Original Article
Constitutively active HCN channels constrain detrusor excitability and modulate evoked contractions of human bladder

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Abstract: Objective: Expression of Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels is reported in bladder, but the functional role remains unsettled. Here, we immunolocalized the HCN1 and HCN4 subtype in human bladder and investigated their functional significance. Methods: Bladder procured from ten organ donors was dissected into mucosa (containing urothelium and submucosa) and detrusor for double immunofluorescence of HCN1 and 4 subtypes with gap junction and neural proteins together with 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, carbachol evoked contractions and TTX sensitive electrical field stimulated (EFS), pre and post-addition of HCN blocker, ZD7288 or the activator, Lamotrigine or the cholinesterase inhibitor, Neostigmine. Results: Double immunofluorescence revealed immunolocalization of HCN1 and HCN4 subtype with calcitonin gene related peptide (CGRP), choline acetyl transferase and gap junction proteins in mucosa and detrusor. Removal of mucosa significantly raised the resting tension and the force of spontaneous contractions upon cumulative addition of ZD7288 in micromolar range relative to Lamotrigine treated strips (P<0.05). ZD7288 [10 nM] did not affect the contractile response evoked by EFS or carbachol, but the addition of ZD7288 [10 nM] in presence of Neostigmine [1 μM] significantly enhanced the atropine and TTX sensitive EFS evoked contractions of mucosa denuded strips. Conclusions: 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 contractions 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 human bladder.

Keywords: HCN channels, immunofluorescence, ZD7288, lamotrigine, spontaneous contraction, nerve evoked contractions, carbachol

Introduction

Given that urodynamics of overactive bladder (OAB) patients is characterized by uninhibited detrusor contractions in filling phase, OAB symptoms are typically managed by diminishing the strength of uninhibited detrusor contractions [1] using two main classes of drugs: muscarinic receptor antagonists and β3 receptor agonists [2]. Importantly, the mechanism of action for β3 receptor agonists and muscarinic receptor antagonists entails the modulation of a second messenger called cyclic adenosine monophosphate (cAMP) [3], which is associated with multiple downstream signaling effects. Intriguingly, one of those effects is a shift in the voltage dependence of hyperpolarization-activated cyclic nucleotide-gated channels (HCN channels) to allow their activation at more depolarized membrane potentials [4]. HCN channels are well known as pacemakers for the rhythmic activity in heart, gut, kidney and brain with the reported expression of four (HCN1-HCN4) isoforms that differ in homomeric or heteromeric subunit composition, activation kinetics and the gating sensitivity to cyclic nucleotides [4]. Although, the expression of HCN channels on detrusor smooth muscle (DSM), urothelium and nerve fibers of mammalian bladder have been reported [1], the functional role of these putative pacemaker channels in bladder remains unsettled [5].

Table 1. Primary and secondary antibodies used for double immunofluorescence of **Figure 1**

Antigen	Code/Identifier	Host	Dilution
C-terminal of HCN1	Abcam Cat# ab229340/RRID: AB_2827414	Rabbit	1:200
HCN4 [SHG 1E5]	Abcam Cat# ab32675/RRID: AB_732770	Rat	1:300
Calcitonin gene related peptide (CGRP) [4901]	Abcam Cat# ab81887/RRID: AB_16584	Mouse	1:50
Choline acetyl transferase (ChAT)	Millipore Cat# AB144P/RRID: AB_2079751	Goat	1:100
α1 subunit of Connexin-43 (Cx43)	Millipore Cat# 05-763, RRID: AB_309975	Mouse	1:100
C-terminus of Connexin 45 (Cx45) clone 8A11.2	Millipore Cat# MAB3100/RRID: AB_94692	Mouse	1:50
Alexa Fluor 488	Molecular probes Cat# A-21202/RRID: AB_141607	Donkey	1:200
Alexa Flour 594	Molecular probes Cat# A-21207, RRID: AB_141637	Donkey	1:200

The purported functional role of HCN channels in bladder is intriguing because unlike rhythmically contracting organs such as heart and the peristaltic function in kidney and gut, bladder generally remains quiescent for the prolonged storage phase of micturition driven by lumbar sympathetic nerves mediated elevation of cAMP in DSM. Given that HCN channels [4] 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 [6], usually exhibited as uninhibited detrusor contractions during the storage phase of micturition. More importantly, HCN channels upon activation indirectly suppress the activation of T and N-type voltage voltage-gated Ca^{2+} channels (VGCC) [7], which are not presumed to play any role in the bladder contraction evoked by carbachol [8] that mimics the voiding contraction. Here, we investigated the immunoreactivity of HCN1 and HCN4 together with gap junction proteins, peptidergic and cholinergic markers [9] in different regions of bladder and assess the effect of pharmacological activation [10] and blockade of HCN channels [1] on the force of contractions evoked by different stimuli.

Materials and methods

Materials

Human bladders from ten organ donors of both genders in the age range of 30-60 years old within 4 hours after 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 about the lower urinary tract symptoms afflicting any of the organ donors 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. Fluoromount-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.

Immunofluorescence

Bladder tissue near the trigone region was separated into mucosa (urothelium and sub-urothelium) and detrusor and immediately cryopreserved for generating 10 μm thick cryosections mounted on glass slides. Sections were first washed in phosphate buffered saline (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 the 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. We previously used these HCN1 and

HCN4 antibodies to detect a single band size of 99 and 130 kDa for HCN1 and HCN4 proteins in the lysates of human bladder by Western blot [1].

