Early electrical remodeling in rabbit pulmonary vein results from trafficking of intracellular SK2 channels to membrane sites

Nazira Özgen a,b,1, Wen Dun b,1, Eugene A. Sosunov a,b, Evgeny P. Anyukhovsky a,b, Masanori Hirose b, Heather S. Duffy b, Penelope A. Boyden b, Michael R. Rosen a,b,c,*

a Center for Molecular Therapeutics, College of Physicians and Surgeons of Columbia University, New York, New York, United States
b Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, New York, United States
c Department of Pediatrics, College of Physicians and Surgeons of Columbia University, New York, New York, United States

Received 30 January 2007; received in revised form 1 May 2007; accepted 2 May 2007

Abstract

Objective: Atrial fibrillation is often initiated by bursts of ectopic activity arising in the pulmonary veins. We have previously shown that a 3-h intermittent burst pacing protocol (BPP), mimicking ectopic pulmonary vein foci, shortens action potential duration (APD) locally at the pulmonary vein–atrial interface (PV) while having no effect elsewhere in rabbit atrium. This shortening is Ca2+ dependent and is prevented by apamin, which blocks small conductance Ca2+-activated K+ channels (SKCa). The present study investigates the ionic and molecular mechanisms whereby two apamin-sensitive SKCa channels, SK2 and SK3, might contribute to the regional APD changes.

Methods: Microelectrode and patch clamp techniques were used to record APDs and apamin-sensitive currents in isolated rabbit left atria and cells dispersed from PV and Bachmann’s bundle (BB) regions. SK2 and SK3 mRNA and protein levels were quantified, and immunofluorescence was used to observe channel protein distribution.

Results: There was a direct relationship between APD shortening and apamin-sensitive current in burst-paced but not sham-paced PV. Moreover, apamin-sensitive current density increased in PV but not BB after BPP. SK2 mRNA and protein levels were quantified, and immunofluorescence was used to observe channel protein distribution.

Conclusions: BPP-induced acceleration of repolarization in PV results from SK2 channel trafficking to the membrane, leading to increased apamin-sensitive outward current. This is the first indication of involvement of Ca2+-activated K+ currents in atrial remodeling and provides a possible basis for evolution of an arrhythmogenic substrate.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Arrhythmias; Ion channels; K channels

1. Introduction

Pulmonary vein–atrial junctions are important sites of ectopy that induces atrial fibrillation (AF) [1,2]. The paroxysms of AF alter atrial electrical properties in a manner that promote AF initiation and maintenance — a process called electrophysiologic remodeling [3]. The major electrophysiologic characteristics of this remodeling are a reduction in the atrial effective refractory period and loss of its adaptation to rate changes [3–5]. In animal models, remodeling is produced by continuous high rate pacing through electrodes located at arbitrary atrial sites. Yet we previously reported that early stages of the remodeling process (within hours) depend critically not only on rate but on site and pattern of ectopic activity [6]. In this study of isolated rabbit atria, we noted that a 3 h intermittent burst pacing protocol mimicking ectopic foci in pulmonary veins alters the atrial repolarization gradient by shortening action potential duration (APD) locally at the pulmonary vein–
atrial interface while having no effect elsewhere in the atrium [6]. When the site of burst rapid pacing was shifted from pulmonary vein to Bachmann’s bundle region, no repolarization changes were seen [6]. We found the burst pacing-induced APD shortening in pulmonary vein region to be calcium-dependent and prevented by nifedipine, ryanodine and apamin [6]. The latter venom blocks small conductance Ca2+-activated K+ channels (SKCa) [7]. These data suggested that SKCa subunits are responsible for APD shortening of pulmonary vein induced by the burst pacing protocol.

