Gynecology & Obstetrics
ISSN: 2161-0932

Rosenbaum et al., Gynecol Obstet 2013, 3:5
DOI: 10.4172/2161-0932.1000171

Research Article Open Access

Relaxant Effect of Cyppa *In Vitro* and Expression Analysis of SK3 Splice Variants in the Myometrium of Pregnant and Non-Pregnant Women

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Abstract

Background and objective: We have recently shown an overall down-regulation of SK3 channels in pregnant human myometrium compared to non-pregnant myometrium. The aim of this study was to investigate whether SK3 splice variants are involved in the down-regulation of SK3 channels. We also tested whether SK channels are implicated in myometrial contractility using CyPPA, a novel and selective SK2 and SK3 channel activator.

Study design: We evaluated the expression of SK3 splice variants at mRNA level by qRT-PCR in myometrium from pregnant (*n*=11) and non-pregnant (*n*=11) women. Isometric tension recordings were performed to assess human myometrial contractility on biopsies obtained at elective caesarean section at term (*n*=6) and from hysterectomy (*n*=6). We investigated the effects of CyPPA (0.1-60 μM) on spontaneous contractions.

Results: The relative quantity of SK3 variant 3 did not differ between myometrium from pregnant and non-pregnant women (*P*=0.332). Variant 2 was not found present at detectable amounts. In contrast, SK3 variant 1 showed a 3-fold down-regulation in pregnant compared to non-pregnant myometrium (*P*=0.002).

CyPPA depressed spontaneous phasic contractions in human myometrial strips of both non-pregnant and pregnant origin. It took place in a concentration-dependent manner (where pIC_{50} = 5.02 ± 0.08 and 5.16 ± 0.15, respectively, and *P*>0.05), with contractions being abolished at 60 μM CyPPA.

Conclusions: We have been successful in demonstrating the presence of SK3 splice variants in human myometrium, for the first time. We also show that SK3 variant 1 is down-regulated in pregnant myometrium, and that it may be responsible for the overall down-regulation of SK3 channels observed in pregnant, as compared to non-pregnant, human myometrium. CyPPA exerts a potent relaxant effect on human myometrial tissue. This suggests that SK3 channels may be a new pharmacological target for the development of tocolytica.

Keywords: CyPPA; Human myometrium; SK channels; qRT-PCR; Splice variants; Pregnant; Non-pregnant

Introduction

Preterm labour is a major medical problem worldwide because of the related mortality and morbidity of the newborn [1]. The etiology of preterm labour is multifactorial and genetic factors seem to be involved [2,3]. Tocolytics, which are medications used to suppress uterine contractions, are regularly used to treat spontaneous preterm labour; however, they have not been shown to reliably extend pregnancy beyond 1-2 days [4,5]. Therefore, better knowledge of the mechanism underlying relaxation of the myometrium would aid finding new strategies for the treatment of preterm labour.

Ion channels play an important role in the regulation of the membrane potential, which is central to myometrial contractility [6]. Among them, potassium channels are the most abundant in the myometrium and are believed to have a key role in maintaining uterine quiescence during pregnancy [7,8]. The family of small-conductance Ca2+ -activated K+ (SK or $K_{Ca}2$) channels consists of three members: SK1, SK2 and SK3 [9]. One important feature of these channels is that they respond to increases in intracellular Ca^{2+} concentrations by stimulating a negative feedback; contributing to the repolarization, and thus the relaxation, of smooth muscle cells.

In the myometrium, previous studies have demonstrated that the parturition of transgenic mice with an over-expression of SK3 channels is compromised [10]. Furthermore, we have previously investigated the effect of a positive SK/IK channel modulator, NS4591, in human myometrium and showed that the activation of these channels reduces myometrial contractility [11]. Lately, CyPPA, a new SK2 and SK3 positive modulator [12], has been demonstrated as having a relaxant effect on the non-pregnant, and pregnant, myometrium of mice in vitro and a successful tocolytic effect in vivo [13].

Another significant aspect of SK3 channels is that their transcript level is regulated during pregnancy. More specifically, we, and others, have shown that SK3 mRNA is present in the myometrium of, and down-regulated in, both pregnant women [14-17], and rodents [18,19].

SK3 channels are encoded by a single gene, *KCNN3*, comprising 9 exons at least. Alternative splicing of SK3 pre mRNA, results in a number of functionally distinct SK3 variants. To our knowledge, at present there are 3 splice variants, each of which encodes a particular SK3 channel proteins [20-22].

