN channels) for activation at more depolarized membrane potentials(Alvarez-Baron et al., 2018). HCN channels are known pacemakers of rhythmic activity in heart and brain with four (HCN1–HCN4) isoforms reported to differ in homomeric or

heteromeric subunit composition, activation kinetics and gating sensitivity to cyclic nucleotides (Alvarez-Baron et al., 2018). Although, the expression of HCN channels on DSM, urothelium and nerve fibers of mammalian bladder have been reported (Kashyap et al., 2015; Xue et al., 2012), the functional role of this putative pacemaker channels in bladder that remains quiescent for prolonged storage phase of micturition remains unsettled.

The initiation of nerve evoked contraction during voiding and uninhibited detrusor contractions i.e. spontaneous contractions of DSM in absence of any neural contractile stimuli(Wu et al., 2002) are dependent on a transient rise in intracellular calcium $[Ca^{2+}]_i$, but the two modes of Ca^{2+} mobilization diverge in their resistance to the antagonism of voltage voltage-gated Ca^{2+} channels (VGCC). The Ca^{2+} mobilization for initiating the nerve evoked or muscarinic receptor (carbachol) evoked contraction is independent of the membrane depolarization(Maggi et al., 1989; Wu et al., 2002), whereas autonomously originating membrane potential oscillations(Hashitani & Brading, 2003) are a prerequisite for spontaneous Ca^{2+} entry(Weng et al., 2012) through high voltage L-type VGCC(Hashitani & Brading, 2003) and the resulting spontaneous contractile activity. Since L-type VGCC are closed at the resting membrane potential of DSM (Sui et al., 2009), a low threshold Ca^{2+} conductance initiated by low voltage VGCC (T-type) (Dave & Manchanda, 2017; Igawa et al., 2014; Sui et al., 2009) is a critical player in the depolarization of DSM from resting voltage of -60 mV to the threshold potential of -40 to -35 mV for the activation of L-type VGCC reflected in the upstroke of action potential. The depolarization to the peak voltage for action potential in DSM inactivates the T-Type VGCC and the long interval of after-hyperpolarization(Petkov et al., 2001) assists in slow recovery from inactivation of T-Type VGCC.

Given that HCN channels(Alvarez-Baron et al., 2018) carry an inward, non-selective cation currents to modulate the membrane conductance of excitable cells at rest to maintain the membrane potential within the activation range, HCN channels expressed in bladder are postulated to be constitutively active for suppressing the spontaneous synaptic drive (Sengupta & Manchanda, 2019), exhibited as uninhibited detrusor contractions during storage phase of micturition. Importantly, activation of HCN channels is shown to indirectly suppress the activation of T and N-type VGCC(Hurtado et al., 2014), which are suggested to be not involved in the carbachol response of bladder (Maggi et al., 1989). Here, we investigated the immunoreactivity of HCN1 and HCN4 together with gap junction proteins, peptidergic and cholinergic markers (Kashyap et al., 2018) in different regions of bladder and assess the effect of pharmacological activation (Poolos et al., 2002) and blockade of HCN channels(Kashyap et al., 2015) on the force of contractions evoked spontaneously, neurogenically or mediated by muscarinic receptor stimulation (carbachol).

Materials & Methods :

Materials

Human bladders from ten organ donors of both genders in the age range of 30-60 years old within 4 hours after donor death were procured from University of Pittsburgh tissue bank in compliance with the tissue bank IRB#0506140 and with an approved protocol #400 of Committee for Oversight of Research and Clinical Training Involving Decedents (CORID). Information whether organ donors suffered from any lower urinary tract symptoms was not available from the tissue bank. Primary and secondary antibodies for double immunofluorescence were obtained from different suppliers (Table 1) and normal donkey serum was procured from Jackson Immunoresearch. Floromount-G with 4',6-diamidino- 2-phenylindole (DAPI) was obtained from Fischer Scientific. Triton X-100, Bovine serum albumin (BSA), ZD7288 (4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride), Lamotrigine, Carbachol and Dimethyl sulfoxide (DMSO) were procured from Sigma Aldrich and Tetrodotoxin (TTX) from Cayman Chemicals. Stock solutions of all drugs except Lamotrigine were prepared in ultrapure (Type 1) water, whereas stock solution of Lamotrigine was prepared in DMSO. All stock solutions were kept at -20°C before use in isometric tension measurments.