Recent studies have confirmed the presence of SKCa channels in human and mouse cardiac myocytes with more abundant channels in the atria compared with the ventricles [8,9]. Each subtype of SKCa channel family, SK1, SK2 and SK3, has a different sensitivity to apamin: SK2 channels are the most sensitive and SK1 channels, the least [10,11]. However no study has revealed how or if these currents are involved in early atrial remodeling. In the present study we hypothesized that SK2 and/or SK3 channels are involved in burst pacing-induced APD shortening in the pulmonary vein region. We asked whether there are changes in apamin-sensitive current and whether any changes of SK2 and/or SK3 that occur are transcriptional and/or posttranslational. As shall be demonstrated for the first time, SK2 channels are present in rabbit atrium and brief periods of pulmonary vein ectopic activity are sufficient to traffic the channel proteins to membrane sites, resulting in increased apamin-sensitive current, electrophysiologic remodeling and the evolution of an arrhythmogenic substrate.

2. Methods

All experiments were performed according to protocols approved by the Columbia University Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85–23, revised 1996).

2.1. Electrophysiological studies

Male New Zealand White rabbits (3 months old, 2–2.5 kg) were anesthetized with sodium pentobarbital (30 mg/kg i.v.), heparinized and the left atria removed, opened, and pinned in a tissue bath, endocardial surface up. The atria were superfused with Tyrode’s solution containing (mmol/L): NaCl 131, NaHCO3 18, KCl 4, CaCl2 0.9, MgCl2 0.5, NaH2PO4 1.8, Na2HPO4 0.47, glucose 5.6, phenol red 0.056 (mM), MEM amino acid and vitamin (GIBCO) pH 6.7. Collagenase (Worthington type II) was added to a final concentration of 1200 U/ml. The tissue was rotated at 37 °C and agitated at a rate 1–2 cycles/s for 35 min. The tissue was then placed in a tube containing 3–4 ml of HEPES-buffered salt solution with 145 mM potassium glutamate 5.7 mM MgCl2 at pH 6.72 and agitated for 10 min at 37 °C. The softened tissue piece was broken up by gentle pipetting with a Pasteur pipette (20 titrations, removal of supernatant and repeated 3 times). The dispersed cells were centrifuged and resuspended in MEM (pH 7.3). Resuspension solution was changed every 15 min for solution containing increasing concentrations of Ca2+ (0 to 0.5 mM). The entire preparation procedure required about 2 h. The cells were maintained in this solution at room temperature until needed for patch-clamping. For patching, an aliquot of cells was transferred onto a poly-lysine coated glass coverslip placed at the bottom of a 0.5 ml tissue chamber mounted on the stage of a Nikon inverted microscope (Nikon Diaphot, Tokyo, Japan). Cells were continuously superfused (2–3 ml/min) with normal Tyrode’s solution containing (in mM): NaCl 137, NaHCO3 24, NaH2PO4 1.8, MgCl2 0.5, CaCl2 2.0, KCl 4.0 and dextrose 5.5 (pH=7.4). The solution was bubbled with 5% CO2/95% O2.

To measure Ca2+-activated K+ currents, patch pipette with resistances 2–3 MΩ was filled with the internal solution (in mM): KCl 140, MgCl2 1.0, EGTA 3, HEPES 10, Mg–ATP 5, Na2–creatinine phosphate 5, pH 7.2. Cells were superfused with a Na+-free solution (mM): N-methyl-D-glucamine Cl 144, HEPES 10, KCl 5.4, CaCl2 5, MgCl2 1.0, pH 7.4, temperature: 30 °C. A voltage clamp protocol including 4
preconditioning depolarizing clamp steps (−70 mV to +20 mV, duration of the pulse=200 ms, repetition rate=1.2 s) was used to load Ca\(^{2+}\) and thus to study currents under similar SR loads. Apamin-sensitive peak currents were assessed using 210-ms voltage step from Vts +30 to +60 mV in 10 mV increments from a holding potential of −70 mV at 0.1 Hz after the preconditioning steps in the absence (control) and presence of apamin (100 nM). As in our studies of Ca\(^{2+}\) dependent chloride currents in canine cells [12], we selected internal and external solutions that would minimize internal Ca buffering. However, as in our earlier ventricular studies, we found that 5 mM and 10 mM EGTA were excessive and buffered almost all Ca\(^{2+}\). Under these conditions we were unable to reliably see any Ca\(^{2+}\) dependent currents. Thus we used 3 mM EGTA in all cell types. Under these conditions we were able to see a difference in the peak current blocked by apamin (apamin-sensitive currents). Finally, we did not measure Ca\(^{2+}\) under the conditions of our patch experiments but did note that the cells were relaxed and could withstand a seal and voltage clamp protocol.

