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Functional link between muscarinic receptors and large-conductance Ca^{2+} -activated K^{+} channels in freshly-isolated human detrusor smooth muscle cells

Shankar P. Parajuli¹, Kiril L. Hristov¹, Qiuping Cheng¹, John Malysz¹, Eric S. Rovner², and Georgi V. Petkov^{1,2,*}

¹Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC 29208

²Medical University of South Carolina, Charleston, SC 29425

Abstract

Activation of muscarinic acetylcholine receptors (mAChRs) constitutes the primary mechanism for enhancing excitability and contractility of human detrusor smooth muscle (DSM). Since the large conductance Ca^{2+} -activated K^{+} ($\text{K}_{\text{Ca}1.1}$) channels are key regulators of human DSM function, we investigated whether mAChR activation increases human DSM excitability by inhibiting $\text{K}_{\text{Ca}1.1}$ channels. We used the mAChR agonist, carbachol, to determine the changes in $\text{K}_{\text{Ca}1.1}$ channel activity upon mAChR activation in freshly-isolated human DSM cells obtained from open bladder surgeries using the perforated whole cell and single $\text{K}_{\text{Ca}1.1}$ channel patch-clamp recordings. Human DSM cells were collected from 29 patients (23 males and 6 females, average age of 65.9 ± 1.5 years). Carbachol inhibited the amplitude and frequency of $\text{K}_{\text{Ca}1.1}$ channel-mediated spontaneous transient outward currents and spontaneous transient hyperpolarizations, which are triggered by the release of Ca^{2+} from ryanodine receptors. Carbachol also caused membrane potential depolarization, which was not observed in the presence of ibuprofen, a $\text{K}_{\text{Ca}1.1}$ channel inhibitor, indicating the critical role of the $\text{K}_{\text{Ca}1.1}$ channels. The potential direct carbachol effects on $\text{K}_{\text{Ca}1.1}$ channels were examined under conditions of removing the major cellular Ca^{2+} sources for $\text{K}_{\text{Ca}1.1}$ channel activation with pharmacological inhibitors (thapsigargin, ryanodine, and nifedipine). In the presence of these inhibitors, carbachol did not affect the single $\text{K}_{\text{Ca}1.1}$ channel open probability and mean $\text{K}_{\text{Ca}1.1}$ channel conductance (cell-attached configuration) or depolarization-induced whole cell steady-state $\text{K}_{\text{Ca}1.1}$ currents. The data support the concept that mAChR activation triggers indirect functional $\text{K}_{\text{Ca}1.1}$ channel inhibition mediated by intracellular Ca^{2+} , thus increasing the excitability in human DSM cells.

Keywords

carbachol; ibuprofen; patch-clamp; ryanodine; spontaneous transient outward currents

*Correspondence to: Georgi V. Petkov, PhD, Professor of Pharmacology, Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Coker Life Sciences Building, Room 609D, 715 Sumter St, Columbia, SC 29208, Phone: 803-777-1891, Fax: 803-777-8356, petkov@sccp.sc.edu.

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Introduction

The physiological role of the urinary bladder is to temporarily store urine and periodically facilitate its release. These functions are subserved by coordinated cyclical contraction and relaxation of detrusor smooth muscle (DSM) and the bladder outlet region [2]. Disruption of DSM function may, in part, contribute to overactive bladder (OAB) syndrome and in some individuals, result in bothersome and medically significant lower urinary tract symptoms, often associated with urinary incontinence [2,1]. The prevalence of OAB increases with age and significantly impairs quality of life [9]. Muscarinic acetylcholine receptor (mAChR) activation is the primary mechanism subserving excitability and contractility of human DSM [7,29]. A better understanding of the mechanism by which mAChRs regulate DSM function at the cellular level is of utmost importance because antimuscarinic drugs are the current mainstay of pharmacotherapy for OAB, and such agents are associated with suboptimal efficacy and substantial collateral side effects elsewhere in the body which limit their clinical utility.

Among the 5 subtypes of mAChRs (m_1 - m_5), which are classified based on molecular and pharmacological properties, the m_2 AChR and m_3 AChRs are felt to be the subtypes primarily responsible for urinary bladder excitability in both experimental animals and humans [35,6]. Even though the m_3 AChRs are less prevalent than the m_2 AChRs, their activation is primarily responsible for the bladder contraction resulting in normal micturition as well as the involuntary bladder contractions which result in the phenotype of urinary urgency and urinary incontinence in many individuals [7,29]. The *in vitro* and *in vivo* activation of m_3 AChRs by acetylcholine is thought to increase inositol triphosphate production, which releases Ca^{2+} from the sarcoplasmic reticulum (SR), and the Ca^{2+} influx that results in the contraction of human DSM [3]. It has also been reported that the activation of mAChRs with carbachol depolarizes the membrane potential in freshly-isolated human DSM cells indicating that mAChRs control the membrane potential of DSM cells [31]. A study on the relative contribution of DSM cell Ca^{2+} influx to mAChR-mediated contraction showed significant species differences in the DSM of humans, pigs, and mice [32]. Differences between human and animal DSM excitability are well documented [25, 10, 32]. Furthermore, most of our knowledge about the electrical properties of DSM has been derived from studies on small experimental animals such as guinea pigs, rats, and rabbits [11]. Since human is the target species of interest for therapeutic intervention, studies on tissues obtained from human donors are of critical importance.

