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SK channel enhancers attenuate Ca^{2+}-dependent arrhythmia in hypertrophic hearts by regulating mito-ROS-dependent oxidation and activity of RyR

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Aims Plasmamembrane small conductance Ca^{2+}-activated K^+ (SK) channels were implicated in ventricular arrhythmias in infarcted and failing hearts. Recently, SK channels were detected in the inner mitochondria membrane (IMM) (mSK), and their activation protected from acute ischaemia-reperfusion injury by reducing intracellular levels of reactive oxygen species (ROS). We hypothesized that mSK play an important role in regulating mitochondrial function in chronic cardiac diseases. We investigated the role of mSK channels in Ca^{2+}-dependent ventricular arrhythmia using rat model of cardiac hypertrophy induced by banding of the ascending aorta thoracic aortic banding (TAB).

Methods and results Dual Ca^{2+} and membrane potential optical mapping of whole hearts derived from TAB rats revealed that membrane-permeable SK enhancer NS309 (2 μM) improved aberrant Ca^{2+} homeostasis and abolished VT/VF induced by β-adrenergic stimulation. Using whole cell patch-clamp and confocal Ca^{2+} imaging of cardiomyocytes derived from TAB hearts (TCMs) we found that membrane-permeable SK enhancers NS309 and CyPPA (10 μM) attenuated frequency of spontaneous Ca^{2+} waves and delayed afterdepolarizations. Furthermore, mSK inhibition enhanced (UCL-1684, 1 μM); while activation reduced mitochondrial ROS production in TCMs measured with MitoSOX. Protein oxidation assays demonstrated that increased oxidation of ryanodine receptors (RyRs) in TCMs was reversed by SK enhancers. Experiments in permeabilized TCMs showed that SK enhancers restored SR Ca^{2+} content, suggestive of substantial improvement in RyR function.

Conclusion These data suggest that enhancement of mSK channels in hypertrophic rat hearts protects from Ca^{2+}-dependent arrhythmia and suggest that the protection is mediated via decreased mitochondrial ROS and subsequent decreased oxidation of reactive cysteines in RyR, which ultimately leads to stabilization of RyR-mediated Ca^{2+} release.

Keywords Ventricular arrhythmia • Small conductance Ca^{2+}-activated K^+ channels • Ryanodine receptor • Reactive oxygen species • Cardiac hypertrophy

1. Introduction

Sudden cardiac death due to ventricular tachyarrhythmias remains the major cause of mortality in the world.[1] There is a well-established link of aberrant intracellular Ca^{2+} homeostasis with enhanced propensity of the heart to arrhythmias in hypertrophy, heart failure, myocardial infarct, and aging.[2–4] Pro-arrhythmic spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR) in these conditions, accompanied by excessive

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production of reactive oxygen species (ROS), is largely ascribed to abnormally high activity of oxidized ryanodine receptors (RyRs), the SR Ca^{2+} release channels.[4–8] During recent years mitochondria, located in close proximity to RyR clusters in cardiac cells, gained recognition as a major source of ROS in diseased, aged, or stressed hearts.[4,9–12] Furthermore, targeted scavenging of mitochondrial ROS was proven to attenuate spontaneous Ca^{2+} release most probably via restoration of RyR redox state.[4,9,13]

Small conductance Ca^{2+}-activated potassium channels (SK) mediate the feedback between intracellular Ca^{2+} dynamics and membrane repolarization in excitable cells.[14–16] The SK channel family consists of three members: SK1 (KCa2.1, KCNN1), SK2 (KCa2.2, KCNN2), and SK3 (KCa2.3, KCNN3) that exhibit high degree of homology and conductance of 5–20 pS. The tetrameric SK channels are voltage independent and their gating is predominantly controlled by intracellular $[Ca^{2+}]$ in the submicromolar range conferred by constitutively bound calmodulin. In ventricular myocytes from healthy hearts plasmalemmal SK (pSK) channels are functionally dormant. However, enhanced SK expression and increased sensitivity to Ca^{2+} (from 500 to 200 nmol/L) was shown in myocytes from failing hearts and from myocardial infarct border zones.[17–22] We recently demonstrated that RyR-mediated SR Ca^{2+} release is both necessary and sufficient for activation of pSK channels.[23] Furthermore we showed that enhanced expression of SK channels plays an anti-arrhythmic role by countering the depolarizing force of the Na^{+}/Ca^{2+} exchanger (NCX) during spontaneous Ca^{2+} release. Despite attenuating arrhythmia trigger in the form of Ca^{2+}-dependent early or delayed afterdepolarizations (EADs and DADs, respectively),[23,24] disease-associated SK upregulation was also shown to promote a substrate for arrhythmia because of action potential shortening and non-uniform tissue expression of the channels.[18–21] It should be noted that previous studies extensively used SK blockers or transgenic mouse models to assess the role of SK channels in arrhythmogenesis.[14,15] However, anti-arrhythmic potential of pharmacological enhancement of endogenous SK channels remains unexplored.

Importantly, in addition to plasmalemmal localization, the presence of functional SKs was demonstrated in the inner mitochondria membrane (mSK). Activation of mSK was found to prevent glutamate-induced oxytosis and mitochondrial dysfunction in a neuronal cell line.[25] Subsequently, mSK channels were discovered in ventricular cardiomyocytes (CMs), where their pharmacological activation protects against ischaemic cardiac injury.[26] In both cases, activation of mSK channels preserves mitochondrial function and reduces production of ROS in cells under stress. Previous studies of cardiac mitochondrial K^{+} channels such as mK_{ATP} and mBK_{Ca} have been mostly focused on their 'pre-conditioning' protection over ischaemic injury,[27–29] whereas the physiological activation and function of these channels are less clear, particularly in light of their steep dependence on [ATP] or mitochondrial membrane potential ($\Delta\Psi m$), respectively. Considering their submicromolar Ca^{2+} sensitivity, it is conceivable that mSK channels can respond to intracellular $[Ca^{2+}]$ and regulate mitochondrial function in healthy or diseased CMs. In this study, we demonstrate that SK channels modulators regulate mitochondrial $\Delta\Psi m$ in myocytes from rat hearts with hypertrophy induced by thoracic aortic banding (TAB)[30,31] and affect mitochondria ROS production. Furthermore, we show that pharmacological enhancement of mSK protects from Ca^{2+}-dependent arrhythmia by decreasing mitochondria ROS production leading to normalization of redox state of RyRs and stabilization of SR Ca^{2+} release.

2. Methods

The tissue, cellular, subcellular, and molecular effects of pharmacological manipulation with SK channels were studied at the organ level and in isolated adult rat ventricular myocytes from sham hearts and hearts with hypertrophy induced by TAB four weeks after procedure. In this well-established commercially available model (Charles River Labs), ligation of the ascending aorta results in pressure overload inducing development of cardiac hypertrophy and enhanced arrhythmogenesis in 4 weeks.[30,31] We studied whole hearts using dual voltage-Ca^{2+} optical mapping. At the cellular level, cytosolic Ca^{2+} changes were monitored using confocal microscopy, and membrane potential was recorded with the whole cell patch-clamp technique using myocytes isolated from TAB hearts. Changes in levels of RNA, protein expression, and protein oxidation were studied using standard approaches.

