BK channel regulation by phosphodiesterase type 1: A novel signaling pathway controlling human detrusor smooth muscle function

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Abstract

Large conductance Ca$^{2+}$-activated K$^+$ (BK) channels are critical regulators of detrusor smooth muscle (DSM) function. We aimed to investigate phosphodiesterase type 1 (PDE1) interactions with BK channels in human DSM to determine the mechanism by which PDE1 regulates human urinary bladder physiology. A combined electrophysiological, functional, and pharmacological approach was applied using human DSM specimens obtained from open bladder surgeries. The perforated whole cell patch-clamp technique was used to record transient BK currents (TBKCs) and the cell membrane potential in freshly-isolated human DSM cells in combination with the selective PDE1 inhibitor, 8-methoxymethyl-3-isobutyl-1-methylxanthine (8MM-IBMX). Isometric DSM tension recordings were used to measure spontaneous phasic and electrical field stimulation (EFS)-induced contractions in human DSM isolated strips. Selective pharmacological inhibition of PDE1 with 8MM-IBMX (10 µM) increased TBKC activity in human DSM cells, which was abolished by subsequent inhibition of protein kinase A (PKA) with H-89 (10 µM). The stimulatory effect of 8MM-IBMX on TBKCs was reversed upon activation of muscarinic acetylcholine receptors with carbachol (1 µM). 8MM-IBMX (10 µM) hyperpolarized the DSM cell membrane potential, an effect blocked by PKA inhibition. 8MM-IBMX significantly decreased spontaneous phasic and nerve-evoked contractions of human DSM isolated strips.

The results reveal a novel mechanism that pharmacological inhibition of PDE1 attenuates human DSM excitability and contractility by activating BK channels via a PKA-dependent mechanism. The data also suggest interactions between PDE1 and muscarinic signaling pathways in human DSM. Inhibition of PDE1 can be a novel therapeutic approach for the treatment of overactive bladder associated with detrusor overactivity.
Introduction

Overactive bladder (OAB), one of the most common forms of lower urinary tract dysfunction, is often associated with increased detrusor smooth muscle (DSM) contractility (2). Currently, the primary option for pharmacological treatment of OAB is antagonists of G-protein-coupled muscarinic acetylcholine (ACh) receptors (mAChRs) (1, 3). However, mAChR antagonists have limited efficacy and undesirable side effects for many OAB patients (1, 3).

It is well established that the large conductance voltage- and Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels are key regulators of DSM excitability and contractility, and therefore are considered as promising novel targets for OAB therapies (19). Depolarization of the DSM cell membrane initiates action potentials, voltage-dependent Ca\(^{2+}\) influx, and spontaneous phasic contractions (19). Depolarization of the cell membrane potential along with the Ca\(^{2+}\) influx during an action potential firing also activates BK channels, which provides a negative feedback to attenuate DSM excitability (19). BK channel activity is regulated by localized intracellular Ca\(^{2+}\) release events from the sarcoplasmic reticulum (SR) ryanodine receptors (RyRs), known as “Ca\(^{2+}\) sparks”. Ca\(^{2+}\) sparks transiently activate BK channels generating spontaneous transient BK currents (TBKCs) (8, 12, 16, 27, 28). TBKCs correspond to spontaneous transient hyperpolarizations in DSM cells (19). The frequencies of TBKCs and related transient hyperpolarizations are tightly correlated in DSM cells, thus contributing to DSM excitability (28).

Previous studies have demonstrated that the activation of mAChRs, the mAChR\(_3\) subtype in particular, leads to an inhibition of BK channel activity in human DSM cells via a Ca\(^{2+}\)-dependent regulatory mechanism (17-19). In contrast, cAMP-dependent activation of PKA
signaling pathways leads to an increase in Ca^{2+} sparks, BK channel activity, and hyperpolarization of DSM cells, thus decreasing DSM excitability and contractility (8, 19, 28).