Appropriate mixtures of primary antibodies were applied to the tissue section and incubated overnight at 4°C. Unbound primary antibodies were removed by thrice washing with PBST containing 1.0% BSA for 5 min at room temperature. Sections were then incubated in the dark for 2 h at 25°C for localizing the primary antibodies binding to HCN1 or HCN4 isoforms with secondary donkey antibodies tagged to Alexa Fluor 488 (1:200) and other markers with secondary antibody tagged to Alexa Flour 594 (1:200). Sections were washed again three times with 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 [9].

Isolated tissue strip studies

Tissue from the dome regions of bladder were kept in ice-cold Krebs salt solution and mucosa intact and denuded strips were generated by separating the mucosa from detrusor 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 in organ bath chambers. The strips were bathed in 20 mL 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% O_2 and 5% CO_2 with pH 7.4, warmed to 37°C [1, 9]. 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 different stimuli was measured within first 24 h after organ collection, which corresponds to the viability window of human tissue.

Spontaneous contractions

Strips were stretched to a basal tension of 10 mN to elicit spontaneous phasic contractions [1]. 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 the cumulative addition of ZD7288 or Lamotrigine. Each concentration addition was separated by 10 min 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 measured after each addition of 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 were expressed as percentages.

EFS frequency response-curves and carbachol response

Equilibrated strips were subjected to EFS protocol with ascending frequency of EFS pulse trains ranging from 0.1 to 32 Hz (one stimulation at each frequency) delivered from constant-current Grass S88 stimulator (Warwick, RI) with the following parameters: pulse amplitude of 20 V, pulse width of 5 ms, and each stimulus duration of 2 s delivered at interval of 30 s [9]. 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 TTX were considered to be evoked by nerve stimulation. Peak contractile responses after the addition of drugs were expressed as a percentage of the maximal response at 32 Hz 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. Maximal carbachol evoked contractile response (100 µM) was measured before and after the addition of ZD7288 [10 nM or 100 µM].

Statistical analysis

Considering the differences in magnitude of contractile response for different strips pre-

sumably due to the variability of DSM content, raw values of spontaneously generated isometric tension of individual strips in presence of ZD7288 or Lamotrigine were expressed as percentage of the values measured just prior to the addition of any drug. Strips not showing any contractile response were excluded as outliers and 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. Post hoc tests were done only if F was significant to ensure there was no variance of inhomogeneity.

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 of HCN4 isoform was strongly co-localized with red immunoreactivity displaying gap junction protein Cx43 in suburothelium, DSM, and in ChAT expressing cholinergic fibers. Compared to HCN1, poor expression of HCN4 was noted in urothelium with strong colocalization in detrusor with ChAT and, Cx43 (**Figure 1**). Manufacturer recommends using higher concentration of HCN1 antibody compared to HCN4, which may explain the stronger immunofluorescence of HCN1 in bladder relative to HCN4. The co-localization of HCN1 with CGRP was higher than with ChAT in suburothelium and detrusor regions. A stronger intensity for Cx43 stain in suburothelium region compared to the unremarkable Cx45 stain localizing to DSM bundles (not shown) suggests that organ donors were unlikely to have suffered from OAB symptoms. Ethical compliance prohibits the disclosure of disease status of donors. Commercially available antibodies for HCN2 and HCN3 did not work well for immunofluorescence in human bladder.

Role of HCN channels in spontaneous contractions

Cumulative addition of ZD7288 in ascending concentrations generated a dose dependent

rise in resting 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 regions 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 ZD7288 ≤ [100 μM]; n=5; two-way ANOVA followed by Tukey's test, *P<0.05; **Figure 2**. Mucosa denuded strips also lacked the bump in the increase of contractility seen in mucosa intact strips with ZD7288 [1-10 μM], presumably due to the release of relaxing factor from urothelium [11].

Since spontaneous contractions consists of complex events of varying amplitude and frequency [2], integral measured by the area under the curve is preferred as an index for the drug effect on amplitude and frequency of spontaneous 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 in these experimental conditions.

Cumulative addition of Lamotrigine in ascending concentrations caused a modest reduction in muscle integral force without any significant difference between mucosa-denuded and mucosa intact strips. A significant reduction in the muscle integral force of spontaneous contractions and the baseline tone with Lamotrigine [10 μM] in mucosa denuded detrusor strips 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 was significantly elevated with ZD7288 [10 μM] relative to Lamotrigine [10 μM]. Tracings at different time scales further illustrate the differences in outcomes from blockade and activation of HCN channels with ZD7288 and Lamotrigine, respectively.

