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Silencing MaxiK Activity in Corporal Smooth Muscle Cells Initiates Compensatory Mechanisms to Maintain Calcium Homeostasis

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Abstract

Introduction—The MaxiK potassium channel is regulated by voltage and intracellular calcium, and plays a critical role in regulating intracellular calcium concentration ($[Ca^{2+}]_i$), which is the ultimate determinant of smooth muscle tone. Tight control of corpus cavernosum smooth muscle (CCSM) tone is critically important and misregulation can result in erectile dysfunction.

Aim—Because of the tight functional linkage of MaxiK and calcium channel activity, the aim of this study was to determine the effects of silencing and pharmacological inhibition of MaxiK on calcium homeostasis and intercellular calcium signaling in CCSM cells.

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Methods—We compared changes in the basal intracellular $[Ca^{2+}]_i$ and parameters defining intercellular calcium wave (ICW) spread in 48 hours MaxiK silenced CCSM cells vs. acute blockade of the channel with iberiotoxin. To analyze changes occurring in gene expression we performed micro-array analysis following MaxiK silencing for 48 hours.

Main Outcome Measures—Changes in Fura-2 fluorescence intensities were measured to evaluate basal $[Ca^{2+}]_i$ levels and ICW parameters. Microarray analysis of global gene expression was performed.

Results—Forty-eight hours after MaxiK silencing the basal $[Ca^{2+}]_i$, the ICW amplitude and spread among CCSM cells were not markedly different in silenced compared to mock transfected controls, whereas short-term blockade significantly increased basal $[Ca^{2+}]_i$ level and amplified Ca^{2+} signaling among CCSM cells. Micro-array analysis showed that several genes within Ca^{2+} homeostasis and smooth muscle tone regulation pathways had significantly altered expression.

Conclusions—Our results indicate that while short-term blockade of the MaxiK channel is associated with an increase in basal $[Ca^{2+}]_i$, Ca^{2+} homeostasis is restored during the 48 hours period following silencing. We hypothesize that the different pathways regulating $[Ca^{2+}]_i$ and CCSM tone are linked through molecular crosstalk and that their coordinated regulation is part of a compensatory mechanism aimed to maintain Ca^{2+} homeostasis and CCSM tone.

Keywords

MaxiK; Smooth Muscle; Calcium Homeostasis; siRNA; Micro-Array; Erectile Tissue Physiology

Introduction

The MaxiK potassium channel is a key modulator of smooth muscle tone. The MaxiK channel is composed of a tetramer of pore forming α -subunits, encoded by the *Slo* gene, which forms the pore through which potassium passes. Each α -subunit is associated with a regulatory β -subunit [1]. Due to its calcium and voltage sensitivity, this channel is activated following depolarization and Ca^{2+} mobilization, relaxing the muscle. Such a process is especially important in control of corpus cavernosum smooth muscle (CCSM) function, where relaxation allows increased blood flow into the penis and consequent erection, whereas CCSM contraction is required for maintenance of the flaccid state. The functional importance of MaxiK channels in the events regulating CCSM tone is evidenced by observations that transgenic mice lacking the gene encoding MaxiK (*Slo*^{-/-} mice) suffer from erectile dysfunction (ED)[2]. Moreover, introduction of the *Slo* gene into the penile corpora can normalize erectile function in animal models of ED [3,4]. These observations led to the development of a gene transfer strategy for ED patients. Phase I clinical studies employing this treatment have provided preliminary evidence of its efficacy [5–7].

Under resting conditions, corporal smooth muscle tone is high, due to activity of multiple pathways that converge on the contractile machinery, including cyclic guanosine monophosphate/ protein kinase G (cGMP/PKG) [8], cyclic adenosine monophosphate/ protein kinase A (cAMP/PKA) and myosin light chain kinase [9,10], and inositol phosphate (IP₃)/ryanodine receptors [11]. A depolarizing stimulus activates MaxiK channels, causing efflux of K⁺ ions, thereby hyper-polarizing the membrane, and closing L-type voltage-

dependent calcium channels. The lowered intracellular Ca^{2+} levels reduce the CCSM contractile state through decreased activation of the calcium–calmodulin signaling complex, which in turn reduces activity of myosin light chain kinase. Given the central importance of Ca^{2+} in regulating smooth muscle tissue function, intracellular Ca^{2+} levels in smooth muscle are controlled by numerous molecular pathways [12]. Furthermore, the interaction of MaxiK with Ca^{2+} channels has been well established in different tissues. The association of MaxiK activity with L-type voltage-gated Ca^{2+} channels has been demonstrated in brain [13,14] and bladder [15,16] and with T-type Ca^{2+} channels in vascular smooth muscle [17].

The aim of this study was to investigate the effects of MaxiK silencing and inhibition in CCSM cells on Ca^{2+} homeostasis and on communication among CCSM through transmission of intercellular Ca^{2+} waves (ICW), which have been postulated to play key roles in the coordination and synchronization of CCSM function [18]. We compared changes in basal intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and ICW parameters in 48 hours MaxiK silenced CCSM cells vs. acute blockade of MaxiK channels with iberiotoxin (IBTX). Our results indicate that silencing of the MaxiK channel activates compensatory mechanisms to maintain calcium homeostasis. We hypothesize that the mechanism of compensation is achieved through molecular crosstalk between pathways regulating smooth muscle tone. To gain insight into the nature of this cross talk, we analyzed global changes in gene transcription that occur following 48 hours of MaxiK silencing, which revealed potential compensatory mechanisms.