Immunofluorescence

Tissue from the trigone region was separated into mucosa (urothelium and sub-urothelium) and detrusor and immediately cryopreserved for generating 10µm thick cryosections mounted on glass slides. Sections were

first washed in PBS and fixed in chilled acetone for 10 min at 4°C. Sections were then incubated with PBS containing 0.4% Triton X-100 (PBST) and 5% normal donkey serum for 30 min at room temperature to block non-specific binding sites before application of primary antibodies. For double immunofluorescence, primary antibodies targeting either HCN1(1:200) or HCN4 (1:300) were diluted and mixed with either CGRP (1: 50) or ChAT (1:100), Cx43 (1:100) or Cx45 (1:50) in PBST containing 5% normal donkey serum. Appropriate mixture of primary antibodies was applied to the section and incubated overnight at 4°C. Unbound primary antibody was removed by thrice washing with PBST containing 1.0% BSA for 5 min at room temperature.

Sections were then incubated in the dark for 2h at 25°C with secondary donkey antibodies tagged to Alexa Fluor 488(1: 200) for localizing the primary antibodies binding to HCN1 or HCN4 isoforms and other markers were individually co-localized with secondary antibody tagged to Alexa Flour 594 (1: 200). Sections were washed again three times at room temperature in PBST containing 1.0% serum and then mounted with anti-fade medium containing DAPI. Double fluorescence was checked with Olympus BX51 microscope, and digital images at low and high magnification were captured using MagnaFire 2.1 software(Kashyap et al., 2018).

Isolated Tissue Strip Studies

Tissue from dome regions of bladder were kept in ice-cold Krebs salt solution and mucosa intact and denuded strips were generated by separating the detrusor from the mucosa by microdissection in pre-oxygenated cold Krebs solution. Mucosa intact and mucosa denuded human bladder strips were mounted vertically between parallel platinum wire electrodes positioned on either side of the bladder strips in organ bath chambers. The strips were bathed in 20mL of Krebs solution composed of 120 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 15.5 mM, 1.2 mM NaH_2PO_4 and 11.5 mM glucose bubbled with 95% O2 and 5% CO_2 with pH 7.4, warmed to 37°C(Kashyap et al., 2015; Kashyap et al., 2018). The isometric contractions were measured using force transducers connected to a bridge amplifier (World Precision Instruments) and digitized by PowerLab Software. Isometric tension in response to following stimuli was measured within first 24 h after organ collection, which corresponds to the tissue viability window.

Spontaneous contractions - Mucosa intact and denuded human bladder strips were stretched to a basal tension of 10 mN to elicit spontaneous phasic contractions(Kashyap et al., 2015). Strips were washed with Krebs solution every 15 min during an equilibration period of 90 min to obtain a stable baseline tension and spontaneous phasic contractions. Strips were incubated with tetrodotoxin (TTX, 1 μM), a neuronal Na^+ channel blocker to exclude the potential contribution of HCN channels expressed on intramural active nerve fibers before cumulative addition of ZD7288 or Lamotrigine in increasing concentrations (1nM -100 μM). Each concentration addition was separated by 10min intervals and responses were expressed as changes in resting tension measured by area under the force-time curve for a 2 min period prior to the addition of next higher concentration of drug. Muscle force at each drug concentration was normalized to the contractile activity in the 2 min time period at the beginning prior to any addition of drug and the values expressed as percentages.