BK channel activity is also regulated by phosphodiesterases (PDEs), which are constitutively active in human DSM (26, 28). The inhibition of PDEs increases intracellular cAMP, activates protein kinase A (PKA), and thus stimulates BK channels to attenuate human DSM excitability and contractility (26, 28). In addition, the mAChR signaling pathways are subjected to positive feedback regulation by PDEs, particularly the Ca^{2+}-activated PDE type 1 isoform (PDE1) (5, 6, 20). PDE1, the only PDE isoform that is activated by Ca^{2+} (5, 6), is considered to be an important mediator of the crosstalk between intracellular cAMP and Ca^{2+} signaling pathways in the regulation of DSM excitability (23). An increase in intracellular Ca^{2+} is often accompanied by a reduction of intracellular cAMP levels due to the Ca^{2+}-dependent PDE1 activation (5, 7). The selective inhibition of PDE1 elevates cellular cAMP levels and TBKC activity in DSM cells, while mAChR activation inhibits TBKCs (17, 19, 28). The mAChR-mediated and Ca^{2+}-dependent PDE1 activation clearly suggests that PDE1 is a highly important regulatory element in human bladder physiology (15, 20).

We recently demonstrated that selective inhibition of PDE1 attenuates guinea pig DSM excitability and contractility by stimulating BK channel activity (19, 28). However, the physiological significance of PDE1 signaling in the regulation of human DSM function has not been fully investigated. As previous investigations have shown differential PDE expression in humans and guinea pigs, it is critically important to determine the functional role of PDE1 directly in human DSM as they are the target species of interest for therapeutic interventions (14, 20). The current research aimed to investigate the regulatory mechanisms by which PDE1-mediated signaling pathways control human DSM excitability and contractility. The data
obtained from human DSM in this study are the foundation for further investigations into the therapeutic potential of selective PDE inhibitors in the treatment of bladder dysfunction (20).

Materials and Methods

**Human DSM tissue collection and single cell isolation.** Human DSM tissue specimens were obtained from routine open bladder surgeries according to protocol Pro00045232, which was reviewed and approved by the Medical University of South Carolina Institutional Review Board. All 16 human DSM specimens were obtained from patients without a preoperative history of OAB and American Urological Association score<8. We used a total of 16 human DSM samples collected from 12 male and 4 female patients, of which 12 Caucasian and 4 African Americans, 46 to 79 years of age (mean 64.0±3.1 years old). DSM strips (~2-3 mm wide and ~6-7 mm long), prepared by removing the mucosa, were used for single DSM cell isolation and isometric DSM tension recordings.

**Human DSM single cell isolation.** Human DSM single cells were freshly isolated by enzymatic digestion using procedures previously described (12, 26). Briefly, 1-2 DSM strips free of mucosa were incubated at 37°C for ~20 min in 2 ml Ca^{2+}-free dissection solution supplemented with 1 mg/ml bovine serum albumin (BSA), 1 mg/ml papain, and 1 mg/ml DL-dithiothreitol. The Ca^{2+}-free dissection solution contained (in mM): 80 monosodium glutamate, 55 NaCl, 6 KCl, 10 D-glucose, 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), and 2 MgCl_2; pH was adjusted to 7.3 with NaOH. The DSM tissues were then transferred to 2 ml of dissection solution supplemented with 1 mg/ml BSA, 0.5 mg/ml collagenase, 0.5 mg/ml trypsin inhibitor, 100 μM CaCl_2 and incubated at 37°C for 7-12 min followed by three washouts with fresh dissection
solution supplemented with 1 mg/ml BSA. Individual cells were released from the DSM tissue by passing the enzyme-treated strips through a fire-polished Pasteur pipette. The freshly-isolated DSM cells were used within 8 hours following isolation.