Methods and Main Outcome Measures

Culture of Rat Corpus Cavernosum Smooth Muscle (CCSM) Cells

Rats were maintained in our AAALAC-accredited animal facilities and all experimental procedures were approved by the Einstein Animal Committee. Rat CCSM cells were isolated as previously described by Jackson et al. [19]. Briefly, corporal tissue was dissected into 1 to 2-mm pieces and cells dissociated by incubating with 30 U/mL papain and 1 mg/mL dithioerythritol for 35 minutes followed by incubation in 1 mg/mL collagenase, 2.5 U/mL elastase, and 1 mg/mL soybean trypsin inhibitor for 25 minutes in a solution containing 137 mM NaCl, 5.6 mM KCl, 1 mM MgCl_2 , 0.42 mM Na_2HPO_4 , 0.44 mM NaH_2PO_4 , 4.2 mM NaHCO_3 , 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mg/mL albumin at 37°C. Enzy-matically dissociated CCSM cells were then plated in cell culture flasks and grown in 1 g/L glucose DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) for no more than three passages.

Human CCSM Explant Cultures

All procedures involving human subjects were approved by the Einstein Ethics Committee. Penile tissue was obtained from the corpus cavernosum of patients undergoing surgery for implantation of penile prostheses. Homogeneous explant cell cultures of human CCSM cells were prepared as previously described [18,20,21]. Briefly, radial sections approximately $3 \times 3 \times 10$ mm were excised from the mid-penile shaft of each patient. Tissue was washed, cut

into 1 to 2-mm pieces, and placed in tissue culture dishes with a minimal volume of DMEM (Gibco, Invitrogen, Carlsbad, CA) with 20% fetal calf serum (Gibco, Invitrogen). Smooth muscle cells that migrated from the explant and underwent division were then detached using a trypsin/ethylenediaminetetraacetic acid (EDTA) protocol [20,22] to establish secondary cultures from the explants.

To ensure the smooth muscle phenotype, both rat and human cultures were immunostained with monoclonal anti-alpha smooth muscle actin fluorescein Isothiocyanate (FITC) conjugated antibody (1:300, in phosphate buffered saline [PBS] 1% bovine serum albumen [BSA]; Sigma-Aldrich, St. Louis, MO, USA); routinely, more than 90% of the cells stained positive.

Silencing of MaxiK in Rat and Human Corporal Cells

Silencing of MaxiK α -subunit was obtained in rat and human corporal cells using Stealth Select RNAi (Invitrogen). Cultured cells were transfected with 30 nM small inhibitory ribonucleic acid (siRNA) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) as a carrier following the manufacturer's instructions. The following RNAi were used for silencing rat and human MaxiK, respectively: rSil1UUACAAG GGCACCAAUGCUGAGAGC; rSil2CCGUGU UUGUGUCUGUAUACUAAAA; hSil1CCGAA GAUAAGAAUCAUCACUAAAA; hSil2CCGA CGGACCUGAUCUUCUGCUUAA. Transfection efficiency was monitored by simultaneous transfection with BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen). To establish the optimal time of silencing, cells were harvested at 8, 24, 32, and 48 hours after transfection and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Once the optimal transfection procedure was established, we omitted the Fluor Red transfection since it does not represent a direct correlation with silencing. Effective silencing was verified by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) at the time of the experiments.

Measurement of Gene Expression by Quantitative RT-PCR

RNA from transfected CCSM cells was used to analyze gene expression by qRT-PCR as previously described [23–25]. Total RNA was reverse-transcribed to first-strand cDNA using Superscript (Invitrogen) following the instructions of the manufacturer. RT products then were amplified using Sybr Green 2X PCR Master Mix (Applied Biosystems, Foster City, CA, USA). qPCR analysis was performed using the 7,300 real-time PCR system (Applied Biosystems). The following primers were used to quantify expression: *rSlo* forward 5'-TACTTCAATGACAATATCCTCAC CCT-3', reverse 5'-ACCATAACAACCACCATC CCCTAAG-3'; *hSlo* forward 5'-TACTTCAATG ACAATATCCTCACCT-3', reverse 5'-ACCA TAACAACCACCATCCCCTAAG-3; *rGAPDH* forward 5'-CCGAGGGCCCACTAAAGG-3', reverse 5'-GCATCAAAGGTGGAAGAATGG-3'; *hGAPDH* forward 5'-CCACCCATGGCAA ATTCCC-3', reverse 5'-TGGGATTTCATT GATGACAAG-3'.

Results from qRT-PCR were presented as threshold cycles normalized to that of the *GAPDH* gene and expression of transcripts were analyzed using comparative crossing threshold (C_t)

method (also known as the 2^{-Ct} method) [26]. This method was applicable because the efficiency of the primers in generating products was found to be close to that of the housekeeping gene, *GAPDH*, which was used to normalize samples.

Western Blot Analysis

Membrane extracts were prepared from 48 hours silenced and control rat CCSM cell cultures. Cells were harvested and washed in phosphate buffered saline (Gibco, Invitrogen) and pelleted by centrifugation at 1,500 g. The cell pellet was lysed in hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing a protease inhibitor cocktail (Sigma-Aldrich) and incubated on ice for 30 minutes. After incubation, lysates were passed through an insulin syringe several times and centrifuged for 5 minutes at 3,000 g at 4°C to pellet nuclei. Membranes were isolated by centrifugation of the supernatant at 14,000 g for 30 minutes, 4°C, and subsequently solubilized in Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) 1%, sodium deoxycholate 1%, sodium dodecyl sulfate 0.1%, Tris 20 mM, NaCl 0.16 mM, EDTA 1 mM, NaFl 15 mM, ethylene glycol tetraacetic acid (EGTA) 1 mM. Protein concentration was determined using BCA Protein Assay (Thermo Scientific, Rockford, IL, USA) and 15 µg of proteins were separated by electrophoresis on NuPAGE gradient 4–12% Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk-0.05% Tween-20 in PBS and incubated with MaxiK (1:25, BD-Transduction Laboratories, San Jose, CA, USA) and α -actin (1:10,000 Sigma-Aldrich) monoclonal antibodies for 48 hours at 4°C. The bound antibodies were detected with horseradish peroxidase-labeled anti-mouse antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature. Enhanced chemiluminescence was performed with SuperSignal West Femto Maximum Sensitivity Substrate (MaxiK) or Pierce ECL Western Blotting Substrate (α -actin; Thermo Scientific).