EFS Frequency response-curves-

For the construction of frequency-response curves, equilibrated strips were subjected to EFS protocol with ascending frequency of applied electric stimuli ranging from 0.1 to 32 Hz (one stimulation at each frequency). Trains of EFS pulses were delivered from constant-current Grass S88 stimulator(Warwick, RI) with the following parameters: pulse amplitude of 20V, pulse width of 5 ms, and each stimulus duration of 2s delivered at interval of 30s (Kashyap et al., 2018). A single concentration of drug was administered in the tissue bath 15 min prior to the construction of frequency response curve. After each frequency response curve, tissues were washed immediately with one exchange of Krebs solution and drugs were re-administered at the appropriate concentration. EFS contractile responses sensitive to 1 μM Tetrodotoxin (TTX) were considered to be evoked by nerve stimulation of EFS. Peak contractile responses after addition of drugs were expressed as a percentage of the maximal response at 32Hz in each strip before any addition of drugs (control). Every experiment replicate included a time control to indicate any natural decay of EFS evoked contractile responses.

Carbachol Evoked Contractile Response

Equilibrated stripes were subjected to cumulative concentration response curve of carbachol to the identify concentration evoking maximal response for each strip (10-100μM). After subsequent washing, maximal carbachol evoked response was repeated before and after the addition of ZD7288 [10nM or 100 μM]. Additional strips were left untreated to monitor any time dependent changes in contractility.

Statistical analysis

Data was analyzed by GraphPad Prism 8.2.1 software (GraphPad Software, Inc., La Jolla, CA) and expressed as mean ± SD. Statistical significance for the effect of drugs on frequency response curve and muscle force integral was analyzed by two-way ANOVA followed by Tukey's test and $p<0.05$ was considered significant

Results :

HCN expression in human bladder- Double immunofluorescence of the separated mucosa (urothelium and sub-urothelium) and detrusor sections of human bladder revealed that green immunoreactivity of HCN1 isoform was co-localized with CGRP containing nerve fibers in apical layer of urothelium, whereas green immunoreactivity HCN4 isoform is strongly co-localized with red immunoreactivity of gap junction protein Cx43 in sub-urothelium, DSM, and in ChAT expressing cholinergic fibers. Unlike HCN1, HCN4 is poorly expressed in urothelium but strongly colocalized with ChAT, Cx43 and Cx45 in detrusor. Stronger staining of HCN1 in all regions of bladder may be due to the use of a slightly higher concentration recommended for immunofluorescence by manufacturer than for HCN4. The co-localization of HCN1 was higher with CGRP than with ChAT in suburothelium and detrusor regions. Staining for Cx43 was stronger in suburothelium region and faint staining of Cx45 was localized to detrusor bundles and appeared to be less marked (not shown), suggesting that organ donors were unlikely to have suffered from OAB symptoms. Ethical compliance prohibits disclosure of disease status of donors. Commercially available antibodies for HCN2 and HCN3 did not work well in our hands.

Spontaneous Contractions : Cumulative addition of ZD7288 in ascending concentrations generated a dose dependent rise in basal tension and the muscle integral force of bladder with the effect being more pronounced in mucosa denuded strips than in mucosa intact strips, which implies that HCN channels expressed in urothelium and sub urothelium region exert a tonic constraint on detrusor excitability. The muscle integral force of mucosa denuded strips treated with ZD7288 [100 μM] was significantly higher than mucosa intact strips exposed to concentrations of ZD7288 [?] [100 μM]; n=5; two- way ANOVA followed by Tukey's test, *$p<0.05$. Since spontaneous contractions consists of complex events of varying amplitude and frequency (Gillespie et al., 2015), integral measured by the area under the curve is preferred as an index for the drug effect on tonic and phasic contractions. To control for the variability of DSM content in the isometric force responses of strips, we normalized the force integral measured in presence of drug to the force measured at pretreatment value. Increase in muscle integral force of mucosa denuded strips in presence of TTX rules out any contribution of HCN channels expressed on nerve fibers and argues for a direct action of ZD7288 on DSM.