Role of HCN channels in neve evoked and carbachol evoked contractions

Given the reported effect of ZD7288 [10 nM] on the nerve evoked contractions of rat bladder
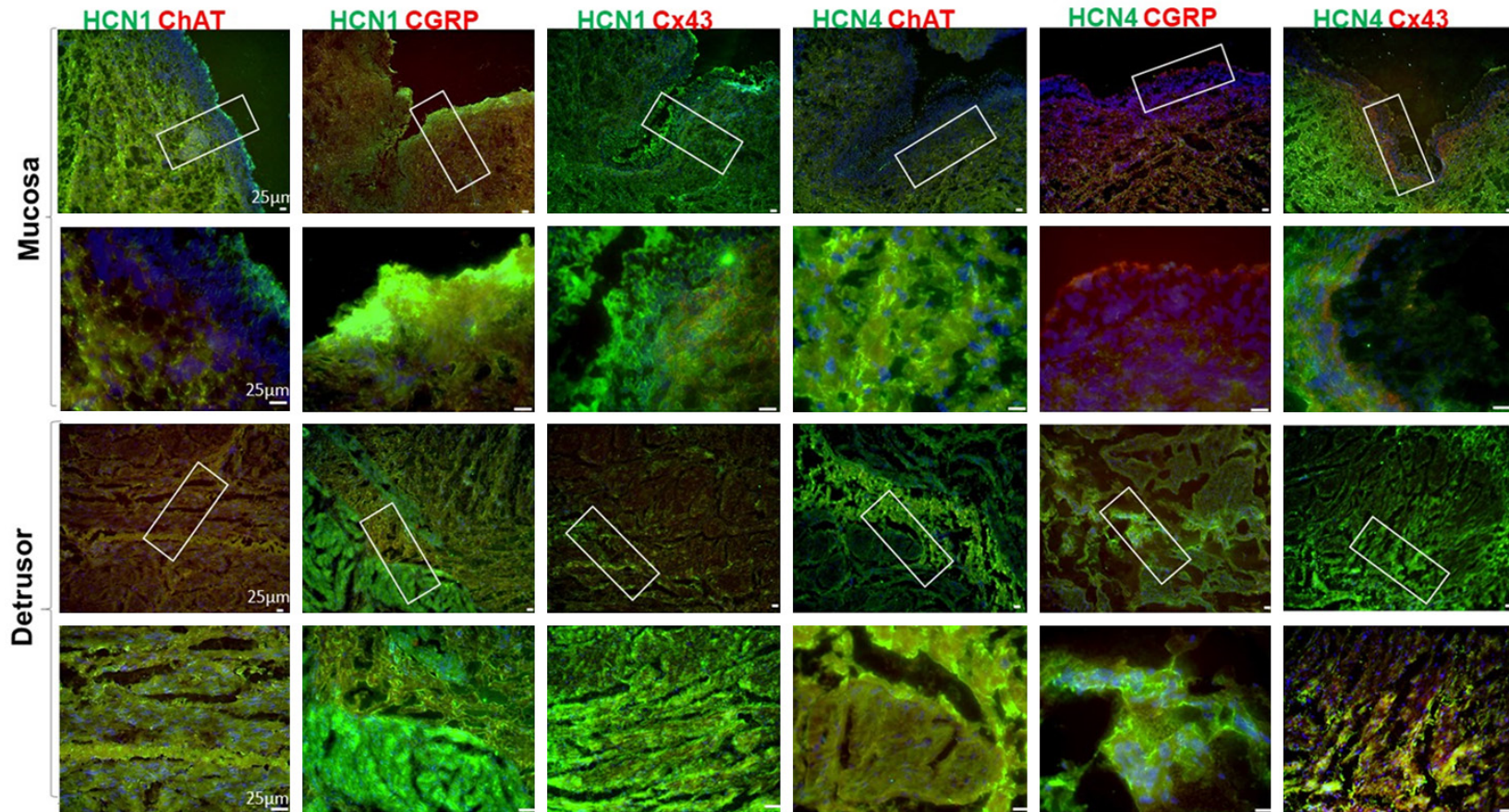
Figure 1. Immunofluorescence of HCN1 and HCN4 in human bladder: Representative double immunofluorescence images for of HCN1 and HCN4 (green stains) of the separated mucosa (urothelium and sub-urothelium) and the detrusor sections of 10 human bladders with primary antibodies and red and green colored secondary antibodies listed in **Table 1** reveals the o-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) 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 the labels on the side and the colocalization of HCN1 or 4 subtypes (green fluorescence) with red fluorescence of either CGRP, ChAT or CX43 is indicated by the color labels on top. Blue fluorescence represents the DAPI stained nucleus. Region outlined by the white box in upper panels is magnified four-fold in respective lower panels with the magnification indicated by scale bar of 25 μm.
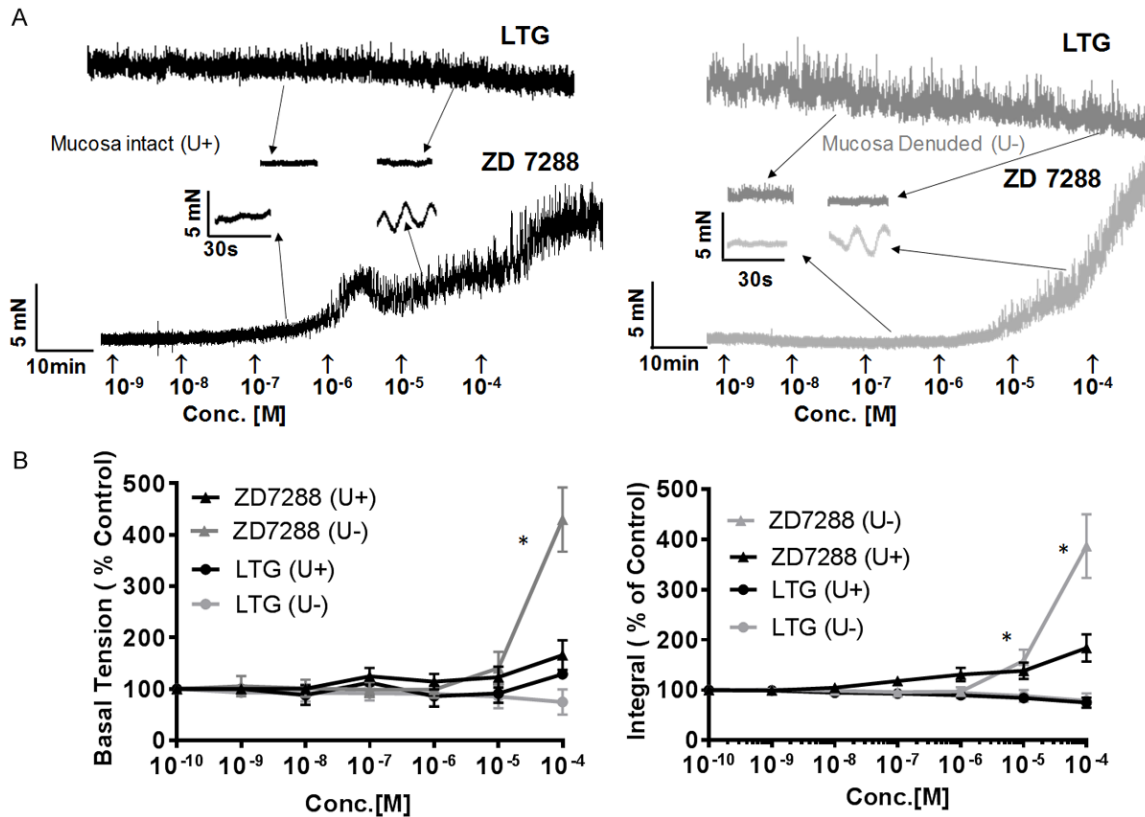
A