### 2.3. Real-time PCR

Total RNA was extracted using the RNeasy midi-kit (Qiagen). First-strand cDNA was generated from 0.2 μg of total RNA using SuperScript First-Standard Synthesis System (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using a LightCycler (Roche) with a reaction mixture composed of 2 μL of the cDNA mixture, 2 μL of LightCycler — Faststart DNA master SYBR Green I (Roche), 4 mmol/L Mg\(^{2+}\), and 0.5 μmol/L of each primer in a final volume of 20 μL. Primer pairs were:

SK2: GCTCGGTCTCGATGAC (forward),
GCAAGAAGAAGAACCAGAAC (reverse),
SK3: TTGCCCAACTCCAGAGC (forward), CAAGCAATGGTCATTTGAATT (reverse),
Cyclophilin A: CCAACACAAACGGCTC (forward), GCACACCGGAACCAA (reverse).

After an initial denaturation at 95 °C for 10 min for amplification was carried out under different conditions for different primers: SK2 — 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 7 s, extension at 72 °C for 8 s, SK3 — 47 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 7 s, extension at 72 °C for 5 s, cyclophilin A — 35 cycles of denaturation at 95 °C for 10 s, annealing at 63 °C for 10 s, extension at 72 °C for 11 s. Fluorescence (arbitrary units) was measured at the end of each extension step and the quantitative comparison of mRNA was done based on a standard curve generated from a 10 times serially diluted cDNA. Data were normalized to the data on cyclophilin A from the same preparation (n=7). Product sequences were verified by their sequencing at the DNA facility of Columbia University, NY.

2.4. Western blot

Tissues were chopped and sonicated in two 30 s bursts in lysate buffer (mmol/L) tris–HCl 20 (pH 7.4), EDTA 10, sodium orthovanadate 0.04, benzamidine 3.2, phenylmethylsulfonyl fluoride 0.1, 1% Triton X-100 and complete proteinase inhibitor (Roche) and incubated on ice for 2 h. After centrifugation at 14,000 rcf for 10 min, supernatants were collected and protein concentration was measured using Bio-Rad DC protein assay. Twenty μg of the whole cell lysate were separated on a 4% to 20% tris-glycine gradient gel (Invitrogen) and transferred to PVDF membranes (BioRad). Membranes were blocked overnight in 5% milk in phosphate buffered saline containing 0.1% Tween 20 (PBST) then incubated with anti-SK2 or SK3 rabbit polyclonal antibody (Alomone, 1:1500) in 5% milk/PBST at RT for 2 h. The membranes were washed in PBST 5×5 min anti-rabbit TrueBlot system (eBioscience) secondary antibody was used to eliminate interference of heavy chain and light chain of endogenous IgG. Membranes were washed for 5×5 min in PBST and immunodetection was performed with an endogenous IgG. Membranes were washed for 5×5 min in PBST and immunodetection was performed with an endogenous IgG. Membranes were washed for 5×5 min in PBST and immunodetection was performed with an endogenous IgG. Membranes were washed for 5×5 min in PBST and immunodetection was performed with an endogenous IgG.

2.5. Immunohistochemistry for SK2 alone or together with SERCA2

Pulmonary vein or Bachmann’s bundle tissues were imbedded in O.C.T. (embedding medium, Tissue-Tek) for immunofluorescence studies. Cryostat sections (12 μM) were fixed in 4% paraformaldehyde for 30 min, and then incubated overnight at 4 °C with SK2 antibody (1:200) alone or together with SERCA2 antibody (1:100, Abcam), as an endoplasmic reticulum marker, in PBS with 0.50% Triton-X and 1% normal goat serum. After incubation sections were rinsed 5×5 min, then incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG, 1:300, and Alexa Fluor 594 goat antimouse IgG, 1:500, Molecular Probes) 90 min, sections were mounted with anti-fade mounting medium which includes DAPI (4′, 6 diamidino-2-phenylindole, VectorLaboratories, Inc.) a counter stain for DNA to visualize nuclei.

