Pharmacologic inhibition of small-conductance calcium-activated potassium (SK) channels by NS8593 reveals atrial antiarrhythmic potential in horses

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BACKGROUND Small-conductance calcium-activated potassium (SK) channels have been found to play an important role in atrial repolarization and atrial fibrillation (AF).

OBJECTIVE The purpose of this study was to investigate the existence and functional role of SK channels in the equine heart.

METHODS Cardiac biopsies were analyzed to investigate the expression level of the most prominent cardiac ion channels, with special focus on SK channels, in the equine heart. Subcellular distribution of SK isoform 2 (SK2) was assessed by immunohistochemistry and confocal microscopy. The electrophysiologic and anti-AF effects of the relative selective SK channel inhibitor NS8593 (5 mg/kg IV) were evaluated in anesthetized horses, focusing on the potential of NS8593 to terminate acute pacing-induced AF, drug-induced changes in atrial effective refractory period, AF duration and vulnerability, and ventricular depolarization and repolarization times.

RESULTS Analysis revealed equivalent mRNA transcript levels of the 3 SK channel isoforms in atria compared to ventricles. Immunohistochemistry and confocal microscopy displayed a widespread distribution of SK2 in both atrial and ventricular cardiomyocytes. NS8593 terminated all induced AF episodes (duration $\geq$15 minutes), caused pronounced prolongation of atrial effective refractory period, and reduced AF duration and vulnerability. QRS duration and QTc interval were not affected by treatment.

CONCLUSION SK channels are widely distributed in atrial and ventricular cardiomyocytes and contribute to atrial repolarization. Inhibition by NS8593 terminates pacing-induced AF of short duration and decreases AF duration and vulnerability without affecting ventricular conduction and repolarization. Thus, inhibition by NS8593 demonstrates clear atrial antiarrhythmic properties in healthy horses.

KEYWORDS Horse; Equine; NS8593; Atrial fibrillation; Pacing; Programmed electrical stimulation; Atrial electrophysiology; Antiarrhythmic drugs; Reverse transcription polymerase chain reaction

ABBREVIATIONS **aEGM** = intra-atrial electrogram; **aERP** = atrial effective refractory period; **AF** = atrial fibrillation; **ANOVA** = analysis of variance; **APD** = action potential duration; **cDNA** = complementary DNA; **dV/dt_{max}** = maximum upstroke velocity; **HR** = heart rate; **IV** = intravenous; **RAA** = right atrial appendage; **RT-PCR** = reverse transcription polymerase chain reaction; **RV** = right ventricular free wall; **S-ECG** = surface electrocardiogram; **SK** = small-conductance calcium-activated potassium; **SK1** = small-conductance calcium-activated potassium channel isoform 1; **SK2** = small-conductance calcium-activated potassium channel isoform 2; **SK3** = small-conductance calcium-activated potassium channel isoform 3; **SR** = sinus rhythm; **$T_{½}$** = plasma concentration half-life

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Introduction

Substantial amounts of data have provided evidence of an important functional role of small-conductance Ca^{2+}-activated K^+ channels (SK1–SK3 channels) in atrial cardiomyocytes. Studies have shown that inhibition of these channels displays atrial antiarrhythmic effects across a number of species.[1–4] SK channels are voltage-insensitive K^+ channels activated exclusively by an increase in free

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intracellular Ca^{2+} that interacts with calmodulin bound to the C-terminal region of the SK channels.[5] Activation of the SK channels results in K^+ outflow, thereby contributing to atrial repolarization.[1,6] Since atrial diastolic sarcoplasmic reticulum Ca^{2+} leak seems to be increased during atrial fibrillation (AF),[7] this may lead to an enhanced activation of SK channels, accelerated repolarization, and consequently shortened action potential duration (APD), which could constitute a proarrhythmic substrate for the maintenance of AF.