In DSM, fast localized SR Ca^{2+} releases from ryanodine receptors (RyRs), also known as Ca^{2+} sparks, activate the large-conductance voltage- and Ca^{2+} -activated K^+ ($K_{Ca1.1}$) channels causing spontaneous transient outward currents (STOCs) [12,13,15]. $K_{Ca1.1}$ channels are key regulators of excitability and contractility in human DSM [15]. $K_{Ca1.1}$ channels maintain the cell membrane potential and generate spontaneous transient hyperpolarizations, shape the spontaneous action potentials, regulate the intracellular Ca^{2+} concentration, and thus are key regulators of DSM cell excitability [25,16,15,33]. Recently, our group has demonstrated that pharmacological inhibition of $K_{Ca1.1}$ channels with iberiotoxin, a selective $K_{Ca1.1}$ channel inhibitor, decreases the whole cell outward currents in freshly-isolated human DSM cells [15]. In contrast, pharmacological activation of the

K_{Ca}1.1 channels with NS1619, a selective K_{Ca}1.1 channel activator, increases the whole cell outward currents and K_{Ca}1.1 channel open probability in freshly-isolated human DSM cells [16,19]. These findings underscore the important functional role of K_{Ca}1.1 channels as main regulators of human DSM excitability. However, the functional link between the clinically relevant observation of mAChRs activation resulting in DSM contraction and the role of the K_{Ca}1.1 channel are largely unknown in humans and only limited to observations in the DSM of other species [23]. Reports from studies on smooth muscle cells isolated from the airway [36], colon [4], and urinary bladder of non-human species [21,23], showed variable results with respect to the activation of mAChRs and the resulting effects on the activity of the K_{Ca}1.1 channels, either activation or inhibition. Since considerable differences exist between species, the results obtained in animal models cannot unconditionally be extrapolated to humans. Moreover, to our knowledge, the potential existence of a functional link between mAChRs and K_{Ca}1.1 channels at the cellular level in human DSM has never been explored.

The principal objective of the present work was to determine if activation of mAChRs decreases K_{Ca}1.1 channel activity in human DSM cells. Using the amphotericin-B perforated whole cell and cell-attached patch-clamp techniques, combined with pharmacological tools, we found that activation of mAChRs with carbachol leads to inhibition of STOCs and spontaneous transient hyperpolarizations, and depolarizes the membrane potential in native human DSM cells. Under pharmacological inhibition of the major cellular Ca²⁺ sources for K_{Ca}1.1 channel activation (with ryanodine, thapsigargin and nifedipine), carbachol did not affect the single K_{Ca}1.1 channel activity or steady-state K_{Ca}1.1 currents. These findings suggest that in freshly-isolated human DSM cells, the muscarinic effects are mediated by intracellular Ca²⁺ rather than a direct effect on the K_{Ca}1.1 channels. The present work in humans supports and logically builds upon our recent study showing similar findings in rat DSM [23]. Because human is the target species of interest for therapeutic intervention, the present study on native human DSM provides a translational link and pharmaco-physiological validation for mAChRs regulation of K_{Ca}1.1 channels complementing our earlier studies on a rodent animal model [23].

Materials and Methods

Human DSM specimen collection

The human DSM tissue studies were reviewed and approved by the Medical University of South Carolina Institutional Review Board (Protocol HR#16918). According to this protocol, human DSM tissues were collected upon written informed consent to participate in this study. We obtained human bladder specimens from 29 patients (23 males and 6 females, average age of 65.9±1.5 years, 21 Caucasians, 6 African-Americans and 2 other) undergoing cystectomy due to bladder cancer. DSM tissues were collected from a tumor-free part of the bladder. No patients had a preoperative history of OAB. Mucosa-free DSM strips of 5-7 mm long and 2-3 mm wide were dissected from the bladder specimens and were used for isolation of single-cells.

Enzymatic isolation of human DSM single-cells

Human DSM single-cells were freshly-isolated as described previously [15, 10, 16, 19]. Briefly, 1-2 DSM strips were incubated for 20-25 min at 37°C in pre-warmed 2 ml dissection solution (DS) supplemented with 1 mg/ml bovine serum albumin (BSA) (Sigma), 1 mg/ml papain (Worthington, Lakewood, NJ), and 1 mg/ml DL-dithiothreitol (Sigma). Next, the tissues were transferred to 2 ml of pre-warmed DS supplemented with 1 mg/ml BSA, 0.5 mg/ml collagenase (type II from Sigma), 0.5 mg/ml trypsin inhibitor (MP Biochemicals, LLC, CA) and 100 μ M CaCl₂, and incubated for 12-15 min at 37°C. The digested DSM tissues were washed 3-5 times with DS supplemented with BSA. DSM tissues were gently triturated with a fire-polished Pasteur pipette to disperse DSM single-cells.