2.6. Immunocytochemistry for SK2 in rabbit pulmonary vein cells

Dissociated pulmonary vein cells were fixed in 4% paraformaldehyde for 30 min, and then blocked in 2% avidin and 2% biotin for 15 min, respectively. After incubation with anti-SK2 antibody (Alomone, 1:100 in 5% goat serum 0.75% Triton X-100 in PBS) overnight at 4 °C, immunofluorescence labeling was done by incubation with biotinylated secondary antibody (1:300) for 90 min followed by treatment with avidin D fluorescein (1:300) for 90 min. Finally, the slides were mounted with anti-fade mounting medium which includes DAPI.

2.7. Microscopy

For both immunohistochemistry and immunocytochemistry, images were viewed using a Zeiss LSM 510 NLO Multiphoton Confocal Microscope. SK2 (green), SERCA2 (red) immunoreactivity and DAPI (blue) were visualized via 488, 543 and 780 nm excitation, respectively. Three
dimensional reconstructions of sections were analyzed using Image J programming to calculate the pixel intensity over background in each section in cytosolic vs. membrane compartments of the reconstructed cells (NIH freeware).

2.8. Statistical analysis

All results are expressed as mean $\pm$ S.E.M. Comparison between group means was done by one-way ANOVA. For apamin-sensitive peak current comparison was done using student’s $t$-test. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Electrophysiological study

Consistent with our previous results [6], transmembrane potential recordings showed that burst pacing progressively and significantly shortened APD within the atrial sleeves in pulmonary vein extending to 1–2 mm beyond the orifice, but not in Bachmann’s bundle (Table 1, Fig. 2A and B) and this shortening was completely blocked by administration of apamin (0.1 μM) (Table 1, Fig. 2A and C). In contrast, no significant APD changes were seen in pulmonary vein or Bachmann’s bundle from sham-paced atria (data not shown).

Moreover, apamin administration during adaptation had no effect on APD at Bachmann’s bundle but significantly prolonged it at pulmonary vein, suggesting the presence of more apamin-sensitive current in pulmonary vein than in Bachmann’s bundle (Table 1, Fig. 2A, C).

To test whether the shortened repolarization in pulmonary vein was the result of increased apamin-sensitive currents, patch clamp studies were performed. A representative experiment in one pulmonary vein cell is shown in Fig. 3A and a family of apamin-sensitive currents is shown in Fig. 3B. Only a few cells were probed using a full IV clamp protocol and

![Fig. 2. Panel A: AP recordings from untreated (Tyrode) and apamin-treated (Tyrode+Apamin, 100 nM) preparations before (Control) and after the 3 h burst pacing protocol. Note that in the presence of apamin there is no effect of the burst pacing protocol on pulmonary vein APD shortening. Panel B shows the summary data for APD50 in pulmonary vein and Bachmann’s bundle for control and at 3 h of burst pacing ($n=12$ preparations, and 12 animals). Panel C shows the summary data for APD50 at pulmonary vein and Bachmann’s bundle for control, apamin and apamin plus 3 h of burst pacing ($n=6$ preparations, and 6 animals). +Ap indicates apamin administration; +Ap+BPP indicates apamin administration plus burst pacing; *, $p<0.05$ vs. control; #, $p<0.05$ vs. +Ap and +Ap+BPP.](image-url)
Fig. 3C shows representative complete IV curves of total current and apamin-sensitive currents in one Sham and one burst-paced pulmonary vein cell. Note the larger current density and a larger apamin-sensitive current in the burst-paced pulmonary vein cell as compared to the Sham. Voltage dependence appeared similar in both settings.

Data for all experiments are summarized in Fig. 3D which demonstrates the average peak apamin-sensitive currents in the different cell groups (n=7 pulmonary vein myocytes obtained from 4 sham animals and 8 pulmonary vein myocytes from 5 paced animals). Of central importance is that burst pacing results in a greater increase in apamin-sensitive current in pulmonary vein than in Bachmann’s bundle (Fig. 3D). There was no difference in the kinetics of decay of the peak apamin-sensitive current in paced pulmonary vein cells (17.2+/-4.1 ms, n=6 cells from 5 animals) versus those of Sham cells (9.8+/-1.1 ms, n=4 cells from 3 animals).