In our present study, we demonstrate that SK3 splice variants are differentially involved in the down-regulation of SK3 channels, and that CyPPA is an effective human myometrial relaxant. Our results suggest that the SK3 gene may play a part in myometrial physiology, and perhaps also in its pathophysiology, with the result that it could represent a new therapeutic approach to controlling preterm labour.

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Received August 22, 2013; **Accepted** September 17, 2013; **Published** September 20, 2013

Citation: Rosenbaum ST, Svalo J, Larsen T, Joergensen J, Bouchelouche P (2013) Relaxant Effect of Cyppa *In Vitro* and Expression Analysis of SK3 Splice Variants in the Myometrium of Pregnant and Non-Pregnant Women. Gynecol Obstet 3: 171. doi:10.4172/2161-0932.1000171

Materials and Methods

Tissue collection

All patients were recruited from the Department of Gynecology and Obstetrics, Copenhagen University Hospital at Holbaek and the experiments were performed at the Smooth Muscle Research Center, Department of Clinical Biochemistry, Copenhagen University Hospital at Koege.

Myometrium was obtained, with informed written consent, from women undergoing elective caesarean section at term not in labour (gestational age between 37 and 40 weeks; mean maternal age 32.2; range 28-40 years; *n*=17), or hysterectomy (mean age 47.2; range 35-54 years; *n*=17). Table 1 and 2 describe the demographic and clinical data of non-pregnant and pregnant subjects, respectively. None of the women included in this study had evidence of underlying disease (hypertension, diabetes, preeclampsia, intrauterine growth restriction, etc.), and all the conceptions in the pregnant group occurred spontaneously. Biopsies were obtained from the midline of the upper lip of the uterine incision (myometrium) at elective caesarean section, or from the same part of the uterus from a macroscopic normal area at hysterectomy.

In the operation theatre, biopsies were immediately placed in PBS (Sigma-Aldrich, Broendby, Denmark) at 4°C for isometric tension recordings or in RNAlater (Qiagen, Denmark) for qRT-PCR experiments and transferred to the Smooth Muscle Research Center. Functional studies were performed immediately upon arrival and biopsies in RNAlater were stored at 4°C overnight, and frozen at -20°C until further processing (< 2 months).

The study has been formally approved by the local ethics committee (SJ-120).

Isometric tension recording

Isometric tension recordings were performed in a special designed myograph (610M, Danish Myo Technology A/S, Aarhus, Denmark). Under a stereo-microscope myometrial strips (3 × 2 mm) were dissected and mounted in 1 ml organ bath containing physiological salt solution (PSS, pH 7.4 in mM: NaCl 119, KCl 4.7, $MgSO_4$ 2.4, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.025, glucose 6.05, $CaCl_2$ 1.6) kept at 37°C and continuously gassed with 95% O_2 and 5% CO_2. Muscle strips were anchored between two stainless steel pins, one of which was connected to a force transducer. Muscle strips were subjected to 6-7 mN tension and allowed to equilibrate for a period of two hours, during which the PSS was changed every 30 minutes. A 20 minute period of stable phasic contractions (pretreatment period) was recorded before addition of CyPPA. Muscle strips that did not develop spontaneous contractions were excluded from the experiment.

Age, years	47.2 ± 5.2
Body mass index, kg/m^2	26.1 ± 5.6
Indication for hysterectomy	Dysfunctional uterine bleeding, leiomioma or cervix dysplasia
Uterus weight	267.6 ± 249.5
Data are presented as mean ± SD	

Table 1: Demographic and clinical data of non-pregnant women.

Maternal age, years	32.2 ± 2.9
Body mass index, kg/m^2	25.2 ± 4.5
Gestational age at delivery, weeks	37 – 40
Indication for caesarean section	Maternal request, previous caesarean section or breech presentation
Birth-weight, kg	3.45 ± 0.7
Data are presented as mean ± SD	

Table 2: Demographic and clinical data of pregnant women.

Concentration-response curves were obtained by adding cumulative concentrations of CyPPA (0.1 - 60 µM) at 20-minute intervals. Time-matched vehicle control experiments were carried out in parallel.

The analogue signal output was digitalized with a Powerlab and visualized and analysed using Chart v7.3.3.5 software (both AD Instruments, Aarhus, Denmark). Myometrial contractions were assessed by calculation of the integral of a period of 20 minutes (mN x min) using the Peak Analysis module of the Chart software (AD Instruments, Aarhus, Denmark). For each strip, data were normalized to the pretreatment period and expressed as a percentage. An average of the measurements from each strip was calculated for the corresponding patient.