Electrophysiology

Rat CCSM cells were plated on coverslips 48 hours prior to recordings. The whole cell patch clamp configuration (see [27]) was performed at room temperature on cells bathed in external solution containing (mM): NaCl 130, HEPES 10, glucose 11, $CaCl_2$ 2.4, KCl 5.9, and $MgCl_2$ 1.2 (pH 7.4) for potassium current recordings; and $BaCl_2$ 10, tetraethylammonium chloride (TEACl) 130, $MgCl_2$ 2, HEPES 10, and glucose 10 (pH 7.4) for recordings of voltage-dependent Ca^{2+} current. Polished patch pipettes (resistance 4–6 MOhms) were filled with solution containing (mM) potassium aspartate 110, KCl 30, NaCl 10, $MgCl_2$ 1, EGTA 0.05, HEPES 10 (pH 7.4), and connected to an Axopatch 1C amplifier (Molecular Devices, Sunnyvale, CA, USA). For recording voltage-dependent Ca^{2+} currents, the pipette solution contained CsCl 110, TEACl 20 (TEA^+), $MgCl_2$ 2, EGTA 10, adenosine triphosphate (ATP) 5, HEPES 10, and glucose 10 (pH 7.4). When recording MaxiK potassium currents, a voltage pulse protocol of 400 milliseconds duration +20 mV increment steps was applied from holding potential of –60 mV to +80 mV. Iberitoxin (Sigma-Aldrich; 100 nM diluted in the external solution) was applied for 2 minutes, and the same voltage pulse protocol was run repeatedly until the drug was washed out. When recording the voltage-dependent Ca^{2+} currents, the protocol consisted of 40-millisecond pulses from –80mV to –40 mV to inactivate voltage-sensitive Na^+ currents if present, followed by 200-

millisecond pulses from -80 to $+80$ in 10 mV increments, each followed by return to -80 mV. All data were normalized by the membrane capacitance and Boltzmann equation was used for fitting. Data were digitally recorded using an Axon Instruments Digitizer; Clampex 8.2 software was used for acquisition and Clampfit 9.0 for analysis (Molecular Devices).

Intracellular Calcium Transients

Changes in cytosolic Ca^{2+} levels were measured as previously described by us [28]. Briefly, cells plated on glass-bottomed MatTek dishes (MatTek, Ashland, MA, USA) were loaded with the ratio-metric Ca^{2+} indicator Fura-2 AM (10 μM , for 45 minutes, at 37°C ; Molecular Probes, Eugene, OR, USA). Fura-2-loaded cells were imaged on an epifluorescence microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) equipped with a CCD digital camera (Photometrics CoolSnap HQ2, Tucson, AZ, USA) and a 20X objective (N.A. 0.45; Nikon). Changes in Fura-2 fluorescence intensities emitted at two excitation wavelengths (340 nm and 380 nm) were acquired at 1.0 Hz using a Lambda DG-4 filter changer (Sutter Instruments, Burlingame, CA, USA) driven by a computer through Metafluor software (Universal Imaging, West Chester, PA, USA). Values of $[\text{Ca}^{2+}]_i$ were determined from regions of interest placed on cells were obtained from Fura-2 ratio images using an in vitro calibration curve.

Intercellular Calcium Wave

CCSM cells plated on MatTek dishes were loaded with Fura-2 AM (10 μM) for 45 minutes at 37°C and changes in Fura-2 fluorescence intensities measured as described above. Intercellular calcium waves (ICWs) were initiated by focal mechanical stimulation of single cells in the center of the microscope field of view, as described previously [29]. The properties of mechanically induced ICW were analyzed in terms of amplitude (peak of response minus basal $[\text{Ca}^{2+}]_i$ were) and efficacy (number of responding cells/total number of cells in field of view).

Microarray Analysis

We silenced the expression of MaxiK α -subunit in cultured human CCSM cells as described above. Total RNA was isolated and cDNA was synthesized by reverse transcription. By qRT-PCR, it was established that the expression of the *Slo* gene was decreased by more than 90% in the siRNA-treated cells. The RNA was then used to perform micro-array analysis of global gene expression using the GeneChip Human Genome U133 Plus 2.0 array from Affymetrix (Santa Clara, CA, USA). Quality control of the RNA, labeling and hybridization to the microarrays were performed by standard Affymetrix protocols by the Albert Einstein College of Medicine Affymetrix Facility. We performed a total of seven micro-array analyses using four chips for the silenced, and three chips for the control human corporal cells. Gene expression in the silenced cells was compared with controls using AffymGUI software, available from <http://www.bioconductor.org> as previously described [23,30]. Data have been submitted to the NCBI Gene Expression Omnibus repository and can be accessed following the link <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jzgpruaqyyiogbc&acc=GSE19672>.

Statistical Analysis

The results are presented as mean $\pm$ standard error (SE). Paired or unpaired Student's *t*-tests were used as appropriate to evaluate differences between two groups, and one-way analysis of variance (*ANOVA*) was used for multiple groups. $P < 0.05$ was considered to indicate statistical significance.