Cumulative addition of Lamotrigine in ascending concentrations caused a modest reduction in muscle integral force of both mucosa -denuded and mucosa intact strips without any significant difference. A significant reduction in the integral of muscle force of spontaneous contractions and the baseline tone was noted at Lamotrigine [10μM] in mucosa denuded detrusor strips, which demonstrates that the activation of HCN channels expressed in DSM modulate the basal tone and autonomous generation of spontaneous activity. The muscle integral force of mucosa denuded strips treated with ZD7288 [10 μM] were significantly elevated relative to the strips treated with Lamotrigine [10 μM]. Tracings at different time scales further illustrate the differences in outcomes following blockade and activation of HCN channels with ZD7288 and Lamotrigine, respectively.

Nerve evoked Contractions - Given the effect of ZD7288 [10nM] on the nerve evoked contractions evoked at 20Hz in rat bladder (Kashyap et al., 2015), we tested the effect of ZD7288 at [10nM] on the frequency

response curve in human bladder. Addition of ZD7288 [10nM] slightly enhanced the EFS contractions evoked at frequencies [?] 8Hz without any significance (Fig.3-4). In contrast to the excitatory effect of ZD7288 [10nM] on evoked contractions, higher concentrations of ZD7288[100 μM] showed an inhibitory effect on nerve evoked contractile response as well as on the carbachol response (supplementary data figure), which implies that HCN channels expressed on post-junctional sites and gap junctions(Aydin et al., 2018; Mader et al., 2018) play a crucial role in increasing the amplitude of nerve evoked contraction and of carbachol response. Stated differently, the action of ZD7288 [100 μM] on HCN channels expressed on post-junctional sites and on gap junctions counters its excitatory effect on HCN channels expressed on nerve terminals expressing ChAT (Fig.1).

Addition of Lamotrigine at the tested concentrations did not produce any remarkable effect on the nerve evoked contractile response suggesting that HCN channels expressed in bladder are constitutively active to maintain membrane conductance within activation range. Testing the effect of ZD7288 or Lamotrigine in the concentration range shown in Fig.2 was precluded by the limited time window of tissue viability and time required for EFS. A hint of excitatory effect for ZD7288 [10nM] on contractions evoked by [?] 16Hz in mucosa denuded strips prompted repeat examination of ZD7288 [10nM] in presence of acetylcholinesterase blockade (Fig.4A). Combined administration of ZD7288 [10nM] with the competitive blocker of acetylcholinesterase, Neostigmine [1 μM] was expected to prolong the excitatory effect of acetylcholine released by EFS [?] 16Hz (Fig.4A-B)(Kashyap et al., 2018). Indeed, we noticed a pronounced enhancement of excitatory effect of ZD7288 [10nM] on nerve evoked contractions, as demonstrated by the significant difference with respect to strips exposed to Neostigmine [1 μM] but not to ZD7288 [10nM] at EFS [?] 16Hz (two- way ANOVA followed by Tukey's test, *$p<0.05$; ***$p<0.001$; Fig.4E). Since this enhancement at EFS [?] 16Hz is sensitive to blockade by TTX [1μM] or Atropine [0.1μM] (Fig.4C-D), we inferred that the ZD7288 [10nM] blocks the HCN channels expressed in ChAT expressing nerve fibers (Fig.1) to increase the EFS[?] 16Hz evoked release of acetylcholine(Kashyap et al., 2018).