B

Figure 2. HCN Channels of Mucosa Constrain TTX-resistant Spontaneous Contractions. (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 [1 μM]. 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 the tension recorded prior to drug application. Since identical concentration range was used for ZD7288 (▲) and Lamotrigine (●), respective concentrations for both drugs are indicated on the x-axis and 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 Lamotrigine [10 μM] significantly reduced the integral force of spontaneous contractions relative to ZD7288 [10 μM]. Shades of the respective tracings in (A) matches with shades of curves in (B).

at 20 Hz [1], we queried its effect on the frequency response curve of human bladder. A slight enhancement in EFS contractions evoked at frequencies ≥ 8 Hz without any significance was noted after the addition of ZD7288 [10 nM] in mucosa denuded strips (**Figures 3A**, **3C** and **4**). However, higher concentrations of ZD7288 inhibited nerve evoked contractions (**Figure 3**) to implicate that ZD7288 [100 μM] blocks the constitutive activity of HCN channels expressed on post-junctional sites and gap junctions [5, 12] in increasing the amplitude of evoked contractile response of bladder (**Figures 3** and **5**). Stated differently, the action of ZD7288 [100 μM] on HCN channels expressed on post-junctional sites and on gap junctions counters a potential excitatory effect on HCN channels expressed on pre-junctional sit-

es of nerve terminals expressing ChAT (**Figures 1** and **6**).