Results

Silencing of the Gene Encoding the MaxiK (Slo) in Rat Corpus Cavernosum Smooth Muscle Cells

Primary rat CCSM cells were transfected with two different Stealth siRNA constructs (rSi11 and rSi12 in Figure 1A) targeting the *Slo* gene encoding the MaxiK α -subunit. The sites of the *Slo* gene targeted by the siRNA are shown in Figure 1A. As shown in Figure 1B the level of expression of the *Slo* gene at 48 hours was compared between nontransfected control cells and cells transfected with siRNA by quantitative RT-PCR analysis. Overall, approximately 80% of knockdown was achieved. Based on the observation that the siRNA rSi11 construct was slightly more effective at silencing *Slo* expression, we used this construct in all other experiments.

Characterization of MaxiK Activity in Silenced Rat CCSM Cells

We confirmed the loss of MaxiK protein after silencing for 48 hours by Western blot analysis and by measuring the channels' activity by patch clamping. Western blot analysis showed that silencing of the *Slo* gene in rat CCSM cells resulted in loss of detectable levels of MaxiK in the membrane fraction (Figure 1C). Patch clamp recordings confirmed a significant down-regulation of MaxiK channel activity in CCSM cells (Figure 1D–G). Mock-transfected control cells, untreated or treated with IBTX (100 nM), were compared to 48 hours MaxiK siRNA-treated cells. Representative recordings are shown in Figure 1D–G. These demonstrate that in CCSM cells, there is a large outward current that is activated by voltage and is significantly IBTX-sensitive (at +20 mV; 35.20 ± 5.1 pA/pF IBTX vs. 74.67 ± 10.83 pA/pF, $P < 0.05$; *ANOVA* followed by Newman–Keuls Multiple Comparison Test). Blockade by IBTX was reversible after rinsing for 3–5 minutes (Figure 1D and F). Rat CCSM cells transfected for 48 hours with MaxiK siRNA showed significantly reduced voltage activated outward currents compared to the mock-transfected control cells (Figure 1E and G; at +20mV; 37.67 ± 9.82 pA/pF MaxiK siRNA vs. 74.67 ± 10.83 pA/pF, control $P < 0.05$; *ANOVA* followed by Newman–Keuls Multiple Comparison Test). The presence of residual K^+ current in the absence of detectable MaxiK protein is likely due to sampling, the electrophysiological recordings being performed on single cells, while the Western blot analysis detects average protein levels in a large pool of cells. Additionally, the residual outward current observed in the presence of IBTX, could be due to the contribution of other potassium channels such as the ATP-dependent K^+ channels. In order to evaluate the effect of the MaxiK siRNA on voltage-sensitive Ca^{2+} channels currents, cells treated with siRNA were voltage clamped 48 hours after silencing and compared with mock-transfected control. Neither voltage nor amplitude of the calcium currents were altered by the siRNA treatment (Figure 1H and I).

Measurement of Basal Intracellular Calcium Levels after Long- and Short-Term Blockade of MaxiK in Rat CCSM Cells

It might be expected that functional deletion of MaxiK would lead to increased $[Ca^{2+}]_i$ due to lack of repolarization that this channel provides. In order to evaluate whether MaxiK silencing for 48 hours or acute treatment with IBTX (100 nM) affected calcium homeostasis in rat CCSM cells, we compared basal Ca^{2+} levels in siRNA-transfected/IBTX-treated cells with those of mock-transfected/untreated cultures. Surprisingly, there was no significant difference between siRNA-silenced cells and mock-transfected controls (mock, 275.4 ± 2.9 nM; silenced, 283.4 ± 4.4 nM). By contrast, when $[Ca^{2+}]_i$ were measured in CCSM cells acutely treated with IBTX, there was no immediate change in basal $[Ca^{2+}]_i$ but a progressive increase in $[Ca^{2+}]_i$ was observed during the 75 minutes duration of treatment ($P < 0.05$ at 45, 60, and 75 minutes; Figure 2). IBTX was ineffective in changing $[Ca^{2+}]_i$ in the siRNA-treated cells, consistent with MaxiK being the target for this drug. These results demonstrate that rapid loss of MaxiK function significantly impacts the intracellular mechanisms of calcium homeostasis, whereas following 48 hours MaxiK silencing, such altered homeostasis is not evident. This absence of altered basal $[Ca^{2+}]_i$ might result from compensatory mechanisms being recruited to maintain calcium homeostasis during the 48 hours of loss of MaxiK activity.

At Shorter Durations of MaxiK Silencing, There Are More Profound Increases in $[Ca^{2+}]_i$ Levels

To test the hypothesis that there is activation of compensatory mechanisms to maintain calcium homeostasis after silencing of MaxiK, we followed the relative changes in basal $[Ca^{2+}]_i$ compared to mock-transfected cells at 8, 24, 32, and 48 hours after siRNA treatment. By quantitative RT-PCR, we verified that the expression level of MaxiK in rat CCSM cells was significantly reduced starting at the 24 hour time-point (Table 1). At 24 and 32 hours after transfection, the basal $[Ca^{2+}]_i$ was significantly higher in silenced rat CCSM cells compared to mock-transfected controls, but decreased to levels comparable to mock-transfected controls after 48 hours (Figure 3). This time-dependent decrease in MaxiK expression was associated with a significant increase in basal $[Ca^{2+}]_i$ levels at 24 and 32 hours after silencing (Figure 3) that, nonetheless, progressively declined with time despite the continued reduction in MaxiK expression (Table 1), reaching $[Ca^{2+}]_i$ values at 48 hours after transfection that were not different from mock-transfected controls. This suggests that over time, there is an adaptation of CCSM cells to the reduction in MaxiK activity.