Effect on Carbachol evoked contractions- Possibility of a post—junctional effect of ZD7288 [10nM] on nerve evoked contractions is ruled out by its lack of any effect on the maximal response of carbachol in mucosa intact or denuded human bladder strips (supplementary data of Fig.6). Consistent with previous reports, maximal response to carbachol(Mader et al., 2018) was opposed by higher concentration of ZD7288 [100 μM]. Since Lamotrigine did not influence the nerve evoked contractions, we did not explore its effect on carbachol response. Importantly, higher concentrations of clinically approved HCN blocker, Ivabradine also reduced the maximal response to carbachol in rat bladder(Aydin et al., 2018). Removal of mucosa is known to enhance the contractile response of detrusor(Guan et al., 2017), intriguingly, the removal of mucosa enhanced the excitatory effect of ZD7288 [10nM] on nerve evoked contractile response (Fig.4) and enhanced the excitatory and inhibitory effect of ZD7288 [100 μM] on spontaneous (Fig.2), and carbachol response (supplementary data), respectively

Discussion:

Overall, these findings characterize the functional role of HCN channels expressed in conjunction with nerve terminals and gap junction proteins in urothelium, sub-urothelium and detrusor region of human bladder. Pharmacological activation and blockade of HCN channels together with physical removal of mucosa containing dense expression of HCN channels demonstrate that constitutively active HCN channels constrain the spontaneous and neurogenic contractions of human bladder in a non-pacemaker fashion.

Immunofluorescence of mucosa separated from human detrusor revealed dense expression of HCN channels in urothelium and sub-urothelium in conjunction with nerve fibers expressing CGRP and ChAT and gap junction protein Cx43. Our findings on mucosa intact and denuded strips not only validate the inhibitory effect of mucosa on detrusor contraction(Guan et al., 2017) whether evoked spontaneously or in response to nerve stimulation or by muscarinic receptor stimulation by carbachol, but also identify the channel that may be play a crucial role in exerting that inhibitory effect. The co-localization of HCN4 and Cx43 in the lamina propria region and of HCN1 with CGRP positive and ChAT positive nerve endings is consistent with the premise for an integrative role for HCN channels in detrusor contractility and in the mechanotransduction of

afferent activity which are amplified by the spontaneous contractions(Aizawa et al., 2017). The expression of faster activating HCN1 subtype(Alvarez-Baron et al., 2018) in CGRP positive terminals and of slower activating HCN4 isoform having higher sensitivity to cAMP in ChAT positive nerve endings of human bladder is in agreement with the reports on dominant expression of HCN1 and HCN4 in primary afferent terminals(Papp et al., 2006) and motor axons(Nodera & Rutkove, 2012), respectively.

The co-localization of HCN1 with CGRP and Cx43 in urothelium and suburothelium, when taken together with the differences in the isometric tension of mucosa intact and denuded strips support the notion of spontaneous contractions originating near the urothelial-sub-urothelial interface and then spreading to the detrusor (Kanai et al., 2007) for mechanotransduction of afferent activity(Aizawa et al., 2017) during bladder volume sensation. Excitatory effect of ZD7288 on detrusor excitability is consistent with the enhanced excitability of pyramidal neurons following a decrease in hyperpolarization-activated currents(Albertson et al., 2011; Poolos et al., 2002). HCN channel inhibition was shown to affect the frequency of action potentials without abolishing the spontaneous electrical activity in Pituitary(Kretschmannova et al., 2012).

Thus, inhibitory effect of HCN channels on autonomously originating membrane potential oscillations in DSM (Hashitani & Brading, 2003) and on the action potential evoked release of neurotransmitters(Huang et al., 2017) may underlie the excitatory effect of HCN blockade on spontaneous and nerve evoked contractions, respectively. Taken together, the co-localization of HCN4 with Cx43 in suburothelium and the significantly higher basal tension and integral force of mucosa-denuded strips upon addition of ZD7288 supports the postulated role of gap junctions as a conduit for transmitting membrane depolarizations between adjacent DSM. Although ZD7288 [10nM] did not influence carbachol response of mucosa intact or denuded strips, significant reduction in the presence of ZD7288[100 μM] implies that HCN channels expressed on urothelium and gap junctions(Mader et al., 2018) are critical for integrating the bladder tone in carbachol response and evoked response. Prominent effect of ZD7288 compared to Lamotrigine on bladder contractility implies that HCN channels expressed on DSM and gap junctions are constitutively active and are suggested to be crucial for spatio-temporal integration of spontaneous excitatory junction potentials (Sengupta & Manchanda, 2019) and the contraction of bladder as a three-dimensional functional syncytium.