Electrophysiology experiments

Freshly-isolated human DSM cells were used for the patch-clamp experiments. DSM cell suspension (0.2-0.5 ml) was dropped into a recording chamber, and cells were allowed to adhere to the glass bottom for ~30 min. Then, DSM cells were washed several times with bath solution to remove cell debris and poorly adhered DSM cells. Distinct, elongated, and bright DSM cells (when viewed under phase-contrast Axiovert 40CFL microscope) with contractile phenotypes were selected for the patch-clamp recordings. A system equipped with Axopatch 200B amplifier, Digidata 1322A, and pCLAMP version 10.2 software (Molecular Devices, Union City, CA) was used for the patch-clamp recordings. The recorded currents were filtered with an eight-pole Bessel filter 900CT/9L8L (Frequency Devices, Inc). The patch-clamp pipettes were prepared from borosilicate glass (Sutter Instruments, Novato, CA), pulled using a Narishige PP-830 vertical puller, and were fire-polished with a Microforge MF-830 (Narishige Group, Tokyo, Japan) to give a final pipette tip resistance of 6-15 M Ω . All patch-clamp experiments were conducted at room temperature (22-23°C).

Perforated whole cell patch-clamp recordings

We applied the amphotericin-B perforated whole cell patch-clamp technique to record STOCs, membrane potential, spontaneous transient hyperpolarizations, and depolarization-induced steady-state K_{Ca}1.1 currents in DSM single-cells. STOCs were measured at the holding potential of -40 mV. Depolarization-induced whole cell steady-state K_{Ca}1.1 currents were recorded by holding the DSM cells at -70 mV and voltage-depolarization was applied from 0 mV to +80 mV in increments of 20 mV for 200 ms and then cells were repolarized back to -70 mV. Stable outward whole cell K_{Ca}1.1 currents were recorded prior to (control) and after the addition of 1 μ M carbachol every 1 min for 6-10 min to examine the effect of carbachol on whole cell steady-state K_{Ca}1.1 currents. DSM cell membrane potential with or without spontaneous transient hyperpolarizations was recorded in current-clamp mode (I=0) of the patch-clamp technique.

Single-channel recordings

Single K_{Ca}1.1 channel recordings were performed using the cell-attached patch-clamp technique. Single-channel recordings were conducted either in the absence or in the presence

of thapsigargin (100-300 nM), a blocker of SR Ca^{2+} ATPase; ryanodine (30 μM), a blocker of RyRs; and nifedipine (1 μM), a blocker of L-type voltage-gated Ca^{2+} channels to eliminate the major known cellular sources of Ca^{2+} for $\text{K}_{\text{Ca}1.1}$ channel activation. We applied the command voltage of -50 mV or -60 mV to determine the effect of carbachol on $\text{K}_{\text{Ca}1.1}$ channel open probability (NP_o). This corresponds approximately to cell membrane potentials of +50 mV or +60 mV, respectively, assuming the cell membrane potential of 0 mV under the recording conditions of high (K^+) for both bath and pipette solutions. In experiments in which the mean $\text{K}_{\text{Ca}1.1}$ channel conductance was determined in the absence or presence of carbachol, the command potential was varied from -20 mV to -80 mV (V_m from +20 mV to +80 mV and the unitary current amplitudes determined for each voltage). Paxilline (500 nM), a selective $\text{K}_{\text{Ca}1.1}$ channel inhibitor, was added as specified.

Solutions and drugs

Freshly-prepared DS had (in mM): 80 monosodium glutamate, 55 NaCl, 6 KCl, 10 glucose, 10 HEPES, 2 MgCl_2 , and the pH was adjusted to 7.3 with NaOH. Physiological solution used for the perforated whole cell patch-clamp experiments contained (in mM): 134 NaCl, 6 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 glucose, 10 HEPES, and pH was adjusted to 7.4 with NaOH. The patch-pipette solution for the perforated patch-clamp had (in mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl_2 , 10 HEPES, 0.05 EGTA, and pH was adjusted to 7.2 with NaOH. Amphotericin-B stock solution was prepared in dimethyl sulfoxide (DMSO) and was added to the pipette solution (200 $\mu\text{g}/\text{ml}$) before the experiment and replaced every 1–2 h. Extracellular (bath) solution used for the single-channel recordings contained (in mM): 140 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, and 4 NaOH (pH=7.35). The patch-pipette solution for single $\text{K}_{\text{Ca}1.1}$ channel recordings had (in mM): 140 KCl, 1.08 MgCl_2 , 5 EGTA, 1 HEPES, and 3.16 CaCl_2 , adjusted to pH 7.2 with NaOH (Ca^{2+} free concentration was calculated ~ 300 nM with WEBMAXC Standard, <http://www.stanford.edu/~cpatton/webmaxcS.htm>, Chris Patton). Carbachol and iberitoxin were dissolved in double distilled water. Stock solutions of ryanodine (Enzo Life Sciences, Inc., NY), nifedipine (Thermo Fisher Scientific, NJ), thapsigargin (MP Biochemicals, LLC, CA), and paxilline (Sigma) were prepared in DMSO or ethanol. The highest concentration of DMSO in the bath solution was 0.21%.