Acute Blockade of MaxiK Increases Intercellular Calcium Signaling in CCSM Cells

We compared mock-transfected control rat CCSM cells with those treated for 48 hours with siRNA and those treated for 30 minutes with IBTX (100 nM) with respect to parameters defining ICW transmission. As shown in Figure 4, acute inhibition of MaxiK, but not chronic silencing of MaxiK significantly enhanced intercellular signaling. The amplitude of calcium wave propagation among neighboring responding cells was significantly greater in mock-transfected control cells following IBTX treatment than in the untreated cells (Figure 4A). By contrast, 48 hours treatment with MaxiK siRNA did not affect the amplitude of ICW spread. Similarly, ICW efficacy, defined as the relative number of responding cells,

was significantly greater in mock-transfected control cells treated with IBTX than without treatment but was not affected by MaxiK knock-down (Figure 4B). These results suggest that short-term blockade of the MaxiK channel by IBTX results in a greater number of responding cells with greater amplitude of response compared to mock-transfected controls. However, these effects were not observed when MaxiK activity was down-regulated for a longer period of time (48 hours).

Silencing of the Gene Encoding the MaxiK (Slo) α -Subunit in Human Corpus Cavernosum Smooth Muscle Cells

The *hSlo* and *rSlo* have ~98% homology [31]. It would thus be expected that silencing of the human MaxiK α and channel blockade would result in changes in basal $[Ca^{2+}]_i$ of primary human CCSM cells that would be similar to those observed for rat CCSM cells. To test this premise, human CCSM cells were transfected with two different Stealth siRNA constructs (hSil1 and hSil2 in Figure 5A) targeting the *hSlo* gene encoding the MaxiK α -subunit. Overall, knock-down of more than 90% of RNA was achieved (Figure 5B). Based on the observation that the siRNA hSil1 construct was slightly more effective at silencing *Slo* expression, we used this construct in all other experiments.

Following the same protocol used for the rat CCSM, we compared the basal $[Ca^{2+}]_i$ of silenced and mock-transfected cells in the absence and presence of IBTX (100 nM) over a total time of 75 minutes (Figure 5C). As in the rat dataset, the basal $[Ca^{2+}]_i$ of silenced cells was not different from that of mock-transfected controls at any of the analyzed time points. Also, similar to the rat CCSM cells, the mock-transfected human cells showed a significantly higher increase in $[Ca^{2+}]_i$ when treated with IBTX.

We have also attempted to compare the ICW parameters in the human cells following silencing or blockade of the MaxiK channel with IBTX. However, the amplitude as well as the number of cells recruited into the ICWs generated in the human CCSM cell cultures was much smaller compared to the ICWs among rat CCSM cells. We could not determine whether there were significant differences between the control cells and cells in which the MaxiK channel was silenced or blocked by IBTX.

Altered CCSM Gene Expression by MaxiK siRNA Treatment

Our measurements of basal $[Ca^{2+}]_i$ and parameters of intercellular calcium signaling suggest that CCSM cells respond to long-term down-regulation of MaxiK channel activity by compensatory changes in genes that regulate smooth muscle tone or $[Ca^{2+}]_i$. As an initial test of this hypothesis, we performed micro-array analysis to identify global gene expression changes following MaxiK silencing for 48 hours. Human CCSM cells were treated with siRNA for 48 hours. Prior to micro-array analysis down-regulation of *Slo* gene expression by >90% was validated using quantitative RT-PCR. The expression level of over 47,000 transcripts and variants were analyzed by the GeneChip Human Genome U133 Plus 2.0 array, of which about 6.6% were significantly changed in expression in our analysis. The *Slo* gene encoding MaxiK was the most down-regulated gene, by approximately 87%. In Table 2, we show a list of selected genes with significantly changed expression levels after MaxiK siRNA treatment that play important roles in regulation of smooth muscle tone. A number of

genes involved in signaling pathways, the Rho kinase pathway, calcium homeostasis, and the contractile apparatus were identified as changed in expression when MaxiK was silenced for 48 hours. Overall, these changes suggest molecular cross talk between MaxiK activity, and several of the changes promote smooth muscle relaxation.

Discussion

Potassium channels modulate corporal smooth muscle membrane potential, and thus, ultimately affect transmembrane calcium flux. Among the several subtypes of potassium channels present in smooth muscle, the MaxiK channel and the meta-bolically gated K^+ channels (KATP) are thought to be the most important modulators of corporal smooth muscle tone [32–34]. In particular, the importance of MaxiK for erectile function is well supported in the literature. For example, MaxiK knockout mice have ED [2] and gene transfer of plasmids expressing MaxiK has been shown to restore erectile function in several animal models of ED [31,35,36]. These findings ultimately led to the development of a gene therapy approach to improvement erectile function, which is being evaluated in clinical trials [7].

In order to investigate the role of MaxiK in the regulation of $[Ca^{2+}]_i$ and intercellular signaling in corporal tissue, we acutely blocked its activity or knocked down its expression and quantified the consequent impact. Functional knock-down of the channel has been proven by electrophysiological recordings. Although currents generated by KATP channels contribute in regulating the erectile function and have been well characterized in human CSSM [32,34], the predominant outward K^+ current in CSSM cells is generated by MaxiK [37].

It has been reported that MaxiK knockout mice develop ED [2] suggesting that compensatory mechanisms for the lack of MaxiK do not, or are insufficient, to overcome this pathological pheno-type. However, a later study in vivo using a conditional knockout model of MaxiK [38] showed that compensation is occurring in bladder. In addition, in an animal model of diabetes, there appear to be changes in the splicing of the *Slo* gene, which encodes the MaxiK α -subunit, from an inactive to an active form, which maybe a compensatory mechanism for the effects of hyperglycemia [39]. Our study therefore supports recent evidence suggesting that compensatory pathways are activated when there are changes in MaxiK expression to maintain smooth muscle tone.

