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Pro-arrhythmic Effect of Blocking the Small Conductance Calcium Activated Potassium Channel in Isolated Canine Left Atrium

Chia-Hsiang Hsueh, PhD, Po-Cheng Chang, MD, Yu-Cheng Hsieh, MD, PhD, Thomas Reher, MA, Peng-Sheng Chen, MD, and Shien-Fong Lin, PhD

Krannert Institute of Cardiology and Division of Cardiology, Indiana University, Indianapolis, Indiana

Abstract

Background—Small conductance calcium activated potassium channels (SK_{Ca}) are voltage insensitive and are activated by intracellular calcium. Genome wide association studies revealed that a variant of SK_{Ca} is associated with lone atrial fibrillation (AF) in humans. Roles of SK_{Ca} in atrial arrhythmias remain unclear.

Objective—The purpose of this study was to determine roles of SK_{Ca} in atrial arrhythmias.

Methods—Optical mapping using isolated canine left atrium was performed. The optical action potential duration (APD) and induction of arrhythmia were evaluated before and after the addition of specific SK_{Ca} blockers, Apamin or UCL-1684.

Results— SK_{Ca} blockade significantly increased APD₈₀ (188 ± 19 ms vs 147 ± 11 ms, $p < 0.001$). The pacing cycle length (PCL) thresholds to induce 2:2 alternans and wave breaks were prolonged by SK_{Ca} blockade. Increased APD heterogeneity was observed following SK_{Ca} blockade, as measured by the difference between maximum and minimum APD (39 ± 4 ms vs 26 ± 5 ms, $p < 0.05$), by standard deviation (12.43 ± 2.36 ms vs 7.49 ± 1.47 ms, $p < 0.001$), or by coefficient of variation ($6.68 \pm 0.97\%$ vs $4.90 \pm 0.84\%$, $p < 0.05$). No arrhythmia was induced at baseline by S1–S2 protocol. After SK_{Ca} blockade, 4 out of 6 atria developed arrhythmia.

Conclusion—Blockade of SK_{Ca} promotes arrhythmia and prolongs the PCL threshold of 2:2 alternans and wave breaks in the canine left atrium. The proarrhythmic effect could be attributed to the increased APD heterogeneity in the canine left atrium. This study provides supportive evidence of GWAS studies showing association of KCNN3 and lone AF

Keywords

Atrial arrhythmia; SK_{Ca} ; action potential duration; repolarization; optical mapping

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Corresponding Author: Shien-Fong Lin, PhD, 1801N. Capitol Ave, E375, Indianapolis, IN 46202, Fax: 317-962-0100, Phone: 317-962-0121, linsf@iu.edu.

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Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and contributes to significant morbidity and mortality.¹ Efficient pharmacological treatment for AF is currently limited and many approaches come with considerable risk of adverse effects. Numerous factors are involved in the pathogenesis of AF.^{2, 3} Specifically, genetic variations in ion channels that predispose to AF have been reported.^{2, 3} The structural and ionic remodeling during AF further leads to the complexity of AF studies. This raises the importance of understanding the role of ion channels in the genesis of AF.

Small conductance calcium activated potassium channel (SK_{Ca}) is voltage independent and is activated by intracellular calcium of submicromolar concentrations.⁴ SK family was first cloned by Adelman and his colleagues in 1996.⁵ It consists of three members (SK1, SK2, and SK3) and is characterized by the specific blockade by apamin.⁶ Although the calcium sensitivity (K_d= 0.6–0.7 μ M) and conductance (9.2– 9.9 pS) are similar among three subtypes, the sensitivity to pharmacological modulation and tissue distribution differs.^{6–9} It has been shown that SK1 and SK2 are predominantly abundant in mouse atria while SK3 expresses equally in atria and ventricles.⁸ In humans, the distribution is different. SK2 and SK3 are more abundant than SK1 in atria.⁹ In the heart, SK_{Ca} plays an important role in action potential duration (APD), contributing to the cardiac repolarization current.^{7, 8} Despite the abundance of SK_{Ca} in the atrium, whether SK_{Ca} plays a role in atrial arrhythmogenesis remains unclear.