Lamotrigine at the tested concentrations did not produce any remarkable effect on the nerve evoked contractile response suggesting that HCN channels expressed in the bladder are constitutively active to maintain the membrane conductance within the activation range. Limited time window of human tissue viability and the time required for EFS precluded the testing of ZD7288 or Lamotrigine in the extended concentration range as shown in **Figure 2**. A hint of excitatory effect with ZD7288 [10 nM] on the contractions evoked by ≥ 16 Hz in mucosa denuded strips prompted repeat examination in presence of acetylcholinesterase inhibitor (**Figure 4A**). Administration of ZD7288
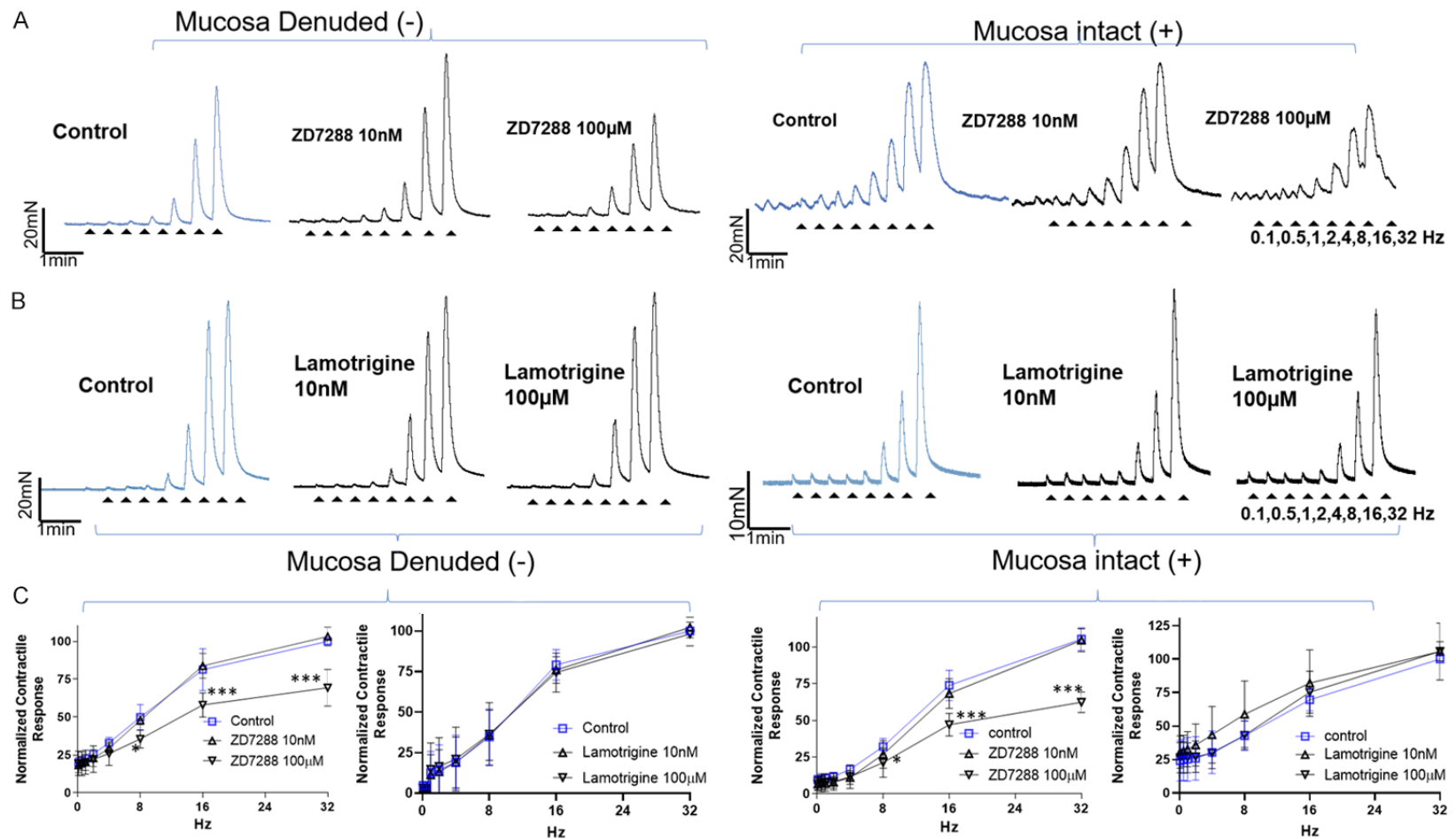
Figure 3. HCN channels of Mucosa Facilitate TTX-sensitive evoked contractions- (A, B) depict the representative tracings of contractions elicited by EFS (20 V trains of 5ms pulses for a duration of 2 s delivered 30 s apart at 0.1-32 Hz) in mucosa intact or denuded strips before and after the addition of ZD7288 [10 nM-100 μM] or Lamotrigine [10 nM-100 μM]. (C) Addition of ZD7288 [100 μM] significantly opposed the contractions evoked at frequencies ≥ 8 Hz compared to the 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 ≥ 16 Hz. Each point in the curve represent mean ± SD of 5 bladder strips. Lamotrigine addition produced an insignificant effect on the mucosa denuded strips and the slight enhancement of contractile response in mucosa intact strips did not reach statistical significance. Color and shades of the respective tracings in (A, B) matches with the color and shades of curves in (C).
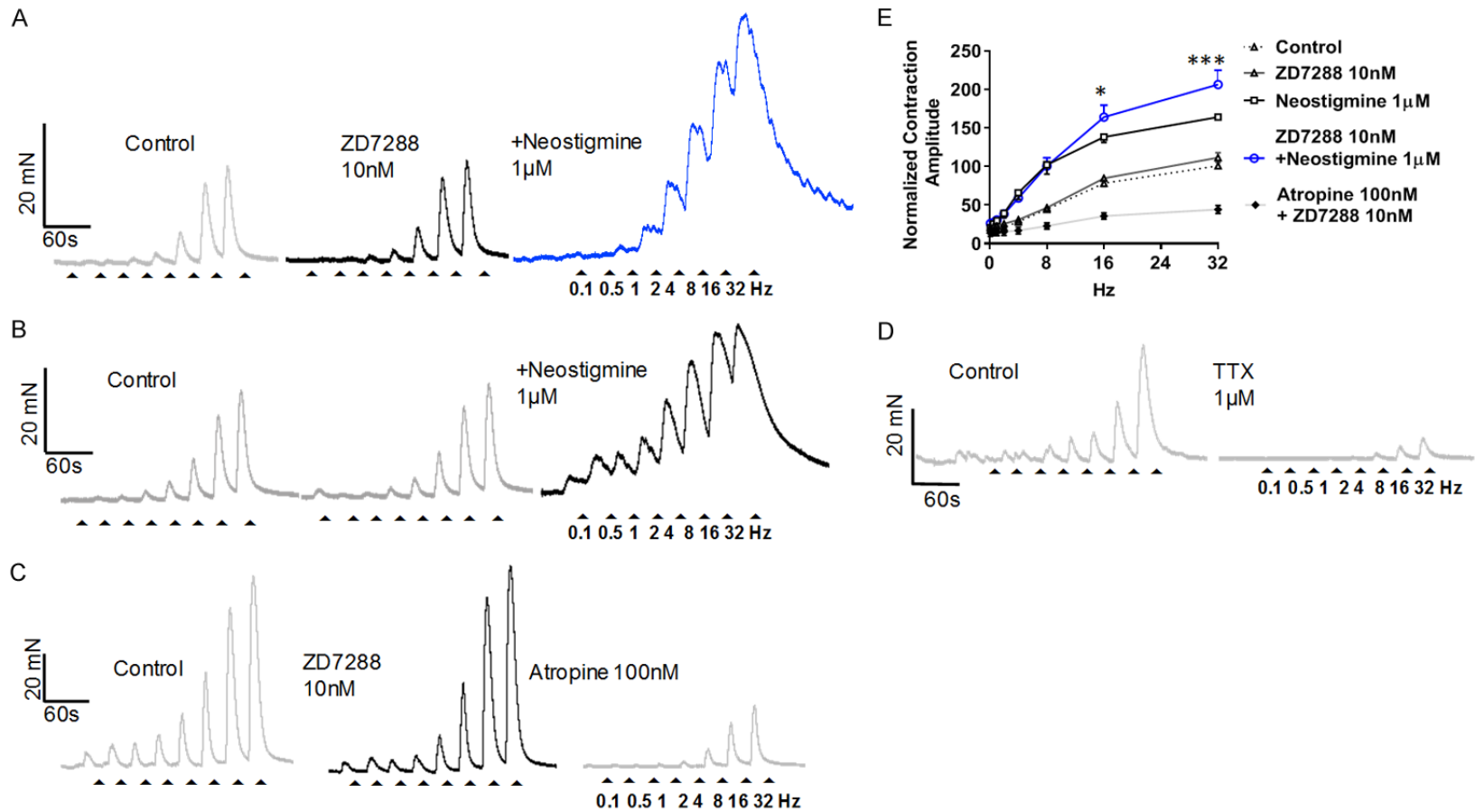