*Patch-clamp electrophysiological recordings.* The amphotericin-B perforated whole cell patch-clamp technique was used for electrophysiological recordings in freshly-isolated DSM single cells as previously described (11-13, 17). Patch-clamp recordings were conducted using an Axopatch 200B amplifier system and Digidata 1440A controlled with pCLAMP 10.2 software (Molecular Devices, Union City, CA). The glass pipettes used for the patch-clamp experiments were made from borosilicate glass (Sutter Instruments, Novato, CA), pulled using a PP-830 vertical puller (Narishige Group, Tokyo, Japan), and polished with a Micro Forge MF-830 fire polisher (Narishige Group). Pipette resistance was 4–6 MΩ. The extracellular solution for whole cell patch-clamp experiments contained (in mM): 134 NaCl, 6 KCl, 1 MgCl_2, 2 CaCl_2, 10 D-glucose, and 10 HEPES; pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl_2, 10 HEPES, and 0.05 Ethylene glycol-bis(2-aminoethylether)-N,N',N''-tetraacetic acid (EGTA), pH was adjusted to 7.2 with NaOH and supplemented with freshly-dissolved (every 1–2 h) 200 µg/ml amphotericin-B. TBKCs were recorded at a holding potential of -20 mV. The membrane potential of DSM cells was recorded in current-clamp mode of the patch-clamp technique ($I_h=0$). All patch-clamp experiments were conducted at room temperature (~22-23 ºC).

*Isometric DSM tension recordings.* Isometric contractions of human DSM isolated strips were measured using a Myomed myograph system (MED Associates, St. Albans, VT) as previously described (11-13, 17). Briefly, DSM strips were secured to isometric force displacement transducers and were suspended in temperature controlled (37°C) water jacketed
tissue baths containing 10 ml physiological saline solution, which was prepared daily and contained (in mM): 119 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, and 11 D-glucose, and was aerated with 95% O2-5% CO2 to obtain pH 7.4. The tension of DSM strips was initially adjusted to 10 mN, and then the strips were washed with fresh physiological saline solution every 15 min during an equilibration period of 45–60 min. To minimize the potential effects of neurotransmitters released from the neurons in the tissue, the experiments on spontaneous DSM contractions were performed in the presence of 1 µM tetrodotoxin, a selective blocker of the neuronal voltage-gated Na⁺ channels.

Nerve-evoked DSM contractions were induced by electrical field stimulation (EFS) using a pair of platinum electrodes mounted in the tissue baths parallel to the DSM strips. The EFS pulse parameters were as follows: 0.75-ms pulse width, 20-V pulse amplitude, 3-s stimulus duration, and polarity was reversed for alternating pulses. After the equilibration period, the DSM strips were subjected to continuous repetitive EFS pulses with a frequency of 20 Hz at 1-min intervals. The EFS pulses were generated using a PHM-152I stimulator.

Data analysis and statistics. The frequency and amplitude of TBKCs, as well as the parameters of the DSM phasic contractions, were analyzed using MiniAnalysis software (Synaptosoft, Inc., Decatur, GA). The threshold amplitude for detecting TBKCs was set at 10 pA. The TBKCs’ frequency was measured in Hz and was normalized to the control value for each cell, which was taken as 100%. The cell membrane potential was analyzed using Clampfit 10.2 (Molecular Devices, Union City, CA). Data were further analyzed with GraphPad Prism 4.03 software (GraphPad Software, Inc., La Jolla, CA). Data were expressed as means±SEM; n=the number of cells or DSM strips, and N=the number of patients. Data were compared for
statistical significance using paired two-tailed Student’s t-test and P<0.05 was considered significant.

Chemicals and drugs. Trypsin inhibitor, BSA, and amphotericin-B were obtained from Thermo Fisher Scientific (Waltham, MA). Papain was purchased from Worthington Biochemical (Lakewood, NJ). H-89, collagenase (type II), and tetrodotoxin citrate were purchased from Sigma-Aldrich (St. Louis, MO). 8-Methoxymethyl-IBMX (8MM-IBMX) was purchased from Tocris Bioscience (Bristol, United Kingdom). Amphotericin-B, H-89, and 8MM-IBMX were dissolved in dimethyl sulfoxide as stock solutions. The final dimethyl sulfoxide concentration was less than 0.1%. All other chemicals were dissolved in double-distilled water.