Genome-wide association studies (GWAS) showed two genetic variations of KCNN3 (gene encoding SK3) to be associated with lone AF.^{10, 11} One of those variations is an intronic single nucleotide polymorphism (SNP) and the other is a synonymous SNP. Although no amino acid is changed by these identified SNPs, it is possible that they are linked to an unidentified non-synonymous SNP in KCNN3 that leads to increased susceptibility of lone AF. Physiological evidence of a role for SK_{Ca} in atrial arrhythmia comes from studies on SK2.^{12, 13} A Potential relationship between SK2 expression and atrial remodeling in AF has been shown. For example, intermittent burst pacing in rabbit hearts increases SK2 mRNA and protein, and the trafficking of SK2 to membrane is increased which leads to increased apamin-sensitive current and shortened APD.¹² Moreover, SK2 knock-out mice show prolonged APD and increased AF inducibility.¹³ To extend these previous studies to large animal models, we used normal canine atria to clarify the role of SK_{Ca} in atrial arrhythmogenesis. We hypothesize that SK_{Ca} plays a protective role in structurally normal atrium and SK_{Ca} blockade promotes arrhythmia.

Methods

The animal protocol was approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine and the Methodist Research Institute, Indianapolis, IN, and conforms to the guidelines of the American Heart Association.

Canine left atrial tissue preparation

Male mongrel dogs weighed 25–30kg were used in this study (N=9 for optical mapping, and N=5 for Western blotting). They were fully anesthetized by isoflurane and were euthanized to obtain hearts by thoracotomy. The atrial tissue preparation was performed as previously described with some modification.¹⁴ Briefly, the heart was harvested and perfused with cardioplegic solution. The cardioplegic solution was composed of (in mM): 129 NaCl, 12 KCl, 0.9 NaH₂PO₄, 20 NaHCO₃, 1.8 CaCl₂, 0.5 MgSO₄, and 5.5 glucose. Left coronary artery was then cannulated through its aortic orifice. Subsequently, the right atria and the ventricles were surgically removed. All open vessels on the border of the remaining left

atrial tissue were ligated. Afterwards oxygenated Tyrode's solution was perfused through the left circumflex. The compositions of Tyrode's solution was as follows (in mM): 125 NaCl, 4.5 KCl, 0.5 MgCl₂, 24 NaHCO₃, 1.8 NaH₂PO₄, 1.8 CaCl₂, 5.5 glucose, and 2% bovine serum albumin, equilibrated with 95% O₂ and 5% CO₂ to maintain a pH of 7.4.

Optical mapping system

The perfusate was maintained at 37°C with a flow rate around 30 ml/min. After stabilizing the preparation, 10 µM voltage sensitive dye, RH237 was added to the perfusate along with 15 µM excitation-contraction uncoupler, blebbistatin. A laser light at 532 nm was used to excite the stained atrial preparation and fluorescence was collected using a CMOS camera (BrainVision, Tokyo, Japan). It recorded voltage fluorescence through a 710 nm long-pass filter. Fluorescence image was captured at frame rate with 2 ms/frame and at 100 × 100 pixels with spatial resolution of 0.35 × 0.35 mm²/pixel for 4s. To determine the rhythm of the atrium, pseudo-ECG was utilized. It was obtained with widely spaced bipolar electrode. The signals were filtered from 0.05 to 100 Hz, and digitized at 1 kHz with AxoScope.

Pacing protocol and Induction of atrial arrhythmia

A bipolar pacing lead was placed at the apex of the appendage with an output at 2.5 times the diastolic threshold. Optical recording was performed after 40 beats of stable pacing at each PCL. The pacing cycle length (PCL) was progressively shortened from 1500ms (1500ms, 1000ms, 800ms, 500ms, 450ms, 400ms, 350ms, 300ms, 280ms, 250ms, 220ms, 200ms, 190ms, 180ms, 170ms, 160ms, etc.) until loss of capture. After five minutes recovery, arrhythmia inducibility was evaluated by a S1–S2 protocol, consisting of continuous 9 beats S1 stimuli followed by an additional S2 beat. An episode with more than two extra beats resulted from reentry were regarded as a successful arrhythmia induction. The same procedure was repeated after 30 minutes of perfusion with 100 nM of apamin (Tocris Bioscience, MN, USA) or 100nM UCL-1684 (Tocris Bioscience, MN, USA). In this study, 5 atria were treated with apamin and 4 were treated with UCL-1684 respectively. The arrhythmia induction protocol was performed in 6 atria among the 9 atria that were optically mapped. Two atria were treated with apamin and four with UCL-1684 respectively.