Not only , ZD7288 has high affinity for HCN channels with reported IC50 of 0.3 μM(BoSmith et al., 1993), it can also directly block T-type VGCC with IC50 of 50μM(Felix et al., 2003). A recent report studied the effect at a single concentration of ZD7288 [50 μM](Mader et al., 2018) on spontaneous contractions of human bladder tissue obtained from cystectomy of bladder cancer patients. Given the IC50 of ZD7288 for HCN channels, it is likely that abrupt rise of basal tension in mucosa denuded strips at higher concentrations of ZD7288 may involve action at sites at other than HCN channels. To exclude the possibility of non-selective action, we studied the effect of ZD7288 on spontaneous and neurogenic contractions at concentrations that were either on higher or lower side of the reported IC50 for HCN channels. Selectivity of ZD7288 for HCN channels is expected to be higher at lower concentrations and the functional impact of ZD7288 [10nM] was easily noticeable on the neurogenic contractions in presence of Neostigmine but not on the spontaneous contractions. Mucosa denudation enhanced the excitatory effect of ZD7288 [10nM] on nerve evoked contractile response, but mucosa denuded strips exhibited an excitatory and inhibitory effect of ZD7288 [100 μM] on spontaneous and carbachol response, respectively

Effect of HCN selective concentrations of ZD7288 [10nM] (BoSmith et al., 1993) on nerve evoked contractions is consistent with the increased frequency of action potentials noted in isolated neurons after HCN channel blockade (Albertson et al., 2011). A prejunctional action of ZD7288 [10nM] on HCN channels(Huang et al., 2017) expressed on ChAT positive cholinergic nerve fibers of bladder (Fig.5). is inferred from the co-localized immunoreactivity of HCN4 and ChAT in sub urothelium and detrusor together with the pronounced TTX-sensitive enhancement of EFS response [?] 16Hz in presence of Neostigmine (which retards the acetylcholine degradation) and the reported dominance of muscarinic signaling at higher frequencies ([?]10 Hz)(Kashyap et al., 2018; Werner et al., 2007). Lower amplitude of maximal response on evoked contractions and upon carbachol addition in presence of ZD7288[100 μM] may be linked to the excitatory action on prejunctional sites being countered by the effect of ZD7288[100 μM] on post-junctional sites and gap junctions.

Prejunctional effect of ZD7288 may also contribute to the excitatory effect of ZD7288[100 μM] on TTX- resistant spontaneous contractions because stretch mediated basal release of neurotransmitters such as adenosine triphosphate (ATP) and ACh from nerve terminal and other sources such as urothelium, suburothelium and interstitial cells is resistant to the application of TTX(Zagorodnyuk et al., 2009). Thus, enhancement of stretch mediated basal release of ATP and ACh(Finney et al., 2007) may contribute to boost the frequency of rhythmic membrane depolarizations which results in augmentation of TTX- resistant spontaneous contractions upon ZD7288 exposure. Unlike the TTX resistant basal release of neurotransmitters, TTX sensitive evoked release of neurotransmitters might explain the inhibitory effect ZD7288 [100 μM] on TTX-sensitive neurogenic contractions.