Fig. 4 demonstrates the relationship between apamin-sensitive currents and changes in APD50 produced with burst pacing. Note that following burst pacing in pulmonary vein...
3.2. mRNA and protein level

To test whether the burst pacing protocol induced changes in SK2 and SK3 transcription levels, we performed quantitative real-time PCR and western blot studies. The burst pacing protocol led to a significant increase in SK2 mRNA levels in pulmonary vein but not in Bachmann’s bundle (Fig. 5A). SK3 mRNA levels did not change significantly after the burst pacing protocol in both pulmonary vein and Bachmann’s bundle (Fig. 5A).

In parallel with the mRNA level change, western blotting showed a significant increase in SK2 protein in pulmonary vein but not Bachmann’s bundle after the burst pacing protocol (Fig. 5B and C). No change in SK3 protein level was induced by the burst pacing protocol in either pulmonary vein or Bachmann’s bundle (Fig. 5B and C).

3.3. Immunohistochemical and immunocytochemical studies of SK2 channels

To visualize the distribution of SK2 channels, we performed immunofluorescence staining of left atrium, pulmonary vein and Bachmann’s bundle frozen sections as well as disaggregated pulmonary vein cells from atria subjected to either the burst pacing protocol or Sham-pacing. Fig. 6A shows the result of co-immunostaining of SK2 channels with SERCA2 protein, as an endoplasmic reticulum marker in sham rabbit left atrium. These data indicate that SK2 protein colocalizes with SERCA2 protein in the endoplasmic reticulum. Fig. 6B and C show representative images of sections and single cells immunostained for SK2 and analyzed by confocal microscopy. Notably in sham-paced pulmonary vein, the SK2 antibody stained intensely in perinuclear regions but minimally in the cytosol or at the plasma membranes (Fig. 6B and C). After the burst pacing protocol, SK2 channel staining significantly increased at sites near or at the membrane as well as in the cytosol in pulmonary vein but not Bachmann’s bundle. Results of quantitative analysis showed that the burst pacing protocol induced a significant increase in staining of SK2 both in the cytosol and membrane in pulmonary vein but not Bachmann’s bundle. Results of quantitative analysis showed that the burst pacing protocol induced a significant increase in staining of SK2 both in the cytosol and membrane in pulmonary vein but not Bachmann’s bundle (bar graphs in Fig. 6B and C). Since Bachmann’s bundle might be considered a specialized tissue, we also asked whether burst pacing might induce changes in SK2 distribution in left atrial free wall. Immunostaining of SK2 channels in Sham and burst-paced left atria showed no difference in channel distribution in these two groups: most of the SK2 channels were located perinuclearly and there was minimal staining in cytosol and membrane (data not shown).

4. Discussion

Atrial fibrillation tends to be a progressive arrhythmia, evolving from paroxysmal to persistent to chronic forms.
AF progression results in part from its effect to induce atrial electrical remodeling, thus creating an arrhythmogenic substrate for its own maintenance [3,13,14]. Initial paroxysms of AF can be triggered by ectopic foci located in the muscle sleeves of pulmonary veins [1,2]. The subsequent advancement of AF to persistence suggests that remodeling of repolarization and refractoriness in the tissues into which the triggered pulmonary venous impulses propagate commences rapidly after the onset of triggered activity.

Although recent studies have shown that SK channels are important in the repolarization of atrial action potentials in mice and humans [8,9], this is the first study focused on the potential association of these channels to the initiation of atrial remodeling. Specifically, we have determined that apamin-sensitive SKCa channels are involved in the repolarization change induced by rapid burst pacing, and have identified SK2 and not SK3 as the culprit channel. Mechanistically, we have determined that the accelerated repolarization results from trafficking of SK2 channel sub-units and that there are the beginnings of transcriptional changes as well.