Proteins Extraction

Left atrium appendage (LAA) was cut into 8 pre-defined pieces and homogenized by POLY-TRON in RIPA buffer containing protease inhibitors (50 mM Tris pH 8.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate 1 mM PMSF, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, and 5 µg/ml aprotinin). Homogenates were incubated on ice for 30min and then centrifuged at 14,000 rpm for 15 minutes. After collection of the supernatant, protein concentration was quantified by Bradford assay (Bio-Rad, Hercules, USA) using bovine serum album (BSA) as the standard.

Western blotting

Protein electrophoresis was done using Bio-Rad mini gel system (Bio-Rad, Hercules, CA) and transferred to PVDF membrane (Millipore, Billerica, MA). The membrane was bathed in TBS with 5% milk for one hour, and probed with anti-KCNN2 antibody (Abcam, ab83733, 1:2500) or anti-GAPDH antibody (Pierce, MA1-22670, 1:5000) overnight. Following primary antibody incubation, the membrane was incubated with HRP-conjugated secondary antibodies (Sigma, 1:5000) for 30 min. Finally, Luminata Crescendo HRP substrate (Millipore, WBLUR100) was added on the membrane according to manufacturer's instructions.

Statistics

Results were summarized by typical parameters such as mean $\pm$ standard deviation and compared using ANOVA test or paired T test. Post-test comparison was conducted by Tukey test (one way) or Bonferroni test (two way). All statistics were calculated by Prism 5.0 (Graphad, CA, USA). P value less than 0.05 was regarded as statically significant.

Results

Effect of SK_{Ca} blockade on action potential duration

Pharmacological blockade of SK_{Ca} was used to study its role in atrial electrophysiology. SK_{Ca} blockade resulted in APD prolongation as observed by optical mapping. The experimental configuration of atrium preparation is shown in Figure 1A. Optical images were captured from the left atrium appendage (LAA). The relationship between APD₈₀ and PCLs was shown in figure 1B and 1C. The differential effects of apamin and UCL-1684 did not show statistical significance ($p=0.994$ by ANOVA). A biphasic relationship between APD₈₀ and PCLs was observed at baseline. APD₈₀ increased proportionally to the PCLs at short and intermediate PCLs. The maximum APD₈₀ occurred at the PCL of 500ms. APD₈₀ gradually decreased as PCLs increased longer than 500ms. After SK_{Ca} blockade, APD₈₀ was generally prolonged at all PCLs. The effect of SK_{Ca} blockade was more obvious at long PCLs (Figure 1B and 1C). At PCL of 1500ms, blocking of SK_{Ca} prolonged the APD₈₀ from 147 ± 11 ms to 188 ± 19 ms ($p<0.001$). However the APD₈₀ at PCL of 250ms were comparable between baseline and SK_{Ca} blockade (129 ± 2 ms vs. 139 ± 3 ms). The percentage of prolongation at PCLs of 1500, and 250ms were $23.54\pm 6.54\%$, and $6.91\pm 2.40\%$ respectively

Increased APD heterogeneity by SK_{Ca} blockade

ADP heterogeneity has been recognized as an important mechanism contributing to reentrant atrial arrhythmia.¹⁵ The representative APD maps in figure 2A are evidence of increased APD heterogeneity by SK_{Ca} blockade. Even at the baseline, APD was not homogeneous. The increased color gradient after SK_{Ca} blockade represents increased heterogeneity. To quantify the ADP heterogeneity, three different measurements were used. In the first measurement of APD heterogeneity, the maximum and minimum APD₈₀ was calculated (Figure 2B). This difference was increased from 26 ± 5 ms to 39 ± 4 ms after SK_{Ca} blockade ($p<0.05$). Standard deviation (SD) generated from optically imaged region was used as the second method to measure ADP heterogeneity (Figure 2C). At baseline, the average SD was 7.49 ± 1.48 ms. After SK_{Ca} blockade, it was increased to 12.43 ± 2.36 ms ($p<0.001$). The last measurement was to calculate the coefficient of variation (COV). This was used to determine the increased SD due to increased APD. Figure 2D showed that COV was also increased from $4.90\pm 0.84\%$ to $6.68\pm 0.97\%$ by SK_{Ca} blockade ($p<0.05$). This suggested that increased SD by SK_{Ca} blockade did not simply result from increased APD but also from increased heterogeneity of APD. All three measurements showed statistically significant increased APD heterogeneity by SK_{Ca} blockade.