Results

Selective pharmacological inhibition of PDE1 with 8MM-IBMX increased TBKC activity in freshly-isolated human DSM cells. 8MM-IBMX is a selective PDE1 inhibitor with IC<sub>50</sub> values for PDE1 below 12 μM (22). 8MM-IBMX (10 μM) significantly increased the frequency of TBKCs to 321.1% of the control value, with no significant effect on TBKC average amplitude (n=7, N=7; P<0.05; Fig. 1A-B). The inhibition of PDE1 increases the cellular cAMP levels and consequently activates PKA (28). H-89 (10 μM), a PKA inhibitor, abolished the potentiating effect of 8MM-IBMX on TBKCs (Fig. 1A). In the presence of 8MM-IBMX, H-89 (10 μM) reduced the mean values for TBKC frequency and amplitude to 11.0±5.7% and 45.6±22.9% of the control, respectively (n=3, N=3). Reduction of TBKC frequency and amplitude to levels below the control implies some endogenous PKA activity as we have recently reported (27).

Carbachol attenuated TBKCs and this inhibitory effect was recovered by 8MM-IBMX. It has been recently reported that activation of the mAChR<sub>3</sub> subtype leads to TBKC inhibition via a
Ca\(^{2+}\)-dependent mechanism (17, 18). Our data show that activation of mAChRs with carbachol (1 µM) significantly reduced the frequency of TBKCs to 33.3±5.6\% of the control value in freshly-isolated human DSM cells (n=5, N=5; P<0.05 carbachol vs. control; Fig. 2). In the presence of 1 µM carbachol, 8MM-IBMX (10 µM) significantly recovered the frequency of TBKCs to 91.0±12.1\% (n=5, N=5; P<0.05, 8MM-IBMX vs. carbachol; Fig. 2). These results suggest that the blockade of PDE1 opposes the inhibitory effect of mAChRs on BK channel activity.

*The pharmacological inhibition of PKA with H-89 eliminated the hyperpolarizing effect of 8MM-IBMX in human DSM cells.* Activation of BK channels upon PDE1 inhibition attenuates DSM excitability (19). Our previous study revealed that the inhibition of PDE1 with 8MM-IBMX (10 µM) hyperpolarized the membrane potential of freshly-isolated human DSM cells by ~12 mV (28). Here, we showed that the inhibition of PKA with H-89 (10 µM) blocked the spontaneous transient hyperpolarizations that correspond to the TBKCs, and depolarized the DSM cell membrane potential from -35.4±10.9 mV under control conditions to -25.7±10.0 mV (n=8, N=8; P<0.05, H-89 vs. control; Fig. 3). In the presence of H-89, 8MM-IBMX did not have any hyperpolarizing effect but rather further depolarized the membrane potential of human DSM cells averaged as -20.4±10.4 mV (n=8, N=8; P<0.05, 8MM-IBMX+H-89 vs. H-89; Fig. 3).

*The pharmacological inhibition of PDE1 with 8MM-IBMX attenuated the spontaneous phasic contractions of human DSM isolated strips.* Human DSM exhibits spontaneous phasic contractions (19). Isometric tension recordings on human DSM isolated strips showed that 8MM-IBMX (10 µM) significantly reduced the amplitude, muscle force, frequency, and muscle tone of the spontaneous phasic contractions to 46.6±14.8\%, 51.3±17.7\%, 41.6±11.0\%, and
78.6±2.5% of the control values, respectively (n=8, N=4, P<0.05; Fig. 4). These data suggest that PDE1 is an important regulator of human DSM spontaneous contractility.