Compared to the excitatory effect of ZD7288 [50-100μM] on spontaneous contractions in bladder(Mader et al., 2018), HCN-dependent pacemaker depolarizations driving the coordinated upper urinary tract peristalsis for urine flow(Hurtado et al., 2014) to bladder is abolished by ZD7288 [50 μM]. Differences in outcomes after blockade of HCN channels expressed in upper and lower urinary tract led us to deduce a non-pacemaking role for HCN channels expressed in human bladder. This inference is also supported by the findings of spontaneous contractions occurring at lower frequency in interstitial cells(Gray et al., 2013) than in DSM of guinea pig. Since pacemaker cells typically contract at a frequency that is higher than the frequency of other cells aligned with the pacemaker cells, a presumed pacemaker role for HCN channels expressing interstitial cells in bladder is unlikely.

Since depolarization of DSM(Wu et al., 2002) is not critical for the muscarinic receptor dependent bladder emptying evoked by parasympathetic nerve(Maggi et al., 1989; Wu et al., 2002), selective modulation of the signaling driving the spontaneous depolarization of DSM through drugs acting on HCN channels holds therapeutic relevance in OAB and in detrusor hyperactivity and impaired contractility (DHIC). Unlike OAB, antimuscarinics are grossly inadequate for DHIC because of their adverse effect on impaired contractility (IC) component of DHIC, which increases the risk for urinary retention, recurrent urinary tract infection and vesicoureteric reflux. Akin to antimuscarinics, antagonism of L-type and T-type VGCC is also not a clinically viable strategy to manage DHIC because FDA approved L-type VGCC antagonists like Nifedipine has the potential to suppress both DH as well as IC component of DHIC, whereas T-type VGCC antagonists l(Igawa et al., 2014; Sui et al., 2009) are not yet available for clinical use.

This findings do shed light on the report of reduced uninhibited detrusor contractions and the increased bladder capacity (Loutochin et al., 2012) of spinal cord injured rat following daily treatment with Lamotrigine, a HCN activator (Poolos et al., 2002). Taken together with the reported suppression of T-type VGCC activity(Huang et al., 2017) with the activation of HCN channels led us to conjecture that HCN channel activation might be a viable strategy for integrating the phasic contractions driving the DH component and for enhancing the bladder tone to evoke a stronger efferent discharge for augmenting the IC component of DHIC.

Conclusions:

Set against the observations of double immunofluorescence and bladder contractility, we infer that HCN channels expressed in bladder are constitutively active to constrain the DSM excitability, modulate the basal and evoked release of neurotransmitters for mechanotransduction of afferent activity and to enable spatio-temporal integration of voiding contraction.

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Legends

Fig. 1: Immunofluorescence of HCN1 and HCN4 in human bladder : Double immunofluorescence of the separated mucosa (urothelium and sub-urothelium) and detrusor sections of human bladder with primary and secondary antibodies listed in Table 1 revealed a co-localization of the faster activating HCN1 isoform (green fluorescence) in the apical layer of urothelium with CGRP (red fluorescence) containing nerve fibers, whereas slower activating HCN4 isoform (green fluorescence) with higher sensitivity to cAMP is poorly expressed in the apical layer of urothelium, but strongly co-localized with gap junction protein Cx-43(red fluorescence) in sub-urothelium, and with ChAT expressing cholinergic fibers in DSM. Bladder regions are identified by labels on the side and the colocalization of HCN 1 or 4 with either CGRP, ChAT or CX43 is indicated by color labels on top. Blue fluorescence is from DAPI stained nucleus. Region outlined by white box in upper panels is magnified four- fold in respective lower panels with magnification indicated by scale bar of 25μm.

Fig. 2: HCN channels of Mucosa Constrain TTX-resistant spontaneous contractions - Panel A- Representative original tracings from mucosa intact (U+; black) and mucosa denuded(U-; grey) strips (U-denuded implies removal of both urothelium and suburothelium) were either treated with activator of HCN channels, Lamotrigine or HCN blocker, ZD7288 in presence of TTX[1μM] . Vertical bar shows tension in mN (millinewtons) and horizontal bar shows time. (B) Ordinate scale represents basal tension and integral force of spontaneous contractions, respectively normalized to tension recorded prior to drug application. Each point in the curve represent mean ± SD of 5 bladder strips. *P < .05, Two- way ANOVA followed by Tukey's multiple comparison test demonstrated that LTG [10 μM] () significantly reduces the integral force of spontaneous contractions relative to ZD7288 [10 μM] ()