Figure 4. HCN Channels of Intramural Nerve Fibers in Bladder Constrain TTX-sensitive Evoked Contractions (A-D) depict representative tracings of contractions elicited by EFS (20 V trains of 5 ms pulses for a duration of 2 s delivered 30 s apart at 0.1-32 Hz) of mucosa denuded strips in presence and absence of ZD7288 [10 nM] alone or together with Neostigmine [1 μM] or Atropine [0.1 μM] or TTX [1 μM], respectively. (E) Force-frequency curves reveal significant enhancement of response evoked in presence of Neostigmine [1 μM] + ZD7288 [10 nM] (blue tracing and blue curve with open circles) vs Neostigmine [1 μM] alone (black tracing and curve with open squares; *P<0.05 at 16 Hz; ***P<0.001 at 32 Hz; Two-way ANOVA, Tukey's Test). Each point for the curve representing Neostigmine [1 μM] +/- ZD7288 [10 nM] represent mean ± SD of 5 bladder strips. Sensitivity of the evoked contractile response at ≥ 16 Hz in presence of ZD7288 [10 nM] to both atropine [0.1 μM] and TTX [1 μM] implies that the prejunctional action of ZD7288 [10 nM] modulates the evoked release of acetylcholine. Color and shades of the respective tracings in panel A-C matches with the color and shades of curves in (E).
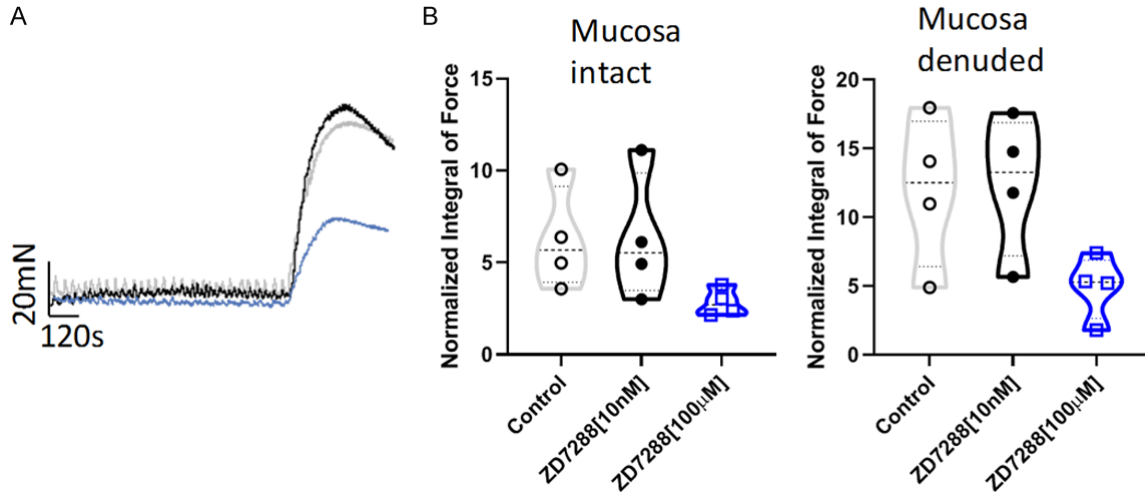
Figure 5. Effect of HCN Channel Blockade on Carbachol Response. (A) depicts the traces for carbachol 100 µM evoked contractile responses of mucosa denuded strips in absence (light grey; control) and in presence of ZD7288 [10 nM] (black tracing) or [100 µM] blue tracing. (B) shows the violin plots for force integral of carbachol [100 µM] response before and after the addition of ZD7288 [10 nM] or [100 µM]. Dots in violin plots represent data from 4 bladder strips. Addition of ZD7288 [10 nM] did not appreciably affect the carbachol evoked response of mucosa intact or denuded strips, but ZD7288 [100 µM] opposed the carbachol response more strongly in mucosa denuded strips. Color and shades of the respective tracings in (A) matches with the color and shades of violin plots in (B).