The inhibition of PDE1 with 8MM-IBMX attenuated the nerve-evoked human DSM contractions. EFS (20 Hz) induces ACh release from parasympathetic nerve terminals which activate DSM mAChRs and mimics nerve-evoked contractions in human DSM isolated strips. 8MM-IBMX (10 µM) significantly decreased the EFS-induced DSM contraction amplitude, muscle force, and tone to 62.6±3.6%, 65.3±4.4%, and 88.9±2.6% of the control values, respectively (n=14, N=4; P<0.05; Fig. 5). These results suggest that the pharmacological inhibition of PDE1 opposes the nerve-evoked contractions in human DSM.

Discussion

The current study revealed a novel mechanism by which PDE1 regulates human DSM function. The data demonstrated that the inhibition of PDE1 increases BK channel currents via PKA-mediated signaling pathways, leading to a decrease in human DSM excitability and contractility (Fig. 6). This is the first study indicating that the constitutively active PDE1 plays a key role in maintaining human DSM contractility.

PDEs, including the Ca^{2+}-activated PDE1, control the basal intracellular cAMP level, and thus PKA activity (5, 6). PDE1 hydrolyzes cAMP and the inhibition of PDE1 increases the cellular cAMP levels which activates PKA and in turn increases BK channel activity in isolated guinea pig DSM cells (28). The current study provided new evidence that the inhibition of PDE1 increased TBKCs in a PKA-dependent manner in human DSM cells (Fig. 1). The activation of BK channels hyperpolarizes the human DSM cell membrane potential (13, 28). The
pharmacological inhibition of constitutive PKA activity abolishes TBKCs and depolarizes DSM cell membrane potential (27) (Figs. 1 and 3). We previously reported that selective PDE1 inhibition hyperpolarizes human DSM cell membrane potential (28). In the current study, we further revealed that this hyperpolarizing effect was PKA-dependent (Fig. 3). These results indicate that PDE1 is an important component of the cAMP/PKA signaling pathways which critically regulates human DSM excitability (Fig. 6). In fact, PDE1 is the only PDE isoform that is activated by mAChR-mediated increase of intracellular Ca$^{2+}$, which provides an important mechanism for the cross-talk between intracellular Ca$^{2+}$ and cAMP signals (6, 15). Our data also suggest potential functional interactions between mAChR-mediated Ca$^{2+}$ and the PDE1-controlled cAMP signaling pathways that regulate the activity of BK channels in DSM (Figs. 2 and 6).

BK channels facilitate the repolarization phase of DSM spontaneous action potentials, which are associated with Ca$^{2+}$ transients and related spontaneous phasic contractions of DSM (10, 19). The highly localized Ca$^{2+}$ releases from SR RyRs, the Ca$^{2+}$ sparks, transiently activate BK channels generating TBKCs and related spontaneous transient hyperpolarizations in DSM cells (19). The elevation of intracellular cAMP, and the subsequent activation of PKA and BK channels hyperpolarize the DSM cell membrane leading to the suppression of spontaneous action potentials and a decrease of Ca$^{2+}$ transients (9). This indicates a close functional interaction between intracellular Ca$^{2+}$ dynamics and cAMP signaling, which are two major regulatory pathways controlling BK channel activity. These data also demonstrate that PDE1 is an important integrator of the Ca$^{2+}$- and cAMP-dependent signaling in human DSM while PDE1 per se is activated by Ca$^{2+}$ (Fig. 6) (5, 6).
Previous studies have demonstrated the key role for Ca\(^{2+}\) in the physiological coupling between PDE1 and mAChRs, a critical mechanism controlling cAMP-dependent signaling (5, 28). ACh, the endogenous mAChR agonist, is the primary excitatory neurotransmitter released from the parasympathetic nerves in human DSM. The activation of mAChR\(_2\) subtypes may lead to the inhibition of adenylyl cyclases by the inhibitory G\(\alpha\)-protein and attenuate cAMP synthesis, thus reducing PKA activity (4, 24). In addition, the mAChR\(_3\)-mediated Ca\(^{2+}\) influx activates PDE1 and increases the hydrolysis of cAMP to further suppress PKA activity (Fig. 6) (6). A decrease in basal PKA activity significantly attenuates BK channels in DSM cells (19, 27). However, this concept needs further investigation as it is particularly important to also study the enzymatic kinetics of PDE1 regarding its regulation by mAChRs in human DSM. In agreement with this concept, the activation of mAChR\(_3\) suppresses BK channels in human DSM cells, which is consistent with our previous findings (17, 18). More importantly, the inhibition of PDE1 substantially recovered TBKC activity, which was suppressed by mAChR\(_3\) activation. These results support the concept for a potential functional interaction between PDE1 and mAChRs in human DSM (Fig. 2) (6). The activation of PDE1 by mAChR\(_3\)-mediated Ca\(^{2+}\) may provide a positive feedback on the attenuation of BK channels by mAChR\(_3\) leading to further increase in intracellular Ca\(^{2+}\) (6). The positive feedback from PDE1 on mAChR signaling is physiologically relevant during micturition, when neuronal ACh release initiates forceful voiding contractions. Our data suggest that in human DSM, PDE1 and mAChRs could function as an integrative signaling pathway (Fig. 6). Thus, combination therapies, consisting of PDE1 inhibitors and antimuscarinics, may represent a novel therapeutic intervention to mitigate the non-voiding contractions that are often associated with OAB. It is also possible that PDE1
activity underlies the low efficacy of antimuscarinic compounds in the inhibition of involuntary DSM contractions (21).