We measured the effect of long- and short-term treatment on the basal intracellular calcium levels and on parameters of ICW propagation. Silencing the MaxiK α -subunit by siRNA in rat CCSM cells led to no detectible changes at 48 hours after treatment, although a time course study demonstrated that MaxiK siRNA caused elevated $[Ca^{2+}]_i$ at 24 hours and 32 hours after treatment, which subsequently recovered to values not different from mock-transfected controls at 48 hours after treatment. By contrast, acute functional blockade of the MaxiK channels by treatment with IBTX caused a progressive increase in $[Ca^{2+}]_i$ over a time period of 75 minutes in rat and human CCSM cells, and increased both the amplitude and efficacy of intercellular calcium signaling in rat CCSM cells.

Given the central role of calcium in regulating smooth muscle tone and the postulated role of intercellular Ca^{2+} signaling in the coordination of corporal smooth muscle tissue function [18,20–22], our findings that acute blockade of MaxiK function is associated with enhanced calcium mobilization and intercellular signaling are indicative that MaxiK-associated changes in CCSM cells calcium homeostasis could be translated into the in vivo setting as impaired control of CCSM tonus and consequent ED. This is in line with several studies previously carried out in airway smooth muscle. Acute blockade of MaxiK channels with the specific blockers IBTX or charybdotoxin increases the baseline contractility, enhancing the activity of contracting agents and decreasing the activity of relaxing agents [40–43].

The time-dependent reversal of basal $[\text{Ca}^{2+}]_i$ and sensitivity of $[\text{Ca}^{2+}]_i$ to stimulation that results from protracted silencing of MaxiK activity in CCSM cells might explain studies carried out in a *Slo*^{-/-} mouse, where tracheal smooth muscle tissue showed a “paradoxical phenotype,” with decreased sensitivity to bronchoconstrictors and enhanced sensitivity to bronchodilators [44], opposite to the effects of short-term MaxiK blockade described by Jones et al. [40–43]. Although cultured CCSM cells are commonly used to perform molecular and biochemical analysis and these results often translate into physiological relevance, the data from our in vitro experiments and micro-array analysis awaits confirmation in animal models. A first evidence of the validity of our observations in vivo is provided by a recent study from Sprossmann et al. [38] where constitutive vs conditional knockdown of MaxiK are compared in mouse models. The authors found that although the constitutive MaxiK^{-/-} mouse presents detrusor muscle hyper-excitability and overactive bladder, the condition was strongly exacerbated in the case of a time-dependent, conditional knock-down of MaxiK. This corresponds to our results showing that at shorter time periods after blocking MaxiK activity basal $[\text{Ca}^{2+}]_i$ is elevated and $[\text{Ca}^{2+}]_i$ is more responsive to stimulation than at longer time periods.

These observations led to our hypothesis that following blockade of MaxiK activity, there is activation of compensatory mechanisms. Micro-array analysis demonstrated that after 48 hours of MaxiK silencing, the expression levels of several genes involved in Ca^{2+} regulation and in the mechanisms of contraction and relaxation of the smooth muscle were significantly changed. Even so, the fold changes in the genes, although significant were (with the exception of the *Slo* gene encoding MaxiK) less than 2-fold. However, the combined effect of all changes could be sufficient to result in the compensation in $[\text{Ca}^{2+}]_i$ that we observe after 48 hours of MaxiK silencing.

Of particular interest, we found up-regulation of the ARHGAP23, 24, and 25 genes coding for guanosinetriphosphatase (GTP)ase-activating proteins that are negative regulators of the Rho family of proteins which mediate smooth muscle contraction [45,46]. GUCY1B2 was up-regulated and encodes the $\beta 2$ subunit of guanylyl cyclase, which is expressed in corpus cavernosum and mediates the effect of NO on smooth muscle relaxation by catalyzing the conversion of GTP to the second messenger cGMP [47]. Interestingly, we also observed down-regulation of the gene coding for phosphodiesterase-5 (PDE5). Orally administered PDE5 inhibitors, such as tadalafil and sildenafil, are commonly prescribed treatments for ED [48–50]. The down-regulation of the PLCD4 and PLCB1 genes coding for the delta4 and beta1 subunits of the phospholipase C enzyme would also promote smooth muscle

relaxation. PLC generates the second messenger IP₃ that controls many cellular processes by inducing intracellular Ca²⁺ mobilization [51]. In agreement with the findings of Brown et al. and Sprossmann et al. [38,52] in the bladders of *Slo*^{-/-} mice, we found up-regulation of the cAMP-dependent protein kinase, PKA, and of the adenylate cyclase, ADCY2 [53]. Interestingly, previous evidence has shown that the compensatory effects activated by a long-term deletion of MaxiK are mediated by an up-regulation of the cAMP/ PKA pathway in bladder [38,52], while in airway smooth muscle compensatory up-regulation involves cGMP signaling [44]. Our results suggest that in the corpora, compensatory changes affect both cAMP and cGMP signaling pathways. In addition, we also detected changes in the expression levels of genes belonging to the PLC/IP₃ pathway.

Besides the changes described above, which tend to favor relaxation, there was also evidence for changes in expression of genes that would favor contraction. Some of our findings are in line with the proteomic analysis performed by Sprossmann et al. [38] in the bladders of *Slo*^{-/-} mice which demonstrated up-regulation of the aldehyde dehydrogenase ALDH3A2 and down-regulation of TMG2. We also found up-regulation of subunits of several voltage-dependent calcium channels, CACAN1D, CACNA1G, CACNA1H, CACNA1I, although no alteration in voltage or amplitude of the calcium currents was detected by electrophysiology (Figure 1H and I). Similarly, Sausbier and coauthors [44] observed that the L-type channel blocker nifedipine abolished carbachol induced phasic contraction in MaxiK^{-/-} tracheal rings, indicating a possible up-regulation of L-type channels, but did not significantly increase voltage gated Ca²⁺ currents. It should be noted that the up-regulation of subunits of several voltage-dependent calcium channels in our study although significant, is not very high, so that this up-regulation may not result in significant changes in the overall calcium current recordings.