RNA extraction and cDNA synthesis

Tissue biopsies were grinded in liquid nitrogen in a mortar, and homogenized in 1.5 ml TriZol (Invitrogen, Denmark) using a rotor-stator homogenizer. Total RNA was extracted from (85-150 mg) grinded tissue using the Trizol method (manufacturer's instruction), including bromochloropropane (VWR, Denmark) for phase-separation. The quantity of extracted RNA was checked spectrophotometrically with a nanophotometer (Implen, VWR, Denmark). To ensure RNA integrity, only samples with an A260/A280 Optical Density (OD) ratio of 1.7-2.0 were used. 1 µg was loaded on a 1% agarose gel containing ethidium bromide for electrophoresis and visualized by a UV-transilluminator (Uvitec, Kem-en-tec, Taastrup, Denmark). RNA (10 µg) samples were purified by DNase treatment in order to remove contaminating genomic DNA. This was done using an RNAse-free DNase kit according to the manufacturer's instructions for rigorous DNA removal (50 µl reaction) (Ambion, Applied Biosystems, Denmark). The quantity and purity of DNase treated RNA was checked spectrophotometrically and with the help of gel electrophoresis.

RNA was reversely transcribed into complementary DNA, (cDNA) with 4.5 µl oligo (dT) primers (100 ng/µl), and reverse transcriptase (1 µl), in a 30 µl reaction volume from 600 ng DNase treated RNA. This was done using the Affinity Script qPCR cDNA synthesis kit (Stratagene, AH Diagnostics, Denmark) as per the manufacturer's instructions. Cycling parameters were 5 min. at 25°C; 15 min. at 42°C; 5 min at 95°C; and 30 min at 25°C using a Mx3000P QPCR system (Stratagene, AH Diagnostics, Aarhus, Denmark). cDNA was stored at -20°C until further processing.

Primers: Primers for the transcript variants of the gene of interest, *KCNN3*, were designed using Primer3 (NCBI), and primer properties were determined by Oligo Analyzer (IDT) software. Primer sets were designed to amplify variant 1-3, respectively. Variant 3 is the only variant that includes exon 4 and the primers were ordered according to the sequence published by Wittekindt et al. [22]. Transcript specific primers were also designed for variant 2. This has an altered N-terminal compared to variant 1 and 3. The primer pair employed for amplification of transcript variant 1 was designed to amplify both variant 1 and 2, as a primer only amplifying variant 1 could not be designed. The primers were ordered from TAG-Copenhagen and purified by MALDI-TOF (Denmark). To ensure primer specificity a BLAST search against the NCBI database was performed, and a melting curve analysis ensured that only one product was amplified. Primer sequences are listed in Table 3. SDHA and TOP1 have formerly been shown to be the most stably expressed genes when comparing myometrium from pregnant and non-pregnant women [17]. Primers for the reference genes SDHA and TOP1 were purchased from Primer Design Ltd (UK).

Gene	Accession number	Primer sequence	Amplicon length (bp)	Amplification efficiency, E (SE)	R^2
KCNN3	NM_002249	F-CGTGTCTGTGAAAGGTACCATGACC	192	1.90 (0.01)	0.988
variant 1+2	NM_170782	R-CACCACGGCCACCACAAGGG			
KCNN3		F- CGCGACATCTGCAAGGACAGT	166	-	-
variant 2	NM_170782	R- AGAGCTGGACTTCACGTGTGTG			
KCNN3	NM_001204087	F-GACCGTCCGTGTCTGTGAAA *	71	1.83 (0.02)	0.984
variant 3	7	R-GGTACCAAGCAGGAAGTGATGAG *			
TOP1	NM_003286	PrimerDesign Ltd	-	1.78 (0.01)	0.996
SDHA	NM_004168	PrimerDesign Ltd	-	1.83 (0.01)	0.993

SDHA: succinate dehydrogenase complex; KCNN3: potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (SK3 protein); TOP1: Topoisomerase (DNA) I

* Primer sequence published by Wittekindt et al. [22]

R^2, correlation coefficient of the slope of the standard curve

Table 3: Stably expressed reference genes, primers and PCR reaction efficiencies.