The data for this early stage of atrial remodeling were obtained from a modified, yet well-characterized in vitro model (isolated rabbit atria) of atrial fibrillation mechanisms [15,16]. The burst pacing protocol used mimics the firing of ectopic foci in pulmonary veins [6]. Conceptually the background for this research evolved from the hypothesis of “sensitivity to the rare” initially developed in neural cell cultures [17,18] which we adapted to the heart [19]. In brief, the concept states that rapid activity originating from sites of intermittent (so-called, “rare”) impulse initiation will more readily take over the activation patterns of sheets of cells than will comparable activity originating from sites of dominant impulse initiation [17–19].

Fig. 5. Panel A: Summary data of real-time PCR for SK2 and SK3 in all groups. There is a significant increase in transcription for SK2 in pulmonary vein and no change in Bachmann’s bundle after burst pacing (*, p<0.05 vs. Sham, n=7). There is no significant change in SK3 transcription in pulmonary vein and Bachmann’s bundle after burst pacing protocol (n=7). Panel B: Summary data of western blots in burst pacing protocol and Sham show that SK2 protein is significantly increased after burst pacing protocol in pulmonary vein but not Bachmann’s bundle (*, p<0.05 vs. Sham, n=3). There is no significant change in SK3 protein level in both burst pacing protocol and Sham at both sites (n=3). Panel C: Representative western blots and loading controls of SK2 and SK3 (above 50 kDa) in pulmonary vein and Bachmann’s bundle.
A goal of the present study was to understand the cellular and molecular mechanisms that might facilitate such behavior in otherwise normal atria which undergo a lifetime of activation via normal sinus rhythm but then can remodel rapidly and often irreversibly when an ectopic focus gains access to the substrate.

The burst pacing protocol (the site- and pattern-specific pacing) we have used in this and previous studies[6,20] induces significant shortening of repolarization in the region of rapid pacing, the pulmonary vein, but not the distant site, Bachmann’s bundle within 3 h. Importantly, this does not mean that 3 h of burst pacing are performed. Rather, (see Fig. 1) because the bursting activity is delivered for only 30 s of each 5 min period of “sinus rhythm,” only 18 min of rapid burst pacing occur during the 180 min of the pacing protocol. The resultant, regionally-delimited shortening of repolarization seen in pulmonary vein but not in Bachmann’s bundle, markedly alters

Fig. 6. Panel A, Co-immunostaining of SK2 protein (green), SERCA2 (red), as an endoplasmic reticulum marker, and nuclei (DAPI, blue) in sham left atrial sections. Note the colocalization of SK2 and SERCA2 protein in the overlay. Panel B: Representative experiments on SK2 immunofluorescence staining (green) for Sham and burst-paced Bachmann’s bundle and pulmonary vein sections. SK2 antibody stained intensely in the perinuclear region of Sham–pulmonary vein sections, and minimally in the cytosol and membrane. In contrast, SK2 antibody stained near or at the membrane as well as in the cytosol in burst pacing protocol–pulmonary vein. In both burst pacing and Sham protocols, Bachmann’s bundle SK2 stained mainly in the perinuclear region. Summary data (bar graphs) show a significant increase in both cytosolic and membrane immunofluorescence intensity after burst pacing protocol of pulmonary vein only (⁎, p<0.05 vs. Sham, n=10). Panel C: SK2 immunofluorescence staining (green) in disaggregated pulmonary vein cells from Sham or burst-paced atria. SK2 antibody stains intensely in the perinuclear region of the Sham pulmonary vein cell, and minimally in the cytosol and membrane. In contrast, SK2 antibody stains near or at the membrane as well as in the cytosol after burst pacing the pulmonary vein cell. Summary data (bar graphs) show a significant increase of immunofluorescence intensity in membrane and in cytosol after burst pacing (n=3) as compared to Sham (n=6) in pulmonary vein (⁎, p<0.05 vs. Sham).
dispersion of repolarization between the two sites [6]. It should be emphasized that while a gradient in repolarization could help to promote reentry between the PV/atria and could help to sustain AF, it would likely not lead directly to initiation of AF.