An expanded Methods section can be found in the Supplementary material online.

3. Results

3.1 *Ex vivo* dual voltage and Ca^{2+} optical mapping

To gain insight into the role of SK channels in arrhythmogenesis at the organ level we performed *ex vivo* whole heart studies using simultaneous membrane potential (Vm) and Ca^{2+} mapping with the voltage sensitive dye RH278 and the Ca^{2+} indicator Rhod-2.[32] Exposure to the β-adrenergic agonist isoproterenol (ISO, 50 nmol/L) induced frequent premature ventricular complexes (PVCs, red arrows) and VT/VF in seven out of seven TAB hearts and one out of eight Sham hearts. Importantly, incubation of TAB hearts with the membrane permeable SK enhancer NS309 (2 μmol/L, 20 min)[33] completely abolished PVCs and ventricular arrhythmia induced by ISO, as seen from ECG recordings in *Figure 1A*. TAB rats showed electrical remodelling resulting in prolongation of APD (*Figure 1B*) in agreement with earlier studies.[30,31] Incubation with NS309 did not shorten APD in TAB rats, while subsequent application of the membrane impermeable SK peptide inhibitor apamin (10 nmol/L)[15,17,18,20] in the presence of NS309 and ISO prolonged APD. These data suggest that SK channels are present in the plasma membrane of ventricular myocytes from TAB rats and the maximal contribution of SK channels to APD is achieved, such that NS309 does not produce any further effect on APD. *Figure 1C* demonstrates representative activation maps recorded in Sham and TAB rat hearts exposed to ISO. The activation maps of VT/VF showed conduction blocks and reentry formation in addition to frequent PVCs. Membrane permeable SK enhancer NS309 alleviates wavebreaks and VT/VF without detectable effects on conduction velocity (*Figure 1C and D*). Subsequent application of apamin did not promote VF despite prolongation of APD (*Figure 1B*) and presence of PVCs (*Figure 1A*, red arrow in the bottom trace).

To study potential effects of SK modulators on spontaneous SR Ca^{2+} release we used burst stimulation followed by pause (*Figure 1E*). Unlike Shams, TAB hearts subjected to this protocol under basal conditions demonstrated elevation in intracellular $[Ca^{2+}]$ during pause (red arrow on the third trace), indicating profound remodelling of Ca^{2+} homeostasis.[34] Exposure to ISO promoted spontaneous elevation of Ca^{2+} in Sham hearts, while in TAB hearts ISO evoked much larger disturbances in Ca^{2+} handling with frequent VF induction. As a result, the stimulation protocol could not be finished in TAB group. Taken together, these results strongly implicate abnormal Ca^{2+} handling as an

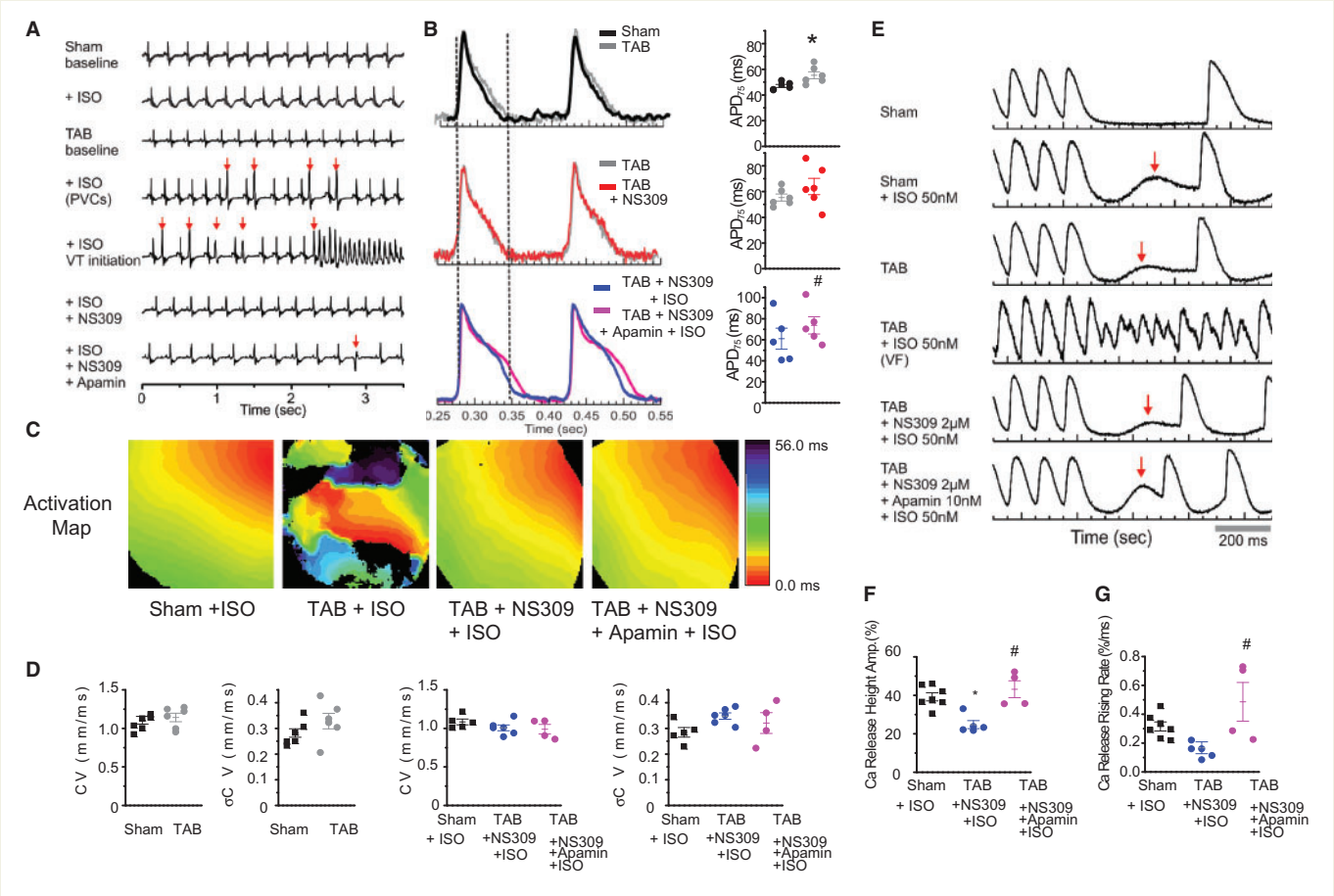

Figure 1 Pharmacological enhancement of SK channels attenuates Ca^{2+}-dependent arrhythmia in *ex vivo* optically mapped hypertrophic hearts. *(A)* Representative ECGs at baseline and in the presence of 50 nmol/L isoproterenol, membrane-permeable SK enhancer NS309 (2 μmol/L) and cell impermeable SK inhibitor apamin (10 nmol/L). *(B)* Representative traces of action potentials and corresponding pooled data, $*^{\#}P < 0.05$, vs Sham and TAB + NS309 + ISO, paired, and unpaired Student's *t*-test where appropriate. *(C)* Representative activation maps in Sham and TAB hearts. *(D)* Pulled data for conduction velocities and conduction velocity heterogeneity, not significant at $P < 0.05$, unpaired Student's *t*-test and One-way ANOVA where appropriate. *(E)* Representative Ca^{2+} traces in hearts paced at 150 ms cycle length before pause. Red arrows indicate spontaneous Ca^{2+} release. *(F, G)* Amplitude and rate of rise of spontaneous Ca^{2+} release during pause. $*^{\#}P < 0.05$, vs. Sham + ISO and TAB + NS309 + ISO, respectively, One-way ANOVA.

underlying cause for an increased arrhythmic potential in hypertrophied TAB rat hearts. Importantly, Ca^{2+} handling in ISO-treated TAB hearts was largely restored with NS309 (*Figure 1E*, *F* and *G*). Consequent application of apamin produced moderate increases in rate and amplitude of spontaneous Ca^{2+} release (*Figure 1E*, *F* and *G*) most likely because of an increased Ca^{2+} influx from the extracellular milieu through L-type Ca^{2+} channels due to APD prolongation. These data suggest that enhancement of SK activity in TAB rat hearts attenuates ventricular arrhythmias primarily due to its stabilizing effect on Ca^{2+} release.