Data analysis and statistics

Clampfit 10.2 (Molecular Device, Union City, CA) and Minianalysis software (Synaptosoft, Inc., NJ) were used to analyze the data. Mean values of the last 50 ms pulse of 200 ms depolarization step of average files (6-10 files) in the absence (control) and in the presence of carbachol were analyzed to evaluate the effect of carbachol on whole cell steady-state $\text{K}_{\text{Ca}1.1}$ currents. Five min of at least 8-10 min stable voltage-clamp or current-clamp recordings prior to application of carbachol were analyzed for control data, and the last 5 min of continuous recordings of 10-15 min after application of carbachol were analyzed to evaluate the effect of carbachol in the absence or presence of iberitoxin on STOCs, spontaneous transient hyperpolarizations, or membrane potential, respectively. The values for single-channel NP_o were obtained using the built-in algorithm in Clampfit, which calculates as $\text{NP}_\text{o} = (T_\text{o}) / (T_\text{o} + T_\text{c})$, where T_o and T_c correspond, respectively, to total open time and closed time during the recording interval. Single-channel events were analyzed

over 3-5-min intervals prior to and after the addition of 1 μM carbachol. The single-channel amplitudes were calculated from all-point histograms using the Gaussian distribution function to qualify the values for closed and open states. GraphPad Prism 4.03 software (GraphPad Software, Inc., La Jolla, CA) and Corel Draw Graphic Suite X3 software (Corel Co., Mountain View, CA) were used for the statistical analyses and data presentation, respectively. Due to high degree of variability in the frequency and amplitude of STOCs in DSM cells, data were normalized with respect to control and are expressed in percentages (%). The data are expressed as mean $\pm$ SEM for the **n** (the number of cells) isolated from **N** (the number of patients). Statistical analyses were performed using the two-tailed paired Student's *t* test. A *P* value <0.05 was considered to be statistically significant.

Results

mAChR agonist carbachol inhibits STOCs in native freshly-isolated human DSM cells

We examined the whole cell $\text{K}_{\text{Ca}}1.1$ currents in freshly-isolated human DSM cells using the perforated patch-clamp technique, which preserves the native physiological environment of the cell, including intracellular Ca^{2+} . Capacitance of human DSM cells used in the present study was 20.0 ± 1.7 pF ($n=46$, $N=24$). Under physiological conditions, freshly-isolated human DSM cells exhibit spontaneous subplasmalemmal releases of Ca^{2+} via SR RyRs, known as Ca^{2+} sparks, which activate $\text{K}_{\text{Ca}}1.1$ channels resulting in STOCs. Because of their iberitoxin sensitivity, STOCs are an indication of the $\text{K}_{\text{Ca}}1.1$ channel activity in human DSM cells [15,33]. DSM cells exhibited STOCs at a holding potential of -40 mV, which is close to DSM cell resting membrane potential recorded with microelectrodes [10,26]. To investigate whether a functional interaction exists between the mAChRs and $\text{K}_{\text{Ca}}1.1$ channels at the cellular level in human DSM cells, we tested the effects of the mAChR agonist carbachol on STOCs. As illustrated in Figure 1, 1 μM carbachol significantly inhibited the amplitude and frequency of STOCs by $56.8 \pm 14.4\%$ and $75.7 \pm 10.3\%$, respectively ($n=9$, $N=8$; $P<0.05$). These data suggest that activation of mAChRs leads to an inhibition of the STOCs in human DSM cells.

mAChR agonist carbachol inhibits spontaneous transient hyperpolarizations and depolarizes the resting membrane potential in freshly-isolated human DSM cells

Next, we sought to explore whether spontaneous transient hyperpolarizations and the cell membrane potential are regulated by the activation of mAChRs in human DSM cells. As illustrated in Figures 2A and 2B, 1 μM carbachol significantly inhibited the amplitude and frequency of spontaneous transient hyperpolarizations by $65.8 \pm 16.8\%$ and $75.9 \pm 14.5\%$, respectively ($n=7$, $N=7$; $P<0.05$). Also, 1 μM carbachol depolarized human DSM cell membrane potential from -24.3 ± 2.4 mV to -19.8 ± 1.9 mV ($n=17$, $N=9$; $P<0.05$; Fig. 2C). This indicates that the mAChRs and $\text{K}_{\text{Ca}}1.1$ channels interact at the cellular level to regulate the cell membrane potential in human DSM.

To further explore the functional link between the mAChRs and $\text{K}_{\text{Ca}}1.1$ channels, we evaluated the effect of carbachol on DSM cell membrane potential in the presence of iberitoxin (200 nM), a selective inhibitor of the $\text{K}_{\text{Ca}}1.1$ channels. As illustrated in Figures 2D and 2E, under conditions of $\text{K}_{\text{Ca}}1.1$ channel pharmacological blockade with iberitoxin,

carbachol (1 μ M)-induced membrane potential depolarization was eliminated ($n=11$, $N=4$; $P>0.05$). These results revealed that the blockade of the $K_{Ca1.1}$ channels prevented the depolarizing effect of carbachol in the human DSM cell. This observation supports that the effect of muscarinic receptor activation on DSM excitability is mediated by the $K_{Ca1.1}$ channels.

mAChR agonist carbachol does not affect the depolarization-induced whole cell steady-state $K_{Ca1.1}$ currents in the presence of thapsigargin, ryanodine, and nifedipine