Detection of SK2 and its relationship to APD prolongation

LAA was divided into 8 regions to study the increased APD heterogeneity by SK_{Ca} blockade (Figure 3A). APD maps from different animals showed different patterns of APD prolongation. Some atria showed significant APD prolongation while others showed little APD prolongation. Western blotting was used to quantify the distribution of SK_{Ca}. The distribution was shown in Figure 3B. After optical mapping, we collected three atria and analyzed the distribution of SK2 and the corresponding percentage of APD prolongation. When curve fitting was performed using all data points, the correlation was statistically

significant with R^2 equaling 0.2388 ($p < 0.05$), suggesting that SK2 participated in our observation of APD change.

SK_{Ca} blockade facilitated development of 2:2 alternans and wave breaks

At rapid PCLs, loci with conduction delay were sometimes observed (Figure 4A) and this phenomenon was exaggerated by SK_{Ca} blockade. The 2:2 alternans initially occurred at regions of conduction delay and eventually spread throughout all regions of the LAA. Wave breaks were even observed in some atria when PCLs were further shortened. At baseline, five atria developed 2:2 alternans followed by wave breaks. Four showed 2:1 capture without any observation of wave breaks. After SK_{Ca} blockade, eight atria developed 2:2 alternans and all showed wave breaks at shorter PCLs. As shown in figure 4C, PCL threshold of for 2:2 alternans was significantly longer by the SK_{Ca} blockade (162 ± 9 ms vs 130 ± 6 ms at the baseline, $p < 0.05$). PCL threshold for wave breaks induction was also longer (153 ± 11 ms vs 118 ± 9 ms at the baseline, $p < 0.05$).

SK_{Ca} blockade promoted development of reentrant arrhythmia

At baseline, we did not observe any episode of arrhythmia. Atrial arrhythmia could be induced in one out of two atria in the presence of apamin, and three out of four in the presence of UCL-1684. As a total, atrial arrhythmia could be induced in four out of six atria after SK_{Ca} blockade. Sustained arrhythmia was observed in one atrium treated with UCL-1684. Optical mapping revealed that arrhythmia emanated from local conduction block and led to the occurrence of reentrant arrhythmia (Figure 5). In panel A, the APD is heterogeneously distributed at baseline. After SK_{Ca} blockade, APD becomes more heterogeneous. This led to partial atrial activation by a premature stimulation (S2) given at the apical region (asterisk on isochrones map).

Discussion

SK_{Ca} blockade in canine atrium

In this study, we used two different SK_{Ca} blockers to study the role of SK_{Ca} in canine atria. The atrial APD prolonged after the addition of specific SK_{Ca} blockers. Western blotting showed the presence and a heterogeneous distribution of SK2. Apamin is an 18 amino acid peptide neurotoxin and is a classical SK_{Ca} blocker. It has been reported that apamin binds to S3–S4 loop of SK_{Ca} and acts as a pore blocker.¹⁶ UCL-1684 is a potent non-peptide SK_{Ca} blocker. It shares a similar mechanism as apamin through regulation of S3–S4 loop.¹⁷ However it exerts its effect by allosteric modulation without binding to S3–S4 loop.¹⁷ The application of blockers with different mechanisms reduces the risk of off-target effect. The results using apamin and UCL-1684 were comparable, suggesting that our observations come from specific SK_{Ca} blockade. Moreover the plotting of SK2 against percentage of APD prolongation yielded a statistically significant correlation. The value of R^2 was small in such a correlation. This could be due to that SK2 is not the only SK_{Ca} in the atrium although it might be the most abundant.