3.2 Effects of SK channel modulators in hypertrophic rat ventricular CMs

To extend our studies to the cellular level we performed whole cell patch clamp experiments and confocal Ca^{2+} imaging (*Figure 2*). We found that 10 min. exposure of the SK2- and SK3-specific enhancer, CyPPA (10 μmol/L),[35] significantly decreased the ISO-induced incidence of EADs, DADs, and underlying spontaneous Ca^{2+} waves (SCWs) in TAB myocytes. Similar stabilizing effect on Ca^{2+} waves was achieved in a separate set of experiments by exposing TAB myocytes to 10 μmol/L NS309 for 10 min, while 10 μmol/L GW542473X (specific SK1 enhancer)[36] showed no effect (Supplementary material online, *Figure S2*). Conversely, the SK inhibitor UCL-1684 (1 μmol/L)[37] promoted SCWs and afterdepolarizations (*Figure 2*). In addition, UCL-1684 significantly prolonged APD80 in TAB cells (Supplementary material online, *Figure S3*). It is conceivable that prolongation of APD upon SK block can contribute to increased spontaneous SR Ca^{2+} release, by promoting Ca^{2+} overload due to the increased Ca^{2+} influx through plasmalemmal L-type Ca^{2+} channels. However, given that SK enhancer CyPPA failed to shorten APD (Supplementary material online, *Figure S3*), the effects of SK modulators on Ca^{2+} waves cannot be fully attributed to changes in activity of plasmalemmal channels alone. This prompted us to focus on the potential roles of SKs recently found in IMM[26] in regulation of Ca^{2+} homeostasis.

3.3 Expression and distribution of SK isoforms in rat ventricular myocytes

Despite a high degree of homology, three known SK channels isoforms (SK1-3) exhibit differential expression patterns in different tissues and in

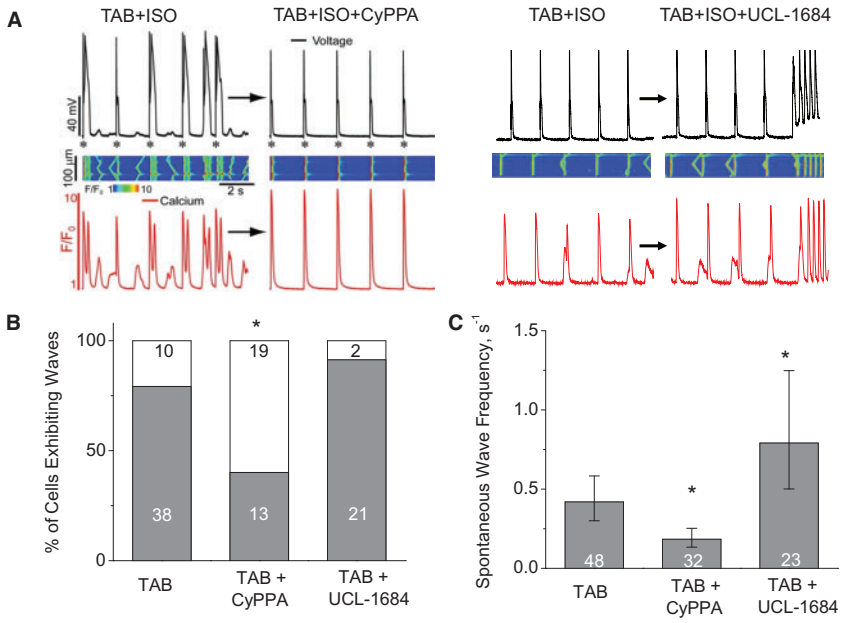

Figure 2 Pharmacological enhancement of SK channels decreases while inhibition increases arrhythmic potential in isolated ventricular myocytes from hypertrophic (TAB) rat hearts. *(A)* Representative simultaneous recording of Ca^{2+} transients and Vm in hypertrophic myocyte exposed to 100 nmol/L ISO before and 10 min after application of SK ehancer CyPPA (10 μmol/L) or SK inhibitor UCL-1684 (1 μmol/L); *(B, C)* Number of ISO-treated hypertrophic cells field-stimulated at 0.5 Hz for 1 min exhibiting Ca^{2+} waves and wave frequency, respectively. Cell numbers are shown in the columns, *$P < 0.05$ vs. TAB, Exact Fisher test and hierarchical linear model where appropriate. Number of TAB animals used was 7.

cardiac disease.[14,15,17,18,38] Furthermore, it is still unclear which SK isoform out of three is expressed in the IMM of CMs.[26] Using quantitative real time PCR (RT-qPCR) and Western blot analysis we assessed intracellular distribution and possible changes in expression of SK isoforms in myocytes from TAB hearts. Our RT-qPCR results confirm that all three isoforms are expressed in rat ventricular myocytes (Supplementary material online, *Figure S1A*). Hypertrophy induced by TAB did not change the mRNA levels of SK1 and SK2, while SK3 levels showed ~two-fold increase ($n = 4$, $P < 0.05$, Student's *t*-test). To assess specificity of anti-SK antibodies we constructed shRNAs to inhibit expression of SK1-3. Freshly isolated rat ventricular myocytes were infected with adenoviruses (MOI 100) carrying shRNA constructs and after 48 h in primary culture cell lysates were subjected to SDS PAGE (*Figure 3A*). SK1 was identified as ~66 kDa band (theoretical molecular weight 59.2 kDa), while SK2 and SK3 were running close to 100 kDa which is higher than their predicted molecular weights (62.2 and 81.4 kDa, respectively). Bioinformatic sequence analysis demonstrates that SK1 and SK2 but not SK3 have N-terminal mitochondria targeting sequences (Supplementary material online, *Figure S4*; also see MitoProt: http://ihg.gsf.de/ihg/mitoprot.html).[39] In line with this prediction, our next experiments using differential centrifugation of cell lysates from isolated ventricular myocytes established SK3 as the major isoform localized to surface membrane, while SK1 was found predominantly in the mitochondrial fraction, and SK2 was present in both (*Figure 3B*). Cytochrome C (Cyt C) and mitochondrial Ca^{2+} uniporter (MCU) were used as markers for mitochondria fraction. Pan-cadherin and α1c and α2 subunits of plasmalemmal Ca^{2+} channels were used as markers of membrane fraction. Analysis of protein expression levels demonstrated that SK3 and pSK2 are significantly reduced in TAB membranes when normalized to pan-cadherin (*Figure 3B and C*). SK1 and mitochondrial SK2 (mSK2) normalized to MCU showed no changes in TABs in comparison to Shams in mitochondrial fraction. Western blot analysis using total cell lysate samples from Sham and TAB ventricular myocytes showed no change in the protein levels of SK1, and SK3, while SK2 was ~50% higher in TABs (*Figure 3D and E*).