In the next series of experiments, we sought to explore how mAChRs activation with carbachol affects the whole cell steady-state $K_{Ca1.1}$ currents in human DSM cells. To test whether carbachol exerts direct effects on $K_{Ca1.1}$ channels by channel phosphorylation or another mechanism, or indirectly via intracellular Ca^{2+} mobilization and influx of Ca^{2+} , we recorded the steady-state $K_{Ca1.1}$ currents under conditions of pharmacological inhibition (with ryanodine, thapsigargin, and nifedipine) of the major known cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation. In the presence of thapsigargin (100 nM), ryanodine (30 μ M), and nifedipine (1 μ M), activation of mAChRs with carbachol (1 μ M) did not change the depolarization-induced steady-state $K_{Ca1.1}$ currents at all voltages from 0 mV to +80 mV. At the highest voltage +80 mV, the $K_{Ca1.1}$ currents were 41.3 ± 7.6 pA/pF and 43.4 ± 9.5 pA/pF in the absence and in the presence of 1 μ M carbachol, respectively ($n=9$, $N=6$; $P>0.05$; Fig. 3). The lack of carbachol-mediated effects on whole cell steady-state $K_{Ca1.1}$ currents supports the idea that carbachol does not interact directly with $K_{Ca1.1}$ channels and suggests involvement of intracellular Ca^{2+} mobilization, or Ca^{2+} influx induced by mAChR activation, which results in the observed indirect attenuation of $K_{Ca1.1}$ channel activity in freshly-isolated human DSM cells.

mAChR agonist carbachol does not change the single $K_{Ca1.1}$ channel activity in the presence of thapsigargin, ryanodine, and nifedipine

To examine whether carbachol exerts any effects on single $K_{Ca1.1}$ channel activity, we conducted single-channel experiments using the cell-attached configuration of the patch-clamp technique. The single-channel recordings were performed under conditions of pharmacological inhibition of all major cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation, the same as in the case of the voltage-step protocol - i.e. in the presence of thapsigargin (300 nM), ryanodine (30 μ M), and nifedipine (1 μ M). The single $K_{Ca1.1}$ channel activities in the absence (Fig. 4A), or in the presence of 1 μ M carbachol (Fig. 4B), and in the presence of both 1 μ M carbachol and 500 nM paxilline (Fig. 4C), were evaluated in human DSM cells pretreated with thapsigargin (300 nM), ryanodine (30 μ M), and nifedipine (1 μ M) at the V_m of +50 or +60 mV. Under these experimental conditions, $K_{Ca1.1}$ channels displayed a relatively low level of average open probability (NP_o) 0.0335 ± 0.015 and unitary single channel amplitude of 6.6 ± 1.0 pA ($n=7$, $N=7$). The addition of 1 μ M carbachol did not have any significant effects on the NP_o or unitary single channel current amplitude. The normalized responses (fold-increases over controls) in the presence of carbachol to those of controls for the NP_o and unitary current amplitude were 1.34 ± 0.40 ($n=7$, $N=7$, $P>0.05$; Fig. 4D) and 0.98 ± 0.03 pA ($n=7$, $N=7$, $P>0.05$; Fig. 4D), respectively. We also determined the mean single-channel conductance by varying the V_m before and after the addition of 1 μ M carbachol, yielding the mean single-channel conductance values

of 133.3 ± 18.4 pS and 136.7 ± 22.9 pS in the absence or presence of carbachol, respectively. This result indicates that carbachol (1 μ M) also did not have a significant effect on the mean single-channel conductance ($n=5$, $N=5$; $P>0.05$, Fig. 4F). In all experiments in which paxilline (500 nM) was applied in the presence of carbachol, no single $K_{Ca1.1}$ channel openings were recorded ($n=3$, $N=3$; Figs. 4C and 4D), confirming that the channel openings were due indeed to $K_{Ca1.1}$ channels. Here, paxilline was used instead of iberiotoxin because this $K_{Ca1.1}$ channel blocker easily crosses the cell plasma membrane, and it can reach and block $K_{Ca1.1}$ channels recorded in the cell-attached configuration.

mAChR agonist carbachol does not alter the single $K_{Ca1.1}$ channel activity in DSM cell-attached patches with all major Ca^{2+} sources intact for $K_{Ca1.1}$ channel activation