Fig.3 : **HCN channels of Mucosa Facilitate TTX-sensitive evoked contractions** - Panel A&B depict representative tracings of contractions elicited by EFS (20V trains of 5ms pulses for a duration of 2s delivered 30s apart at 0.1-32Hz) in mucosa intact or denuded strips before and after the addition of ZD7288 [10nM-100μM] or Lamotrigine[10nM-100μM]. Panel C- Addition of ZD7288 [100μM] significantly opposed the contractions evoked at frequencies [?]8Hz compared to response prior to addition of ZD7288 (controls, blue tracing with open squares) (*p<0.05; ***p<0.001 Two-way ANOVA, Tukey's Test) in mucosa intact or denuded strips with lower p value for contractions evoked [?]16Hz. Each point in the curve represent mean ± SD of 5 bladder strips. Lamotrigine addition produced an insignificant effect on mucosa denuded strips and the slight enhancement of contractile response in mucosa intact strips did not reach significance.

Fig.4 : **HCN channels of intramural Nerve Fibers in bladder Constrain TTX-sensitive evoked contractions** Panel A-D depict representative tracings of contractions elicited by EFS (20V trains of 5ms pulses for a duration of 2s delivered 30s apart at 0.1-32Hz) of mucosa denuded strips in presence and absence of ZD7288 [10nM] alone or together with Neostigmine [1μM] or Atropine[0.1μM] or TTX[1μM], respectively. Panel E- Force-frequency curves reveal significant enhancement of response evoked in presence of Neostigmine [1μM] + ZD7288 [10nM] (blue tracing and blue curve with open circles) vs Neostigmine [1μM] alone (black tracing and curve with open squares; *p<0.05 at 16Hz; ***p<0.001 at 32Hz; Two-way ANOVA, Tukey's Test). Each point in the curve of Neostigmine [1μM] +/- ZD7288 [10nM] represent mean ± SD of 5 bladder strips. The evoked contractile response at [?]16Hz in presence of ZD7288 [10nM] is sensitive to both atropine [0.1 μM] and TTX [1μM], which implies that prejunctional action of ZD7288 [10nM] modulates the evoked release of acetylcholine.

Fig.5 : **Schematic illustration for proposed non-pacemaker function of HCN channels in human bladder** : HCN channels expressed on nerve terminals, gap junctions, mucosa and DSM converge functionally for spatio-temporal integration of voiding contraction and for constraining spontaneous and evoked contractions. HCN channels expressed on pre-junctional sites of nerve terminals constrain the release of neurotransmitters presumably by attenuating the activity of N-type VGCC, whereas HCN channels expressed on post-junctional sites of mucosa, gap junctions and DSM constrain the spontaneous contractions by attenuating the activity of T-type VGCC, which are a critical player in the depolarization of DSM from resting voltage of -60 mV to the threshold potential of -40 to -35 mV for the activation of L-type VGCC and

Ca^{2+} influx into DSM for spontaneous contraction.

Supplementary data

Fig.6 : **Effect of HCN Channel Blockade on Carbachol Response** - Panel A depicts the traces for carbachol 100μM evoked response of mucosa intact strips in absence (light grey; control) and in presence of ZD7288[10nM] (black tracing) or [100μM] dark grey tracing. Panel B and C shows the violin plots for force integral of carbachol [100μM] response before and after the addition of ZD7288[10nM] or [100μM]. All the data points are represented by dots in violin plots. Addition of ZD7288[10nM] did not appreciably affect the carbachol evoked response of mucosa intact or denuded strips, but ZD7288 [100μM] opposed the carbachol response more strongly in mucosa denuded strips.

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