While it might have been interesting to chart the temporal aspects of change in message, protein and current, subdividing the 18 min time course of pacing would not only have been difficult with regard to quantifying magnitude, but would likely not have been very informative. More important is to understand the transition that occurs between the rapid events that we describe here and the more long-term aspects of remodeling that occur over hours–months–years of repetitive bursting activity. It should be noted that changes in repolarization secondary to trafficking of SK2 channels are not viewed by us as the trigger for an arrhythmic event, but rather as the response to a period of burst pacing (which itself is the trigger) that then facilitates the further expression of the arrhythmia.

The changes that occur appear to be calcium-dependent as they are prevented by nifedipine and ryanodine [6]. Since SK1 current is not apamin-sensitive, this result is consistent with a role for the calcium- and apamin-sensitive SK2 and/or SK3 channels in the evolution of action potential shortening. However, the observation that rapid burst pacing affects the pulmonary vein site more than the Bachmann’s bundle site not only implies that SK2 and/or SK3 channels are involved in this process, but that there is a regional dispersion of their presence and/or that of the factors that activate them.

SK channels are present in many excitable and non-excitable cells including brain, peripheral nervous system, and smooth, skeletal and cardiac muscle [21–26]. Calmodulin constitutively binds to SK channels and functions as a Ca\(^{2+}\) sensor: in response to Ca\(^{2+}\) binding to calmodulin, channel opening occurs with time constants of activation of 5–15 ms [27]. SK channels show a steep dependence on intracellular Ca\(^{2+}\) concentration and can be activated by submicromolar concentrations of intracellular Ca\(^{2+}\) in a voltage-independent manner [28]. Hence, even subtle changes in intracellular Ca\(^{2+}\) concentration might have profound physiological consequences. Although the structure and the nature of Ca\(^{2+}\) gating of the three SK channels are similar, the sensitivities of the channel isoforms to apamin are different, with SK2 > SK3 > SK1 [10,22,29]. SK channels have been implicated in many physiological processes including regulation of secretion, smooth muscle tone, firing pattern in neuron excitable cells, the repolarization phase of cardiac action potentials and in afterhyperpolarization induction following action potentials in neurons [8,9,11,30,31].

Electrophysiological data from our single cell studies demonstrated significantly greater apamin-sensitive current in pulmonary vein than Bachmann’s bundle cells. It should be noted that the SK2 currents we record are transient and outward. This differs from the results of others [8,9] who have reported steady-state apamin-sensitive currents in a setting of Ca\(^{2+}\) chelation. Our studies were designed to minimally perturb the sarcoplasmic reticulum Ca\(^{2+}\) release that occurs with voltage clamp steps so that Ca-dependent, apamin-sensitive currents could be reported during the dynamic change in Ca\(^{2+}\).

Thus the apamin-sensitive current we report here is a transient outward current presumably reflecting the transient global Ca\(^{2+}\) release of atrial cells.

Our voltage clamp results are consistent with our microelectrode studies in which apamin significantly prolonged APD in pulmonary vein but not Bachmann’s bundle cells. A significant relationship between the change in action potential duration and the size of the apamin-sensitive current is seen in the pulmonary vein region, and there was a greater increase in apamin-sensitive current in burst-paced pulmonary vein than in either sham pulmonary vein or in Bachmann’s bundle. Even allowing for the potential impact of the cell disaggregation protocol on the currents subsequently recorded, it is clear that an apamin-sensitive current is present and is functional in the pulmonary vein more than in Bachmann’s bundle.

Our molecular studies also support a role for SK2 channels in the pulmonary vein and not Bachmann’s bundle in the setting of burst pacing-induced acceleration of repolarization. Quantitative PCR showed that burst pacing-induced a significant increase of SK2 trancription in the pulmonary vein, but not Bachmann’s bundle. Consistent with the mRNA results, burst pacing also induced a significant increase in the SK2 protein level in pulmonary vein alone. In contrast to the SK2 results, neither SK3 message nor protein changed during the protocol in either region. These results suggest a prominent role for the SK2 channel in pulmonary vein APD shortening induced by burst pacing.