In the last series of experiments, the effects of carbachol (1 μ M) were examined in seven cell-attached human DSM patches ($N=6$) at the assumed V_m of +50 or +60 mV measured in the absence of ryanodine, thapsigargin, and nifedipine. The $K_{Ca1.1}$ channels displayed a low basal level of activity, NPo of 0.1159 ± 0.1078 ($n=7$, $N=6$) and unitary single channel amplitude of 6.6 ± 1.1 pA ($n=7$, $N=6$). Both of these parameters were not significantly different from those obtained in the presence of the blockers of Ca^{2+} sources ($P>0.05$). The addition of carbachol (1 μ M) did not significantly change the NPo or the unitary channel amplitude with the normalized ratio of 1.40 ± 0.48 ($n=7$, $N=6$; $P>0.05$) or 0.96 ± 0.02 pA ($n=7$, $N=6$; $P>0.05$; Fig. 5), respectively. Hence, under the experimental recording conditions, carbachol (1 μ M) did not change the $K_{Ca1.1}$ channel open probability or unitary single channel amplitude measured in the absence or presence of ryanodine, thapsigargin, and nifedipine. It is noteworthy that the recorded single $K_{Ca1.1}$ channels displayed relatively low NPo, which is indicative of very low intracellular Ca^{2+} levels (<200 nM) in the vicinity of the channels measured. In contrast, the localized Ca^{2+} levels within Ca^{2+} spark sites can reach 10 μ M [12] and single $K_{Ca1.1}$ channels exposed to such high levels are expected to exhibit a very high level of activity, supported by a recent finding in excised human DSM patches with elevated intracellular Ca^{2+} [19]. Under the recording conditions for the cell-attached configuration, we did not observe any simultaneous openings of at least three $K_{Ca1.1}$ channels, which, by definition, are indicative of STOC generation [24,13]. This was not surprising since we estimated the chance of recording $K_{Ca1.1}$ channels precisely at the Ca^{2+} spark site to be very low. As there are $\sim$ three active Ca^{2+} spark sites per DSM cell with each spark encompassing an area of $\sim 15 \mu m^2$ and a DSM cell surface area of at least $\sim 2000 \mu m^2$, the recording of Ca^{2+} spark-dependent single $K_{Ca1.1}$ activity with the cell-attached configuration can be achieved only on rare occasions ($\sim 2.25\%$) [24,18]. Thus, the most practical experimental method to assess the effects of carbachol on Ca^{2+} spark-potentiated $K_{Ca1.1}$ channels is with the perforated whole cell patch clamp – as we have done and described in Figures 1 and 2 – indicating a net attenuation of $K_{Ca1.1}$ channel activity dependent on intracellular Ca^{2+} handling but not via a direct effect.

Discussion

The present study for the first time revealed that activation of mAChRs suppresses STOCs and spontaneous transient hyperpolarizations, and depolarizes human DSM cell membrane potential. Furthermore, the lack of mAChR-induced effects on single $K_{Ca1.1}$ channel

properties and steady-state $K_{Ca1.1}$ currents, in the absence of the major known cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation, suggests a contribution of intracellular Ca^{2+} rather than a direct effect of carbachol on the $K_{Ca1.1}$ channels in DSM cells. Thus, the present study provides a novel mechanistic insight into mAChR-mediated regulation of $K_{Ca1.1}$ channels in native freshly-isolated human DSM cells.

Previously, our group and other investigators demonstrated that STOCs are controlled by the SR Ca^{2+} release from RyRs and these STOCs are suppressed directly upon inhibition of RyRs with ryanodine in DSM cells of experimental animals [34,27,5,12,13] and humans [15]. In the present study, the amplitude and frequency of STOCs were significantly inhibited upon the activation of mAChRs with carbachol (Fig. 1). These data support the concept that carbachol-induced inhibition of STOCs may be caused by either direct inhibition of the RyRs or by a decrease in the SR Ca^{2+} concentration load due to SR Ca^{2+} -pump blockade. An indirect inhibitory effect of acetylcholine on $K_{Ca1.1}$ channels by depletion of the 1,4,5-triphosphate-sensitive Ca^{2+} stores has been demonstrated in neuroblastoma cells [28]. In addition, phosphatidylinositol 4,5-bisphosphate (PIP2) may directly potentiate the $K_{Ca1.1}$ channels in DSM cells as shown previously in recombinant cells [30]. Another study on rat DSM cells, in agreement with present findings in human DSM cells, has reported that pharmacological activation of mAChRs with carbachol leads to inhibition of the $K_{Ca1.1}$ currents [23]. In contrast to earlier reports, here we examined the effects of carbachol on human DSM $K_{Ca1.1}$ channels measured with the cell-attached single channel technique under conditions of intact and inhibited major sources of Ca^{2+} for $K_{Ca1.1}$ channel activation, and examined how mAChR activation affected $K_{Ca1.1}$ -channel dependent STOCs, spontaneous transient hyperpolarizations and membrane potential in freshly-isolated (not-cultured) human DSM cells. Examination of the responses directly in human cells or tissues including DSM is required as the data from experimental animals does not necessarily directly translate to humans and shows interspecies differences [25].

Previous studies demonstrated a link between mAChR activation and enhancement of DSM cell excitability. Using intracellular microelectrode recordings, mAChR activation with a higher concentration of carbachol (10 μ M) caused substantial depolarization in DSM tissues of experimental animals and humans, and a robust increase in action potential frequency [10]. A transient hyperpolarization followed by a sustained depolarization of membrane potential coincident with the activation of mAChRs has been reported in freshly-isolated human DSM cells [31]. It has also been suggested that RyR activity can regulate the membrane potential in guinea pig DSM through modulation of $K_{Ca1.1}$ channel activity [14]. Our group has previously shown that blocking the $K_{Ca1.1}$ channels with iberiotoxin significantly depolarizes the membrane potential of human DSM cells and suppresses the spontaneous transient hyperpolarizations [15]. The present results are consistent with those findings because 1 μ M carbachol caused a small but significant depolarization of DSM cell membrane potential, and these depolarizing effects were eliminated by blocking the $K_{Ca1.1}$ channels with iberiotoxin (Figs. 2A and 2B). One interpretation of these results is that both iberiotoxin and the mAChR agonist carbachol inhibit $K_{Ca1.1}$ channel activity, and thus depolarize DSM cell membrane potential. Furthermore, this carbachol-induced membrane potential depolarization may subsequently activate the L-type voltage-gated Ca^{2+} channels and enhance the action potential firing and related phasic contractions in human DSM.