[10 nM] together with a competitive blocker of acetylcholinesterase, Neostigmine [1 µM] was expected to prolong the excitatory effect of acetylcholine released by EFS ≥ 16 Hz [9]. Indeed, we noticed a pronounced enhancement of excitatory effect of ZD7288 [10 nM] on nerve evoked contractions (**Figure 4A**, **4B**), as demonstrated by the significant difference from the strips exposed to Neostigmine [1 µM] without the addition of ZD7288 [10 nM] at EFS ≥ 16 Hz (two- way ANOVA followed by Tukey's test, *P<0.05; ***P<0.001; **Figure 4E**). Since this enhancement by ZD7288 [10 nM] at EFS ≥ 16 Hz is sensitive to independent addition of TTX [1 µM] or Atropine [0.1 µM] (**Figure 4C**, **4D**), we inferred that the ZD7288 [10 nM] blocks HCN channels expressed on ChAT expressing nerve fibers (**Figure 1**) to increase the EFS ≥ 16 Hz evoked release of acetylcholine [9].

Discussion

Overall, these findings characterize the functional role of HCN channels expressed in conjunction with the nerve terminals and gap junction proteins expressed in urothelium, sub-urothelium and detrusor regions of human bladder. Although the experimental evidence with Lamotrigine (a HCN activator) in bladder contractility studies may be equivocal, the experimental evidence with ZD7288 (HCN blocker)

in bladder contractility studies performed with or without the mechanical removal of mucosa-characterized by dense expression of HCN channels-led us to infer that HCN channels are constitutively active to constrain the spontaneous and neurogenic contractions of human bladder in a non-pacemaker fashion.

The 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, which supports the contractility analysis of mucosa intact and denuded strips reported here. Differences in the isometric tension of mucosa intact and denuded strips in light of the double immunofluorescence of HCN1 with CGRP and Cx43 in mucosa supports that spontaneous contractions originating near the urothelial-sub-urothelial interface spread to the detrusor [13] for the mechanotransduction of afferent activity [14] and bladder volume sensation. Therefore, the reported enhancement in the contractile response of mammalian detrusor after mucosa is removed [15] accords with the excitatory effect of ZD7288 [10 nM] on the nerve evoked contractile response and the enhancement in excitatory and inhibitory effect of ZD7288 [100

µM] on spontaneous and carbachol response, respectively after the removal of mucosa. Our findings led us to profess that HCN channels expressed in mucosa [11] may participate in the inhibitory influence of mucosa on the detrusor contraction, irrespective of the stimuli eliciting the contractile response [15]. Furthermore, the co-localization of slower activating HCN4 isoform having higher sensitivity to cAMP with Cx43 in the lamina propria region and of faster activating HCN1 subtype [4] with CGRP positive and ChAT positive nerve endings is in agreement with the reports of dominant HCN1 and HCN4 expression in primary afferent terminals [16] and motor axons [17], respectively.

The excitatory effect of ZD7288 on the detrusor excitability at higher concentrations shown here is consistent with the increased excitability of pyramidal neurons in brain following a decrease in hyperpolarization-activated currents [10, 18]. Blockade of HCN channels is known to affect the frequency of action potentials without eliminating the spontaneous electrical activity in Pituitary [3]. Based on our findings, we postulate that excitatory effect of HCN blockade on the spontaneous and nerve evoked contractions of bladder is linked to the constraining effect of HCN channels on the autonomous origination of membrane potential oscillations in DSM [19] as well as on the action potential evoked release of neurotransmitters [20].

Indeed, the effect of HCN selective concentrations of ZD7288 [10 nM] [21] on the nerve evoked contractions is consistent with the increased frequency of action potentials following blockade of HCN channels in isolated neurons [18, 22]. A prejunctional action of ZD7288 [10 nM] on the HCN channels [20] expressed on ChAT positive cholinergic nerve fibers of bladder can be inferred from the co-localized immunoreactivity of HCN4 and ChAT in sub urothelium and detrusor. The inference is also supported by the pronounced TTX-sensitive enhancement of EFS response ≥ 16 Hz in presence of Neostigmine (which retards the acetylcholine degradation) and the reported dominance of muscarinic signaling at higher frequencies (≥ 10 Hz) [9, 23]. The TTX-resistance of stretch mediated basal release of neurotransmitters [24] such as adenosine triphosphate (ATP) and ACh from nerve terminals,

urothelium, suburothelium and interstitial cells suggests that pre-junctional action of ZD7288 [10-100 µM] may also enhance the stretch mediated basal release of ATP and ACh to boost the frequency of rhythmic membrane depolarizations for augmenting the TTX- resistant spontaneous contractions. Compared to the TTX resistant basal release of neurotransmitters involved in spontaneous contractions, the inhibitory effect ZD7288 [100 µM] on TTX-sensitive neurogenic contractions is explained by the TTX sensitive evoked release of neurotransmitters.

A postulated role for gap junctions as a conduit for the transmission of membrane depolarizations across adjacent DSM cells is supported by the co-localization of HCN4 and Cx-43 in suburothelium and by the significant rise in the resting tension and integral force of mucosa-denuded strips following the addition of ZD7288. A significant reduction in EFS evoked response and of carbachol evoked contractile response [5, 12] in presence of ZD7288 [100 µM] suggests that HCN channels expressed on urothelium and gap junctions [5] are constitutively active to integrate the rise in bladder tone evoked by the stimuli of EFS or carbachol through spatio-temporal integration of spontaneous excitatory junction potentials [6], a prerequisite for the contraction of bladder as a three-dimensional functional syncytium (**Figure 6**). Taken together, our findings support the premise of HCN channels sub serving an integrative role in detrusor contractility and the mechanotransduction of afferent activity as well as its amplification by spontaneous contractions [14].