Mathematical modeling of human atrial action potential using 12 different transmembrane currents has shown a biphasic relationship between APD and PCL.¹⁸ This resembles our observations of biphasic APD–PCL relationship at baseline. SK_{Ca} blockade transformed it into monophasic, suggesting the importance of SK_{Ca} in atrial APD regulation and APD adaption. SK_{Ca} blockade prolonged APD especially at long PCLs. This was consistent with our previous finding in failing rabbit ventricles (unpublished data). We have previously found that the effect of SK_{Ca} blockade is most obvious at very long and very short PCL. The effect of SK_{Ca} blockade is minimal at intermediate PCLs. This study does not compare APDs shorter than 250ms due to the occurrence of alternans. Theoretically, tachycardia

would facilitate the accumulation of intracellular calcium that activates SK_{Ca}. Hence it would be expected to contribute to considerable cardiac repolarization power during tachycardia.

Ion channel remodeling during AF promotes APD shortening.³ It is generally accepted that pharmacologically prolonged APD might prevent AF. Interestingly, the results of SK_{Ca} blockade showed paradoxical effects. It promoted arrhythmia despite APD prolongation.

Increased APD heterogeneity by SK_{Ca} blockade

We observed APD heterogeneity throughout the LAA. SK_{Ca} blockade significantly enhanced APD heterogeneity. Increased APD heterogeneity might lead to increased susceptibility of arrhythmia.¹⁵ Our finding clearly supports this phenomenon. After SK_{Ca} blockade, APD became more heterogeneous. When an additional stimuli (S2) was applied, only a few regions could be activated while the rest of LAA remained in effective refraction period (ERP). This led to an uneven pattern of atrial activation and eventual reentry arrhythmia.

Appearance of local conduction delay and arrhythmia induction

The occurrence of 2:2 alternans was reported to be the result of diffusion current between regions of 1:1 capture and 2:1 capture.¹⁹ Although we could not identify any regions with 2:1 capture within the optically imaged area, we found that there were certain regions that showed conduction delay during rapid pacing. The occurrence of 2:2 alternans initiated from these regions and spread to the other regions if PCLs was further reduced. Blockade of SK_{Ca} aggravated the development of local conduction delay and hence prolonged the PCL threshold of 2:2 alternans. This might be the result of increased APD heterogeneity by SK_{Ca} blockade. It has been shown that local conduction abnormality will lead to disturbance in propagation, wave breaks and reentry arrhythmia.^{20, 21} Similar phenomena were observed in this study. SK_{Ca} blockade promoted the atria to develop wave breaks and reentrant arrhythmia. The PCL threshold for induction of wave breaks was longer after SK_{Ca} blockade. Reentrant arrhythmia was only seen by SK_{Ca} blockade. Furthermore, failure of proper APD adaption might contribute to a substrate of arrhythmia.²² SK_{Ca} might be involved in the process of APD adaption since its blockade increased APD heterogeneity. Consequently, SK_{Ca} blockade facilitated the development of local conduction delay, wave breaks, and reentrant arrhythmia.

Conclusion

This study provides supportive evidence of GWAS studies showing association of KCNN3 and lone AF.^{10, 11} We show that SK_{Ca} plays a protective role against atrial arrhythmia, and SK_{Ca} blockade promotes atrial arrhythmia. Our results are in agreement with the observation that SK2 KO mice have increased AF susceptibility.¹³ On the other hand, Diness et al. suggested that pharmacological inhibition of SK_{Ca} prevents the onset and maintenance of AF in isolated rat and rabbit hearts.²³ Such a result is opposite to our current findings in canine atria and might be due to difference in species. Canine atrium has been considered as a good animal model for human atrial arrhythmias. However further works are required to extend the current findings to humans.

Study Limitation

We have reported distinct transmural expression of SK2 in failing human ventricles.²⁴ It might be possible that atrium also expresses differential SK2 channels transmurally. Due to technical limitation of the optical mapping measurement, it is not feasible to perfectly match area of tissue for Western blotting and APD analysis. However, the curve fitted with SK2

does yield a statistically significant correlation. We did not systematically test the difference of APD distribution with respect to pacing site. It is unclear whether pacing the atrium at difference locations alters the APD distribution.