3.4 Modulators of SK channels affect mitochondrial membrane potential in ventricular CMs

We next determined if fluctuations in intracellular [Ca^{2+}] could modulate mSK activation to modify mitochondrial membrane potential ($\Delta\Psi m$) using freshly isolated rat CMs with plasmamembrane permeabilized with saponin. Increasing cytosolic [Ca^{2+}] from 50 nmol/L to 1 μmol/L and 50 μmol/L resulted in relatively slow Ca^{2+} uptake by mitochondria measured using mitochondria-entrapped Ca^{2+} indicator Rhod-2 (Supplementary material online, *Figure S5A*). To prevent myocyte contraction at high [Ca^{2+}]cyt cells were pre-incubated with Cytochalasin D (10 μmol/L for 10 min).[6] Parallel experiments with mitochondria stained with 1 nmol/L voltage sensitive dye TMRE showed small depolarization of $\Delta\Psi m$ even at 1 μmol/L [Ca^{2+}]cyt, consistent with K^+ influx due to activation of mitochondrial K^+ channels with high affinity to Ca^{2+} and ability to open at very high $\Delta\Psi$ (Supplementary material online, *Figure S5B*). Application of UCL-1684, a specific SK inhibitor (1 μmol/L), attenuated the drop in $\Delta\Psi m$ evoked by 1 μmol/L [Ca^{2+}]cyt (*Figure 4A and B*). Importantly, neither UCL-1684 (1 μmol/L), nor SK activator CyPPA (10 μmol/L) had dramatic effects on mitochondrial Ca^{2+} uptake (Supplementary material online, *Figure S6A* and *B*). These data imply that

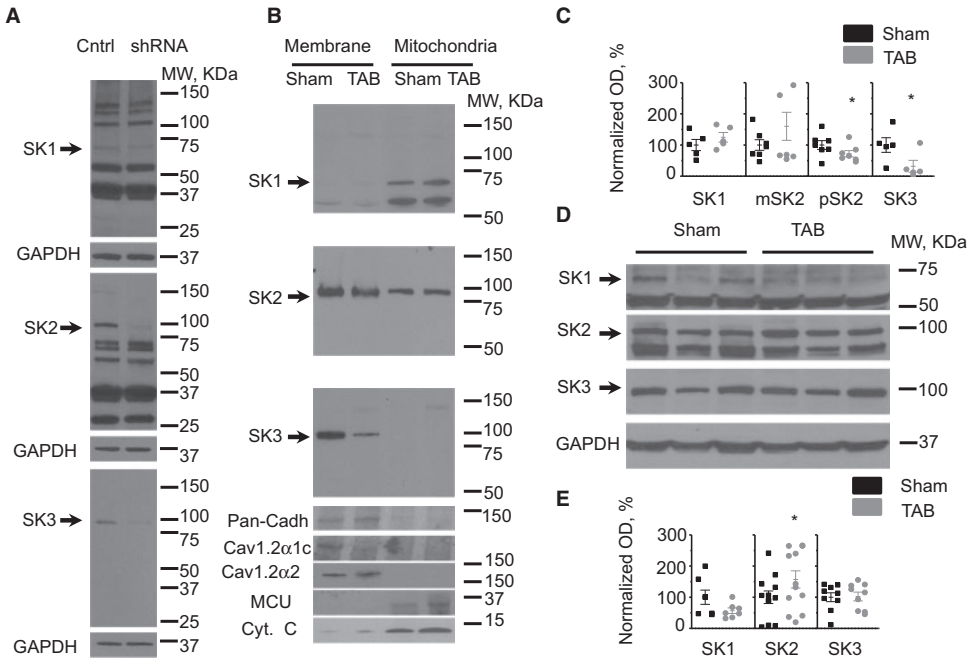

Figure 3 Expression and subcellular distribution of SK isoforms in ventricular myocytes from Sham and TAB rat hearts. *(A)* Identification of SK1, SK2, and SK3 using specific shRNAs expressed in cultured rat ventricular myocytes for 48 h. *(B)* SK channels distribution in membrane and mitochondrial fractions. Cyt C and MCU were used as the markers of mitochondria fraction. Pan-Cadherin and α1c and α2 subunits of L-type Ca^{2+} channels were used as the markers membrane fraction. *(C)* Pooled data for normalized optical density (OD, %) of SK1-3 Western Blot analysis in membrane and mitochondrial fractions from isolated myocytes from 5 to 7 Sham and TAB hearts, $^*P < 0.05$, Student's *t*-test. Surface membrane SKs (pSK2 and SK3) were normalized to Pan-Cadherin and mSKs SK1 and mSK2 were normalized to MCU. *(D, E)* Representative western blots and pooled data for OD of SK1-3 in unfractionated myocytes. GAPDH was used as loading control. $^*P < 0.05$, $n = 7–12$, Student's *t*-test.

Ca^{2+}-mediated drop in $\Delta\Psi$m at least in part is conferred by the activation of SK channels. Pharmacological activation of mito-K^+ channels is expected to induce small $\Delta\Psi$m depolarization.[25,29] We were unable to detect statistically significant changes in $\Delta\Psi$m upon application of SK activators in concentrations near reported EC_{50}s (less or ~10 μmol/L).[33,35,36] However, application of 100 μmol/L CyPPA (selective to SK2 and SK3) resulted in ~40% depolarization of $\Delta\Psi$m as measured by TMRE fluorescence in permeabilized cells (*Figure 4C and D*). Depolarization of IMM evoked by 25 μmol/L of CyPPA or NS309 was fully reversible by consequent application of membrane permeable SK inhibitor UCL-1684 (1 μmol/L, Supplementary material online, *Figure S7C* and *1D*) but not by application of membrane impermeable apamin (100 nmol/L, Supplementary material online, *Figure S7A* and *B*). Taken together, the effects of SK enhancers are most consistent with the activation of mSK channels, allowing K^+ influx and depolarization of $\Delta\Psi$m.[27–29] Importantly, incubation of permeabilized myocytes with the SK1-specific SK activator GW542573X (100 μmol/L) failed to produce changes in $\Delta\Psi$m. The latter suggests that the presence of SK1 in mitochondrial fraction (see *Figure 3B*) does not result in proper retainment of the functional channel in the IMM, which leaves SK2 as the only functional SK subtype out of three targeted to mitochondria. For a mechanistic link between mSK channel activation and mitochondrial function in TAB rats, we measured the effect of the membrane-permeable SK inhibitor UCL-1684 (1 μmol/L) on $\Delta\Psi$m in permeabilized Sham and TAB CMs. As shown in *Figure 5A and B*, UCL-1684 resulted in a small (~10%) but significant hyperpolarization of $\Delta\Psi$m in myocytes from TAB rats, while no effect was detected in Sham cells. This observation is consistent with mSK channels being activated at baseline in diseased hearts, causing a small depolarization of $\Delta\Psi$m which is reversible by SK inhibition. Importantly, data presented in *Figure 5C and D* suggest that mSK channels were not maximally activated in TAB cells because both NS309 (selective to SK1, SK2, and SK3) and CyPPA (selective to SK2 and SK3) at high concentrations were able to evoke $\Delta\Psi$m depolarization. Interestingly, the SK1-selective activator GW542573X failed to produce any changes in $\Delta\Psi$m in TAB cells (*Figure 5C and D*) consistent with no effect on SCWs (see Supplementary material online, *Figure S2*).