ZD7288 has high affinity for HCN channels with the reported IC50 of 0.3 µM [21], but it can also directly block T-type VGCC with IC50 of 50 µM [25]. A recent report studied the effect of ZD72288 at a single concentration of [50 µM] [5] on the spontaneous contractions of human bladder tissue obtained from cystectomy of bladder cancer patients. Even after the potential impact of malignancy on the functional response is disregarded, the reported excitatory effect of ZD7288 [50 µM] in the bladder of HCN1 deficient mice raises questions on the specificity of ZD7288 for HCN channels beyond the concentration of 0.3 µM [21]. Findings reported here are consistent with the lack
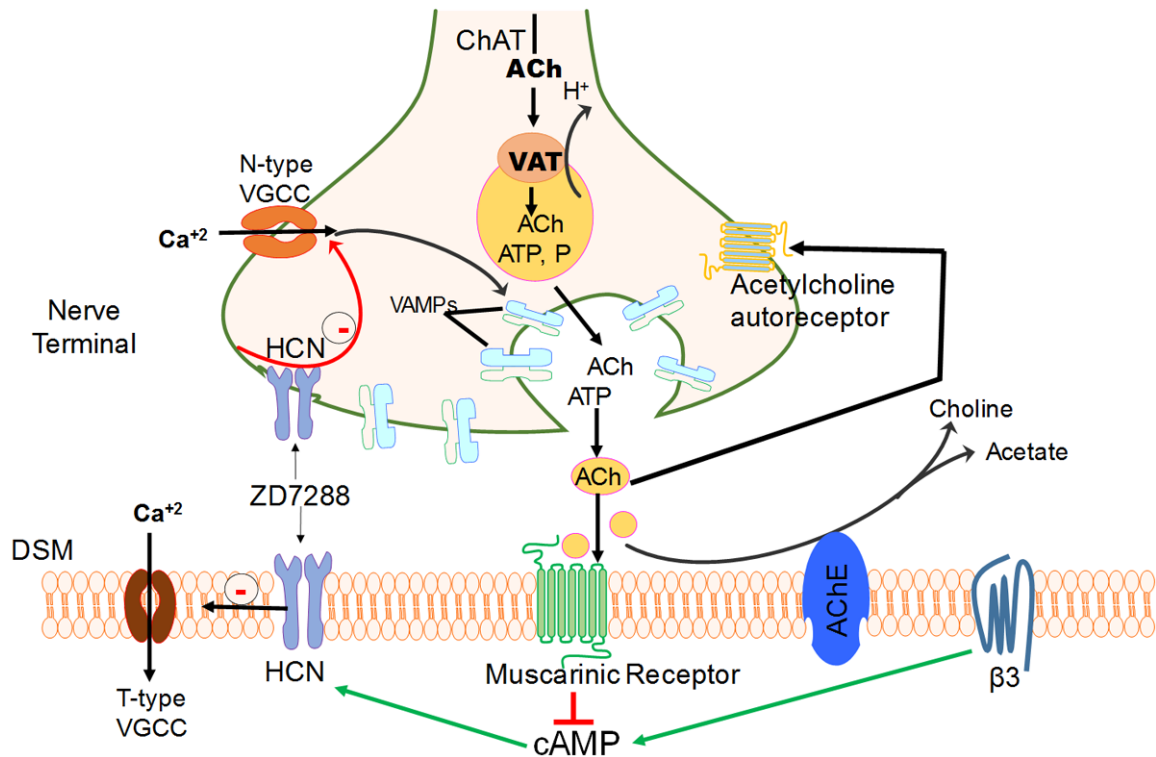
Figure 6. Schematic illustration for proposed non-pacemaker function of HCN channels in human bladder: HCN channels expressed on nerve terminals, gap junctions, mucosa and DSM converge functionally for the spatio-temporal integration of voiding contraction and for constraining the spontaneous contractions. HCN channels expressed on pre-junctional sites of ChAT expressing nerve terminals constrain the release of neurotransmitters such as ACh and ATP 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, a prerequisite for the activation of L-type VGCC leading to the Ca^{2+} influx into DSM for spontaneous contractions.

of any effect of Lamotrigine [30 μM] on EFS or carbachol evoked contraction [26]. The specificity of Lamotrigine [10-100 μM] for HCN channels [18, 22] is supported by the specific blockade of its anticonvulsant effect by the clinically approved HCN blocker, Ivabradine [27]. Although, Lamotrigine is also shown to block sodium channels at 91 μM [28] and stabilize the presynaptic neuronal membrane to hinder the basal release of excitatory neurotransmitters involved in spontaneous contractions and directly inhibit the excitatory action of serotonin at 0.24-0.47 mM [29].

Although, ZD7288 [50 μM] abolishes the HCN-dependent pacemaker depolarizations driving the coordinated upper urinary tract peristalsis necessary for unimpeded flow of urine [7] to the bladder, we and others have observed excitatory effect of ZD7288 [10-100 μM] on the

spontaneous contractions of mammalian bladder [5]. Differences in the functional outcomes after blockade of HCN channels expressed in upper and lower urinary tract led us to deduce a non-pacemaking role for HCN channels in human bladder. Our inference is also supported by the occurrence of spontaneous contractions at lower frequency in interstitial cells [30] than in the DSM of guinea pig.

Depolarization of DSM is critical for the spontaneous contractions [31], but muscarinic receptor dependent bladder emptying evoked by parasympathetic nerve is suggested to be independent of DSM depolarization [31]. Although antimuscarinics work well for the majority of OAB patients, they are grossly inadequate for detrusor hyperactivity and impaired contractility (DHIC) owing to their adverse effect on impaired contractility (IC) component of DHIC, which can increase the risk for urinary reten-

tion in older adults [32, 33], recurrent urinary tract infection and vesicoureteric reflux. Likewise, L-type VGCC antagonists such as Nifedipine also have the potential to suppress both DH and IC component of DHIC as well as T-type VGCC antagonists [34, 35] which are not yet available for clinical use.

Our findings shed light on the mechanism for the reported reduction of uninhibited detrusor contractions and increased bladder capacity [36] in spinal cord injured rats following daily treatment with Lamotrigine, a HCN activator [10]. When taken together with the suppression of T-type VGCC activity [20] by the agents activating HCN channels, we surmise that HCN channel activation may be a viable strategy for integrating the phasic contractions driving the DH component and to augment the bladder tone for evoking a stronger efferent discharge to augment the IC component of DHIC. Future studies on mice with targeted or global deletion of HCN subtypes can advance our understanding about the role of HCN function in mammalian bladder.

Conclusions

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

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Disclosure of conflict of interest

None.

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