Recently, using the excised-patch single-channel recordings, our group has demonstrated that activation of $K_{Ca1.1}$ channels with NS1619 enhanced the $K_{Ca1.1}$ channel open probability without altering the mean $K_{Ca1.1}$ channel conductance in freshly-isolated human DSM cells [19]. In the present work, we explored whether activation of mAChRs with carbachol affects the single $K_{Ca1.1}$ channel properties in native freshly-isolated human DSM cells using the cell-attached single-channel recording technique. Under conditions of pharmacological inhibition of all major cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation, carbachol did not alter the $K_{Ca1.1}$ channel NPo and single-channel conductance values (Fig. 4). These results suggest a role of intracellular Ca^{2+} in the mAChR-mediated regulation of $K_{Ca1.1}$ channels in human DSM cells. Our data further demonstrated that mAChR activation with carbachol did not alter the depolarization-evoked steady-state $K_{Ca1.1}$ currents under conditions of pharmacological inhibition of the major cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation (Fig. 3), consistent with the results of the single $K_{Ca1.1}$ channel recordings (Fig. 4). It has been demonstrated that mAChR-mediated Ca^{2+} mobilization due to Ca^{2+} influx and Ca^{2+} release are the key events in mediating contractions of DSM in humans, pigs, and mice [32]. In the present study, carbachol had a significant inhibitory effect on STOCs and cell membrane potential in the presence of all cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation. However, carbachol did not have any direct effects on NPo, single-channel conductance, or steady-state $K_{Ca1.1}$ currents under conditions of pharmacological inhibition of the major cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation (Figs. 3 and 4). In addition, under the experimental conditions of intact intracellular Ca^{2+} sources favoring the recording of low probability of opening of $K_{Ca1.1}$ channels -indicative of low Ca^{2+} levels in the range of 100-200 nM in the vicinity of the channels-carbachol (1 μ M) did not change the NPo or unitary single channel amplitude. These findings are similar to the results obtained in the presence of the blockers of the Ca^{2+} sources. Furthermore, the Ca^{2+} dependency for activation of $K_{Ca1.1}$ channels determines that the effects of Ca^{2+} are stimulatory only at relatively high levels of Ca^{2+} reaching micromolar concentrations [8,22]. At lower Ca^{2+} concentrations (<300 nM), the half-maximum constants for $K_{Ca1.1}$ channel activation remain unchanged [8,22]. As mAChR activation does not increase the global Ca^{2+} concentrations at the steady-state levels – at which we determined the effects on single channel activities and voltage-evoked steady-state currents – above approximately 300 nM [20], we did not detect any increases in $K_{Ca1.1}$ channel activities that could be solely due to the increase in intracellular Ca^{2+} alone. Our data support the concept that mAChRs and $K_{Ca1.1}$ channel activities are functionally coupled to intracellular Ca^{2+} levels, which dependent on the L-type voltage-gated Ca^{2+} channels, SR Ca^{2+} -pumps, and RyRs in DSM cells. When localized Ca^{2+} levels reach high concentrations achieved during Ca^{2+} sparks they activate STOCs. Furthermore, the present findings in human DSM cells are consistent with a recent study, which reported that mobilization of intracellular Ca^{2+} is critical in mAChR-mediated $K_{Ca1.1}$ channel regulation in rat DSM cells [23]. It has also been suggested that protein kinase-C (PKC) regulatory pathways are involved in DSM function [2,7,17]. The m_3 AChR activation leads to diacylglycerol production, which activates PKC. PKC may cause direct inhibition of the SR Ca^{2+} -pumps and/or RyRs, collectively resulting in suppression of STOCs as shown in the present study.

In conclusion, our results for the first time reveal that pharmacological activation of mAChRs with carbachol inhibits STOCs, suppresses spontaneous transient hyperpolarizations and depolarizes the cell membrane potential in native freshly-isolated human DSM cells. We provide evidence that the carbachol inhibitory effect on the $K_{Ca1.1}$ channel in human DSM cells involves intracellular Ca^{2+} . Collectively, these findings strongly suggest that there is a functional indirect link between the mAChRs and $K_{Ca1.1}$ channels in human DSM cells.

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Figure 1. Pharmacological activation of mAChRs with carbachol leads to an inhibition of the amplitude and frequency of STOCs in freshly-isolated human DSM cells

A) A representative original recording from a DSM cell illustrating the inhibitory effect of 1 μM carbachol on the amplitude and frequency of STOCs. **B)** Data summary illustrating the inhibitory effects of carbachol (1 μM) on the amplitude and frequency of STOCs ($n=9$, $N=8$; $*P<0.05$). The data were normalized to control values (prior to carbachol addition) taken as 100% and were presented as percentages (%). The bath solution contained 134 mM NaCl, 6 mM KCl, and 2 mM CaCl_2 , and the pipette solution contained 110 mM potassium aspartate, 30 mM KCl, 10 mM NaCl, and <100 nM $[\text{Ca}^{2+}]$ (nominal Ca^{2+} and 0.05 EGTA). For full listing of composition for solutions, see Materials and Method section. STOCs were recorded at a holding potential of -40 mV.