A previous clinical trial for a PDE1 inhibitor, vinpocetine, on OAB patients who did not respond to antimuscarinic treatment, showed that this drug significantly improved the clinical symptoms of urge incontinence and a low compliance bladder (25). In our study, isometric DSM tension recordings demonstrated that the inhibition of PDE1 can significantly suppress the spontaneous phasic contractions in human DSM isolated strips (Fig. 4). Furthermore, EFS-induced contractions, which are primarily caused by neuronal ACh release, were also significantly attenuated by PDE1 inhibition (Fig. 5).

In conclusion, our data provide mechanistic evidence that pharmacological inhibition of PDE1 may represent a novel therapeutic approach for the treatment of OAB by attenuating DSM excitability and contractility through PKA-dependent BK channel activation (Fig. 6). The inhibition of PDE1 can be an effective alternative to suppress detrusor overactivity in OAB patients who do not respond to antimuscarinic treatment (20, 25), and in patients with neurogenic detrusor overactivity (11). These investigations are an important step in validating PDE1 as a potential novel therapeutic target for OAB (20). We hope that this initial study will stimulate interest towards the development of more potent and selective PDE1 inhibitors for urological applications.
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References


**Abbreviations**

8MM-IBMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine;

BK channel, large conductance voltage- and Ca$^{2+}$-activated K$^+$ channel;

BSA, bovine serum albumin;

cAMP, cyclic adenosine monophosphate;

DSM, detrusor smooth muscle;

EFS, electrical field stimulation;

EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid;

HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N'-

(2-ethanesulfonic acid);

mAChR, muscarinic ACh receptor;

OAB, overactive bladder;

PDE, phosphodiesterase;

PKA, protein kinase A;

RyR, ryanodine receptor;

SEM, standard error of the mean;

SR, sarcoplasmic reticulum;

TBKC, transient BK current.
Figure Legend

Figure 1. The pharmacological inhibition of PDE1 increased the TBKCs in human DSM cells in a PKA-dependent manner. (A) An original patch-clamp recording illustrating that 8MM-IBMX (10 μM) increased the TBKCs and H-89 (10 μM) abolished the potentiation effect of 8MM-IBMX on TBKCs in a freshly-isolated human DSM cell. (B) Summary data showing that 8MM-IBMX (10 μM) significantly increased the TBKCs frequency without a significant effect on the average amplitude of TBKCs (n=7, N=7; *P<0.05).