3.5 Effects of SK modulators on mitochondrial ROS production and RyR hyperactivity in hypertrophic CMs

Excessive ROS production by mitochondria is a hallmark of hypertrophy, heart failure or aging; conditions that are often accompanied by proarrhythmic disturbances in Ca^{2+} handling. Our data demonstrate that increased ROS production is likely responsible for the increased frequency of RyR-mediated SCWs in CMs from TAB rats, as direct scavenging of mitochondria derived ROS with mito-TEMPO (mitochondria specific-scavenger, 10 μmol/L, 10 min.)[4,13] also inhibits the proarrhythmic SCWs (Supplementary material online, *Figure S8*). The monobromobimane (mBB) fluorescence assay shows that incubation of hypertrophied CMs with CyPPA or NS309 reduces thiol-oxidation of a protein band with MW similar to RyRs (*Figure 6A and B*). Experiments using

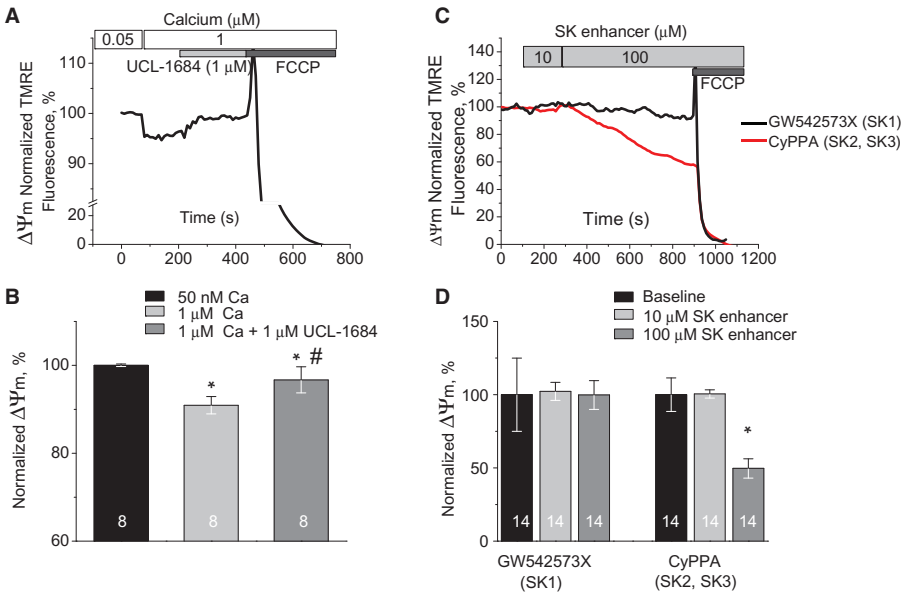

Figure 4 Pharmacological enhancers and inhibitors of SK channels modulate ΔΨm in Sham myocytes. *(A)* Representative recording of changes in mitochondrial membrane potential in response to increase in intracellular Ca^{2+} from 50 nmol/L to 1 μmol/L followed by application of 1 μmol/L UCL-1684, a selective SK inhibitor. Myocytes from healthy hearts were permeabilized with saponin and immobilized with 10 μmol/L Cytochalasin D. TMRE signal was normalized to minimum fluorescence obtained by application of 50 μmol/L FCCP and presented as a % of baseline. *(B)* Pooled data for normalized TMRE signal, $*^{\#}P < 0.05$ vs. 50 nmol/L $[Ca^{2+}]$cyt and 1 μmol/L $[Ca^{2+}]$cyt, respectively, hierarchical linear modelling. Numbers of cells are presented in columns; number of Sham animals was 3. *(C)* Representative recordings of ΔΨm in permeabilized cells exposed to GW542573X (selective SK1 enhancer, black) and CyPPA (selective SK2 and SK3 enhancer, red); *(D)* Pooled data for C, $*P < 0.05$ vs. baseline, hierarchical linear modelling. Numbers of cells are presented in columns; number of Sham animals was 5.

immunoprecipitated channels from isolated TCMs further confirmed that incubation with SK enhancers decrease oxidation of RyRs (Supplementary material online, *Figure S10*) consistent with our hypothesis that mSK enhancement eliminates SCWs by reducing ROS production. Furthermore, experiments using the mitochondria-specific ROS indicator MitoSOX demonstrated that inhibition of SK channels with UCL-1684 increased ROS production, while enhancers NS309 and CyPPA reduced it in freshly isolated TAB CMs (*Figure 6C*).

To assess the direct effects of SK activation on RyR-mediated SR Ca^{2+} leak, we performed measurements of Ca^{2+} sparks in myocytes permeabilized with saponin to eliminate any potential contribution of effects caused by plasma membrane SKs that can confound interpretation of the results. As shown in *Figure 7*, Ca^{2+} spark frequency is increased, while SR Ca^{2+} content measured using rapid application of 20 mmol/L caffeine is decreased in myocytes from TAB rats vs. Shams. Ten-minute pre-incubation with 10 μmol/L CyPPA or NS309 resulted in higher SR Ca^{2+} load in TAB myocytes and in case of NS309, which is more potent SK enhancer[35] significantly reduced Ca^{2+} spark frequency. It is well known that RyR activity is controlled by $[Ca^{2+}]$ not only at its cytosolic activation sites, but at luminal sites as well.[40,41] Oxidation of RyRs was linked to the enhanced sensitivity of RyRs to intra-SR $[Ca^{2+}]$ resulting in substantial decrease in SR Ca^{2+} content.[7] A decrease and even lack of increase in spark frequency at higher SR Ca^{2+} loads in SK-enhancer-treated TAB myocytes is consistent with at least partial stabilization of RyRs due to restoration of their luminal Ca^{2+} sensitivity. This recovery of RyR function might be sufficient to substantially reduce propagating SCWs in myocytes from hypertrophic hearts (see *Figure 2*, Supplementary material online, *Figure S2*) and thereby propensity to Ca^{2+}-dependent arrhythmia.

4. Discussion

Recently SK channels gained recognition as an attractive new target for treatment of malignant cardiac arrhythmias.[14–16] Previous reports were focused on delineating the role of aberrant expression/function of pSK channels and the potential cardioprotective role of mSK channels is only beginning to emerge.[26] In this work, using rat model of pressure-overload-induced hypertrophy,[30,31] we provide the first evidence that mSK channels modulators can influence SR Ca^{2+} release via regulation of mitochondrial ROS production, which determines oxidative state and activity of RyRs in diseased hearts.