Figure 2. Pharmacological activation of mAChRs with carbachol leads to an inhibition of the amplitude and frequency of the spontaneous transient hyperpolarizations and depolarizes human DSM cell membrane potential

A) A representative original recording from a DSM cell illustrating the inhibitory effects of carbachol (1 μ M) on the amplitude and frequency of spontaneous transient hyperpolarizations and the effects of membrane potential depolarization. **B** and **C)** Data summary illustrating the inhibitory effects of carbachol (1 μ M) on the amplitude and frequency of spontaneous transient hyperpolarizations (n=7, N=7; *P<0.05) and cell membrane potential (n=17, N=9; *P<0.05), respectively. **D)** A representative original

recording from a human DSM cell illustrating that carbachol-induced DSM cell membrane potential depolarization was blocked by 200 nM iberiotoxin. E) Data summary illustrating the lack of carbachol (1 μ M) effect on human DSM cell membrane potential in the presence of 200 nM iberiotoxin (n=11, N=4; P>0.05). The data were normalized to the control values (prior to carbachol addition) taken as 100% and were presented as percentages (%). The bath solution contained 134 mM NaCl, 6 mM KCl, and 2 mM CaCl₂, and the pipette 110 mM potassium aspartate, 30 mM KCl, 10 mM NaCl, and < 100 nM [Ca⁺] (nominal Ca²⁺ and 0.05 EGTA). For full listing of solution composition see Materials and Method section. The membrane potential recordings were made with the current-clamp mode (I=0). IbTX= iberiotoxin.

Figure 3. mAChR agonist carbachol does not affect the depolarization-induced whole cell steady-state $K_{Ca1.1}$ currents under conditions of pharmacological inhibition of the major cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation in freshly-isolated human DSM cells

A) Representative original recordings illustrate the depolarization-induced whole cell steady-state $K_{Ca1.1}$ currents in the absence (control) and in the presence of carbachol (1 μ M). **B)** The current-voltage relationship curve summarizes the lack of carbachol effects on the whole cell steady-state $K_{Ca1.1}$ currents ($n=9$, $N=6$; $P>0.05$). Steady-state $K_{Ca1.1}$ currents were recorded in the presence of ryanodine (30 μ M), thapsigargin (100 nM), and nifedipine (1 μ M). The bath solution contained 134 mM NaCl, 6 mM KCl, and 2 mM $CaCl_2$, and the pipette 110 mM potassium aspartate, 30 mM KCl, 10 mM NaCl, and <100 nM $[Ca^{+}]$ (nominal Ca^{2+} and 0.05 EGTA). For full listing of solution composition see Materials and Method section.

Figure 4. Carbachol does not alter single $K_{Ca}1.1$ channel activity recorded with cell-attached configuration in freshly-isolated human DSM cells under conditions of pharmacological inhibition of the major cellular sources of Ca^{2+} for $K_{Ca}1.1$ channel activation

A-C) Representative recordings of single $K_{Ca}1.1$ channel currents measured at V_m of +60 mV prior to and after the addition of 1 μ M carbachol or 500 nM paxilline, a selective inhibitor of the $K_{Ca}1.1$ channels. In this example, NP_o values were 0.00051 and 0.00060 in the absence (control: **A**) and in the presence of 1 μ M carbachol (**B**), respectively. Subsequent addition of 500 nM paxilline (**C**) completely inhibited the single $K_{Ca}1.1$ channel activity. **D)** Data summary for the effects of carbachol (1 μ M) on mean $K_{Ca}1.1$ channel open

channel probability (NP_O) and unitary single channel amplitude ($n=7$, $N=7$; $P>0.05$) and carbachol + paxilline ($n=3$, $N=3$; $P<0.05$), respectively. **E**) A representative graph in a single cell-attached recording illustrates the lack of an effect of $1\text{ }\mu\text{M}$ carbachol on the mean single $K_{Ca1.1}$ channel amplitude and conductance. **F**) Data summary illustrating the lack of an effect of $1\text{ }\mu\text{M}$ carbachol on the mean single-channel conductance ($n=5$, $N=5$; $P>0.05$). All single-channel recording experiments were carried out in the presence of thapsigargin (300 nM), ryanodine ($30\text{ }\mu\text{M}$), and nifedipine ($1\text{ }\mu\text{M}$). The bath and pipette solutions contained $140\text{ mM [K}^+]$, and 2 mM and $\sim 300\text{ nM free [Ca}^{2+}]$, respectively.

Figure 5. Carbachol elicits no change in single $K_{Ca1.1}$ channel activity recorded with cell-attached configuration in freshly-isolated human DSM cells under conditions of low level of channel activity and intact major cellular sources of Ca^{2+} for $K_{Ca1.1}$ channel activation **A-B)** Representative recordings of a low level of activity of single $K_{Ca1.1}$ channels, measured at V_m of +50 mV prior to and after the addition of 1 μ M carbachol. In this example, NP_o values were 0.00974 and 0.01154 in the absence (control: **A**) and in the presence of 1 μ M carbachol (**B**), respectively. **C)** Data summary depicting the lack of significant effects by carbachol (1 μ M) on mean $K_{Ca1.1}$ channel open channel probability (NP_o) and unitary single-channel amplitude ($n=7$, $N=6$; $P>0.05$). The bath and pipette solutions contained 140 mM K^+ , and 2 mM and ~ 300 nM free $[Ca^{2+}]$, respectively.