Quantitative real-time polymerase chain reaction: qRT-PCR was performed using the Brilliant SYBR Green QPCR Master Mix and Mx3000P QPCR system (both from AH Diagnostics, Aarhus, Denmark). Reactions were carried out in 12.5 µl reaction volumes containing 30 ng of cDNA (10 ng/µl) (20 ng of cDNA for SK3.1+2); 6.25 µl brilliant SYBR green mastermix; 0.25 µl (10 pmol/µl) of each primer; and 3.25 µl RNase-free H_2O. Amplification was detected using SYBR green fluorescent dye with the following amplification conditions: i) one 10 min pre-amplification cycle at 95°C; ii) 40 amplification cycles including 30 sec (denaturation) at 95°C; 60 sec (annealing) at 60°C; and 60 sec (extension) at 72°C; iii) 60 sec end-amplification cycle at 95°C, followed by increasing temperature from 55°C to 95°C with continuous reading of fluorescence data (dissociation curve).

Standard curves were performed for the reference genes, and for the gene of interest *(KCNN3)*, by 2-fold serial dilution of a cDNA pool of myometrium (0.58-75 ng), respectively.

No template, and no reverse transcription, controls were performed for each sample and primer pair when appropriate in order to monitor for contamination or primer-dimers. A $\Delta Cq > 5$ between sample and noRT control ensured that the samples were not contaminated by genomic DNA. All reactions on samples were carried out in triplicate, whereas standard curves were performed in duplicate. Cq values were calculated from the exponential phase of amplification when crossing threshold using MxPRO 4.1 software (AH Diagnostics, Aarhus, Denmark). Cq values were transformed into relative quantities using the gene-specific PCR amplification efficiency by geNormPlus software (qbasePLUS, Biogazelle, Primer Design Ltd, UK). A geometric mean of the reference genes, SDHA and TOP1, was employed as the normalization factor in order to calculate the relative expression level of SK3 transcript variants within the two patient groups. Amplicons were sequenced in order to ensure SK3 variant specificity (Beckman Coulter Genomics, UK), and a BLAST search against the NCBI database confirmed that only the gene of interest was amplified.

Data analysis and statistical procedures

The maximal effect (Max, %) was obtained at 60 µM CyPPA (highest concentration tested). The concentration of drug required to elicit half of the maximal effect is known as the IC_{50}. pIC_{50} is defined as -log IC_{50} and was determined by non-linear regression.

Data are expressed as mean ± SE/SEM as appropriate and n refers to the number of patients. Data were tested for statistical differences with Unpaired Student´s *t-t*est or two-way ANOVA and post hoc Bonferroni for comparisons of means where appropriate. $P<0.05$ was considered statistically significant.

For the qRT-PCR experiments, group differences were analyzed using the Mann-Whitney test (qbasePLUS software) and values were presented as mean (95% CI). Statistical significance was designated as $P<0.05$.

Curve fitting of concentration-response curves, statistics and graphical presentation were generated using GraphPad Prism version 5.04 (GraphPad Software, San Diego California USA).

Drugs and chemicals

CyPPA (N-Cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine) (Tocris Bioscience, Denmark) was prepared on the day of experimentation using DMSO. Serial dilutions were made using PSS and maintained at room temperature for the duration of the experiment.

Results

Effects of CyPPA on myometrial contractility

Following equilibration, myometrial strips exhibited a consistent and regular pattern of spontaneous contractions that lasted for the duration of the experiments. For this reason it was not necessary to induce contractions by chemical stimulance.

We investigated the effects of CyPPA on uterine contractions on isolated myometrial strips from non-pregnant and pregnant women. Representative recordings are shown in Figure 1. On exposure to CyPPA (0.1-60 µM) a concentration-dependent reduction of the contractility was recorded (Figure 2). CyPPA completely abolished contractions at 60 µM in both groups. The calculated pIC_{50} value for CyPPA was 5.02 ± 0.08 and 5.16 ± 0.15 for non-pregnant and pregnant myometrium, respectively ($P>0.05$). In both groups the maximal effect was 100% ($n=6$ in each group).