4.1 SK modulation and ventricular arrhythmia

Previous studies using bee-venom peptide apamin, a specific SK inhibitor, demonstrated that inhibition of SK channels in rabbit hearts with heart failure induced by rapid pacing can attenuate arrhythmia via prolongation of APD and reducing heterogeneity, thus limiting reentry.[16,18,19,20] However, experiments in single cells from human and canine failing hearts demonstrated that apamin application not only further prolongs already long APD caused by electrical remodelling, but also induces profound instabilities in repolarization suggesting an anti-arrhythmic role of SKs.[17] Furthermore, we recently showed in rat myocytes with

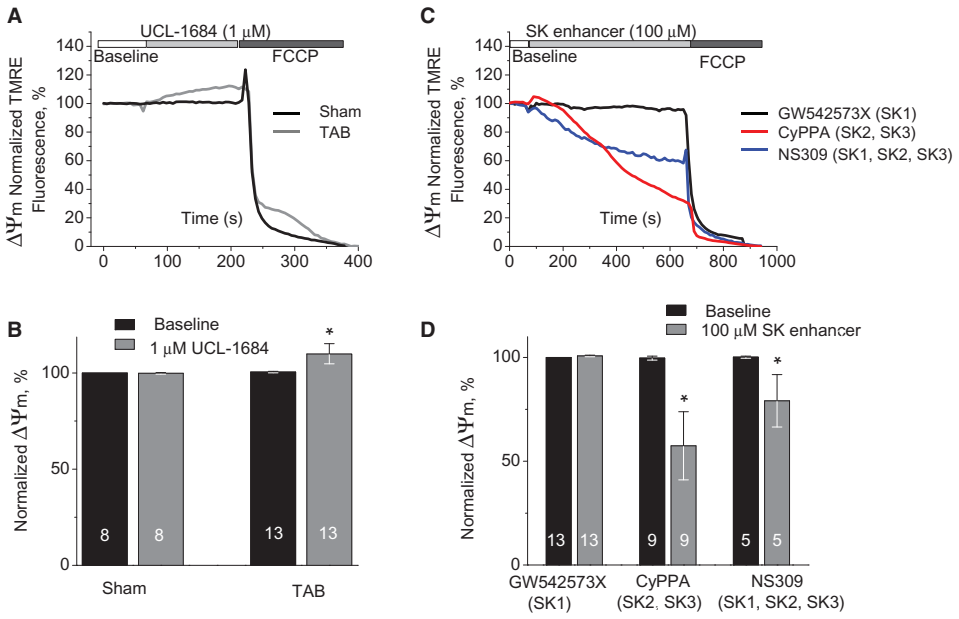

Figure 5 Pharmacological inhibition of SK channels increases while enhancement decreases ΔΨm in TAB myocytes. *(A)* Representative recording of changes in ΔΨm in response to SK channels block by 1 μmol/L UCL-1684 in permeabilized Sham (black) and TAB (grey) myocytes. *(B)* Pooled data for A, *$P < 0.05$, hierarchical linear model. Numbers of cells are presented in columns, number of Sham animals was 4, and number of TABs was 5. *(C)* Representative recordings of ΔΨm in permeabilized cells exposed to 100 μmol/L GW542573X (selective SK1 enhancer, black), CyPPA (selective SK2 and SK3 enhancer, red), and NS309 (enhancer of SK1, SK2, and SK3, blue). *(D)* Pooled data for C, *$P < 0.05$ vs. baseline, hierarchical linear model. Numbers of cells are presented in columns; number of TABs was 6.

adenovirus-mediated overexpression of SK2 that pro-arrhythmic spontaneous Ca^{2+} release effectively induces repolarizing SK current which attenuate NCX-driven afterdepolarizations.[23] Accordingly, in failing rabbit hearts, apamin exacerbated EADs associated with disturbances in Ca^{2+} cycling.[24] Taken together, results of these studies imply that in conditions accompanied with aberrant Ca^{2+} handling, enhancement rather than inhibition of SK channels must play a protective role by limiting Ca^{2+}-dependent triggering events in the form of EADs and DADs. Indeed, our experiments in whole hearts and single cells demonstrate beneficial anti-arrhythmic effects of SK enhancers and pro-arrhythmic effects of SK inhibitors (*Figures 1 and 2* and Supplementary material online, *Figure S2*). SK inhibitors prolonged APD in TABs providing an evidence of the presence of functional SK channels in plasma membrane of TAB CMs (*Figure 1B*, Supplementary material online, *Figure S3*). Remarkably, SK enhancers NS309 and CyPPA (which increase SK activity by sensitizing channels to Ca^{2+})[35] failed to shorten APD both in whole hearts (*Figure 1B*) and in isolated CMs (Supplementary material online, *Figure S3A*). This implies that pSK channels in TABs are already hypersensitive to Ca^{2+}, which is similar to human[18] and rabbit HF.[19] SK-enhancer-mediated leftward shift to Kd to [Ca^{2+}] as high as 200 nmol/L characteristic of cardiac disease is unlikely to cause any additional effects on APD, because SK channels will be already open during Ca^{2+} transient when [Ca^{2+}]cyt reaches micromolar range. Lack of effects of membrane-permeable NS309 and CyPPA also implies that their anti-arrhythmic effects are largely mediated by mSKs through regulation of SR Ca^{2+} release. The latter is further confirmed by the inability of membrane-impermeable apamin to reverse protective stabilization of Ca^{2+} cycling by the membrane permeable NS309 in TAB rat hearts despite apamin-induced prolongation of APD (*Figure 1*).

4.2 Expression and distribution of SK channels in ventricular myocytes

In addition to enhanced sensitivity of pSKs to Ca^{2+}, increased expression levels of the channels were demonstrated in humans and several animal models of heart failure and myocardial infarct.[16–22] Our results using RT-qPCR demonstrate that SK1 and SK2 mRNA levels are unchanged and SK3 mRNA levels are ~two-fold higher in isolated CMs from TABs than Shams. However, Western blot analysis shows no change in SK3 levels in whole CMs lysates (*Figure 3D and E*). One possibility why SK3 protein levels remain unchanged in TAB CMs at increased mRNA levels is translation interference by microRNAs. To test this possibility we assessed the expression levels of muscle-specific microRNA 499 (miR-499), showed to target SK3.[42] We found that the levels of miR-499 are significantly higher in TABs vs. Shams (Supplementary material online, *Figure S1B*). SK1 protein levels were unchanged in CMs which is consistent with RT-qPCR results, while SK2 was moderately ~50% higher in TABs vs Shams despite no change in mRNA levels. Our results markedly differ from reported two-to four-fold upregulation of SK2 and SK3 protein levels in tissues from rat,[43] human and canine failing hearts.[15,17] This discrepancy can be attributed to the existence of SK channels in cell types other than myocytes that are present in cardiac tissue and also undergo disease-associated remodelling. For example, SK channels are likely present in fibroblasts[44] and endothelial cells[45] and can contribute to substantial increase in expression levels detected in ventricular tissues from HF patients. Moreover, our experiments with fractionation of CMs demonstrated significant reduction of pSK2 and SK3 (*Figure 3B and D*). Ren *et al.*[46] demonstrated that phosphorylation of SK2 by protein kinase A at three C-terminal serines leads to the channel removal from the plasma