Figure 2. Activation of mAChRs with carbachol inhibited TBKCs and the subsequent blockade of PDE1 with 8MM-IBMX reversed the inhibitory effect of carbachol on the TBKCs in human DSM cells. (A) An original patch-clamp recording of TBKCs illustrating that carbachol (1 μM) inhibited the TBKCs and the subsequent addition of 8MM-IBMX (10 μM) recovered the TBKCs. (B) Summary data showing that carbachol (1 μM) significantly inhibited the frequency of TBKCs and the subsequent addition of 8MM-IBMX (10 μM) reversed the inhibitory effect of carbachol on TBKCs (n=5, N=5, *P<0.05, carbachol vs. control; #P<0.05, 8MM-IBMX vs. carbachol; NS-non-significant).

Figure 3. The pharmacological inhibition of PKA with H-89 blocked the hyperpolarizing effect of 8MM-IBMX in DSM cells. (A) An original current-clamp recording showing that the inhibition of PKA with H-89 abolished the spontaneous transient hyperpolarizations and depolarized human DSM cell membrane potential. In the presence of H-89 the inhibition of PDE1 did not have any hyperpolarizing effect. (B) Summary data illustrating that the inhibition of PKA with H-89 (10 μM) significantly depolarized human DSM cell membrane potential (n=8, N=8; P<0.05, H-89 vs. control). The subsequent inhibition of PDE1 with 8MM-IBMX (10 μM)
did not have any hyperpolarizing effect on DSM cell membrane potential in the presence of H-89.

**Figure 4. The pharmacological inhibition of PDE1 with 8MM-IBMX attenuated the spontaneous phasic contractions of human DSM isolated strips.** (A) An original recording illustrating that 8MM-IBMX (10 μM) reduced the spontaneous phasic contractions of human DSM strips. (B) Summary data showing that the inhibition of PDE1 with 8MM-IBMX (10 μM) significantly reduced the amplitude, muscle force, frequency, and the tone of human DSM spontaneous contractions (n=8, N=4; *P<0.05).

**Figure 5. The pharmacological inhibition of PDE1 with 8MM-IBMX attenuated the nerve-evoked contractions in human DSM isolated strips.** (A) An original recording illustrating that 8MM-IBMX 10 (μM) reduced 20 Hz EFS-induced DSM contractions. (B) Summary data showing that 8MM-IBMX (10 μM) reduced the amplitude, muscle force, and tone of 20 Hz EFS-induced human DSM contractions (n=14, N=4; *P<0.05).

**Figure 6. Schematic representation of the proposed cellular signaling pathways regulating DSM excitability and contractility.** The activation of mAChR3 increases the IP3R activity, depletes the SR Ca2+, reduces RyR activity in the SR, and consequently attenuates TBKCs. The activation of mAChR2 inhibits adenylyl cyclases and reduces cellular cAMP level. The increase in the cytosolic Ca2+ activates PDE1 and further reduces the cellular cAMP and Ca2+ in the SR, which exerts a positive feedback on mAChR-mediated signaling pathways. AC, adenylyl cyclase; BK, the large conductance voltage- and Ca2+-activated K+ channel; CaV, L-type voltage-gated Ca2+ channel; IP3R, inositol trisphosphate receptor; mAChR2 and mAChR3, type 2 and type 3 muscarinic acetylcholine receptor, respectively; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.
Figure 1

(A) Graph showing data with H-89 (10 μM) and 8MM-IBMX (10 μM) treatments.

(B) Bar graph comparing control with 8MM-IBMX treatment, showing significant differences in frequency but not amplitude.
Figure 3

A

B

Membrane Potential (mV)

Control

H-89

H-89 + 8MM

*
Figure 4

A

8MM-IBMX (10 μM)

5 mN
5 min

B

Control (%)

Amplitude Force Frequency Tone

*
Figure 5

A

8-MM-IBMX (10 μM)

2 mN

20 min

B

Control (%)

0 25 50 75 100

Amplitude Force Tone

* *
Figure 6