Whereas the changes in mRNA and protein levels would suggest longer-term alterations in apamin-sensitive current, they do not necessarily explain the steady progression of APD shortening during the 3 h burst pacing protocol. In immunofluorescence studies of tissue preparations (Fig. 6B) migration of positively staining material occurred from perinuclear sites to a more general cytoplasmic distribution in pulmonary vein only. In addition, the single myocyte experiments (Fig. 6C) show that the distribution achieved with rapid burst pacing was primarily at the membrane and/or the immediate sub-membranous region. We could not perform western blots using membrane preparations to test the presence of altered SK2 channel protein after burst pacing because the preparations are too small (each one about 3 mm²) for adequate membrane preparation. That the SK2 channel protein was likely inserted in the membrane is suggested by the increase in current and the decrease in action potential duration, both of which are dependent on localization of channel protein in the membrane and its resultant functionality.

Although there is an alternative interpretation for the cytosolic staining might be a slower recycling of the membrane channels due to slower degradation by proteosomes, all our results are strongly suggestive of altered protein trafficking. Supporting this suggestion is the presence of two potential endoplasmic reticulum retention domains at the SK2 carboxyl terminal: one is the KXXX motif at position 436–439; the other site is the RXR site at 552–554. Although, there
are also potential endoplasmic retention motifs, RXR motifs, at SK3 sequences these are located close to the amino terminal. Licata et al. observed that when SK2 channels are overexpressed in yeast, SK2 protein does not reach the plasma membrane, its normal destination; rather, it remains largely in the endoplasmic reticulum. Adding a putative translocation sequence alters the intracellular distribution significantly with enhanced trafficking out of the endoplasmic reticulum [32]. Using SK2 channels expressed in COSm6 cells, Lee et al. suggested that calmodulin, a putative SK channel beta subunit, is required for cell-surface expression of the SK2 channel [33]. Another study found that surface expression of SK2 is suggested that calmodulin, a putative SK channel beta subunit, is required for cell-surface expression of the SK2 channel [33]. Lee et al. demonstrated that both SK2 channels and L-type Ca\(^{2+}\) channels colocalize in T tubules in the sarcolemma via interactions with alpha-actinin 2 in cardiac myocytes, and the function of SK2 channels in atrial myocytes is critically dependent on the normal expression of Ca\(_{1.3}\) channel [35]. All these studies suggest a complex series of steps contributing to SK2 channel expression at cell membranes.

In conclusion, the 3 h protocol mimicking an intermittently bursting focus in pulmonary vein accelerates repolarization in the region of rapid pacing but not distally. APD shortening at the pulmonary vein region results from SK2 channel trafficking to the membrane leading to upregulation of apamin-sensitive outward current. The downstream result of the action potential shortening would be facilitation of propagation of triggered impulses arising in the pulmonary veins to the bulk left atrial myocardium, facilitating still further evolution of atrial remodeling. Within this framework, we can speculate that SK2 channels maintained at intracellular sites are available for rapid deployment to the cell membrane. When a stress (such as triggered activity or pacing) occurs, beta sub-units, and/or chaperone proteins or phosphate residues might bind to the SK2 channel and transport it to the membrane. This is conceived as a “fast response” system that would not require time for synthesis of new protein when the environment changes.

Our data also suggest that ionic and molecular mechanisms responsible for the initial steps of atrial remodeling induced by site- and pattern specific pacing may be different from those found in the models with short-term [28,29] and long-term [30–32] continuous rapid pacing from a single atrial site. The latter involve transcriptional and translational changes and are consistent with the alteration of SK2 transcriptional activity that is seen in addition to trafficking during the 3 h protocol. Importantly, the sequence of processes from trafficking to transcription sets up a means whereby a rapid response element can segue into a sustained response of the same target protein (in this case, the SK2 channel). Knowledge of the ionic and molecular mechanisms of the early phases of remodeling in settings such as these should help to create strategies for preventing the occurrence of AF by countering the development of a substrate that facilitates evolution of the arrhythmia.

Acknowledgements

This work was supported by NIH grants HL67131, HL28958 and HL66140. We thank Laureen Pagan for her careful attention to the preparation of the manuscript and Sebila Kratovac for her technical assistance with western blotting studies.

References