The data presented in this study add to the increasing evidence that compensatory mechanisms are activated when MaxiK is ablated in smooth muscle tissue for an extended period of time. In this study, we show that acute blockade of MaxiK strongly impacts the mechanisms of intra-cellular calcium homeostasis and intercellular communication through transmission of calcium waves. These results lead to our overall conclusion that compensatory mechanisms are activated to maintain calcium homeostasis. Our micro-array analysis provides preliminary identification of possible gene candidates involved in Ca²⁺ homeostasis and smooth muscle tone regulation that would form the molecular basis of compensatory pathways activated through the loss of MaxiK activity. Our results do not rule out the possibility that the compensation process may include calcium-independent pathways affecting smooth muscle tone. A comprehensive understanding of the molecular and biochemical changes that account for compensation would require probing both transcriptional and post-transcriptional processes, perhaps using a proteomic approach.

Nevertheless, our micro-array analysis demonstrates that a multitude of compensatory pathways is activated, suggesting a high degree of redundancy is present in the CCSM, which may explain the efficacy of several different therapeutic approaches for the improvement of erectile function. Our evidence supports the hypothesis that erectile function is the result of a complex molecular crosstalk between several players involved in Ca²⁺ regulation and thereby smooth muscle tone regulation. We hypothesize that ED only

results from an imbalance in the equilibrium of several of these molecular pathways, but only one needs to be restored to improve regulation of CCSM tone and thus erectile function.

Changes in the expression and activity of the MaxiK gene as a result of aging and diabetes are known to play a significant role in the development of ED [39,54]. Current available therapies still require improvement and are not effective on patients suffering from diseases such as diabetes and metabolic syndrome [55]. A better understanding of the molecular consequences of changes in MaxiK expression could lead to the identification of novel treatment strategies and therapeutic targets.

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Figure 1.

Silencing of the *Slo* gene in rat CCSM cells. (A) Diagram indicating the locations of the *Slo* gene targeted by the siRNA constructs. (B) RNA from rat CCSM cells transfected with two different siRNA sequences (rSil1 and rSil2) was extracted at 48 hours after transfection, and quantitative RT-PCR performed. Expression levels were determined using the comparative crossing threshold (C_t) method with *GAPDH* to normalize between samples. Mean changes in the level of *Slo* expression in five experiments were compared to untreated control CCSM cells. Error bars represent the standard errors of the mean (SEM). (C). Western blot analysis

shows knock-down of the MaxiK protein in rat CMSC treated for 48 hours with siRNA. (D) Blockade of MaxiK outward currents recorded in rat CCSM cells following perfusion with 100 nM iberiotoxin followed by rinsing (N = 7). Data were normalized with respect to membrane capacitance and plotted as a current (I)-voltage (V) relationship. $P < 0.05$ for MaxiK Currents IBTX vs. mock-transfected control from +20 to +80 mV. Significant decrease of MaxiK outward currents is shown after 48 hours of silencing. (F) Whole cell recordings representative of outward currents recorded under control conditions, after Iberiotoxin and after rinsing (wash out). (E) Current-voltage relationship of outward currents recorded in mock transfected CCSM cells (N = 7) and CCSM cells after 48 hours treatment with MaxiK siRNA (N = 11). $P < 0.05$ for mock-transfected control vs. MaxiK siRNA cells from +20 to +80 mV. (G) Representative traces of whole cell recording in a MaxiK siRNA treated cell. (H) Amplitudes of voltage-gated Ca^{2+} channel currents in MaxiK siRNA treated and mock transfected cells. Peak inward current was measured in whole cell configuration, using Ba^{2+} as charge carrier, and plotted as a current-voltage relationship (N = 8 for mock-transfected control and N = 7 for MaxiK siRNA treated). (I) Representative traces of a recording of the Ca^{2+} channel current in response to voltage pulse paradigm shown in the inset. Bars represent $\pm$ standard error of the mean (SEM).

Figure 2.

Basal $[Ca^{2+}]_i$ after treatment with MaxiK siRNA or IBTX. Basal $[Ca^{2+}]_i$ was measured ratiometrically in Fura 2AM loaded cells every 15 minutes for a total period of 75 minutes. Cells where MaxiK had been silenced for 48 hours were compared to mock-transfected controls and to cells treated with IBTX at the time of the experiment. Values measured from each group of cells have been normalized for the corresponding $[Ca^{2+}]_i$ at time 0. The $[Ca^{2+}]_i$ of IBTX treated cells was significantly higher than $[Ca^{2+}]_i$ in the other groups at 45, 60, and 75 minutes. No significant difference was detected between mock-transfected controls, 48-hours-silenced and 48-hours-silenced cells treated with IBTX. Overall, $[Ca^{2+}]_i$ was monitored in an average of over 200 cells for each group. Error bars represent standard error of the mean (SEM), asterisk indicates statistical significance ($P < 0.05$).

Figure 3.

Basal $[Ca^{2+}]_i$ levels of rat CCSM cells at different periods of time after treatment with MaxiK siRNA. Basal $[Ca^{2+}]_i$ was measured in MaxiK siRNA- and mock-transfected cells at 8, 24, 32, and 48 hours from transfection. To follow the changes in cytosolic calcium over time, the $[Ca^{2+}]_i$ values for MaxiK siRNA-treated cells were normalized to $[Ca^{2+}]_i$ of mock-transfected controls at each time point. Overall, changes in $[Ca^{2+}]_i$ were monitored in an average of more than 200 cells for each group. Error bars represent standard errors of the mean (SEM), asterisks indicate statistical significance ($P < 0.05$).

Figure 4.