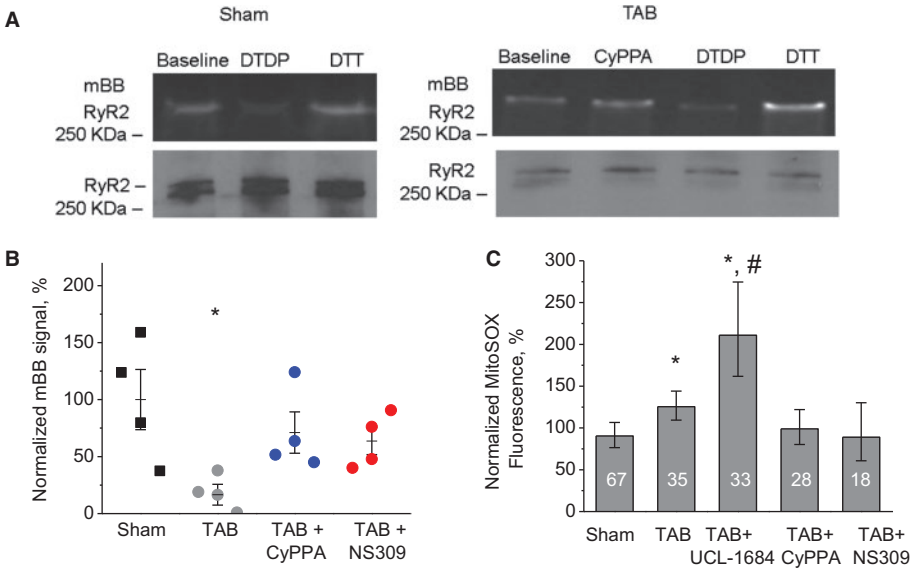

Figure 6 Reversal of RyR oxidation in TAB myocytes by SK-enhancers via attenuation of mitochondrial ROS. *(A)* CyPPA reduced thiol-oxidation of RyR in TAB ventricular myocytes. Cells were incubated with 10 µmol/L CyPPA for 10 min before lysis. ROS scavenger dithiothreitol (10 mmol/L) and oxidant 2,2′-dithiodipyridine (200 µmol/L) were used to obtain minimal and maximal oxidation for normalization. Top: Representative fluorescence images of oxidation-sensing dye mBB. Bottom: RyR western for normalization. *(B)* Pooled data for A. Additional group of cells was treated with 10 µmol/L NS309 for 10 min in the same way as CyPPA, $^{*}P < 0.05$ vs. Sham and $^{\#}P < 0.05$ vs. TAB, $n = 4$ hearts for both Sham and TAB, one-way ANOVA. *(C)* SK modulators alter rate of ROS production measured with MitoSOX in CMs. Cell numbers are shown in the columns. $^{*}P < 0.05$ vs. Sham and $^{\#}P < 0.05$ vs. TAB, hierarchical linear model. Number of Sham animals was 3; number of Tab animals was 4.

membrane. It is possible that in conditions when blood levels of catecholamines are increased like in HF, plasmalemmal localization of SK2 and structurally similar SK3 channels is diminished rather than increased as previously thought.

Our further experiments with CMs fractionation confirmed presence of SK channels in mitochondria of ventricular myocytes as described by Stowe *et al.*[26] However, this study did not provide definite answer which SK isoform resides in the IMM in CMs. We demonstrate that SK1 and SK2 subtypes are present in the mitochondrial fraction in rat CMs, while SK3 is restricted to the plasmalemma (*Figure 3B*). These results are in agreement with bioinformatics prediction, which suggested that SK1 and SK2 but not SK3 have N-terminal mitochondria-targeting sequences (MitoProt[39]: http://ihg.gsf.de/ihg/mitoprot.html, 26 January 2017, date last accessed; Supplementary material online, *Figure S4*). SK1 and SK2 localized in mitochondria show no change in expression levels in TABs (*Figure 3C*). Importantly, the bioinformatics programme also predicts that SK2 exhibits much higher probability of mitochondria targeting than SK1 (0.90 vs. 0.17, respectively). In line with this, our data show lack of effect of SK1-specific activator GW542573X on $\Delta\Psi$m (*Figures 4C, D, 5C and D*) and SCWs (Supplementary material online, *Figure S2*). Although we cannot completely rule out potential role of SK1, our results establish SK2 as the major SK isoform residing in the inner mitochondrial membrane.

4.3 SK channels and mitochondrial ROS

Cardioprotection mediated by mitochondrial K_{ATP} and BK Ca^{2+}-activated channels is well established, while the cardioprotective potential of mSK channels is only beginning to emerge. BK and K_{ATP} channel openers are known to reduce damaging ROS production by mitochondria (under stress conditions), decrease arrhythmic potential, and improve overall cardiac function after ischaemia-reperfusion or hydroxyl radical-induced stunning in failing hearts.[14,27–29,47] Recently Stowe *et al.*[26] demonstrated that pharmacological pre-conditioning using SK enhancers resulted in dramatically improved function of guinea pig hearts subjected to ischaemia-reperfusion. This improvement was linked to normalized mitochondria bioenergetics accompanied by reduced levels of superoxide production. Our pharmacological analysis confirms presence of functional mSKs in ventricular myocytes responsive to changes in $[Ca^{2+}]$ within the physiological range, determined in experiments assessing $\Delta\Psi$m (*Figure 4*). Furthermore, increased activity of mSK (*Figure 5A and B*) may be an adaptive response to preserve mitochondria function as inhibition of mSK channels in hypertrophic myocytes results in increased mito-ROS production measured with MitoSOX (*Figure 6C*). Importantly, pharmacologic enhancement of mSK channels in TAB CMs reduced mito-ROS rendering them similar to control CMs (*Figure 6C*). It should be noted that activation of mito-K^+ channels during pre-conditioning was well established to increase rather than decrease mito-ROS emission by accelerating electron flux along the respiratory chain. In line with this, our experiments revealed that treatment of rat myocytes from healthy hearts with 10 µmol/L CyPPA increased rate of ROS production (not shown). Interestingly, our experiments showed that neither SK enhancer nor SK inhibitor can dramatically affect mitochondrial Ca^{2+} uptake in TAB CMs (Supplementary material online, *Figure S6*), thus SK-mediated changes in mito-ROS production are likely not dependent on changes in mito-Ca^{2+} homeostasis. Apparently, the exact mechanisms of mSK- mediated protection in cells from diseased hearts,