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Figure 1. Effect on atrium by SK_{Ca} blockade

A. Configuration of isolated canine left atrium preparation and corresponding APD change. The black square identifies the optically imaged region. The corresponding APD is shown in the left panel. Note that the pattern of APD prolongation is very heterogeneous. LOM, Ligament of Marshall. LAA, left atrium appendage. **B. Relationship between PCL and APD₈₀.** A biphasic relationship between APD and PCL was observed at baseline. APD increased as PCL increased from 250ms to 500ms. When PCL was longer than 500ms, APD shortened. SK_{Ca} blockade abolished this phenomenon (N=9, 5 with apamin treatment and 4 with UCL-1684 treatment). Comparison was performed between baseline and SK_{Ca} blockade (apamin and UCL-1684). Star (*) denotes the post test p-values following ANOVA test. **C. Percentage of APD prolongation by SK_{Ca} blockade at different PCLs.** SK_{Ca} blockade showed a tendency to prolong APD in all PCLs. The effect was most significant at long PCLs. Star (*) denotes the p-values from post test when compared with 1500ms *, p<0.05, **, p<0.01. ***, P<0.001

Figure 2. Increased APD heterogeneity by SK_{Ca} blockade

A. Examples of APD map at different PCLs. The orientation of LAA is illustrated by the diagram. Increased APD heterogeneity is demonstrated by increased color gradient in APD maps after blockade by UCL-1684. ΔAPD reveals heterogeneous prolongation of SK_{Ca} blockade. **B, C, and D. Quantification of APD heterogeneity.** All three measurements indicate that SK_{Ca} blockade increases APD heterogeneity (N=9, 5 with apamin treatment and 4 with UCL-1684 treatment). Comparison was performed between baseline and SK_{Ca} blockade (apamin and UCL-1684). Star (*) denotes the post test p-values following ANOVA test. *, p < 0.05. **, P < 0.01. ***, P < 0.001

Figure 3. Detection of SK2 and its relationship with APD prolongation

A. Top left is an illustration showing the 8 predefined regions which the LAA was dissected into. **Top right** shows percentage of APD prolongation by SK_{Ca} blockade in different regions (n=9, 5 with apamin treatment and 4 with UCL-1684 treatment). Insets are representative Δ APD maps from two different LAAs showing heterogeneous APD prolongation by SK_{Ca} blockade. **B. SK_{Ca} protein expression at different regions** (n=5). A representative Western blotting image is shown in the inset. **C. Relationship of SK2 proteins and APD prolongation.** The abundance of SK2 protein is plotted against the percentage of corresponding APD prolongation (n=3). Among them, #1 and #2 were treated with apamin while #3 was treated with UCL-1684. The formula and value of R^2 are displayed. The plotting from all points yields a significant linear correlation with R^2 equaling to 0.2388 ($p=0.0154$). *, $p<0.05$., ***, $P<0.001$

Figure 4. Development of 2:2 alternans and wave breaks

A. Representative traces of 2:2 alternans. Data were acquired from an atrium at baseline. Corresponding isochronal maps and APD maps from beat 1 and beat 2 are shown. Note that there is a region showing conduction delay (#4 and #5). The site that developed 2:2 alternans lies within the region showing conduction delay. **B. Ratio of consecutive beats showing conduction efficiency.** Beat 2/beat 1, beat 4/beat3, and beat 6/beat5 from Figure 3A are plotted. The conduction efficiency decreases gradually from #1 to #5. **C. SK_{Ca} blockade prolonged the PCL threshold of 2:2 alternans and wave breaks.** Wave breaks could only be induced in 5 atria at baseline while it was induced in all atria after SK_{Ca} blockade. *, $p < 0.05$. ***, $P < 0.001$

Figure 5. Induction of reentry arrhythmia by SK_{Ca} blockade

A. APD maps and the corresponding ΔAPD map from the same atrium showing the pattern of APD change after SK_{Ca} blockade. Maps were generated from PCL of 1500ms.

B. Representative arrhythmia induced by S1–S2 protocol. Additional pacing (S2) after a stimuli was used to induce arrhythmia. S1, regular pacing at 400ms, S2, additional pacing 160ms after a S1 pacing. No arrhythmia could be induced at baseline while 4 out of 6 atria developed arrhythmia after SK_{Ca} blockade. **a) Isochronal maps showing the conduction patterns.** Star symbol (*) indicates the pacing site. Only parts could be activated, as revealed by S2 isochrone. **b) Corresponding voltage maps and phase maps.** Arrows in voltage map show the propagation of reentry. Time label represents time interval after S2 pacing.