Determination of SK3 transcript variant mRNA expression levels in human myometrium

The relative amount of SK3 transcript in myometrium from pregnant and non-pregnant women was determined by normalizing to the geometric mean of SDHA and TOP1. These reference genes have formerly been evaluated by the geNorm algorithm as the most stably expressed genes when comparing myometrium from pregnant and non-pregnant women [17]. In the present study the reference target stability measure M was 0.34 ± 0.12 which corresponds to stably expressed genes ($M<0.5$). The relative quantity of SK3 transcript variant 3 in pregnant and non-pregnant myometrium was 0.81 (0.56-1.19) and 1.00 (0.34-1.57), respectively (mean (95% CI), $n=11$). Thus, the relative quantity of SK3 transcript variant 3 did not differ between myometrium from pregnant and non-pregnant women (P=0.332) (Figure 3a). In contrast, SK3 transcript variant 2 was not expressed at

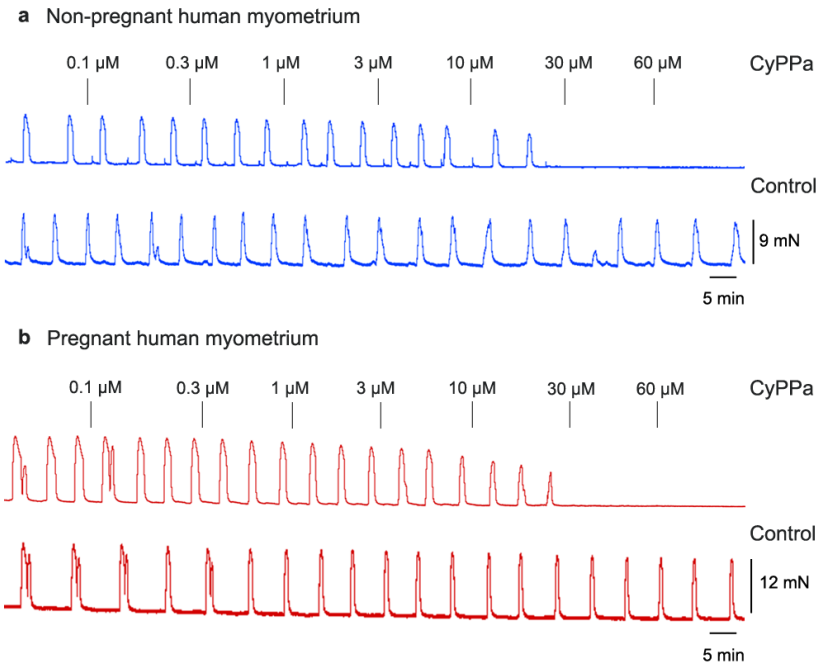

Figure 1: Representative myograph recordings demonstrating the effect of cumulative additions of CyPPA on spontaneously contracting human myometrial strips from non-pregnant (a) and pregnant (b) women and time matched vehicle controls.

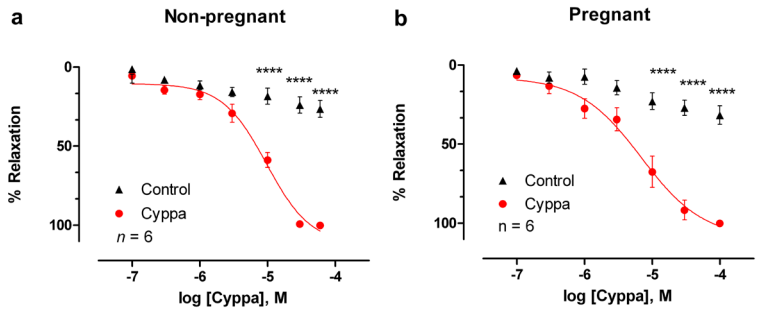

Figure 2: The concentration-response curves show the relaxant effect of cumulative additions of Cyppa on myometrial contractions from non-pregnant (a) and pregnant (b) women. Time-matched vehicle controls are shown for comparisons. Values are normalized to pretreatment, %. **** $P<0.001$ compared to corresponding control values (Two way ANOVA, Bonferroni post hoc t-test).

detectable amounts (Cq>34) when employing 30 ng of input cDNA. Interestingly, when evaluating the expression level of SK3 transcript variant 1 (co-amplified with variant 2), the relative level of mRNA transcript was 0.36 (0.25-0.53) and 1.00 (0.64-1.57), in pregnant and non-pregnant myometrium, respectively (Figure 3b). Accordingly, a 3-fold down-regulation was observed in pregnant compared to non-pregnant myometrium (P=0.002).

Discussion

Our study confirms that SK3 channels are expressed and down-regulated in the human myometrium. More specifically, with the help of qRT-PCR we have elucidated which splice variants are involved in the overall down-regulation of SK3 channels in myometrial tissue. We have also demonstrated that SK channels can be pharmacologically targeted in order to depress the phasic uterine contractions of pregnant, and non-pregnant, women *in vitro*.