Genome-wide association studies have linked variants in the KCNN3 gene, which encodes the SK isoform 3 (SK3) channel, to an increased risk of AF in human patients.[8] Overexpression of SK3 has been shown to be proarrhythmic,[9] and studies of experimental AF in various animal models have reported that inhibition of SK channels has antiarrhythmic properties.[1–4] Because SK channels have not been shown to make a considerable contribution to the repolarization of the ventricular action potential,[4,6,10–12] except in failing hearts[13] and acute myocardial infarction,[14] pharmacologic inhibition of SK channels has been proposed as a potential atrial-selective target for treatment of AF. SK channels have been described as functionally important in atrial repolarization in humans,[6,10] dogs,[1] rabbits,[4] guinea pigs,[4] rats,[3,4] and mice,[10] but the existence and possible role of SK channels in the equine heart have not yet been investigated. Horses have an extraordinary cardiac capacity, an impressive ability to increase cardiac output in response to increased requirements, and a relatively large heart compared to other mammals. Horses are under high parasympathetic tone at rest and have an impressive heart rate (HR) span that ranges from approximately 30 bpm at rest to 220 bpm during maximal performance. The heart of the horse is unique, but with respect to AF, the horse shares multiple features with humans. Most importantly, AF is a common arrhythmia in both species. Like humans, horses develop AF both with and without detectable underlying structural heart disease; therefore, the horse may constitute an interesting large-animal AF model.[15]

The compound NS8593 is a potent and relatively selective negative modulator of SK channels that has shown antiarrhythmic potential in multiple species.[1–4,6,16] The negative modulatory effect of NS8593 is exerted via a concentration-dependent rightward shift of the concentration-response curve for Ca^{2+}, making SK channels less sensitive to free intracellular Ca^{2+}.[16,17] NS8593 blocks all 3 SK channel isoforms with equal potency.[16]

The present study was designed to investigate the presence and functional role of SK channels in the equine heart. Specifically, we focused on studying SK isoform mRNA expression and SK protein distribution as well as the electrophysiologic and anti-AF effects after treatment with NS8593 in anesthetized horses.

Materials and methods

Animals

A total of 13 horses were included in the study. Of these, 6 were slaughter horses included in the molecular part of the study and 7 were Standardbred horses included in the *in vivo* part.

Quantitative reverse transcription polymerase chain reaction

In order to investigate the regional expression level of the most prominent calcium, sodium, and potassium ion channels, with special focus on the expression and distribution of SK channels in the equine heart, quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed. Steady-state mRNA transcription from the following genes was investigated: CACNA1C (Cav1.2), SCN5A (Nav1.5), KCND3 (Kv4.3), KCNIP2 (KChiP2), KCNA5 (Kv1.5), KCNH2 (ERG1), KCNQ1 (Kv7.1), KCNJ2 (Kir2.1), KCNJ3 (GIRK1), KCNJ5 (GIRK4), KCNN1 (SK1), KCNN2 (SK2), and KCNN3 (SK3). Cardiac tissue (right atrial appendage [RAA], left atrial appendage, right ventricular free wall [RV], left ventricular endocardium, left ventricular mid-myocardium, and left ventricular epicardium) from 6 slaughtered horses (1 stallion and 5 mares) were included in the *in vitro* study population (age 9.6 ± 2.3 years). Inclusion criteria were a history of absent cardiac-related diseases of any kind. Detailed information regarding the collection of tissue is provided in the Online Supplemental Appendix, including Table A1. The tissue samples were homogenized in Precellys24 (Bertin Technologies, Paris, France) and purified with Tri Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Chloroform was added to separate the RNA fraction from DNA and proteins. RNA was extracted and washed several times with a 70% alcohol solution. Successful RNA extraction was evaluated on 1% agarose gels to verify the presence of the ribosomal RNA 28S and 18S. RNA concentration was manually estimated using the RNA ladder as reference (150 ng/μL). Furthermore, RNA was reverse transcribed and copied into a complementary DNA (cDNA) sequence, using random sequence oligonucleotide primers. cDNA was synthesized from 2 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Naerum, Denmark). Quantitative RT-PCR was performed using TaqMan Gene Expression (Life Technologies, Naerum, Denmark) with specific probes and primers targeting the cDNA sequences of interest (see Online Supplemental Appendix). The efficiency of each assay was evaluated as previously described.[18] Each of the 13 genes of interest from the 6 described cardiac regions from 6 hearts was analyzed in triplets on the 7300 real-time PCR System (Applied Biosystems). All data were normalized to the highly conserved gene expression of ACTB (β-actin). In each experiment, 3 sample-free wells were included, testing the plate for DNA contamination. Relative gene expression was analyzed using the $2^{-\Delta Ct}$-method.[18,19]

Immunohistochemistry and confocal microscopy

To confirm the expression and determine the subcellular distribution of SK2 protein, immunohistochemistry with confocal microscopy using thin slices of equine RAA and