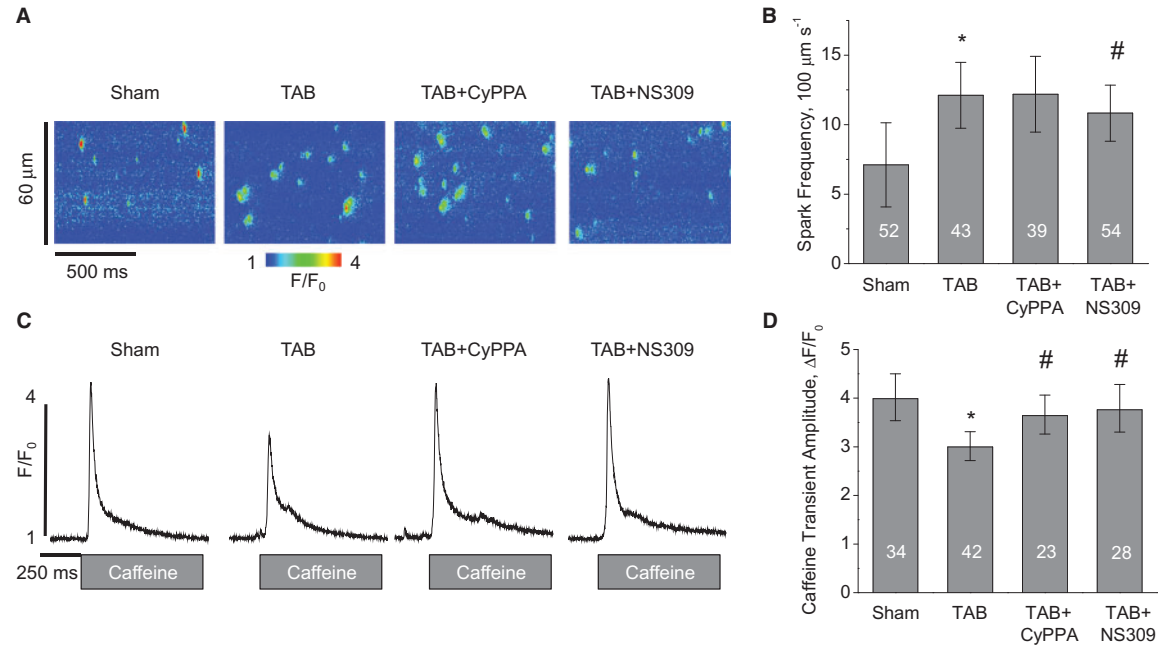

Figure 7 SK enhancement stabilizes RyRs in TAB myocytes. Pre-incubation of TAB myocytes with 10 µmol/L CyPPA or 10 µmol/L NS309 for 10 min normalizes SR Ca^{2+} content in permeabilized TAB myocytes. *(A, B)* Representative line scan images and pooled data for spark frequency, respectively. *(C, D)* Representative traces of caffeine-induced Ca^{2+} transients (20 mmol/L) and pooled data for caffeine-induced Ca^{2+} transients amplitude, respectively. Ca^{2+} sparks and Ca^{2+} transients were measured in saponin-permeabilized myocytes using Fluo-4. Cell numbers are shown in the columns, $*^{\#}P < 0.05$ vs. Sham and TAB respectively, hierarchical linear model. Number of Sham animals was 4; number of TAB animals was 6.

where ROS levels are already high, are yet to be determined and their delineation will require extensive efforts in the future.

4.4 SK channels and RyR-mediated Ca^{2+} release

Overactive RyRs play a central role in arrhythmias associated with hypertrophy, heart failure, MI, and aging.[4–7] Excessive activity of RyRs in these conditions is routinely attributed to posttranslational modifications including phosphorylation at RyR's CaMKII and PKA sites and oxidation of reactive cysteines.[8,40,41] Reducing agents were shown to produce stabilizing effects on RyRs resulting in attenuation of SR Ca^{2+} leak, pro-arrhythmic SCWs and Ca^{2+} alternans in many experimental settings.[5–8,13] We recently demonstrated that incubation of ventricular myocytes derived from old rabbit hearts with mitochondria-specific ROS scavenger mito-TEMPO normalizes RyR redox state and thereby SR Ca^{2+} release,[4] which corroborates the theory of a tight control of RyR function by mitochondria mediated by ROS.[4,10,11,13] Our data demonstrate that pharmacological enhancement of SKs reverses oxidation of RyRs in myocytes from hypertrophic hearts via normalization of ROS production by mitochondria (*Figure 6*, Supplementary material online, *Figure S10*), which results in stabilization of RyR-mediated Ca^{2+} release (*Figure 7*), attenuation of pro-arrhythmic SCWs at the single cell level (*Figure 2*, Supplementary material online, *Figure S2*) and rescue of Ca^{2+} homeostasis in the whole heart experiments (*Figure 1*). Importantly, while blockade of pSK channels produces anti-arrhythmic effects in certain conditions by reducing arrhythmic substrate, our data imply that usage of membrane-permeable SK inhibitors may affect mitochondrial function thus promoting Ca^{2+}-dependent triggers for arrhythmia.

5. Limitations

Usage of pharmacological modulators of ion channels and small peptides can raise concerns of their specificity and potential off-target effects. To address these concerns we performed experiments in rat myocytes infected with adenoviral constructs carrying rSK2 and maintained in primary culture for 48 h. SK2 overexpression significantly reduced the number of SCWs (Supplementary material online, *Figure S9*) and mito-ROS measured with MitoSOX (SK: 842 ± 99 a.u., $n = 4$; Control: 1338 ± 80 a.u., $n = 5$, $P < 0.05$, Student's *t*-test), similar to the effects of pharmacological activators of SK2 in freshly isolated TAB cells. The other limitation is that we cannot completely rule out a possible role of pSK channels in the regulation of mitochondrial function and thereby Ca^{2+} release. Finally, presented data does not provide the exact mechanism by which mSKs can regulate mitochondrial ROS production, which is the subject of future research.

6. Conclusions

Our results suggest that functional upregulation of SK channels in hypertrophic hearts serve protective, albeit incomplete, roles against Ca^{2+}-dependent arrhythmia via two independent mechanisms (*Figure 8*): (i) Plasmalemmal SKs protect from Ca^{2+} overload by shortening APD limiting Ca^{2+} influx and attenuate NCX-mediated EADs and DADs during SCWs; (ii) mSKs stabilize RyR-mediated Ca^{2+} release by reducing mitochondrial production of ROS. We conclude that facilitation of mSK channels in diseased hearts may serve as a new strategy to treat Ca^{2+}-dependent arrhythmia in hypertrophy and HF.

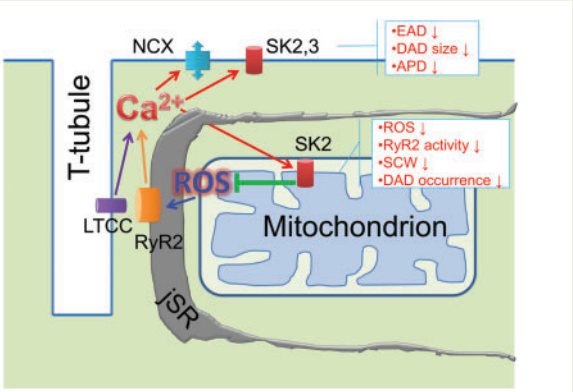

Figure 8 Schematic presentation of proposed cellular mechanisms of SK-mediated protection from Ca^{2+}-dependent ventricular arrhythmia. (i) Functional upregulation of SK2 and 3 in the plasma membrane limits disease-related prolongation of APD and reduces Ca^{2+}-dependent EADs and DADs countering I_{NCX}; (ii) Functional upregulation of mitochondria-targeted SK2 attenuates disease-related increase in mito-ROS. Pharmacological enhancement of SK2 reduces mito-ROS resulting in normalization of the oxidative state of RyR, stabilization of RyR-mediated Ca^{2+} release and abolishment of DAD-driving SCWs.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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