In the course of our research, we evaluated 3 splice variants of SK3 channels of which variant 1 (SK3-1A) was seen to be down-regulated 3-fold in late pregnant human myometrium, as compared to non-pregnant tissue. Interestingly, the expression of variant 1 follows the overall expression of SK3 transcript [17], which is why we propose that it may be the variant responsible for the overall down-regulation of the SK3 channels. In contrast, variant 3 (SK3_ex4) was expressed in equal amounts in pregnant, and non-pregnant, myometrium, and variant 2 (SK3-1C) was found not to be expressed to any detectable degree in either of the groups.

We suggest that the identification of a specific expression profile of SK3 splice variants in the human myometrium would give an insight in the myometrial physiology and perhaps pathophysiology, for example at preterm labour. Due to the difficulty in obtaining preterm human myometrial tissue, we cannot investigate the expression profile

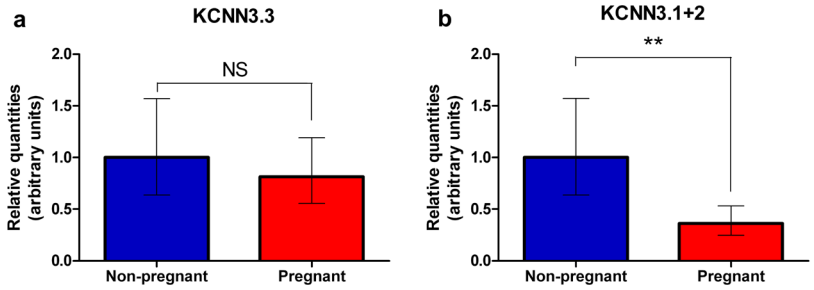

Figure 3: SK3 splice variant expression measured by qRT-PCR. The relative expression of SK3 splice variants is normalized to the geometric mean of the most stable reference genes, SDHA and TOP1. Bar chart showing expression of SK3 variant 3 (a) and variant 1 (co-expressed with variant 2) (b) in non-pregnant (blue) and pregnant (red) human myometrium. Values were presented as mean (95% CI), Mann-Whitney test and * P<0.05.

in this group. Nevertheless, we believe that the present results give some directions for future studies aimed at further elucidating the mechanisms behind the SK3 splice variants in the myometrium.

Having characterized the expression profile of myometrial SK3 splice variants, we tested the effect of CyPPA, a novel SK2 and SK3 positive modulator. Hence, this study shows that CyPPA effectively relaxes myometrial strips originating from both pregnant, and non-pregnant, women. We have previously studied the effect of NS4591 on human myometrial contractility and observed a depression of uterine contractions [11]. However, as this compound does not distinguish between SK channel subtypes and as it activates intermediate-conductance Ca^{2+}-activated K^+ (IK) channels, it cannot fully describe the role of SK channels in human myometrium. On the contrary, CyPPA, a subtype-selective positive modulator of SK channels, has shown selectivity for SK2 and SK3 channels which makes it more appropriate for the aim of this study. We found that CyPPA-related suppression of phasic uterine contractions is concentration-dependent, and that it is equally potent in pregnant and non-pregnant human myometrium. We therefore propose that the mechanism of action of CyPPA could be through activation of splice variant 3, which is expressed in similar amounts in both groups.

Lately, SK channels have been the focus of interest as they have been shown to constitute attractive new pharmacological targets for several diseases [23,24]. Our laboratory, among others, have studied the effect of SK channels modulators on detrusor muscle and showed that it may be a potential target for the treatment of overactive bladder syndrome [25-27]. Taken together, we believe that SK3 channels modulation offers a therapeutic possibility for the treatment of uterine diseases such as preterm labour.

In a clinical context it is important to minimize the toxicity by maximizing the specificity of the therapeutic agent for the target organ. We therefore forward the hypothesis that the identification of a myometrial-specific SK3 splice variant would be conducive to the development of novel and more selective, therapeutics for the management of preterm labour. However, additional research is required to investigate the SK channel profile in human myometrium at preterm, and to confirm that SK channel manipulation can prevent preterm uterine contractions in humans.

In summary, our results show that CyPPA effectively relaxes human uterine muscle strips and that there are two quantifiable SK3 splice variants in human myometrium, with splice variant 1 being responsible for the overall down-regulation of SK3. These findings support the hypothesis that pharmacological activation of SK3 channels reduces human myometrial contractility, and as such may represent a new therapeutic approach for controlling preterm labour.

Acknowledgment

The authors would like to thank Majken M. Janssen at the Smooth Muscle Research Center, Department of Clinical Biochemistry, Koege Hospital for her outstanding technical assistance.

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