Effects of acute MaxiK blockade (30 minutes iberiotoxin) and prolonged MaxiK knock-down (48 hours silencing) on parameter of intercellular calcium wave (ICW) propagation among rat CCSM cells. (A) Amplitudes of the Ca^{2+} response in cells recruited into the ICW that was triggered by focal mechanical stimulation of a single cell. (B) Efficacy of the ICW spread, calculated as the number of responding cells relative to the total number of cells in the field. Changes in $[\text{Ca}^{2+}]_i$ were monitored in an average of more than 400 cells for each group. Error bars represent standard errors of the mean values (SEM), asterisks indicate statistical significance ($P < 0.05$). Representative recordings of changes in $[\text{Ca}^{2+}]_i$ observed in cells recruited into the ICW spread induced in mock-transfected (C) and in IBTX-treated mock-transfected CCSM cultures (D).

Figure 5.

Silencing of the *Slo* gene in human CCSM cells. (A) Diagram indicating the locations of the *Slo* gene targeted by the siRNA constructs. (B) RNA from human CCSM cells transfected with two different siRNA sequences (hSil1 and hSil2) was extracted at 48 hours after transfection, and quantitative RT-PCR performed. Expression levels were determined using the comparative crossing threshold (C_t) method with *GAPDH* to normalize between samples. Mean changes in the level of *Slo* expression in five experiments were compared to untreated control CCSM cells. Error bars represent the standard errors of the mean (SEM). (C) Basal $[Ca^{2+}]_i$ was measured ratiometrically in Fura 2AM loaded cells every 15 minutes for a total period of 75 minutes. Cells where MaxiK had been silenced for 48 hours were compared to mock-transfected controls and to cells treated with IBTX (100 nM) at the time of the experiment. Values measured from each group of cells have been normalized for the corresponding $[Ca^{2+}]_i$ at time 0. Basal $[Ca^{2+}]_i$ of silenced cells was not different from those of mock-transfected controls at any of the analyzed time points. Mock-transfected human cells showed a significantly higher increase of intracellular Ca^{2+} when treated with IBTX.

Table 1

MaxiK expression levels after treatment with MaxiK siRNA for different periods of time. Rat CCSM cells were treated in triplicates with siRNA and the expression levels of MaxiK at 24, 32, and 48 hours from transfection were determined by quantitative RT-PCR. Results are expressed as means $\pm$ standard errors

	24 hours	32 hours	48 hours
<i>Slo</i> expression levels	42% $\pm$ 0.10	37% $\pm$ 0.01	17% $\pm$ 0.09

Table 2

Microarray analysis of changes in gene expression after silencing MaxiK. Statistically significant changes were selected and classified by molecular function. *P* values and the fold-change relative to control (FC) are indicated. Triangular marks signal up- and down-regulation. The *Slo* gene (coding for MaxiK) is the most down-regulated gene. Gene changes favorable to smooth muscle relaxation are highlighted.

SLO	Potassium large conductance calcium-activated channel, su	<i>P</i> value	FC
PRKACG	Protein kinase, cAMP-dependent, catalytic, gamma	0.034	1.32 ▲ cAMP/PKA pathway
ADCY2	Adenylate cyclase 2 (brain)	0.042	1.59 ▲
CHRM2	Cholinergic receptor, muscarinic 2	0.02	0.39 ▲
CHRM3	Cholinergic receptor, muscarinic 3	0.04	1.33 ▼
GUCY1B2	Guanylate cyclase 1, soluble, beta 2	0.048	1.31 ▲ cGMP/PKG pathway
NOS3	Nitric oxide synthase 3 (endothelial cell)	0.034	1.34 ▲
PDE5A	Phosphodiesterase 5A, cGMP-specific	0.052	0.57 ▼
PLCD4	Phospholipase C, delta 4	0.006	0.6 ▼ IP3/PLA pathway
PLCB1	Phospholipase C, beta 1 (phosphoinositide-specific)	0.036	0.53 ▼
IMPA2	Inositol (myo)-1 (or 4)-monophosphatase 2	0.016	0.34 ▼
INPP4A	Inositol polyphosphate-4-phosphatase, type 1, 107kDa	0.007	1.36 ▲
ITPR3	Inositol 1, 4, 5-triphosphate receptor, type 3	0.055	1.57 ▲
CAMKK1	Calcium/calmodulin-dependent protein kinase kinase 1, alpl	0.043	1.23 ▲
CALML3	Calmodulin-like 3	0.054	1.35 ▲
CALD1	Caldesmon 1	0.015	0.63 ▼
CAMK2B	Calcium/calmodulin-dependent protein kinase (CaM kinase)	0.019	1.45 ▲
ARHGAP23	Rho GTPase activating protein 23	0.052	1.29 ▲ Rho/Rho kinase pathway
ARHGAP25	Rho GTPase activating protein 25	0.055	1.45 ▲
ARHGAP24	Rho GTPase activating protein 24	0.019	1.61 ▲
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3	0.037	1.5 ▲ Ca2+ homeostasis
ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	0.045	1.3 ▲
CACNA1G	Calcium channel, voltage-dependent, alpha 1G sub	0.043	1.38 ▲ Ca2+ channel
CACNA1I	Calcium channel, voltage-dependent, alpha 1I sub	0.032	1.43 ▲
CACNA1D	Calcium channel, voltage-dependent, L type alpha 1D sub	0.031	1.45 ▲
CACNA1H	Calcium channel, voltage-dependent, alpha 1H sub	0.013	1.47 ▲

		<i>P</i> value	FC	
MKL2	MKL/myocardin-like 2	0.047	1.51	▲ Smooth muscle gene expression
CASR	Calcium-sensing receptor	0.055	1.44	▲ Extracellular Ca2+ homeostasis
NPR1	Natriuretic peptide receptor A/guanylate cyclase A	0.05	1.38	▲
ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	0.004	0.26	▼ Aldehyde dehydrogenase activity
TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-gam)	0.027	0.53	▼ Intra/extracellular matrix organization